**University of Bath**

UNIVERSITY OF
**BATH**

**PHD**

**An investigation into the loss of antigen-specific lytic activity in cytolytic T cell hybridomas**

Woodland, David L.

*Award date:*
1986

*Awarding institution:*
University of Bath

[Link to publication](Link to publication)

Max-Planck-Institut für Immunbiologie, Freiburg im Breisgau, West Germany.


# AN INVESTIGATION INTO THE LOSS OF

# ANTIGEN-SPECIFIC LYTIC-ACTIVITY IN

# CYTOLYTIC T CELL HYBRIDOMAS


submitted by David L. Woodland

for the degree of PhD

of the University of Bath

1986


## COPYRIGHT

*D L Woodland*

D.L. Woodland

UMI Number: U362851

UMI

Dissertation Publishing

ProQuest

# SUMMARY

There are very few reports in the literature of antigen-specific cytolytic T cell hybridomas (CTH). The aim of the work presented in this report was to examine the hybridomas produced in fusions with cytolytic T lymphocytes (CTL) and ascertain the reasons for the loss of antigen-specificity.

Several fusions were performed between the AKR thymoma, BW5147, and three types of CTL, namely (i) CTL generated in a secondary in vitro mixed lymphocyte culture (ii) a long term cloned CTL and (iii) aged killer cells. Growing hybridomas were generated in all of these fusions but none of them expressed the stable antigen-specific lytic-activity of the parental CTL. However, in all fusions, hybridomas were found which expressed a non-specific lytic activity for P815 mastocytoma cells in the presence of the mitogenic lectin phytohaemagglutinin (PHA). This activity was further investigated by the isolation of both lytic (P815/PHA) and totally non-lytic hybridoma clones and was shown to resemble that of classical CTL in as much as it resulted in a reduced viability of P815 cells i.e. cytolymic effects were excluded.

The reasons for the loss of antigen-specific lytic-activity were investigated both with freshly prepared hybridomas and with the previously isolated clones. It was clearly demonstrated by flow-cytometry and poly-acrylamide gel-electrophoresis that loss of Lyt-2 antigen and/or T cell antigen-receptor is not primarily responsible for the consistent loss of antigen-specific lytic activity in CTH.

Several of the lytic CTH clones were back fused with various types of CTL. These fusions were very inefficient and the hybridomas generated were unstable and did not express stable antigen-specific lytic-activity.

MY PARENTS

## ABBREVIATIONS

| | |
|---|---|
| AK | aged killer (cell) |
| ConA | concanavalin A |
| ConA-SN | 24 hour supernatant from rat spleen cells stimulated with concanavalin A. |
| CTH | cytolytic T cell hybridoma |
| CTL | cytolytic T lymphocyte |
| DMEM | Dulbecco's modified Eagles's medium |
| DNA | deoxyribonucleic acid |
| E | effector cell (CTL) . |
| EDTA | ethylenediamine-tetra acetate |
| f | frequency |
| FCS | foetal calf serum |
| HAT | hypoxanthine, aminopterin, thymidine |
| HEPES | N-2-hydroxyethylpiperazine-N-thanesulphonic acid. |
| H-2 | murine major histocompatibility complex |
| H-Y | male antigen |
| IFNγ | interferon gamma |
| IL-2 | interleukin-2 |
| K | antibody dependent cytolytic cell |
| LFA-1 | lymphocyte function associated antigen 1 |
| LGL | large granular lymphocyte |
| MHC | major histocompatibility complex |
| MLC | mixed lymphocyte culture |
| mRNA | messenger ribonucleic acid |
| NK | natural killer (cell) |
| Ou | ouabain |
| p | probability (chi-squared analysis) |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| PHA | phytohaemagglutinin |
| RNA | ribonucleic acid |
| SBA | soybean agglutinin |
| SDS | sodium dodecylsulphate |
| SP | 3-(p-sulphophenyldiazo)-4-hydroxyphenyl acetic acid |
| T | target cell (cytolytic assay) |
| TCR | T cell antigen-receptor |
| TG | thioguanine |
| TNP | trinitrophenol |
| Tris/HCl | tris-(hydroxymethyl-) aminomethane buffer |

# CONTENTS

# 1. INTRODUCTION

## 1.1    Cytolytic T lymphocytes

It has long been known that tissue grafted from one individual to another is quickly rejected. However, it was not until 1954 that Mitchison and colleagues, first showed that in most cases this rejection was due to cells of the immune system. As the various cellular subsets of the immune system were established it soon became clear that tissue rejection was dependent on cells which had matured in the thymus i.e. T cells (Brunner and Cerottini, 1971). It now appears that this response is mediated by cytolytic  T lymphocytes (CTL) which are derived from stem cells generated in the bone-marrow and which mature (or are processed) in the thymus. They strongly express the T cell marker Thy-1 and also typically express the CTL marker Lyt-2 when mature. For a long time, the co-expression of Thy-1 with Lyt-2 was a partial definition of CTL.

The function of CTL is to lyse and thereby destroy cells bearing foreign antigens. This is the basis for the rejection of transplanted (i.e. foreign) tissue. However, tissue transplantation is an unnatural occur*e*ance in nature and the CTL lineage is thought to have primarily evolved for the destruction of virally infected cells and tumour cells.

The recognition of antigen by CTL is not a simple interaction. In normal responses CTL do not recognise antigen alone but only in conjunction with self major histocompatibilty (MHC) antigens (Zinkernagel and Doherty, 1974). This mechanism has probably evolved as a way of directing the response of CTL to appropriate sites. In addition, certain CTL are able to lyse cells

bearing foreign MHC molecules in the absence of self MHC molecules; it is probably this subpopulation of CTL which mediate tissue rejection. This response is thought to be due to a cross-reaction since foreign MHC molecules may conceivably appear as self MHC molecules plus antigen to CTL (for review see Nabholz and MacDonald, 1983). The antigen specificities of CTL are clonally distributed and the total repertoir is carried in a large number of individual clones.

Upon activation by recognition of appropriately presented antigen, CTL proliferate to enhance the lytic response. This proliferation is dependent upon the lymphokine interleukin-2 (IL-2) which is produced by helper or inducer T cells. It is now possible to maintain CTL in in vitro culture by fulfilling both of the requirements for antigen and IL-2. However, it is thought that CTL kept under these conditions partially transform and are not typical of "normal" CTL (Nabholz et al., 1980a).

Under certain circumstances, other cells within lymphoid populations may also be cytolytic. These cells are not readily classified as T cells and are collectively termed null cells because of their lack of expression of classical immune-cell markers. Killer (K) cells mediate cytotoxicity in concert with classical immunoglobulin-G, and natural killer (NK) cells lyse a variety of tumour cells without prior activation and without the need for specific antibody.

In recent years there has been considerable interest in understanding the mechanisms underlying the interactions of CTL with target cells. Many studies have been performed both on CTL themselves and on other types of lytic cells of the immune system such as neutrophils, macrophages, K cells and NK cells. It now seems that, with perhaps the exception of neutrophils and macrophages, all of these systems conform to certain general principles and in the case of

NK cells and CTL may even be identical except for the mode of target recognition. These studies have all been greatly facilitated by the development of a simple and reliable assay for lytic activity, the chromium release assay, in which target cell death is measured by the release of chromium-51 (Brunner et al., 1968).

Certain features of the NK and CTL mediated lytic mechanisms have now become very clear. It is well established that these systems require viable and metabolically active cells since metabolic inhibitors inhibit the lytic mechanism (Quari et al., 1982). Moreover, lysis of the target cell does not result in the concomitant lysis of the effector cell, such that CTL are able to recycle and lyse multiple targets (Zagury et al., 1975; Martz and Benacerraf 1976). The lytic mechanism is dependent on cell to cell contact and is clearly antigen specific since bystander cells of inappropriate specificity are not lysed.

At present, the sequence of events associated with CTL mediated cytolysis can be grouped into four principle stages (Berke, 1980);

(i)  Effector to target recognition and binding. This stage occurs within minutes and is magnesium ion dependent.

(ii)  "Programming for lysis" or "lethal hit". The lethal hit is delivered within seconds and is calcium ion dependent. (reviewed Martz et al., 1982b).

(iii)  CTL independent lysis of the target cell. The target may take up to two hours to die.

(iv)  CTL recycling. The CTL can recycle between 20 mins and 1 hour.

The division into distict binding and lytic stages has led to the general consensus that binding and lysis involve distinct molecules of the CTL. These two aspects are discussed independently.

## 1.2    The lytic-mechanism

After the T cell antigen-receptor (TCR) has been engaged and the CTL activated, the effector cell undergoes a sequence of events which culminate in the delivering of a lethal-hit to the target cell. The precise nature of these events is, as yet, unclear but recent studies have provided many important clues.

Morphological studies of lysis by CTL, NK and K cells have revealed that the effector cell cytoplasm undergoes a pronounced rearrangement after target cell binding such that most of the organelles become localised between the nucleus and the target cell (Bykovskaja, 1978 a,b). This rearrangement occurs during the thirty minutes following target cell binding. In parallel, there is a period of increasing membrane contact between the effector and target cells and there is often a pronounced interdigitation of the cytoplasmic processes and membranes of the two cells, presumably to further increase the area of contact (Ryser et al., 1977). Geiger and colleagues, 1982, have also described the asymmetric distribution of the CTL cytoplasm in conjugates by immunofluorescent labelling of the cytoskeletal protein tubulin. Similar results were obtained with regard to actin and it is clear that there is extensive cytoskeletal rearrangement in CTL after conjugate formation. This rearrangement is probably responsible for the movement of cell organelles to the site of lysis.

There is much evidence to suggest that the lytic mechanism involves a secretory process. Zagury, 1982, has shown that acid phosphatase, origially located in the lysosomal granules of CTL, is deposited at effector-target junctions after the rearrangement of the cytoplasm. Other materials which have osmophillic properties have also been shown to be deposited into the

intracellular space (Bykovskaja et al., 1978b). In addition, the requirement for calcium ions by both CTL and NK cells suggests a secretory mechanism since an increase in cytoplasmic free calcium is typical for most secretory processes.

Granules have been observed in the cytoplasm of large granular lympocytes (LGL) and CTL in most electron microscopic studies which seem to be involved in secretory processes (Zagury et al., 1975; Bykovskaja et al., 1978b; Matter et al., 1979). Millard and collegues, 1984, first purified cytoplasmic granules from a rat LGL tumour cell and found that they had a potent lytic effect on a wide variety of target cells (see also Henkart et al., 1984). Lytic granules have subsequently been purified from NK cells and CTL which are able to lyse several tumour targets with YAC-1 and EL4 cells being particularly sensitive (Podack and Konigsberg, 1984; Podack et al., 1984). The ability of the granules to lyse numerous targets demonstrates that the antigen specificity of CTL does not reside in the granules themselves. The activity of the granules is rapid requiring less than five minutes at room temperature and has the same calcium requirements as the CTL/NK lethal-hit. However, the lytic-activity is not stable in the presence of calcium ions and decays within minutes. With granule specific antisera it has been shown that, within ten minutes after target cell binding, the cytolytic granules become positioned towards the effector-target contact area. It is likely that the reorientation of the cytoskeleton is responsible for bringing the granules to the site of conjugation where they release their contents by directed secretion onto the target membrane (Podack, 1985). Indeed, immunofluoresence studies have also provided strong evidence for early secretion of granule antigens that subsequently appear to associate with the target cell.

Quantitatively, it seems that granule mediated lysis may account for most if not the entire cytolytic principle of CTL and NK cells. Blumenthal and colleagues, 1984, have shown that LGL tumour granules induced the deposition of membrane associated cylindrical structures on target membranes in a rapid process requiring calcium ions. Electron microscopy of liposomes exposed to these LGL granules in the presence of calcium showed that they were penetrated by cylindrical structures with a diameter of 15nm. Two types of similar pore like structures have been described on target cells after incubation with CTL (Dennart and Podack, 1983). The larger class (termed polyperforin-1) has a diameter of 16 nm and the smaller class (termed polyperforin-2) has a diameter of 5-7 nm. There are a number of similarities between these structures and those formed by polymers of the complement protein C9.

The insertion of pores into the target cell membrane is consistent with the theory that target death is induced by colloid osmotic lysis and destruction of the membrane potential of the target cell. But there are clearly other mechanisms involved since tumour targets which have received a lethal hit undergo a series of cytoplasmic bulging movements termed zeiosis or membrane blebbing (Sanderson, 1976). Moreover, Russell and colleagues, 1980, 1982, have clearly demonstrated that there is rapid hydrolysis of the target cell nuclear deoxyribonucleic acid (DNA) during CTL mediated lysis. This digestion generates DNA fragments of 200 base-pairs (or multiples thereof) and the process requires calcium ions. However, this DNA digestion is probably only a secondary event to cell lysis since cytolytic granules can readily lyse erythrocytes or other enucleated targets. At present, the origin of this DNAse activity is unclear. The activity probably has an anti-viral role by destroying viral DNA in an infected cell.

## 1.3    The binding of antigen by CTL

### 1.3.1    The T cell antigen receptor complex

The lytic granules of CTL appear to be non-specific in that they are able to lyse a variety of targets regardless of the antigen-specificity of the CTL from which they were derived. Thus, the antigen-specificity of CTL must reside elsewhere. The nature of this antigen-specificity is intriguing since it has been demonstrated that T cells need to co-recognise antigens in association with MHC encoded molecules for activation to occur. CTL require class I MHC molecules whereas helper (or inducer) T cells require class II MHC molecules. As a consequence of this complexity, no simple ligand binding assay exists and therefore, the TCR has proved elusive to characterise. However, in the last three years, the TCR has been identified both by monoclonal antibodies and by the isolation of copy-DNA clones encoding for the receptor (Allison et al., 1982; Haskins, et al., 1983; Meuer, et al., 1983; Samelson et al., 1983; Yanagi, et al., 1983; Chien et al., 1984; Hedrick et al., 1984; Saito et al., 1984). It appears that the TCR is a heterodimer composed of two polypeptide chains linked together by interchain disulphide bonds. The two chains can be distinguished by isoelectric focussing gels; the acidic chain is designated as α and the neutral/slightly-basic chain as ß. Each chain is a transmembrane glycoprotein with a core size of 32 kd (glycosylated forms approx. 40 kd) and composed of two domains, one variable and one constant. Indeed, there is a great deal of similarity to antibody molecules both in physical structure and in amino-acid sequences.

Like the immunoglobulin genes, the genes encoding the TCR are assembled from separate segments, one of which encodes the invariant constant (C) domain while two or three other segments known as the variable (V), diversity (D) and joining (J) segments encode the variable domain. Diversity in the TCR

appears to be generated by several mechanisms:-

(i) The presence of numerous and diverse V, D and J segment genes in the germ-line (reviewed Hood et al., 1985). It seems that the V gene pools of the TCR $\alpha$ and ß chains are not as large as those for immunoglobulin heavy chain genes but that this is compensated for by larger numbers of J and D segment genes.

(ii) Random combination of different V, D and J segments (Hood et al. 1985). In addition, the recombination mechanism allows, in principle, the joining of both D to D segments and V segments directly to J segments, neither of which is possible in the immunoglobulin heavy chain pool. This versatility, however, has not been demonstrated in practice.

(iii) Junctional flexibility due to variability in the sites at which the gene segments may be joined (Milissen et al., 1984) This mechanism also operates in B cells.

(iv) Diversification resulting from the random addition of nucleotides to either end of the D gene segment during the process of joining to the V and J gene segments (Kavaler et al., 1984; Siu et al., 1984). This mechanism also operates in B cells.

(v) $D_\beta$ gene segments can be joined to the $V_\beta$ gene segments in all three translational reading frames (Goverman et al., in press). In B cells, each D gene segment is joined to a V gene segment in a single translational reading frame.

(vi) Somatic mutation. Augustine and Sim, 1984, have presented evidence for somatic mutation in the ß chain of the TCR of a cell recognising MHC determinants. However, these findings have, as yet not been substantiated. Certainly, somatic mutation is a mechanism for the diversification of immunoglobulin genes.

(vii) The association of rearranged α and β chains generates new specificities. At present, the structure of the antigen-binding cleft of the TCR and how it corr*e*lates with the recognition of antigen in the context of MHC molecules is unclear.

In addition to the α and β chains, a third TCR gene has been described and is referred to as the γ chain (Saito et al., 1984, Hood et al., 1985). The mouse γ gene pool contains three J segments, three C segments (only one of which seems to be transcribed in mature T cells) and several functional V segments (Hayday et al., 1985). Although the γ genes have been shown to rearrange, no cell suface products have been found. The role of the γ chain (if it is expressed) remains highly speculative.

Recent evidence suggests that the human TCR is associated with additional proteins termed the T3 complex. This complex is found only on T cells (both T8 (Lyt-2) and T4 positive) and is comprised of three invariant proteins, two glycoproteins of 20 kd and 25 kd, and a non-glycosylated protein of 20 kd (Borst et al., 1983; Kanellopoulos et al., 1983). Monoclonal antibodies that recognise and bind to the 20 kd T3 glycoprotein can either stimulate or inhibit T cell activation (van Wauwe et al., 1980; Reinherz et al., 1980). Under certain circumstances, monoclonal antibodies that immunoprecipitate the human 20 Kd T3 glycoprotein co-precipitate the clonotypic α and β chains of the TCR as well as the other two polypeptides of the T3 complex (Reinherz et al., 1983; Oettgen et al., 1984). Subsequent experiments with bifunctional cross-linking reagents have demonstrated a close association of the human β chain with the 25 kd T3 polypeptide (Brenner et al., 1985). A similar complex has recently been found to be associated with the murine TCR (Allison and Lanier, 1985; Samelson and Schwartz, 1984) and a copy-DNA clone isolated for one of the

components (van den Elsen et al., 1985). This complex, also termed T3 in mouse, has been described by Samelson and colleagues, 1985, as being comprised of the α and β TCR heterodimer with two glycoproteins of 25 kd (γ) and 21 kd (δ) and two non-glycosylated peptides of 26 kd (ε) and 16 kd (ξ). The ξ chain appears to possess an intrachain disulphide bond and the chain exists in the complex as a disulphide linked homodimer.

At present, the role of T3 in cellular activation is under intense study. There is much evidence that rapid influxes of calcium are involved in the activation of T cells (Freedman and Raff, 1975; Allwood et al., 1971; Shapino et al., 1985) and Oettgen and colleagues, 1985, have shown that stimulation of T3 in human leukemic cells leads to an increase in the concentration of cytoplasmic calcium ions. They suggest that the T3 complex forms a calcium-ion channel since the 20 kd non-glycosylated ε protein has some properties that are similar to those described for other plasma membrane ion channels. Such calcium ion channels are known to be involved in the response of cells to several hormones (see Carafoli and Penniston, 1985 for review). Samelson and colleagues, 1985, have shown that the δ chain of murine T3 is phosphorylated on a serine residue in response to mitogenic-lectin or antigen. In contrast, both δ and ε chains are phosphorylated in response to treatment with phorbol 12-myristate 13-acetate, a potent activator of T cells. It is suggested that these proteins may play a role in the transduction of the signals in T cell activation. Protein kinase C has also been implicated in this mechanism by Cantrell and colleagues, 1985.

Taken together, the data suggest that both helper cells and CTL are activated by a common mechanism involving the TCR and T3 complex. Presumably, stimulation of the TCR results in a signal to the T3 which opens an ion-channel causing an increase in the cytoplasmic calcium-ion

concentration (although this may also, in part, be due to a release of calcium from internal reserves). In turn, an amplifying cascade of phosphorylating enzymes is activated resulting finally in the delivering of a message to the nucleus although, at present, little is known of the processes involved. This proposed mechanism of activation is highly speculative.

## 1.3.2 Lyt-2/3

The Lyt-2 and Lyt-3 antigens were initially defined using absorbed mouse alloantisera and shown to be coded by closely linked genes (Boyse et al., 1968; 1971). CTL were found to strongly express Lyt-2 (Cantor and Boyse, 1977) and it was subsequently shown that T cells can be essentially divided into two mutually exclusive subsets, namely CTL which are Lyt-2 positive and T4 negative and helper (inducer) T cells which are Lyt-2 negative and T4 positive (Swain, 1983).

In 1980, Fan and colleagues demonstrated that monoclonal antibodies specific for Lyt-2 block CTL-target cell conjugation and reverse previously completed recognition/adhesion events (also Shinohara et al., 1981; Hayot et al., 1982). High concentrations of the mitogenic lectin concanavalin A (ConA) overcome this inhibition (Meuer et al., 1982). It was proposed by Swain, 1981a, b, that expression of Lyt-2 on mature functional T cells is necessary for recognition of class I MHC antigens. This proposal has gained considerable strength from parallel findings in the human system with antibodies specific for T8, the human homologue of Lyt-2. MacDonald and colleagues, 1982, have suggested that Lyt-2 stabilises the interaction of the TCR with antigen but that this stabilisation is unnecessary if the receptor is of a sufficiently high affinity or is present at high density. It remains unclear whether Lyt-2 interacts with class I MHC molecules or not.

Surface labelled thymic Lyt-2/3 antigen consists of a major protein of 70 kd which upon reduction resolves into two species of 38 kd ($\alpha$) and 35 kd ($\alpha'$) and a minor protein of 30 kd (ß). The $\alpha$ chains carry the Lyt-2 epitopes and the ß chain carries the Lyt-3 epitopes. The Lyt-2 gene has recently been sequenced and the different $\alpha$ chains have been shown to be derived from alternative splicing of a single mRNA species (Zamoyska et al., 1985). In the mouse, the Lyt-2 antigen has two alleles termed Lyt2.1 and Lyt2.2.

### 1.3.3 Lymphocyte function associated antigen-1

Murine lymphocyte function associated antigen-1 (LFA-1) is expressed on both B and T lymphocytes and on 80% of bone marrow cells; there are no allelic forms (Kürzinger et al., 1981). Monoclonal antibodies raised against LFA-1 readily block the lytic activity of CTL against target cells seemingly by preventing effector to target conjugation and suggesting a role in cell adhesion (Davignon et al., 1981). Martz and colleagues, 1982a, suggest that the unusual requirement for magnesium ions by CTL in order to bind to target cells may be due to a requirement for these ions by LFA-1.

Work by Springer and colleagues, 1982, has shown that LFA-1 is composed of a 180 kd $\alpha$ chain and a 94 kd ß chain.

### 1.4 Other surface molecules involved in the CTL lytic-mechanism

CTL function has been shown to be blocked by several other monoclonal antibodies against T cell surface markers but as yet most of these remain undefined. One example is T200 which is involved in the NK lytic mechanism and also appears to play a role in CTL-mediated lysis (Lefrancois and Bevan,

1985). Other examples include LFA-2, LFA-3 and human T11 which are generally involved in T cell activation.

## 1.5    The role of mitogenic lectins in mediating non-specific lysis by CTL

Under appropriate conditions CTL are able to lyse target cells non-specifically in the presence of mitogenic lectins such as ConA and phyto-heamagglutinin (PHA) (Formann and Möller, 1973; Bevan and Cohn, 1975). It was initially thought that lectin-dependent cytolytic-activity was due purely to the agglutination of effector to target cells but it later became clear that the lectin played an additonal role in activating the CTL (Green et al., 1978). Berke and colleagues, 1981a,b, have presented further evidence against a trivial ("glueing") role of mitogenic lectins. They showed that ConA and PHA mediated non-specific lysis most effectively when lectin treated target cells were allowed to react with untreated CTL but not so effectively when lectin treated CTL were allowed to react with untreated target cells. They interpreted these finding as being due to a lectin induced alteration of molecules on the surface of the target cell allowing it to be recognised by the CTL.

Recently, much work has been carried out to ascertain the binding sites of mitogenic lectins on T lymphocytes. Sitkovsky and colleagues, 1984, have demonstrated that ConA binds to several relevant murine T cell surface antigens including T200, Lyt-2 and the α and ß chains of LFA-1. Several other unidentified molecules also bound ConA and two of these had molecular weights of 42 kd and 46 kd which probably represent the α and ß chains of the TCR. In addition, Karellopoulos and colleagues, 1985, claim that ConA but not PHA binds the 20 kd chain of the human T3 complex. However, this point is disputed by two other laboratories claiming that PHA does bind to human T3

(Valentine et al, 1985; Bolhuis and van de Griend, 1985). Certainly, all data are [~~is~~]

in agreement with or suggestive that both PHA and ConA bind·to the TCR

(Chilson et al., 1984; Sitkovsky et al., 1984).

Taken together, these [~~this~~] data would suggest that both ConA and PHA activate

CTL by binding to the TCR, T3 and/or other molecules involved in cellular

activation. It is intriguing to speculate that the binding of lectin to LFA-1 or

Lyt-2 could inhibit lytic activity (as has been shown by monoclonal antibodies)

and be responsible for the unidirectional effect of lectin in mediating lysis

(Berke et al., 1981a, b). This point remains to be tested.


## 1.6    Aged killing activity


A recent technical advance in the study of lytic mechanisms has been the

use of lymphokines to allow the continuous in vitro growth and cloning of CTL

and NK cells. In some cases, these cells have aquired unexpected patterns of

target cell specificities including some which appear to be different from any

previously described (Grim et al., 1982; Simon et al., 1984). Most surprisingly,

it has been shown that cloned CTL can become convincingly NK like in their

target cell recognition, morphology and surface markers (Acha-Orbea et al.,

1983; Binz et al., 1983; Brooks, 1983; Meretta et al., 1984; Wilson and

Shortman, 1985).

The acquisition of a new specific lytic activity against P815 mastocytoma

cells in long-term murine CTL clones has been termed aged killing (AK)

activity by Simon and colleagues, 1984. This activity is distinct from the NK

·or "NK like" activity of other long term T cell lines and clones by its exclusive

specificity for P815 targets and by its sensitivity to inhibition with anti-Lyt-2

monoclonal antibodies. The development of AK activity has been shown to

corrélate with the rearrangement and expression of a new mRNA in the cytoplasm coding for the ß chain of the TCR (Epplen et al., 1986). This new ß chain has been shown to be composed of identical V, D and J region genes ($V_{\beta AK}$, $D_{\beta AK}$, $J_{\beta AK}$) in several independent AK cell lines and it is interesting to note that the $V_{\beta AK}$ region is highly homologous to the human ß chain variable region YT35 which is expressed in at least two human T cell leukemia lines, MOLT 3 and Jurkat (Yoshikai et al., 1984; Rinaldy et al., 1985). Jankovié et al. (in press) have shown that AK cells require the presence of TCR in order to express any form of lytic activity, whether specific or non-specific, and that co-expression of Lyt-2 with the TCR is necessary for specific lytic-activity against P815.

## 1.7 Cytolytic T cell hybridomas

Whereas T cell hybridomas have proven and continue to prove very useful for studying helper T cell function, there are suprisingly few reports in the literature of cytolytic T cell hybridomas (CTH). Interestingly, nearly all of the CTH described do not appear to represent classical H-2 restricted or allo-reactive CTL, but instead represent "AK like" cells or are unusual in some other aspects of their antigen recognition properties. These hybridomas are reviewed here.

Theoretically, CTH offer great advantages over normal CTL clones for molecular genetic and biochemical studies. Normal CTL clones require the continuous presence of antigen and growth factors (usually IL-2) which tend to interfere with most studies unless extensive purification procedures are performed. Moreover, cloned CTL tend to grow very slowly and are relatively unstable. Hybridomas, on the other hand, should grow quickly to high densities in the absence of contaminating antigen and growth factors.

The earliest reports of attempts to generate CTH appeared in 1977 and 1978

shortly after the advent of the fusion technique. In all cases, CTH were sought

in populations of hybridomas generated from mixed lymphocyte cultures (MLC)

(Goldsby et al., 1977; Köhler et al., 1977; DiPauli and DiPauli, 1978;

Grützmann and Hämmerling, 1978; Ruddle, 1978 and Simpson et al., 1978). No

hybridomas were found in these populations that were able to lyse relev$\overset{a}{\text{e}}$nt

target cells either specifically or non-specifically in the presence of ConA.

Köhler and colleagues, 1977, claimed that a single hybridoma clone lysed P815

cells after a fusion between cells from a secondary MLC (C57BL/6 against

BALB/c) and BW5147. However, the lytic-activity of this clone was unstable

and disappeared within two weeks.

It was not until later that reports first started to appear of lytically active

CTH. These hybridomas were derived from essentially three types of CTL i.e.

CTL generated in MLC cultures, cloned CTL with H-2 restricted or allo-

reactivity and cloned CTL with "AK like" or unusual activity.

## 1.7.1 Hybridomas generated from cells produced in mixed lymphocyte cultures

Several lytic CTH clones are reported to have been derived from MLC.

Kaufmann and colleagues, 1981a, b, produced CTH from CTL generated in an

MLC between BALB/c or CBA spleen responder cells and EL4 (H-$2^b$) tumour

stimulator cells. Approximately, 5-20% of the hybridoma cultures generated

lysed EL4 in the presence of PHA and 1-2% of cultures lysed EL4 in its

absence. Altogether, this laboratory managed to isolate five rather unstable

hybridoma clones which appeared to specifically lyse EL4 in the absence of

lectin. However, in order to observe significant lysis, they had to treat the

EL4 targets with neuraminidase to remove sialic acid from the cell surface

and in most experiments both effector and target cells were treated. More-over, the titration curves of these clones were highly unsatisfactory since the lytic activity is reduced to almost zero at effector to target ratios of 40:1 resulting in bell shaped curves. The hybridomas were antigen-specific in as much as they lysed several H-2$^b$ tumour targets but not tumour targets of other haplotypes. The antigen recognised is probably not H-2$^b$ because H-2$^b$ ConA blasts are not lysed unless PHA is present in the assay. Lytic activity was temperature and calcium ion dependent, was not mediated by soluble factors and was inhibited by monoclonal antibodies reactive against H-2D$^b$ and LFA-1 but not Lyt-1 or Lyt-2 determinants (Eshhar et al., 1982; Kaufmann et al., 1982 and Kaufmann and Berke, 1983). In another MLC system, Whittacker and colleagues, 1982, fused BW5147 with spleen cells from CeH/HeJ mice which were lytically active against syngeneic cells infected with reovirus type 1. Several hybridoma clones were generated of which half were able to lyse virally infected syngeneic targets in the presence of ConA or PHA. However, they were unable to demonstrate lectin independent lytic activity unless they used assays involving effector to target ratios of 1000:1 and 6 hour incubation periods. Interestingly, some of their hybridoma clones possessed a degree of specificity in that they would lyse virally infected syngeneic targets, but not uninfected syngeneic targets in the presence of ConA. This lectin-dependent specificity is unusual and atypical of classical CTL.

Greenstein and colleagues, 1984, report generating a hybridoma in a fusion between BW5147 and MLC cells (reactive against a synthetic peptide) which expresses the helper cell antigen T4 but is MHC class 1 restricted in its activity. In addition to the secretion of IL-2, this hybridoma will also lyse H-2$^d$ tumour targets, such as P815 and A20, but not tumour targets of other H-2 haplotypes (including YAC-1 cells) or BALB/c ConA blasts. The lytic activity

expressed by this hybridoma clone is strong but the titration curves are bell-shaped. Antibodies against $H-2^d$ determinants are able to block this lytic activity and, interestingly, a T4 negative variant of the hybridoma is no longer lytic.

Similar multifunctional hybridomas have been generated by Blanckmeister and colleagues, 1985, in a fusion between splenic T cells from mice immunised with bovine serum albumin and a hybridoma cell (111/4T, derived from the fusion of CBA ConA blasts with BW5147). Many of these hybridomas are capable of suppressing the antigen specific proliferation of lymph-node cells from CBA mice immunised with bovine serum albumin and two clones were generated which additionally expressed lytic activity. These hybridoma clones lysed a variety of tumour targets of various haplotypes including YAC-1 ($H-2^d$) although the range of activity is not typical of NK cells. The parental hybridoma 111/4T was not cytolytic.

## 1.7.2 Hybridomas genrated from cloned CTL

In the second type of fusion, cloned CTL with classical lytic activity have been used. Such clones require continuous antigen stimulation in vitro and the presence of IL-2.

Nabholz and colleagues, 1980b, have generated hybridomas from a fusion between BW5147 and a CTL clone called CSP.2 which is derived from C3H mice ($H-2^k$) and lyses 3-(p-sulphophenyldiazo)-4-hydroxyphenylacetic acid (SP)-modified syngeneic blast cells in an MHC restricted fashion. Hybridomas which had developed in the absence of ConA-SN showed no lytic activity whereas several of those which had developed in the presence of ConA-SN showed lytic activity against SP-modified syngeneic blasts but not against

syngenic blasts alone. The authors claim that these hybridomas are classically MHC restricted but the lytic activity of the hybridomas against tumour targets is not reported. Haas and Kisielow, 1985b, have generated IL-2 independent variants from one of these hybridomas (SPH1) and fused it in a secondary fusion with two types of CTL clone which recognise fluoroscein either in the context of H-2D$^d$ (DFL.2) or in the context of any H-2 class 1 haplotype (CFL.1). These experiments were designed to examine the question of whether MHC and antigen recognition was determined by either one or two receptors. The hybridomas generated possessed lytic activities either identical to that of the individual parental cells, or occasionally, to the lytic activities of both parental cells together. However, no hybridomas with cross-specificities of different MHC restriction with different antigens were observed suggesting that antigen and MHC recognition is mediated by a single receptor.

Silva and colleagues, 1983, have reported a series of fusions between classical allospecific CTL clones (specific for H-2$^d$ or H-2$^k$) and a rat thymoma, W/Fu (C58NT)D. In most of these fusions they claim that no cytolytic activity was detected but in one fusion with a CTL clone called C10 (derived from C57BL/6 mice and specific for H-2$^d$) they detected lytic activity against P815 cells (H-2$^d$) but not against H-2$^d$ ConA blasts or other tumour cells. With isolated clones they showed that this lytic activity is not transmitted by a soluble factor and is dependent on calcium ions. These cytolytic hybridomas were only derived from cultures which had developed in the presence of ConA-SN and it was shown that some factor(s) present in ConA-SN directly controls the expression of lytic activity. Thus, if these hybridomas are cultured without ConA-SN their lytic activity disappears but can be reinduced by subsequent culture in the presence of ConA-SN. The hybridomas generated in these fusions express high levels of the Lyt-2 antigen,

but no OX8 antigen which is the rat homologue of Lyt-2. In addition, antibodies specific for Lyt-2 are capable of inhibiting this activity against P815 whereas the same antibodies do not inhibit the parental clone C10 in the same manner. The authors suggest that this provides further evidence for the theory that Lyt-2 is required in the lytic process to enhance cell binding if, and only if, the TCR has low affinity for its antigen.

### 1.7.3 Hybridomas generated from cloned "AK like" CTL

In the third type of fusion, several groups have used cloned CTL with unusual or "AK like" activities. One such CTL clone is B6.1.2 which originates from C57BL/6 mice and was initially active against syngeneic male ConA blasts (H-Y in the context of H-2D$^b$) and H-2$^d$ ConA blasts of either sex (von Boehmer et al., 1979). This clone was always maintained in the presence of its specific antigen and IL-2. However, the lytic activity against H-Y later disappeared and was replaced by a lytic activity against the BALB/c myeloma S194 (H-2$^d$) and to a lesser extent against P815 (H-2$^d$) (Nabholz et al., 1980 and Berebbi et al., 1983). This variant of the original clone retains its dependence on IL-2 but is no longer dependent upon any known antigen for the maintenance of its lytic activity against S194. Nabholz and colleagues, 1980, fused this CTL clone with the AKR thymoma AKR/A and found that 75% of the hybrids which developed in the presence of ConA-SN were capable of non-specific lysis of S194 in the presence of ConA. Several cultures, which were later cloned, showed specific (lectin independent) activity against S194. Only non-lytic hybridomas were found in cultures which had developed in the absence of ConA-SN and the authors suggest a corrolation between morphology, adherence, lytic activity and the presence or absence of ConA-SN. Thus,

non-lytic hybridomas resemble the thymoma parent in that they are round in shape and non adherent whereas lytic hybridomas morphologically resemble B6.1.2 and are adherent. The "thymoma like" non lytic hybridomas develop both in the presence and absence of ConA-SN but "CTL like" lytic hybridomas develop only in the presence of ConA-SN.

Similar results were obtained when Conzelmann and colleagues, 1982, fused the same clone B6.1.SF1 with the rat thymoma, W/Fu (C58NT)D. However, in this case, the non-lytic variants which had originated in cultures without ConA-SN could be induced to lyse S194 targets by the addition of ConA-SN to the culture medium. Induction took place in 2-3 days. This inducible lytic-activity is identical to that described previously in hybridomas generated by Silva and colleagues between a classical CTL clone and the same rat thymoma (Silva et al., 1983).

Erard and colleagues, 1984, of the same group later identified a factor which they named cytotoxicity inducing activity which has a molecular weight of 12-18 kd, resembles the lymphokine interleukin-1 and acts synergistically with IL-2 to induce cytolytic activity in the hybridomas. It has recently been shown by Masson and colleagues, 1985, that the induction of lytic activity in these hybridomas corrélates with the presence or absence of cytolytic granules and polyperforin 1 which is a protein involved in the construction of membrane lesions in the target cell during the lethal hit. Thus, hybridomas with induced lytic activity contain polyperforins 1 and 2, cytolytic granules and granule associate proteins, whereas non-lytic hybridomas lack cytolytic granules and polyperforin 1 but contain polyperforin 2.

B6.1.2 has also been fused with a polyoma virus transformed fibroblast line (Berebbi et al., 1983 and Foa et al., 1984). Several hybridoma clones were isolated which grew in the absence of ConA-SN and had specific lytic activity

against S194. However, this lytic activity was very weak (12% at 50 effectors per target) and the authors were unable to titrate this activity. These hybridomas are Lyt-2 negative.

Another CTL clone with non-classical cytolytic activity is CFL.1 which has already been mentioned, (see also Haas et al., 1980). This clone specifically lyses fluoroscein-modified targets expressing several independent H-2 haplotypes but it does not lyse fluoroscein-modified targets which lack class 1 MHC antigens. These cells are dependent on ConA-SN for growth but do not require constant antigen restimulation for the maintenance of their lytic activity. After fusion with BW5147, hybridomas were found only in cultures containing ConA-SN and several clones were derived which are specifically lytic for fluoroscein-modified CBA ConA blasts (Haas and Kisielow, 1985a). The lytic hybridomas produced interferon γ (IFN-γ) in response to either appropriate antigen (fluoroscein-modified CBA ConA blasts) or ConA, indicating that they express the TCR of one or both of the parental cells.

Taken together, these reports demonstrate that the generation of CTH with classical MHC restriction patterns is very difficult with standard fusion procedures. In most cases the hybridomas generated have unusual specificities for tumour targets or have very weak or lectin dependent activity.

In most of the reports cited, the authors prove their cells to be hybridomas by demonstrating co-expression of surface antigen specific for both parental cells. However, although the hybridomas are shown to possess properties typical of classical CTL such as sensitivity to inhibition by appropriate antibodies, ethylenediamine-tetra-acetate (EDTA) and temperature, it is never clearly demonstrated that the activity observed really results in target death. This is a particularly important point bearing in mind the reports of cytolymic *
effects by FischerLindahl and colleagues, 1982. Certainly, some of the so-

* see section 3.6.3

called specific targets employed in these reports are also optimal cytolymic

targets.

To date, the most promising candidates for CTH representing classical CTL

are the hybridomas described by Nabholz and colleagues, 1980b, specific for

the hapten SP in the context of H-2K$^k$ and the hybridomas described by Haas

and colleagues, 1985b,c, which lyse either SP in the context of H-2K$^k$ or

fluoroscein in the context of H-2D$^d$.


## 1.8 Aims of the present thesis


The aims of the work presented in this thesis were threefold:-

(i)   To determine the reasons behind the difficulties in generating CTH
      which maintain antigen-specific lytic-activity.

(ii)  To apply this knowledge to develop techniques for the routine genera-
      tion of antigen-specific CTH.

(iii) To use the hybridomas generated to investigate the CTL lytic-
      mechanism.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Chemicals

Standard chemicals of high purity were bought from Merck, Roth, Serva and Sigma. Chemicals for gel-electropheresis were mainly obtained from LKB.

#### 2.1.2 Experimental animals

C3H ($H$-$2^k$), C57BL/6 ($H$-$2^b$), DBA/2 ($H$-$2^d$) and (C57BL/6 x AKR) $F_1$ mice of both sexes were obtained from the specific-pathogen-free breeding facilities of the Max-Planck-Institute for Immunobiology, Freiburg. Mice were used between the ages of 6 and 24 weeks.

Spraque-Dawley rats were bought from Mus-Rattus.

### 2.2 Cell culture methods

#### 2.2.1 Culture media and solutions

(i) Phosphate buffered saline (PBS)

PBS was prepared as a 10mM solution of sodium phosphate, pH 7.2, containing 150mM NaCl, 2.5mM KCl, 0.5mM MgCl. $6H_2O$ and 0.1mM $CaCl_2$.

(ii) Inactivated foetal calf serum (FCS)

FCS (Roth) was inactivated by incubating at 57°C for 30 min.

(iii)    Complete RPMI medium

RPMI 1640 (Gibco) was supplemented with 10% $^V$/v heat in-activated FCS (Roth), 100 µg/ml kanamycin (Gibco), 10 µg/ml tylosine (Flow), 20 µM 2-mercaptoethanol (Roth), 2mM L-glutamine (Gibco) and 25mM HEPES buffer (N-2-Hydroxyethyl-piperazine-N- thanesulphonic acid, Gibco), pH 7.3 and used at 37°C in 5% $CO_2$ in humidified air. For some cell lines, this basic medium was supplemented with 10% $^V$/v ConA-SN.

(iv)    Complete Dulbecco's Modified Eagle's Medium (DMEM)

DMEM (Gibco) was supplemented with 10% $^V$/v heat inactivated FCS (Roth), 100 µg/ml kanamycin (Gibco), 10 µg/ml tylosine (Flow), 2mM L-glutamine (Gibco) and 25mM L-asparagine (Merck), pH 7.3, and used at 37°C in 7% $CO_2$ in humidified air. For some cell lines, this basic medium was supplemented with 10% $^V$/v ConA-SN.

## 2.2.2   In vitro cell culture

(i)    Tumour cells and hybridomas

Tumour cells and hybridomas were grown as bulk cultues in flasks (Nunclone). Cells were subcultured every 2-4 days as necessary. P815, RBL-5 and RDM4 were cultured in complete RPMI medium, clone 96 was cultured in complete RPMI medium supplemented with 10% $^V$/v ConA-SN, BW5147 and EL4 were cultured in complete DMEM and hybridomas were cultured in complete DMEM both with and without 10% $^V$/v ConA-SN as appropriate.

(ii)   BT 7.4.1

BT 7.4.1 was grown in 2 ml cultures (Nunclone, 24 well culture trays) in complete RPMI medium supplemented with 10% $^V$/v ConA-SN and $5 \times 10^6$ irradiated (2200 RAD), TNP modified, ♀ C57BL/6 spleen stimulator cells. Cells were subcultured weekly by diluting the cells 1:5 into fresh medium and adding fresh stimulator cells. The cells were not purified at each subculture.

(iii)   Anti H-Y MLC

Female C57BL/6 mice were immunised intraperitoneally with $2 \times 10^7$ ♂ spleen cells in 0.2ml of RPMI 1640 with supplements. Three to four weeks later, the mice were killed by cervical dislocation and a single cell suspension prepared from the spleen. One $\times 10^6$ of the primed responder cells were cultured with $3 \times 10^6$ stimulator cells (irradiated C57BL/6 ♂ spleen cells, 2200RAD) in complete RPMI medium supplemented with 10% $^V$/v ConA-SN in 2 ml cultures (Limbro, 24 well culture trays).

2.2.3   Storage of cells (freezing and thawing)

Samples of $5 \times 10^6$ cells were suspended in 1ml of RPMI 1640 or DMEM (as appropriate) supplemented with 20% $^V$/v heat inactivated FCS (Roth) and 10% $^V$/v Dimethyl-sulphoxide (Roth). Samples were put into small vials which were slowly cooled to -70°C (cooling rate < 1°C/min) in polystyrene boxes and the following day transferred to liquid nitrogen for permanent storage.

Cells were recovered by quickly thawing the samples, washing once in appropriate medium and then putting into flask culture; in the case of BT 7.4.1, stimulator cells were also supplied immediately after thawing.

## 2.3 Preparatory methods

### 2.3.1 Estimation of cell numbers

Cell numbers were estimated on a standard haemocytometer. Viable cells were distinguished by their exclusion of trypan blue dye (0.16% $^w$/v trypan-blue, Serva, 150nM NaCl).

### 2.3.2 Preparation of spleen cells

Mice and rats were killed by cervical dislocation and the spleens removed into RPMI medium supplemented with 2% $^v$/v heat inactivated FCS. Single cell suspensions were prepared by gently teasing the spleens through fine guage sieves.

Erythrocytes were removed by suspending the cells at approximately $5 \times 10^7$ cells per ml in ammonium chloride solution (15mM ammonium chloride, Merck, 16mM Tris/HCl, pH 7.5) for 1 min. at 20°C and subsequently washed three times. When necessary, the cells were irradiated with 2200 RAD to prevent their proliferation.

### 2.3.3 Preparation of ConA-SN

A single cell suspension of unirradiated spleen cells from Spraque-Dawley rats was cultured at $5 \times 10^6$ cells/ml in complete RPMI medium supplemented with 5 µg/ml ConA (Pharmacia) for 24 hours at 37°C, 5% $CO_2$ in humidified air. After incubation, cells were removed by centrifugation and the ConA inactivated by the addition of 20 mg/ml α methyl-D-mannoside (Roth). The supernatant was aliquoted and stored at -20°C.

2.3.4 **Preparation of ConA blasts**

A single cell suspension of unirradiated spleen cells was cultured at $2 \times 10^6$ cells/ml in complete RPMI medium supplemented with 5 μg/ml ConA (Pharmacia) for 2 or 3 days in flasks (Nunclone) at 37°C, 5% $CO_2$ in humidified air. Blasts were purified over a Ficoll-Paque$^{TM}$ bed prior to use. Yields of blast cells ranged from 25% to 70% of the input cell number.

2.3.5 **Purification of cells with Ficoll-Paque $^{TM}$**

(i)     Ficoll-Paque$^{TM}$ solution.

9.239 g Ficoll (Pharmacia) was mixed with 25 ml Urovison (Schering) and 119.4 ml distilled $H_2O$ for 60 min at 20°C. The density was corrected when necessary with distilled $H_2O$ to 1.077g/ml.

(ii)    Separation of cells

Blast cells were separated from normal or dead cells by purification over Ficoll-Paque$^{TM}$. Between $1 \times 10^6$ and maximally $1 \times 10^8$ cells were suspended in 5 ml of appropriate medium which was then layered over 4 ml of Ficoll solution in a 15 ml centrifugation tube (Falcon). Tubes were centrifuged (approx. 700g) for 15 min and the blast cells subsequently recovered from the interphase. Cells were washed three times before use.

2.3.6 **Modification of cells with trinitrophenol**

Cells were suspended at $3 \times 10^7$ cells/ml in labelling solution (3mM 2,4,6-trinitrobenzene sulphonic acid, Serva, in PBS, pH 7.4, pH corrected with 2M NaOH) for 30 minutes at 37°C. Cells were washed three times prior to use.

### 2.3.7 Trypsin treatment

Cells were suspended at $5 \times 10^6$/ml in PBS containing 10% $^V$/v heat inactivated FCS (Roth) and 1.8 mg/ml trypsin (Trypsin-TPCK, EC. 3.4.21.4, Worthington, 200 U/mg) and incubated for 30 min at 37°C. After digestion, cells were washed three times in appropriate medium and stored on ice prior to use.

### 2.3.8 Neuraminidase treatment

Cells were suspended at $1 \times 10^7$/ml in PBS containing 10% $^V$/v heat inactivated FCS (Roth) and 75 U/ml neuraminidase (Behringwerke AG, EC. 3.2.1.18) for 15 min at 37°C. After digestion, cells were washed three times in appropriate medium and used immediately.

### 2.3.9 Induction of 6-thioguanine resistance into cells

Cells were suspended at $1 \times 10^4$ cells per 200 µl culture in appropriate medium supplemented with 2 µg/ml 6 thioguanine (6TG, Sigma) in 96-well, round-bottomed microtitre plates (Nunclone). The cells were cultured under normal conditions and the concentration of 6TG was raised in steps to 20 µg/ml according to the rate of adaptation of the cells. Cells which grew readily at 20 µg/ml 6TG were tested for their inability to grow in HAT medium (Section 2.3.10) and subsequently cultured in the absence of 6TG.

### 2.3.10 Cell fusion procedure

The fusion procedure used was essentially as described by Galfre and colleagues, 1977. Appropriate numbers of each partner cell were mixed and washed 3 times in fusion medium (DMEM without supplements).

Mixed cells were centrifuged (approx. 200g) and the pellet slowly resuspended by adding 0.8 ml of polyethylene glycol 1500 (or 4000) (PEG, 50% $^W$/v in fusion medium, Serva) over a period of 30 seconds at 37°C. Cells were gently agitated for a further 30 seconds at 37°C before adding 20 ml of prewarmed fusion medium dropwise over 5 minutes. Fused cells were washed once with complete DMEM and distributed into 1 ml cultures (24 well culture trays, Costar) in complete DMEM either with or without 10% $^V$/v ConA-SN; see section 2.3.3. Forty-eight parallel control cultures were also prepared containing mock-fused cells ($1-5 \times 10^5$ per culture) in complete DMEM either with (24 cultures) or without (24 cultures) 10% $^V$/v ConA-SN. The following day, 1 ml of 2xHAT medium (1xHAT = 100µM hypoxanthine (H), Sigma; 0.4 µM aminopterin (A), Sigma; 16 µM thymidine (T), Sigma; 0.5 mM ouabain (Ou), Sigma, in complete DMEM $\pm$ 10% $^V$/v ConA-SN) was added to all cultures to select only for the growth of hybridoma cells.

On day 4, and when necessary day 8, after fusion, 1 ml of medium from each culture was replaced by 1 ml of fresh 1xHAT medium. After 10-12 days, the hybridomas were transferred to HT medium (100 µM H and 16 µM T in complete DMEM $\pm$ 10% $^V$/v ConA-SN) and cultured for at least a further two weeks in this medium. Hybridomas were finally cultured in complete DMEM both with and without 10% $^V$/v ConA-SN. If the hybridomas were cloned relatively soon after fusion, HT medium was normally used.

2.3.11 **Isolation of hybridoma clones**

Hybridoma clones were isolated by limiting dilution. Graded numbers of cells were seeded into several groups of 200 µl cultures with complete DMEM either with or without 10% $^V/v$ ConA-SN as appropriate and feeder cells (2-5x10$^5$ irradiated (C57BL/6 x AKR)F$_1$ spleen cells) in round or flat bottomed microtitre plates (Nunclone, Costar). Typically, each group consisted of 96 or 192 cultures with cell concentrations ranging from 0.3 to 10 hybridoma cells per culture. For freshly generated hybridomas, HT medium was frequently used (see section 2.3.10). Growing hybridomas appeared within 10 to 14 days and were picked from groups seeded with low cell numbers showing less than 10% of the cultures positive for growing cells. There is a greater than 95% probability that such cultures are derived from a single cell i.e. are clonal. Selected clones were subcultured in 2 ml cultures (24 well trays, Costar) for 5 to 10 days before finally transferring to flask culture.

2.3.12 **Extraction of total cellular ribonucleic acid**

Total cellular ribonucleic acid (RNA) was extracted according to Maniatis and colleagues, 1982. Essentially, cell pellets of 1.0-5.0x10$^8$ cells were suspended in 5 volumes of lysis buffer (4.0 M guanidine isothiocyanate, 0.5% $^W/v$ N-lauryl sarcosinate, 25 mM sodium citrate, pH 7.0, and 100 mM 2-mercaptoethanol, Roth). The lysate was layered onto a cushion of 5.7 M caesium chloride, 100 mM EDTA, and was centrifuged at 35000 rpm for 20 hours at 20°C in a Beckman SW 50-1 rotor. The RNA pellet was dissolved in H$_2$O and reprecipitated with ethanol. Reprecipitated RNA pellets were dissolved in H$_2$O at a concentration of 5 µg/ml (the amount of RNA was estimated according to the cell number assuming RNA content of 4 pg per cell).

## 2.4    Analytic Methods

### 2.4.1    Estimation of cellular growth frequencies

(i)    Experimental

Growth frequencies were estimated by limiting dilution analysis. Essentially, graded numbers of cells were seeded into several groups of cultures with not less than 24 cultures per group (200 $\mu$l cultures for less than $10^{-4}$ cells/culture, round bottom microtitre plates, Nunclone, and 2 ml cultures for larger cell numbers, 24 well trays, Costar). Occasionally, irradiated syngeneic spleen feeder cells were also included in each culture at a constant cell number. All cultures were examined microscopically every third day for growing cells and a cumulative record kept of the number of cultures containing growing cells per group.

(ii)    Data analysis

If the only limiting factor in the experiment is the number of growing cells seeded per culture then the data will conform to single-hit kinetics and the frequency of growing cells can be estimated by application of the Poisson distribution (see Lefkovits and Waldmann, 1979). The general form of the Poisson formula gives the probability, Fn, that a culture contains n growing cells:-

$$Fn = \frac{\mu^n e^{-\mu}}{n!} \qquad (1)$$

where $\mu$ is the average number of growing cells per culture. The

frequency of growing cells within a population is calculated from the zero order term:-

$$Fo = e^{-\mu} \qquad (2)$$

or $\quad -lnFo = \mu \qquad (3)$

Thus, there is a linear relationship between the logarithm of the fraction of cultures without growing cells (negative cultures) and the average number of growing cells seeded per culture. For each experiment the log of the fraction of negative cultures is plotted against the total number of cells seeded per culture. From equation 3 it can be calculated that the dose of cells which on average contains one growing cell ($\mu = 1$) is the x value determined from this plot corresponding to a y value of -1 (37% negative wells).

Straight lines were fitted to the data points by chi-squared minimisation (Taswell, 1981) and tested for significance, p, where p is the probability of obtaining the given result. Data is normally rejected below the 5% level of significance i.e. $p < 0.05$

### 2.4.2 Cytolytic assay

Cells were assayed for cytolytic activity in a 4 hour chromium-51 release assay at various effector to target ratios.

(i) Preparation of target cells

Five $\times 10^6$ target cells were suspended in 250 $\mu$l Na$^{51}$CrO$_4$ in PBS (1 mCi/ml, NEN) and 500 $\mu$l assay medium (RPMI, 10% $^v$/v heat inactivated FCS and 5 mM HEPES, pH 7.4) and incubated for 90 minutes at 37°C in air. Labelled target cells were washed 3 times prior to use. The specific labelling ranged from 0.5 to 1.5 cpm/cell depending on the cells used.

(ii)    Assay

Two $x10^3$ chromium-51 labelled target cells were mixed with appropriate numbers of effector cells in 200 µl of assay medium in round bottom microtitre plates (Nunclone). Plates were centrifuged at approx. 50g for 5 min prior to incubation at 37°C in 5% $CO_2$ in humidified air for 4 hours. After incubation, plates were centrifuged at approx. 200g for 10 min and subsequently 100 µl of supernatant removed for counting (Packard, gamma counter). Percent specific lysis was calculated according to the following formula:-

$$\frac{\% \text{ specific lysis}}{(\text{specific}^{51}\text{Cr release})} = \frac{\text{exp - LC}}{\text{HC - LC}} \times 100 \qquad (4)$$

where exp is the cpm obtained from the sample, HC is the high control (cpm obtained from cells incubated for 4 hours in 2M HCl) and LC is the low control (cpm obtained from cells incubated for 4 hours in assay medium). For normal titrations, each effector to target ratio was prepared in triplicate and the results averaged. For the screening of hybridoma cells generated in fusions, 100 µl samples were taken directly from cultures; these samples contained between $2x10^4$ and $2x10^5$ hybridoma cells representing effector to target ratios of 10:1 to 100:1.

(iii)   Lectin facilitated assay

For the lectin facilitated assays, either 2% $^V$/v PHA (Gibco), 10 µg/ml ConA (Pharmacia) or 30 µg/ml soybean agglutinin (SBA, Pharmacia) was added to the target cells prior to the addition of effector cells. The final concentration of lectins in the assay were PHA, 1% $^V$/v; ConA, 5 µg/ml; SBA 15 µg/ml.

(iv)    Inhibition by EDTA

EDTA (Sigma) was added to the assay at a concentration of 10mM.

(v)    Test system for the detection of toxins (section 3.6.3.2).

Various numbers of effector cells were prepared as four groups (as for a normal titration assay, i.e. triplicates) in 200 $\mu$l assay medium either with (groups 3 and 4) or without (groups 1 and 2) 1% $^V$/v PHA in round bottom microtitre plates (Nunclone). Plates were incubated at 37°C in 5% $CO_2$ in air for 4 hours. After incubation, plates were centrifuged at 200g for 10 minutes and then 100 $\mu$l samples of supernatants were removed into duplicate microtitre plates. Plates containing cells were washed 3 times with assay medium and thereafter, all plates (both with cells and with supernatants) were treated as for a normal cytolytic assay. Thus, $2 \times 10^3$ chromium-51 labelled target cells in 100 $\mu$l assay medium either with (groups 2 and 4) or without (groups 1 and 3) 2% $^V$/v PHA were added to appropriate plates containing either cells or supernatants and incubated 4 hours at 37°C.

## 2.4.3 Flow cytometry

(i)    Cell staining

Cells were washed 3 times in PBS and samples of $5 \times 10^5$ cells suspended in 125 $\mu$l analysis medium (RPMI, 2% $^V$/v FCS and 0.02% $^W$/v $NaN_3$) containing first-antibody (see below) at saturating dilutions in microtitre plates (Nunclone). Samples were incubated for 30 min on ice and then washed 3 times in analysis medium. Cells were subsequently suspended in 100 $\mu$l of approp-

riately diluted FITC coupled anti-rat-immunoglobulin or anti-mouse-immunoglobulin second-antibody in analysis medium for 30 min on ice. Finally, cells were washed 3 times in analysis medium and then suspended in 1ml PBS supplemented with 2% $^V$/v FCS and 0.02% $^W$/v NaN$_3$. Controls were prepared as described above except that cells were not stained with the first-antibody.

(ii)    Analysis

Approximately $2x10^4$ cells were analysed at $2x10^3$ cells per second on a cytofluorograph (50H, computer system 2150, Ortho Diagnostic Systems) with an argon laser (Lexel) set at 250 mW and 488 nm.

Light scatter gain and fluorescence gain were set at 1.2 and 6.5 respectively. Dead, or very small cells were detected by light scatter and gated out of the analysis. Control cell samples, stained only with FITC labelled second-antibody, were analysed first to determine regions of positive and negative staining (represented as bars on flow cytometric diagrams)

(iii)   Antibodies

First-antibodies were a) mouse-anti-Thy1.1 (NEN) used at a dilution of 1:400, b) mouse-anti-Thy1.2 (Olac, code 5B5) used at 1:400, c) mouse-anti-Lyt2.1 (NEN) used at 1:200, d) mouse-anti-Lyt2.2 (from Dr. U. Hämmerling, Sloan-Kettering-Institute, NY, USA) used at 1:10 e) rat-anti-Lyt-2 (56-6.7, Ledbetter and Herzenberg, 1978) used at 1:5 and f) mouse-anti-H-2 class I (mainly H-2$^b$) (9C5, obtained by fusion of spleen cells of an AKR mouse repeatedly immunised with clone 96 cells with

X63.Ag8.653 cells, K. Eichmann, personal communication) used undiluted.

Second antibodies were FITC coupled goat-anti-mouse Immunoglobulin (F(ab)$_2$, Medac) and goat-anti-rat-immunoglobulin (F (ab)$_2$, Medac).

## 2.4.4 Two-dimensional, reduced non-reduced, sodium dodecyl-sulphate poly acrylamide gel electrophoresis (SDS-PAGE)

(i) Radiolabelling of cells

Cell surface proteins were labelled by lactoperoxidase-catalysed iodination using a modification of the method described by Hubbard and Cohn (1975). Five $x10^7$ washed cells were suspended in 1 ml of PBS with 250 μl of Enzymobead$^{TM}$ reagent (Preswollen for 1 hr in H$_2$O, Bio-Rad) and 1.5 mCi $^{125}$I$^-$ (Na$^{125}$I, Amersham). The iodination was carried out at room temperature by adding, eight times, 50 μl of a 1% $^W$/v aqueous solution of ß-D-glucose (Sigma) at two minute intervals. After the last addition of ß-D-glucose the reaction was allowed to proceed for an additional 30 min and then stopped by adding a large volume (ca. 40 ml) of ice cold PBS containing 5mM KI and 0.02% ($^W$/v) NaN$_3$. Labelled cells were washed a further five times with PBS containing 10mM iodoacetamide (Merck) and 0.02% $^W$/v NaN$_3$.

(ii) Lysis of cells

Radiolabelled cells were lysed by suspending in lysis buffer (50mM Tris/HCl, pH 8.0, 150mM NaCl, 1% $^V$/v Nonidet P-40 (LKB), 1mM EDTA, 2mM phenylmethylsulphonyl fluoride (Serva), 10mM iodoacetamide (Merck and 0.02% $^W$/v NaN$_3$) for 45 min on

ice. Cell lysates were centrifuged for 15 min in a airfuge (Beckmann) at approx. $1 \times 10^4$g. Supernatants were stored at - 70°C.

(iii)     Two dimensional gel electrophoresis (SDS-PAGE)

Two dimensional (non-reduced/reduced) SDS-PAGE employed the protocol described by Goding and Harris, 1981. The first dimension consisted of a tube gel (0.25x15 cm) with 7.5% acrylamide SDS. Tubes were siliconised to facilitate gel removal. The sample volume was 50µl and contained 10µl of labelled cells (corresponding to $1.5 \times 10^6$ cells) in sample buffer (250mM Tris/HCl pH 6.8, 10% $^V$/v glycerol, 3% $^W$/v SDS and 0.01%$^W$/v bromophenol blue). Gels were run in electrophoresis buffer (250mM Tris/HCl pH 8.7, 200mM glycine, 0.1% $^W$/v SDS at 1mA per tube constant current (60-250 V) for 3 hr. When the bromopheral blue marker reached the bottom, the gel was removed and equilibrated for 1 hr at 25°C in sample buffer with 5% $^W$/v 2-mercaptoethanol and 50mM dithiothreitol. The second dimension was performed using a 10% acrylamide SDS slab gel (15.0x14.5x0.1 cm). Gels were run in electrophoresis buffer with additional sodium thioglycolate, 5mM, in the upper resevoir at 40mA constant current (60-250 V) for 5 hours. Afterwards, the gels were stained for 1 hr with 0.25% $^W$/v coomassie brilliant blue, 40% $^V$/v methanol and 8% $^V$/v acetic acid in $H_2O$. Destaining was carried out overnight in 10% $^V$/v acetic acid and 30% $^V$/v methanol in water. The gels were dried under vacuum at 80°C for approximately 1 hr. Autoradiographs were carried out for 1 to 7 days as appropriate at -80°C on Kodac XAR-5 films

using an intensifier screen and the films were developed and fixed according to the manufacturers recommendations.

Molecular weights were determined by reference to the mobility of low molecular weight standard proteins applied to the same gel (Pharmacia)

2.4.5 Northern hybridisation analysis

(i)     Electrophoresis of RNA

Total cellular RNA (10 μg) was denatured with formaldehyde and electrophoresed in 1.4% agarose gels containing formaldehyde (Thomas, 1980). After ethidium bromide staining and photography under UV, RNA was blotted onto Genescreen membranes (NEN) for hybridisation.

(ii)    Hybridisation

Oligonucleotide probes were radiolabeled using $\gamma$ $^{32}$P-ATP and T4 polynucleotide kinase as described by Miyada et al. (1985). Unicorporated $\gamma^{32}$P-ATP was separated from the oligonucleotide by either chromatography on the anion exchange resin DE52 or by polyacrylamide gel electrophoresis in the presence of 7 M urea. Hybridisation of Northern blots included a 1h prehybridisation step in a solution containing 5xSSPE (20xSSPE: 174g NaCl, 27.6g $NaH_2PO_4H_2O$, 7.4g EDTA per litre), 10x Denhardt's solution (50x Denhardt's: 10g/l Ficoll, 10g/l polyvinylpyrrolidone, 10g/l BSA in $H_2O$) 0.1% SDS and 10μg/ml denatured E.coli DNA. For the 3 hour hybridisation step this solution was replaced by a hybridisation solution composed as before except that 5x Denhardt's solution was used and the synthetic oligonucleotide probe was added to a final concentration of $1 \times 10^6$ cpm/ml.

Prehybridisation and hybridisation were done 5°C below the theoretical dissociation temperature calculated according to Miyada et al. (1985). Hybridisation was carried out at 60°C for 3 hours. Blots were washed after hybridisation in 6xSSC (20xSSC: 175.3g NaCl, 88.2g sodium citrate per litre) at 21°C three times for 15 minutes followed by a stringent wash at 5°C below the theoretical dissociation temperature for one minute in 6xSSC. Blots were stripped prior to the next hybridisation by 2-3 washes at 60°C in 0.1xSSC or 5mM EDTA for 15 minutes. Blots were exposed for one day using an intensifying screen.

(iii)   Probes

Oligonucleotide probes specific for the variable region of the AK TCR ß chain ($V_{\beta AK}$) and the constant regions of the $\beta_1$ ($C_{\beta 1}$) and $\beta_2$ ($C_{\beta 2}$) isotypes of the TCR were hybridised with RNA blots at 57°C, 67°C and 67°C respectively (Epplen et al. in press).


$V_{\beta AK}$  5' GGCATCCCTGAATCATCTAT

$C_{\beta 1}$   5' TACAGGGTGGCTTTCCCTAGCAG

$C_{\beta 2}$   5' TATAGGGTGCCTTCCCCAGTAG

## 3.    RESULTS

### 3.1    Cell lines used in these studies

#### 3.1.1    BW5147

BW5147 is a thymoma derived from AKR (H-$2^k$) mice which expresses Thy1.1 and H-$2^k$ but not Lyt-2 or T4. The variant used in these studies is resistant to ouabain and deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT, EC. 2.4.2.8) which makes it sensitive to HAT selection medium. Since the CTL used in these fusions were ouabain sensitive, it was possible to positively select against both parental cells by culturing them in the presence of HAT and ouabain directly after fusion. Hybridoma cells inherit both HAT and ouabain resistance from the parental cells and therefore grow readily in this selective medium.

BW5147 is not cytolytic.

#### 3.1.2    Secondary mixed lymphocyte culture : anti H-Y

CTL active against the male antigen H-Y were generated in a classical MLC (Gordon et al., 1975; Simpson, 1982). Female C57BL/6 mice were primed interperitoneally with male C57BL/6 spleen cells. Three weeks later, spleen cells from these primed animals were put into culture with irradiated male C57BL/6 spleen stimulator cells in the presence of ConA-SN. These cultures developed a strong lytic-activity against male ConA blasts which peaked on day 5 (figure 1). A strong lytic-activity also developed against P815 mastocytoma cells (H-$2^d$) probably due to the well documented cross-reactivity between H-$2^d$ and H-Y (von Boehmer et al., 1979). No activity was detected

against control syngeneic female ConA blasts. The total lytic-activity of these cultures was revealed by determining the lysis of the sensitive target P815 in the presence of PHA which is known to circumvent the requirement for specific-antigen recognition; this peaked on day 5.

### 3.1.3 BT 7.4.1

BT 7.4.1 is a murine cytolytic T cell clone which specifically lyses cellular targets bearing the hapten TNP in the context of $H-2K^b$. This clone was derived by H.U. Weltzien (Max-Planck-Institut für Immunbiologie, Freiburg) from an in vitro primary response of spleen cells to TNP-modified syngeneic cells according to the method of Shearer, 1974. Female C57BL/6 spleen cells were initially put into culture with irradiated antigen (TNP-modified syngeneic female spleen cells) in the presence of ConA-SN (2 ml cultures with $1 \times 10^6$ responder cells and $3 \times 10^6$ TNP-modified syngeneic stimulator cells). There-after, lines were developed which possessed a strong lytic activity against TNP-modified syngeneic blast cells or tumour cells. These lines require weekly restimulation with antigen and the continuous presence of ConA-SN for the maintenance of their antigen-specificity. BT 7.4.1 is a clone derived from one of these lines by limiting-dilution; the final cloning was performed at 0.3 cells/wells.

At the time of these studies BT-7.4.1 had been in continuous tissue-culture for over 2 years without any loss of activity or specificity. Like the parental line it is dependent upon antigen and ConA-SN for the maintenance of antigen-specificity and proliferation. It expresses both Thy1.2 and Lyt2.2 demonstra-ting that it is of cytolytic T cell origin and the alleles correspond to those of C57BL/6 mice. The cells are irregular in shape and adhere very strongly to the

plastic surfaces of normal tissue culture plates. Figure 2 shows the lytic-activity of BT 7.4.1 against a variety of target cells and demonstrates the H-2 restricted nature of this activity against TNP-modified cells. Lytic-activity is found only against RBL-5 tumour cells and C57BL/6 ConA blasts (both H-2$^b$) which have been modified with TNP whereas there is no activity against TNP-modified blasts (or tumours) of other haplotypes or against unmodified cells.

BT 7.4.1 is HAT resistant and Ou sensitive.


## 3.1.4 Clone 96


Clone 96 is a murine cytolytic T cell line of uncertain origin which was obtained from P. Krammer (Deutsches Krebs Forschung Zentrum, Heidelberg, Krammer et al., 1983). The cells express Thy1.2, H-2$^b$ and Lyt2.2 alloantigens indicating that they may have originated from C57BL/6 mice. The original cytolytic specificity and H-2 restriction of clone 96 is unknown and in this work the cells are assumed to be representatives of aged killer (AK) cells based on the grounds that:-

(i) they specifically lyse the DBA/2 mastocytoma P815 (Simon et al., 1984).

(ii) they did not show lytic activity against P815 three years ago, 1982 (Krammer et al., 1983).

(iii) their activity against P815 is inhibited by monoclonal antibodies against Lyt-2 (Simon et al., 1984).

(iv) they express messenger RNA (mRNA) for the $V_{\beta AK}$ region of the TCR (see section 3.7.3).

The AK activity of clone 96 is stable in that it does not require continuous antigen restimulation either for proliferation or maintenance of specificity. The line requires IL-2 (in the form of ConA-SN) for proliferation although mutants which grow independently of IL-2 arise at a frequency of $2.5 \times 10^{-3}$. Figure 3 shows the lytic activity of clone 96 against P815 both in the presence and absence of PHA. The lytic activity of clone 96 against other tumour targets will be discussed in section 3.6.2.

These cells are HAT resistant and Ou sensitive.

## 3.2 An investigation into the optimal conditions for cytolytic T cell fusions

In order to study the effects of fusing cytolytic T cells with BW5147 it was first necessary to attempt to optimise the fusion conditions. Several experiments are presented here, the results of which determined the practical aspects of all the major fusions performed later.

The investigation required a simple cellular system which could be readily manipulated and clone 96 was ideal for this purpose due to its tumour like growth. A pilot fusion of clone 96 and BW5147 indicated that the fusion frequency lay in the region of $2.5 \times 10^{-5}$ (data not shown). Further fusions were then performed in which the effects of the molecular weight (MW) of PEG used, the number of cells fused, pretreatment of cells with trypsin and addition of ConA-SN and feeder cells were studied. In all of these experiments a measure was made of the fusion efficiency under different condition by using limiting-dilution analysis. It is important to note that the frequency measurement is based on the number of surviving hybridomas and is, of course, lower than the actual fusion frequency.

The hybridomas generated in these fusions were routinely assayed for lytic-activity against P815 mastocytoma cells. Lytic-activity was found in all experiments irrespective of the conditions tested; this activity is studied in more detail in sections 3.5 and 3.6.

### 3.2.1 Frequency of BW5147 revertants

For later discussion it was necessary to determine whether the frequency of reversion of BW5147 from a HAT sensitve (6TG resistant) to a HAT-insenstive variant, under fusion conditions, is low enough to affect the experimental results. It may be possible that a homologous fusion between two or more BW5147 cells could result in an additive effect whereby normally low levels of the enzyme HGPRT are raised above a threshold at which the hybrids may survive in HAT medium. Therefore, $3 \times 10^8$ BW5147 cells were treated with PEG 1500 and distributed into 2 ml cultures with $1 \times 10^6$ cells per culture in medium containing HAT. The cultures were examined visually for growing cells every week over a period of two months. No growing cells were found in these cultures (data not shown) indicating that the revertant frequency under fusion conditions is probably less than $3 \times 10^{-9}$. It should be noted that all fusions were also internally controlled by mixing both fusion partners in the absence of PEG in order to test for the presence of revertants or the failure of the selection medium. No growing cells were ever found in these control cultures.

### 3.2.2 PEG 1500 versus PEG 4000

It is thought that the molecular weight (MW) of PEG has an effect on its efficiency as a cell fusing agent depending on the type of cell being fused. PEG 4000 (MW = 4.0 kd) is normally used for B cells and occasionally for T cells although PEG of lower molecular weight (PEG 1500, 1.5 kd; PEG 1000, 1.0 kd) is often preferred for T cell fusions. In cytolytic cell fusions a variety of molecular weights of PEG have been successfully used i.e. PEG 1000,

Nabholz et al., 1980; Berebbi et al., 1983; PEG 1500, Kaufmann et al., 1981a and PEG 4000, Haas and Kiselow 1985a. However, the suitability of using either low or high molecular weight PEG has not been ascertained for these types of fusion. Therefore, the relative fusion efficiencies of PEG 1500 and PEG 4000 were compared in the clone 96 x BW5147 test system. Twentyfour $x10^6$ BW5147 and $24x10^6$ clone 96 cells were incubated with 0.5 ml of either PEG 1500 or PEG 4000. The cells were then distributed into 2 ml cultures under limiting dilution conditions at 24 wells per group and ranging from $3.8x10^3$ to $4.8x10^5$ of each parental cell type per well. The number of wells supporting growing hybrids was assessed once a week by microscopic examination. Fig 4 shows the semi-logarithmic plot of the cumulative data, measured after four weeks, and the results of a chi-squared test. It is clear that the points appear to form curved lines indicating positive co-operativity, i.e. multi-hit kinetics and consequently it is not possible to accurately estimate the fusion frequencies in these fusions. However, the general shapes of the curves are very similar suggesting that, at least for this particular system, the choice of PEG 1500 or PEG 4000 makes very little difference to the fusion frequency.

## 3.2.3  Cell concentration during fusion

Different laboratories which use PEG as a cell fusing agent employ a variety of different parental cell ratios and concentrations. In this particular study it seemed inappropriate to examine the effect of different parental cell input ratios on the fusion frequency because the test system involves fusing two actively dividing cell types in which the frequency of dividing cells (which are thought to fuse preferentially), is approximately equivalent suggesting an

optimal parental cell input ratio of 1:1. However, the concentration of cells which are subjected to the fusion procedure may affect the fusion fequency. To test this hypothesis an experiment was performed in which the ratio of clone 96 cells to BW5147 cells was maintained at 1:1 but various total numbers of cells, ranging from $1 \times 10^6$ to $1 \times 10^8$, were fused in a constant volume of 0.8 ml PEG. In the case of $1 \times 10^6$ cells, two fusions were performed and then pooled to increase the number of cells put into the limiting dilution assay. Directly after fusion, the cells were distributed under limiting dilution conditions into 2 ml cultures with between $3.1 \times 10^3$ and $1.0 \times 10^6$ of each parental cell type per culture as appropriate so that the relative fusion frequencies could be estimated. Growing hybrids were recorded by microscopic examination once every week. Figure 5 shows the semi-logarithmic plots of the cumulative data as determined four weeks after fusion. In all cases the points form positively co-operative curves indicating multi-hit kinetics. When straight lines are fitted to the data, the p values obtained from a chi-squared analysis are found to be unacceptably low. However, certain trends can be seen in this data. Fusion with low cell numbers appears to be inefficient as evinced by the fusions involving $1 \times 10^6$ (data not shown) and $3 \times 10^6$ cells in which no hybridomas developed. In contrast, fusions with relatively higher cell numbers, namely $3 \times 10^7$ and $1 \times 10^8$, generated hybridomas at frequencies of the order of $1 \times 10^{-4}$ to $1 \times 10^{-5}$ although reliable frequency estimates cannot be obtained.

On the basis of these results it was decided to use only higher cell numbers ($> 3 \times 10^7$) in future fusions.

## 3.2.4  Trypsin treatment

In early reports where cells were taken from MLR cultures and fused with tumour cells it was found that, although many hybridomas were generated, no cytolytic-activity could be detected (Ruddle, 1978; DiPauli and DiPauli, 1978; Simpson et al., 1978; Köhler et al., 1977 and Grützmann et al., 1978). Köhler and colleagues suggested that this may be due to lysis of the tumour partner by the CTL induced by the non-specific "glueing effect" of PEG. To circumvent this problem, Kaufmann and colleagues, 1981 a,b, transiently deactivated the lytic-mechanism of cytolytic cells before fusion by treating the cells with trypsin. This treatment is known to temporarily remove proteins from the cell surface which are required in the lytic process (Todd, 1975). Hybridomas were generated in these experiments both with lytic capacity and with some degree of antigen-specificity. In contrast, Nabholz and colleagues, 1980, have generated cytolytically active and antigen-specific hybridomas without inactivating their CTL prior to fusion.

The effects of trypsin treatment were tested on the model fusion of clone 96 and BW5147. Preliminary experiments were performed to demonstrate the effect of trypsin on both the growth and lytic-activity of clone 96. In figure 6 the growth of trypsinised and non-trypsinised clone 96 is compared over five days. Quite clearly, the growth remained unaffected by this treatment. Figure 7 shows the effect of trypsin on the lytic-activity of clone 96 over a period of two days. Immediately after treatment, the specific activity of clone 96 towards P815 was reduced to 15% of its original level. However, when the same cells were put into culture and reassayed on day one or day two after trypsin treatment, the lytic-activity fully recovered.

To examine the effects of trypsin inactivated clone 96 on the fusion frequency, a limiting-dilution experiment was performed. Essentially, $1 \times 10^8$ clone 96 cells were either left untreated or pretreated with trypsin and then fused with $1 \times 10^8$ BW5147. The products were distributed under limiting-dilution conditions into 2 ml cultures with 24 cultures per group and between $2 \times 10^3$ and $2 \times 10^6$ of each parental cell type per culture. Wells supporting growing hybrids were recorded microscopically once a week. The cumulative results of this experiment after four weeks are shown as a semi-logarithmic plot in figure 8. As previously observed, the results indicate that multi-hit kinetics operate such that the fusion frequency cannot be accurately determined. When a straight line is fitted to the data and a chi-squared analysis performed, the p value is found to be unacceptably low (p = 0.081). The result do suggest, however, that the fusion frequency is not drastically altered by pretreating the CTL with trypsin prior to the fusion.

In addition to affecting the fusion frequency, Kaufmann and colleagues, 1981 a,b, claim that trypsin treatment somehow promotes the generation of hybridomas which are antigen-specific and cytolytic. To test this hypothesis, the hybridomas produced in this experiment were tested for specific lytic activity against P815 cells. Hybridomas were found in both fusions with lytic activity against P815 regardless of whether or not clone 96 had been treated with trypsin prior to the fusion (data not shown). It can be argued, however, that the test system employed here, involving AK cells, is somewhat unusual. Whereas this system is adequate for addressing questions such as fusion frequency, it is probably inappropriate for questions of antigen-specificity and cytolytic activity because it is unclear whether the AK lytic-mechanism is identical to that of classical CTL. In fusions with more "normal" CTL, such as those generated in a secondary MLC, antigen-specific cytolytic-activity is not

readily observed in the hybridomas generated. Such a system would be far more appropriate to examine the question of whether trypsin treament promotes the generation of CTH which can specifically and functionally recognise relevant antigen. Thus, a secondary MLC was initiated between irradiated male stimulator cells and primed female responder cells of C57BL/6 mice as described in section 3.1.2. Cells were harvested on day four and enriched over Ficoll-Hypaque. This population is highly enriched for CTL which recognise and lyse male C57BL/6 ConA blasts. Two fusions were performed in which the MLC cells were either left untreated or were treated with trypsin prior to fusion with BW5147. In neither fusion were hybridomas found which specifically lysed male C57BL/6 ConA blasts, indicating that, at least in this case, trypsin does not promote the generation of antigen specific CTH. For further fusions it was considered unnecessary to use trypsinated CTL.

## 3.2.5 Syngeneic feeder cells

One possible explanation for the positively co-operative curves obtained in the previous limiting-dilution experiments is that newly-created hybridomas at low density may require the presence of other cells for stabilisation. Silva and colleagues, 1983, found large differences in CTH growth frequencies depending on whether feeder cells were used or not although they did not perform full limiting-dilution analyses. Thus, the effect of feeder cells was tested by examining the growth frequency of freshly prepared clone 96 x BW5147 hybridomas in the absence or presence of various numbers of irradiated syngeneic spleen cells. Twelve $\times 10^7$ BW5147 cells were fused with $12 \times 10^7$ clone 96 cells in a single fusion and the resulting cell populations were divided

into three groups. Each group was distributed into 2 ml cultures under limiting

dilution conditions at 24 wells per group and ranging from $2.2 \times 10^3$ to $8 \times 10^5$ of

each parental cell type per culture. One $\times 10^6$ or $5 \times 10^6$ irradiated syngeneic

(AKR x C57BL/6) $F_1$ spleen feeder cells were added to each well of the first

two groups and the third group was left as a control without feeder cells. The

number of wells with growing hybridomas was assessed once a week by

microscopic examination. The cumulative results, after four weeks in culture,

are shown as semi-logarithmic plots in fig. 9. Straight lines are fitted to these

curves and tested by the chi-squared test. The p values of these fusion

frequencies appear to be highly acceptable but it should be noted that they are

calculated on only three points as the 100% positive values cannot be included

in the test. If one takes a "best case" example of one well out of 24 wells being

negative for these points, then the p value drops below 0.01 demonstrating

that the curves do not follow single-hit kinetics. Therefore, it is not possible

to obtain truely limiting conditions with single-hit kinetics by the addition of

feeder cells. Moreover, the curves also suggest that the presence or absence of

feeder cells does not drastically affect the growth frequency of hybridoma

cells.

### 3.2.6 ConA-SN

A second possible explanation for the positively co-operative limiting-dilu-

tion curves seen in these fusion experiments is that the hybridomas generated

secrete a soluble factor which they require for their own growth or stabilisa-

tion. This possibility can be tested by examining the effects of adding a crude

mixture of lymphokines (ConA-SN) to the cultures directly after fusion.

Certainly several laboratories have noticed that the various factors which are

present in ConA-SN can effect the growth frequencies of freshly prepared CTH (Nabholz et al., 1980; Berebbi et al., 1983; Silva et al., 1983). For example, Berebbi and colleagues, 1983, found that, in their system, no hybridomas developed in the absence of ConA-SN suggesting a total dependence on exogenous factors. Therefore, the effect of ConA-SN on the development of hybrids between clone 96 and BW5147 was examined.

Four $x10^7$ BW5147 cells were fused with $4x10^7$ clone 96 cells and distributed into 2 ml cultures under limiting dilution conditions with 24 cultures per group and ranging from $3.2x10^3$ to $4x10^5$ of each parental cell type per culture both in the presence and absence of ConA-SN (10%). Hybridoma growth was assessed at weekly intervals by microscopic examination and fig. 10A shows a semi-logarithmic plot of the cumulative data after four weeks. A straight line is clearly obtained in the presence of ConA-SN and this is shown to be statistically significant by a chi-squared test ($0.1 < p < 0.25$). However, as usual, in the absence of ConA-SN, multi-hit kinetics operate resulting in a curved line which is not significant by chi-squared analysis ($p < 0.01$). The approximate fusion frequencies were $7.6x10^{-6}$ ($p < 0.01$) without ConA-SN and $3.4x10^{-4}$ ($0.1 < p < 0.25$) with ConA-SN showing that the addition of exogenous factors strongly enhances the frequency of growing hybridomas.

The experiment was repeated in the presence of syngeneic (AKR x C57Bl/6) $F_1$ feeder cells ($5x10^5$ per culture). The semi-logarithmic plot of the data from this experiment is shown in fig. 10B. The results are almost identical to those obtained without feeder cells in that ConA-SN both induces single-hit kinetics and increases the frequency of growing hybridomas. The approximate growth frequencies in this experiment were $2.3x10^{-5}$ ($p < 0.01$) in the absence of ConA-SN and $2.3x10^{-4}$ ($0.5 < p < 0.75$) in the presence of ConA-SN.

A summary of the hybridoma growth frequencies of several BW5147 x clone 96 fusions both in the presence and absence of ConA-SN is shown in table 1. There appears to be a two to ten fold increase in growth frequency when ConA is supplied immediately after fusion. All further fusions were performed both in the presence and absence of ConA-SN.

### 3.3 Fusion between BW5147 and cell from a secondary MLC raised against male antgen (H-Y)

In this fusion, cells were taken from day four of an in vitro secondary MLC between C57BL/6 female responder cells and irradiated C57BL/6 male stimulator cells. Day four was chosen because at this time the cultures are still actively growing and display strong cytolytic activity against the expected antigen, C57BL/6 male ConA blasts, and P815 mastocytoma cells (H-2$^d$) (section 3.1.2). These activities are stronger on day five of the MLC but at this time the cells are not proliferating as quickly and therefore perhaps not optimal for fusion.

Twelve x10$^7$ MLC cells (enriched over Ficoll-Hypaque) were fused with 1.2x10$^8$ BW5147 and distributed into 2 ml cultures with between 1.3x10$^5$ and 1.4x10$^6$ of each parental cell type per culture and 24 cultures per cell concentration. Parallel cultures were set up in the presence and absence of 10% ConA-SN. Wells with growing hybridomas were recorded every third day by microscopic examination and assays for cytolytic activity were done twice weekly using C57BL/6 ConA blasts (male) and P815 cells as targets both with and without the lectin PHA in the assay. PHA was used in these assays to identify hybridomas which had other specificities or which had lost all specificity whilst retaining cytolytic activity.

Figure 11 shows the number of growing cultures per group (o) and the cytolytic activity of each culture for P815 in the presence of PHA determined on day 13 after fusion. Each dot represents the cytolytic activity of one culture. The hybridomas clearly possess a strong cytolytic activity when measured against the highly sensitive target P815 in the presence of PHA. However, this activity appears to be restricted to P815/PHA because the hybridomas did not induce lysis of P815 alone, H-Y ConA blasts or H-Y ConA blasts in the presence of PHA (data not shown). Thus it appears that the hybridomas have not retained their antigen specificity whereas they have maintained their lytic capacity. It is unusual that there is no lysis of B6 male ConA blasts in the presence of PHA since the non-specific lytic activity of the cells should be revealed on any target cell coated with an appropriate lectin. This result may be due to the relative insensitivity of the H-Y blasts to lysis (as compared to P815) although in controls these blast cells were shown to be readily lysed by the TNP specific CTL clone, BT 7.4.1, in the presence of PHA.

The effect of ConA-SN on the hybridomas appears to be an overall increase in the degree of non-specific lytic activity (measured against P815/PHA) as compared to those hybridomas grown in the absence of ConA-SN (figure 11 panels A and B). This was a consistent finding in similar assays performed on later days (data not shown). It remains unclear whether this is due to a higher proportion of lytic (to non lytic) cells or to the hybridomas reaching a higher maximal density with ConA-SN.

Frequencies of growing hybridomas in this fusion were $1.8 \times 10^{-6}$ (p = 0.13) without and $5.6 \times 10^{-6}$ (p = 0.56) with ConA-SN showing that its presence triples the number of surviving hybridomas. These data do not show multi-hit kinetics as has been found in the BW5147 x clone 96 series of fusions although the addition of ConA-SN seems to result in more significant limiting dilution

curves (higher p values). Thus, as in clone 96 x BW5147 fusions, ConA-SN both increases the frequency of growing hybridomas and provides better conditions for the growth of these hybridomas.

All of the hybridomas generated in this fusion were non-adherent and irregular in shape.

### 3.4   Fusion beteeen BW5147 and a long term CTL clone, BT 7.4.1

One possible explanation for the failure to generate antigen-specific CTH between BW5147 and heterologous T cells recovered from a secondary MLC is that the latter contain many antigen non-specific activated T cells which could, for unknown reasons, fuse preferentially to BW5147. The use of a well-characterised long term CTL clone such as BT 7.4.1 (section 3.1.3) should circumvent this problem since all the cells are functionally identical. Therefore, $4\times10^7$ BT 7.4.1 CTL (enriched over Ficoll-Hypaque) were fused with $4\times10^7$ BW5147 cells and distributed into 2 ml cultures with $2\times10^5$ of each parental cell type per culture both in the presence and absence of 10% ConA-SN.

After 16 days growing cells were observed in 77 out of 96 cultures supplemented with ConA-SN and in 58 out of 96 cultures without ConA-SN. These figures correspond to growth frequencies of approximately $1.3\times10^{-5}$ and $8\times10^{-6}$ respectively. This result is typical of a series of fusions between BT 7.4.1 and BW5147.

Figure 12 shows a histogram of the non-specific cytolytic-activity of these cultures against P815 in the presence of PHA determined on days 12 (panels A and C) and 16 (panels B and D) after fusion. The upper panels, A and B, show the activity of hybridomas grown in the absence of ConA-SN and the

lower panels, C and D, in the presence of ConA-SN. As described for the fusion between MLR cells and BW5147 (section 3.3), the presence of ConA-SN in the medium appears to enhance both the growth frequency and the lytic potential of the hybridomas.

The hybridomas showed no lytic activity against P815 alone or against the expected antigen, namely TNP modified RBL-5 cells, in either the absence or presence of PHA. As a control, BT 7.4.1 was shown to lyse TNP modified RBL-5 cells both with and without PHA (data not shown). This effect is similar to that described for MLR x BW5147 cytolytic hybridomas (section 3.3) which did not lyse C57BL/6 male ConA blasts despite the addition of PHA to the assay.

Since the parental cells, BT 7.4.1, are dependent on the continuous presence of antigen for the maintenance of antigen specificity it was considered possible that the hybridomas may have lost their antigen specificity due to the lack of antigen in the cultures. Subsequent culture of these hybridoma populations in the presence of $5 \times 10^5$ irradiated, TNP-modified, C57BL/6 spleen cells did not result in the recovery of antigen specific lytic activity. Similarly, in a separate fusion between BT 7.4.1 and BW5147 where $5 \times 10^5$ TNP modified C57BL/6 irradiated spleen cells were added to the cultures immediately after fusion, no antigen specific lytic activity was observed (data not shown).

All of the hybridomas generated in these fusions were irregular in shape and resembled the parental BT 7.4.1. However, unlike the parental cells, they were not adherent. The "thymoma like" type of hybridoma described by Nabholz, 1980, in simular fusions, were not found.

Several lytic and non lytic hybridoma clones were isolated from this fusion and are characterised in sections 3.6 and 3.7.

## 3.5    Fusion between BW5147 and an aged killer cell, clone 96

The maintenance of an antigen-specific cytolytic T cell line such as BT 7.4.1 requires the continuous presence of antigen. Withdrawal of antigen from such lines results in the loss of the original antigen specificity. It is possible that the acquisition of autonomous growth in T cell hybridomas has a similar effect as the withdrawal of antigen i.e. loss of selection for antigen recognition.

To test this hypothesis several fusions were performed between BW5147 and the AK T cell, clone 96, which has stable cytolytic activity for P815 target cells but which grows independent of antigen in the presence of ConA-SN in a highly stable manner (section 3.1.4).

Thus, clone 96 and BW5147 $(4 - 8x10^7)$ were fused and cultured under limiting dilution conditions in 2 ml cultures both in the presence and absence of 10% ConA-SN. Wells with growing hybridomas were recorded twice weekly and assays for cytolytic activity were performed every day against P815 both with and without PHA.

Figure 13 shows the results from two independent but representative experiments. Panel A shows the lytic activity of hybridomas on day 14 after fusion measured against P815 alone. Each dot represents the lytic activity of each culture, and the left and right sections compare hybridomas grown in the absence and presence of ConA-SN. The number of wells (from 24) containing growing cells was determined after three weeks and is indicated on the same figure (o). As can been seen, the hybridomas expressed a relatively strong antigen-specific lytic-activity against P815. This activity appeared to be stronger when the hybridomas were grown in ConA-SN and the difference was apparent in assays performed on several other days. However, this antigen-specific lytic-activity was unstable and slowly disappeared over the following

two weeks. It was never possible to isolate clones which retained this activity against P815, despite extensive cloning, in this and other experiments.

Panel B shows the results of a second experiment of identical format except that the cells were assayed for activity against P185 in the presence of PHA to detect the total P815 and non-specific lytic capacity of the hybridomas. This assay was performed on day 10 after fusion. The total lytic activity is stronger than that against P815 alone (c.f. panels A and B) and this was a consistant finding in several experiments. In addition, the presence of ConA-SN appeared to increase the overall lytic-activity of the hybridomas. This finding was also consistant for assays performed on other days suggesting that the effect is not simply due to the cells growing to different maximal densities with and without ConA-SN.

The non-specific lytic activity is very stable and, unlike the specific P815 activity, is readily retained by individual clones. Several such clones were isolated by selecting in limiting dilution for either strong PHA dependent lytic activity against P815 or for no lytic activity whatsoever. Interestingly, clones possessing no cytolytic potential could only be obtained from cells which had developed in the absence of ConA-SN. These clones are characterised in sections 3.6 and 3.7.

The frequencies of growing hybridomas in these and other experiments are difficult to estimate because of the problems of positive co-operativity in the limiting dilution curves. The results suggest, however, that two to four times as many hybridomas grow when ConA-SN is present after the fusion as compared to its absence.

Morphologically, the freshly prepared hybridoma populations were highly irregular and resembled the BW5147 parent. Like both parental cells, they

were not adherent. Cytolytic clones derived from these cultures also retained this morphology whereas non-lytic clones were much more regular in shape and tended to resemble the clone 96 parent. They also tended to be smaller and grow more rapidly than the lytic hybridomas.

## 3.6 Analysis of hybridoma clones

### 3.6.1 Isolation of lytic and non-lytic hybridoma clones and analysis of cell surface antigens

In order to examine the lytic activity of the hybridoma cells generated in these experiments it was first necessary to derive individual clones. Thus, both lytic (defined as lysing P815 in the presence of PHA) and non-lytic clones were isolated by limiting-dilution from populations of cells generated in fusions with clone 96 and BT 7.4.1; a selection of these clones are shown in table 2. These clones are non-adherent and grow with a division time of 12-20 hr to densities of $5 \times 10^5$ - $3 \times 10^6$ cells per ml with viabilities of 98% or greater.

All of the lytic clones described were derived from cultures supplemented with ConA-SN and were, therefore, initially maintained in the presence of ConA-SN. However, they were later shown to be independent of ConA-SN for proliferation (trypan blue exclusion assay, data not shown) and lytic activity. As an example, the lytic activity of two such clones are shown in figure 14. Panels A and C show the lytic activity of clones 17B6 (BW5147 x clone 96) and 22E1 (BW5147 x BT 7.4.1) respectively which had been cultured in the presence of ConA-SN prior to the assay. Panels B and D show the lytic activities of the same two clones after four weeks of continuous culture in the presence or

absence of ConA-SN demonstrating that ConA-SN has no effect on the lytic activity of the cells. All clones were subsequently cultured in the absence of ConA-SN.

Non-lytic clones were derived from cultures both with and without ConA-SN but none of these clones display any dependence on ConA-SN for proliferation (trypan blue exclusion assay, data not shown).

The clones mentioned in table 2 were analysed for the expression of typical T cell surface antigens by flow cytometry, table 3. All of the hybridoma clones strongly express the Thy1.1 allele derived from the BW5147 parental cell but only lytic hybridoma clones co-express the Thy1.2 allele of the CTL parent. All of the hybridomas tested express the H-2D$^b$ determinant typical of the CTL parental cells. Thus, all of the clones discussed in this report are demonstrated to be hybridomas by the co-expression of Thy1.1, typical of the BW5147 parent and of either Thy1.2 and/or H-2D$^b$ typical of the CTL parent.

None of the hybridoma clones appears to express Lyt-2 determinants typically expressed on CTL and thought to be involved in the lytic mechanism.

### 3.6.2 The lytic activity of the hybridoma clones against various target cells and in the presence of other mitogenic and non mitogenic lectins

Mitogenic lectins such as ConA and PHA are known to facilitate the lysis of irrelevant targets by CTL (Formann and Möller, 1973; Bevan and Cohn, 1975). In order to compare the properties of the cytolytic hybridomas with normal CTL, a series of experiments were performed in which various other targets and lectins were used. The results of these experiments are shown in figures 15 and 16.

A clear contrast can be seen in the activities of the parental cells, clone 96 and BT 7.4.1. The clone BT 7.4.1 behaves as a classical CTL in that it readily

lyses a range of irrelevant target cells of different H-2 haplotypes in the presence of 1% PHA (figure 15) and ConA (data not shown). Clone 96, on the other hand, will only lyse P815 cells (its specific target) in the presence of 1% PHA. The activity of all the hybridoma clones, whether derived from BT 7.4.1 or clone 96 is similarly restricted to P815 in the presence of 1% PHA (figure 15). These results cannot be completely explained by differential susceptibility to lysis by various target cells because, firstly, all targets are tumour cells which tend to be readily lysed and, secondly, they are shown to be lysed by BT 7.4.1 in the presence of PHA (figure 15).

If the concentration of PHA is titrated with various targets, then in some experiments, slight lytic activity can be found in clone 96 against RBL-5 and RDM4 at higher PHA concentrations but not against EL4 (data not shown). In contrast, no activity against other tumour targets was found in the hybridoma clones when the concentration of PHA was titrated.

Con A is another mitogenic lectin which is known to reveal non-specific lysis against irrelevant target cells. Therefore, ConA was tested on clone 96 and the clone 96 x BW5147 hybridoma clones for its ability to facilitate the lysis of P815. As a control, the non mitogenic lectin soya-bean agglutinin (SBA) was used. The results of this experiment (figure 16) clearly show that ConA cannot mediate the lysis of P815 by the hybridomas at 5 μg/ml. Moreover, The specific lysis of P815 by clone 96 was actually slightly enhanced by ConA at this concentration. If the concentration of ConA in the assay was increased to 20μg/ml it was found that there was a slight augmentation of the lytic activity of clone 96 for P815 and a very weak lytic activity for P815 was found in the clone 96 x BW5147 hybridoma clones (5-8% lysis at 50:1, data not shown). The non mitogenic lectin SBA totally inhibited the specific lysis of clone 96 against P815 and did not reveal the lytic activity of the hybridoma

clones against P815. Taken together these results suggest that clone 96 and the hybridomas behave basically like classical CTL in the presence of ConA and PHA but that their activity is weak. Thus, the differences observed are probably quantitative rather than qualitative.

The role of mitogenic lectins in the non-specific lysis of target cells by CTL is unclear. It was initially assumed that merely the close contact between effector and target cells was enough to initiate lysis. However, later work showed that other processes were involved including probably the stimulation of the effector cell via the antigen receptor (Chilson et al., 1984; Sitkovsky et al., 1984). In most of this work, the target cell was always pre-incubated with the lectin. However, in a series of experiments, Berke and co-workers (1981a, b) have shown that the effect of the lectin is undirectional in as much as effector cells precoated with lectin are much less efficient at lysing irrelevant target cells. Similarly, pre-incubation of both target and effector cells with lectin results in reduced target cell lysis. It is possible that this unidirectional effect is due to the binding of lectin to function associated molecules on the CTL which results in the inhibition of lytic activity. For example, monoclonal antibodies specific for LFA-1 or Lyt-2 can inhibit lytic activity and it is reasonable to assume that lectins could do the same. Based on the finding that PHA and ConA do not reveal non-specific lytic activity in the hybridomas against targets other than P815, an experiment was performed to see whether the effects described by Berke and co-workers, 1981a, b, also occurred in this system. Essentially, either effectors or targets or both effectors and targets were incubated with PHA for five to ten minutes prior to the assay. In all other respcts the lysis assay was as described in the materials and methods. Clone 96, BT 7.4.1 and the hybridoma clones derived from both of these cell types were tested and the results are shown in figure 17. Only clone 96 lysed

P815 in the total absence of PHA (o) but all cells lysed the target P815 when it had been preincubated with PHA (●). The non-specific lytic activities of clone 96 and BT 7.4.1 were partially inhibited when the effector cells (Δ) or both effector and target cells (▲) were pretreated with PHA. In the case of clone 96 this effect was quite pronounced ($\approx 50\%$) whereas in BT 7.4.1 this inhibition was much smaller (10-15%). The hybridoma clones appeared to behave in a different manner; pretreatment of either the effector or the target cells with PHA prior to the assay did not affect the relative lytic activity whereas there was a 50% inhibition of lytic activity when both the effector and target cells were pre-incubated with PHA. These results suggest that the lytic mechanism of the CTH is not identical to that of the parental CTL and that this this difference may be due to the loss of a function associated antigen on the CTH.

### 3.6.3 The lytic activity of hybridoma clones resembles that of bone fide CTL

The cytolytic behaviour of the hybridoma clones is rather unusual in that they do not readily lyse tumour targets other than P815 in the presence of PHA or ConA. It is therefore necessary to demonstrate that the lysis induced by the hybridomas resembles that of bone fide CTL.

The classical lytic event is well documented and can be typically divided into four basic stages, namely (i) effector: target recognition and binding (ii) calcium ion dependent programming for lysis or lethal hit (iii) CTL independent lysis of the target cell and (iv) CTL recycling (Berke, 1980). The precise mechanism of lysis remains unclear but it is known that cell contact is required and that lysis is not induced by the transfer of a soluble toxic factor.

The lytic activity of the hybridoma clones was tested for features typical of CTL induced lysis i.e. (i) sensitivity to EDTA which drastically reduces the

concentration of calcium ions and inhibits the lethal hit, (ii) sensitivity to temperature which affects all stages of lysis and (iii) the involvement of a soluble toxic factor. In addition, FisherLindahl and colleagues, 1982, describe an effect which they term a "cytolymic effect" whereby chromium-51 release is not necessarily corrȯlated with target lysis and death in the standard cytolytic assay. P815 is reported as being a particularly sensitive cytolymic target. Therefore, the hybridomas were tested for their ability to lyse P815 in a novel type of assay which measures lysis by directly determining the viability of the target cells after exposure to the effector cells.

### 3.6.3.1 The requirement for calcium ions and a temperature of 37°C

It is well documented that cytotoxic T cells require both the presence of calcium ions and a temperature of 37°C to successfully deliver a lethal hit to a target cell (reviewed Martz et al., 1982b). These requirements are independent of whether the lytic mechanism is induced by specific recognition of the appropriate antigen or by the binding of a non-specific mitogenic lectin. One would expect, therefore, that the activities of BT 7.4.1 and clone 96 hybridomas are sensitive to alterations in the temperature and to the addition of 10mM EDTA which chelates and drastically lowers the concentration of calcium ions in free solution. Thus, the parental cells BT 7.4.1 and clone 96 and their respective lytic hybridoma clones were tested for their lytic activity against P815 with PHA in the presence or absence of 10mM EDTA and at 21°C or 37°C. The results are shown in fig. 18. They clearly demonstrate that, in these respects, the lytic activity of the hybridoma clones is similar to that of bone fide CTL because both lower temperatures and the removal of free calcium ions completely inhibits their lytic activity.

## 3.6.3.2 The requirement for cellular contact; lysis is not mediated by toxins

A critical stage of the lytic mechanism of normal CTL is effector:target recognition and the resultant cell contact. If cell contact is in any way prohibited, then a lethal hit is not delivered to the target. It may be possible that the hybridoma clones do not rely on cell contact but instead secrete a toxin which is responsible for the observed target lysis, in which case the lytic mechanism would vary from that of bone fide CTL.

Experiments were therefore performed to see whether normal hybridoma supernatants or supernatants from hybridomas stimulated with PHA were cytotoxic for P815 cells. Full details of the experimental procedure are given in the materials and methods. Essentially, supernatants were taken from cells which had been preincubated for four hours in the presence or absence of PHA and then both the supernatants and the washed cells were tested for lytic activity against P815 both with and without PHA. The results of this experiment are shown in figures 19 and 20. It can be clearly seen that all of the cells except BW5147 showed lytic activity against P815 with PHA as expected (fig. 19 and 20, A panels). The hybridoma clones that had been preincubated with PHA (B panels) also lysed P815 cells alone. This finding is not unusual since, despite extensive washing, the hybridoma cells probably retain PHA on their surface which can facilitate lysis (see section 3.6.2).

None of the supernatants from these cells induced lysis of P815 under any conditions. Thus, the lytic activity of these hybridomas is not mediated by a soluble toxin.

## 3.6.3.3 The viability of the target cells is reduced

In order to fully discount the possibility that the hybridoma clones are inducing cytolymic effects it is necessary to demonstrate that target cell lysis occurs, i.e. that target cell viability is reduced. FischerLindahl and colleagues, 1982, did this by examining the capacity of target cells to proliferate after treatment with effector cells. In these experiments the effector cells were irradiated (thereby preventing their proliferation) and the target cell proliferation was assessed by tritiated thymidine uptake. They demonstrated that some of their T helper clones were able to induce chromium-51 release from P815 and EL4 target cells in the presence of PHA without affecting the viability of these target cells. However, some of these results were difficult to interpret and in the case of their hybridomas (helper type) they could not show cytolymic effects in several experiments although they suspected that a cytolymic effect was in fact occurring.

Similar difficulties were encountered in this laboratory using this test system (M.M. Simon, personal communication) due to the relative insensitivity of the assay (the tritiated thymidine uptake assay itself is not insensitive but in these experiments the target cells have to proliferate for several days before assay and thus small differences in viability are difficult to differentiate).

A different approach was chosen to detect cytolymic effects in the cytolytic hybridomas based on the fact that P815 is known to have a very high cloning efficiency (H.U. Weltzien, personal communication). Essentially, the viability of target cells after incubation with effector cells and PHA was measured by limiting dilution analysis.

A pilot experiment was performed to demonstrate that the plating efficiency of P815 was indeed 100% despite the presence of PHA which would have to be present in a normal lytic assay. P815 cells at $2x10^3$ cells/well were incubated with or without 1% PHA for 30 minutes and then diluted (without washing) for the limiting dilution assay. Graded numbers of PHA treated or untreated P815 cells were seeded into 200 $\mu$l cultures with 24 cultures per group both with and without irradiated syngeneic DBA/2 spleen cells at $1.3x10^4$ per culture. The number of wells supporting growing cells was determined microscopically every three days. The cumulative results after four weeks are shown in the form of limiting dilution curves (figure 21). The growth frequency in all cases was 1.0 (100% plating or cloning efficiency) regardless or whether PHA or feeder cells were present or not. This indicates that PHA does not interfere with the viability of P815 and that the inclusion of feeder cells is unnecessary.

The assay was then used to determine whether clone 96 x BW5147 hybridoma clones are able to reduce the apparent viability of P815 cells. The initial cytolysis phase of the experiment was performed as for a chromium-51 release assay except that the P815 target cells were not labelled with chromium-51 and the effector cells were irradiated to 4000 Rads; this had been previously shown to inhibit the growth of both clone 96 and hybridomas without effecting their lytic potential (data not shown). All cultures were comprised of $2x10^5$ effectors and $2x10^3$ target cells (to maximise lysis) both with and without PHA. After a standard incubation time of four hours, wells were pooled and diluted based on the assumption that each culture contained $2x10^3$ viable P815 cells. The limiting dilution cultures contained between 0.1 and 30 cells per well at serial three fold dilutions with 24 wells per group. Growing cultures were determined every three days by microscopic examination. Parallel

chromium-51 release assays were performed on day 0 with the same irradiated effector cells for comparison and to confirm that these cells were still able to induce chromium-51 release. The limiting dilution curves assessed from cumulative data after four weeks are shown in figure 22, and the chromium-51 release assays in the insets. Irradiation of the effector cells did not influence the lytic activity of the cells.

The viability of P815 was not reduced in control cultures where either no cells or BW5147 cells were used as effectors since the growth frequency was 1.0 or less. These curves are used as control curves.

Clone 96 reduced the viability of P815 cells both in the absence of, and to a greater extent, in the presence of PHA [f = 0.64 uncorrected to the controls (p = 0.89), 36% lysis, and f = 0.15 (p = 0.68), 85% lysis, respectively]. This corrolates closely with the chromium-51 release assay in which clone 96 lyses P815 to 35% in the absence of PHA and to 85% in the presence of PHA with 100 effector cells per target. The limiting dilution curves for P815 cells which had been incubated with the clone 96 x BW5147 hybridomas, 17E4 and 17F8, corr*e*lates with the controls indicating that the hybridomas have had no effect on the viability of P815 in the absence of PHA. However, when PHA was added to the assay the viability of P815 was affected since the limiting dilution curves are shifted as compared to the controls. These curves appear to be co-operative but they nevertheless suggest that the viability of P815 has been reduced. If straight lines are fitted to the data, growth frequencies of 0.23 (p< 0.05), 77% lysis, for 17E4 and 0.4 (p = 0.068), 60% lysis, for 17F8 are found. These data correspond to the standard chromium release assay where 17E4 and 17F8 do not release chromium-51 from P815 in the absence of PHA but induce a 50% chromium-51 release at 100 effectors per target in the presence of PHA.

Taken together, these results indicate that, at least in clone 96 and clone 96 x BW5147 hybridomas, the release of chromium-51 from P815 corrolates with loss of viability of the P815 cells. This excludes the possibility that the hybridomas are inducing cytolymic effects. It does not, however, formally prove that the hybridomas are capable of actually lysing the target cells since cytostatic effects are not excluded.

## 3.7    Reasons for the loss of antigen-specificity in cytolytic hybridomas

### 3.7.1    A specific lytic activity is not revealed in cytolytic hybridomas by neuraminidase treatment of the target cells.

It has been reported by Kaufmann and co-workers, 1981 a,b, that they were able to generate CTH which weakly lysed EL4 in an antigen specific manner. This activity was enhanced if sialic acid residues were cleaved from the surface of the target cells by neuraminidase. Sialic acid is known to mask antigenic determinants (for review see Schauer, 1985) and removal of these moieties by neuraminidase will often enhance the apparent cytolytic activity of CTL by exposing more antigen on the target cell surface and thereby either enhancing effector to target binding or effector activation (Brondz et al., 1971).

Based on the findings of Kaufmann it was important to test whether the hybridomas generated in the fusions presented here failed to lyse their specific targets due to either a reduced concentration of TCR on the hybridoma surface or reduced affinity of the TCR for its antigen. Therefore, P815 cells or TNP-modified RBL-5 cells were treated with neuraminidase and then tested as targets for the hybridomas in specific and lectin dependent cytolytic assays. The results are shown in figure 23. It can be clearly seen that the hybridomas

22E1 (BW5147 x BT 7.4.1) and 17E4 (BW5147 x clone 96) do not lyse their specific targets, RBL-5/TNP and P815 respectively in the absence of PHA, regardless of the neuraminidase treatment (open symbols). However, the hybridomas do lyse P815 in the presence of PHA and there seems to be a slight enhancement of this activity when P815 is pretreated with neuraminidase. These results indicate that the lack of specific lytic activity by the hybridomas is not due to the masking of antigenic determinants by sialic acid. Other lytic hybridoma clones have also been tested on neuraminidase treated targets and none of them showed specific lytic activity (data not shown).

## 3.7.2 Expression of Lyt-2 on freshly prepared hybridomas

As described in section 3.6.1, cloned hybridomas never expressed significant levels of the Lyt-2 antigen which one would expect to have been transmitted by the parental CTL. Jancovié and colleagues (in press) claim that the expression of Lyt-2 is essential for the specific cytolytic activity of AK cells and there is a large body of literature stressing the importance of Lyt-2 for assisting the binding of effector to target cells (Swain, 1981a, b; MacDonald et al., 1982). Thus, it is possible that the cloned hybridomas have lost their specific activity due to the loss of Lyt-2. In order to investigate whether CTH can express Lyt-2 at all, hybridomas were generated in a fusion between BW5147 and cells taken from a secondary MLC of C57BL/6 female against C57BL/6 male spleen cells (described in section 3.1.2) and analysed for Lyt-2 expression early after fusion. On day 23, wells containing hybridomas with high PHA dependent cytolytic activity against P815 were pooled from plates initially seeded with $5 \times 10^5$ cells/culture in either normal medium or medium supplemented with 10% ConA-SN. Each group was tested for cytolytic activity

(against P815+PHA and C57BL/6 male ConA blasts) and expression of Thy1.1, Thy1.2 and Lyt-2 alloantigens by flow-cytometry. Both populations of pooled cells co-expressed the Thy-1 antigens indicating that they are hybridomas (data not shown). The cytolytic-activity and expression of Lyt-2 on the hybridomas are shown in figure 25, panels A and D, and figure 26, panels A and D. Clearly, those hybridomas which developed in the presence of ConA-SN do not express Lyt-2. However, those hybridomas cultured in the absence of ConA-SN can be divided into two distinct subpopulations which either do or do not express Lyt-2. The lytic activity of these two hybridoma populations is restricted to P815 and PHA as found previously (section 3.3) and does not appear to be affected by the presence or absence of ConA-SN.

Taken together, these data suggest that (i) loss of Lyt-2 expression does not explain loss of antigen specific lytic activity and that (ii) a factor present in the ConA-SN affects the expression of Lyt-2.

It was interesting to see whether Lyt-2 expression could be regulated by manipulating the culture conditions. Each group of hybridomas was split into two, forming four new groups (1 to 4) which were cultured for a further three weeks. Groups 1 and 4 were cultured for the duration of the experiment in medium without and with ConA-SN respectively. Cells in group 2, which had developed in the absence of ConA-SN, were transferred to medium with ConA-SN and cells in group 3, which had developed in the presence of ConA-SN, were transferred to medium without ConA-SN. The proliferation of these cells immediately after the transfer was measured by trypan-blue exclusion tests over the following three days (fig. 24) and the cytolytic activity and Lyt-2 expression of each group was measured after three weeks (figs. 25 and 26). The proliferation rate of each group of cells was equivalent although group 1 cells, which had always been cultured in the absence of ConA-SN, appeared to have

a lag period. Lyt-2 expression was only observed in those hybridomas that had never been exposed to ConA-SN (group 1). Moreover, these hybridomas were clearly divided into two subpopulations which either did or did not express this antigen. Lyt-2 expression was not induced in cells which had previously been cultured with ConA-SN and were then cultured in medium without ConA-SN, group 3. The lytic activity of the hybridomas in all four groups generally decreased during this period. There appeared to be no correlation between the lytic activity and Lyt-2 expression of these populations.

The switching of Lyt-2 expression in these hybridomas can be explained by one of two alternative mechanisms. Firstly, it is possible that certain factors present in ConA-SN permanently repress the expression of Lyt-2 in these cells. Secondly, the presence of ConA-SN may enhance the growth of the Lyt-2 negative subpopulation such that it overgrows the other population. To test these possibilities, clones were derived (at 0.5 cells/well) from populations of hybridomas which had never been exposed to ConA-SN (group 1). Two such clones were found to strongly co-express the Thy1.1, Thy1.2, H-2K$^b$ and Lyt2.2 alleles. One hundred percent of the cells within each clone strongly expressed Lyt-2. When the clones were cultured in medium both with and without ConA-SN there was no change in the expression of Lyt-2. This suggests that the apparent regulation of Lyt-2 expression in the uncloned hybridoma populations occurs by a selective mechanism.

### 3.7.3 Expression of the T cell antigen receptor on cytolytic hybridoma clones

One possibility to explain the loss of specific antigen-recognition function in cytolytic hybrids was the partial or total loss of expression of the TCR. Thus, hybridomas were tested for the expression of this receptor both at the protein and mRNA levels.

The TCR is a disulphide linked heterodimer consisting of two polypeptides each of approximately 45 kd. Due to the fact that very few disulphide-linked dimeric glycoproteins exist on the T cell surface, the TCR can be visualized by separating a total lysate of $I^{125}$ surface labelled T cells by two dimensional non-reduced/reduced SDS electrophoresis. The TCR appears as a spot at a characteristic position (1 dim: 90 kd, 2 dim: 45 kd) underneath a diagonal which contains the bulk of the non-reducable surface proteins.

Figures 27 and 28 show examples of analysing a number of lytic and non-lytic hybridoma clones and their parental cells by this method. BW5147 (figures 27 and 28, panel a) appears negative for TCR whereas both BT 7.4.1 (figure 27, panel b) and clone 96 (figure 28, panel b) clearly possess a characteristic large spot lying just off the diagonal at approximately 43 kd. In both cases, the TCR spot appears itself as a short diagonal ranging from 40-50 kd probably due to differential glycosylation. In addition, in both parental cells, a faint spot can be seen lying off the diagonal and under the TCR at approximately 35 kd. This spot is characteristic for the Lyt-2 antigen. Both of these spots in the parental cell lines have been shown to directly correspond to the TCR and Lyt-2 by immunoprecipitation of $I^{125}$ surface labelled material with antisera and monoclonal antibodies specific for TCR and Lyt-2. (D. Janković personal communication). The results obtained from the analysis of both lytic and non-lytic CTH are shown in panels c and d of both figures. It can be clearly seen that the lytic hybridomas have a weak but characteristic TCR spot at around 43 kd whereas non lytic hybridomas appear to be negative for TCR. Of eight hybridomas tested, it was found that all four lytic hybridomas expressed TCR whereas three out of four non-lytic hybridomas were either negative for or expressed only very low levels of TCR not detectable within the sensitivity of this system (the one exception is the BW5147 x BT 7.4.1

hybridoma, 15C1, which is non-lytic but expresses TCR). These results are summarised in table 4. Taken together, the results suggest that TCR expression on the cell surface is necessary for lytic-activity.

None of the hybridomas appears to express cell surface Lyt-2 antigen within the level of detection of this system. This correlates to the data from the flow-cytometry experiments for Lyt-2 expression presented in table 3.

Although the two-dimentional SDS gels readily demonstrate whether the TCR is present on the cell surface or not they do not provide information on the specificity of the receptor. An oligonucleotide probe was available which hybridises with the $V_{\beta AK}$ region of the TCR of clone 96 and other AK cells (Epplen et al., in press) and therefore allowed the examination of TCR specificity. Thus, total cellular RNA extracts were prepared from BW5147, clone 96 and four clone 96 x BW5147 hybridomas (both lytic and non lytic) and were tested in Northern blot hybridisation experiments for expression of $V_{\beta AK}$ mRNA transcripts. In addition, the same samples were tested with oligonucleotide probes for the $C_{\beta 1}$ and $C_{\beta 2}$ regions of the TCR. The results of these Northern blots are shown in figure 29 and show that both parental cell lines, clone 96 and BW5147, express mRNA transcripts of approximately 1.3 kb which hybridise with the $C_{\beta 2}$ but not the $C_{\beta 1}$ probe. Three of the hybridomas tested express $C_{\beta 2}$, but not $C_{\beta 1}$, mRNA transcripts and one non-lytic hybridoma (19A6) does not express any ß transcripts whatsoever. Only clone 96 and lytic hybridomas, but not BW5147 or non-lytic hybridomas, express $V_{\beta AK}$ transcripts. These data suggest that the ß chain of the TCR which is demonstrably expressed on the surface of lytic hybridomas has the same specificity as that of the parental clone 96. Formally, it remains unclear whether the ß chain protein expressed on the cell surface is derived from BW5147 or clone 96 transcripts. Moreover, no data are available on the

expression fo the $V_{\alpha AK}$ chain in cytolytic hybridomas which would presumably be required in conjunction with $V_{\beta AK}$ to generate full AK specificity of the TCR. However, the data does demonstrate that normal TCR transcripts derived from CTL partners can be found in the cytoplasm of CTH and it would be reasonable to assume that these can be expressed as protein on the hybridoma cell surface.

The fact that non-lytic hybridomas express $C_{\beta 2}$ mRNA transcripts in the cytoplasm, but not $V_{\beta AK}$ transcripts suggests that the $V_{\beta AK}$ gene has been either lost or is no longer expressed. Thus the $C_{\beta 2}$ transcripts observed could be derived either from BW5147 or from a further rearrangement of clone 96 TCR ß genes. These hybridomas have clearly inherited one or more features of BW5147 which prevent the expression of TCR on the cell surface.

## 3.8    Secondary fusions

The results of the primary cytolytic cell fusions described in sections 3.3, 3.4 and 3.5 suggest that BW5147 is unsuitable as a fusion partner, perhaps due to its supposed "helper cell" nature (Hämmerling, 1977). EL4 is another tumour cell which has been used in CTL fusions (DiPauli and DiPauli; 1978) with negative results; like BW5147, EL4 is also of helper T cell origin. It is therefore important to try to find a more compatable fusion partner for the generation of CTH. Such a partner cell should be of cytolytic T cell origin and should preferentially express Lyt-2. Clone 96 is a clear candidate for a partner cell since it grows continuously, retains a specific lytic aparatus and is presumably of cytolytic T cell (Thy-1$^+$, Lyt-2$^+$, T4$^-$) lineage. Several attempts were made to induce HAT sensitivity into clone 96 by selective mutagenesis with 6-thioguanine (6TG) but all of these attempts were unsuccessful.

Other potential fusion partners are the hybridomas described in section 3.6. These cells have the advantage that, as well as growing continuously in tissue culture and retaining some vestige of lytic mechanism, they may also retain some of the membrane characteristics of BW5147 that make it an efficient fusion partner. The disadvantage of these cells is that they do not express Lyt-2. A clone 96 x BW5147 hybridoma (17E4) and a BT 7.4.1 x BW5147 hybridoma (22 E1) were successfully made HAT sensitive by treatment with 6TG. These hybridomas already retained a resistance to ouabain which had been inherited from the BW5147 parent. The cytolytic activity of these hybridomas subclones (17E4H1 and 22E1C3) is shown to have been unaffected by the mutagenic procedure, figure 30.

In the first type of fusion the BW5147 x clone 96 hybridoma 17E4H1 was fused with either BT 7.4.1 or clone 96. Four $x10^7$ 17E4H1 cells and $4x10^7$ of either clone 96 or BT 7.4.1 were fused with PEG 1500 and distributed into 2 ml cultures with $2x10^5$ of each parental cell type per culture both with and without 10% ConA-SN. In the BT 7.4.1 x 17E4H1 fusion, no growing cells were detected and after seven weeks this fusion was abandoned. In the clone 96 x 17E4H1 fusion, sixteen wells contained growing cells but many of these died after three weeks leaving only six cultures which could be tested; two growing in the absence of ConA-SN and four growing in its presence. These cells appeared very large and irregular in shape under the microscope, were very slow growing and proved difficult to adapt to flask culture. Some of the more unstable cultures, which died before they could be tested, consisted of giant cells perhaps created by the fusion of three or more cells.

All cultures tested showed strong non-specific lytic-activity against P815 in the presence of PHA (table 5). Only one culture, which had developed in the presence of ConA-SN (20-6D4), showed a weak activity against P815 alone

(15% at an approximate E:T of 50:1). This cell was unstable and proved difficult to clone. Of the few clones that were obtained, none of them showed this specific lytic activity. This behaviour is reminiscent of the original fusions between BW5147 and clone 96 in which lytic activity was transiently observed against P815. Moreover, the original line, which was probably clonal anyway, slowly lost its specific lytic potential. All of the clones derived from 20-6D4 retained strong non-specific lytic activity against P815 in the presence of PHA.

In the second type of fusion, the HAT sensitive variant of the BW5147 x BT 7.4.1 hybridoma, 22E1C3, was fused with BT 7.4.1. Five x$10^7$ 22E1C3 cells and 5x$10^7$ BT 7.4.1 cells were fused by PEG 1500 and distributed into 2 ml cultures with 2.5x$10^5$ of each parental cell type per culture both with and wtihout 10% ConA-SN. Seven wells were found to contain growing cells after seventeen days (all in the presence of ConA-SN) but only five of these remained stable during culture. The hybridomas generated in this fusion appeared very similar to the parental 22E1C3 in morphology and were not adherent. However, they were larger than the parental cells and grew relatively slowly i.e. one division every 24 hours. No giant cells were observed as with the 17E4H1 x clone 96 fusion. None of the five hybridomas tested showed lytic activity against either P815 or the expected antigen, TNP-modified RBL-5 cells. However, all hybridomas strongly lysed P815 but not TNP-modified RBL-5 in the presence of PHA (table 5).

Table 1

Summary of the fusion frequencies obtained from various clone 96 x BW5147 fusions in the presence and absence of ConA-SN.

| Fusion no | No of cells fused[1] | Feeder cells/well[2] | Fusion Frequency[3] | |
|-----------|----------------------|----------------------|---------------------|-----------------|
| | | | (-)ConA-SN | (+)ConA-SN |
| 8 | $2.4 \times 10^7$ | - | $1.5 \times 10^{-5}$ | - |
| 9 | $12.0 \times 10^7$ | - | $5.3 \times 10^{-5}$ | - |
| 9 | $12.0 \times 10^7$ | $1 \times 10^6$ | $4.8 \times 10^{-5}$ | - |
| 9 | $12.0 \times 10^7$ | $5 \times 10^6$ | $5.3 \times 10^{-5}$ | - |
| 10 | $4.0 \times 10^7$ | - | $0.8 \times 10^{-5}$ | $34.0 \times 10^{-5}$ |
| 11 | $4.0 \times 10^7$ | $5 \times 10^5$ | $2.3 \times 10^{-5}$ | $23.0 \times 10^{-5}$ |
| 12 | $4.0 \times 10^7$ | - | $3.3 \times 10^{-5}$ | $3.2 \times 10^{-5}$ |
| 17 | $8.0 \times 10^7$ | - | $6.6 \times 10^{-5}$ | $11.0 \times 10^{-5}$ |
| $\bar{x}$ | - | - | $3.7 \times 10^{-5}$ | $19.0 \times 10^{-5}$ |

1) The number of cells fused refers to only one of the parental cells. The ratio of clone 96 cells to BW 5147 cells in the fusions was always maintained at 1:1.

2) Feeder cells were (C57BL/6 x AKR/J) $F_1$ irradiated spleen cells.

3) The fusion frequency is the proportion of one of the parental cells which fused to create viable hybridomas.

Table 2

Summary of the origins and cytolytic activities of the various hybridoma
clones presented in this report.

| Clone | CTL Parent[1] | Cytolytic activity[2] |
|---|---|---|
| 15C1 | BT 7.4.1 | − |
| 16C3 | " | − |
| 22E1 | " | + |
| 22F7 | " | + |
| 22G8 | " | + |
| | | |
| 19A1 | Clone 96 | − |
| 19A6 | " | − |
| 19B8 | " | − |
| 17B6 | " | + |
| 17E4 | " | + |
| 17F8 | " | + |

1) The tumour partner was always BW5147

2) Lytic activity refers to the ability of the clone to lyse P815 target cells in the
presence of PHA. Clones are scored + if they can induce greater than 10%
specific chromium-51 release at 100 effectors per target.

Table 3

Analysis of the expression of T cell surface antigens on hybridoma clones and parental

cells by flow-cytometry.

| Monoclonal antibody[2] | Percentage of cells stained[1] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BW5147 | BT7.4.1 | Clone 96 | 15C1 | 16C3 | 22E1 | 22F7 | 22G8 | 19A1 | 19A6 | 19B8 | 17B6 | 17E4 | 17F8 |
| m-control[3] | 5.7 | 3.7 | 2.9 | 6.0 | 5.9 | 4.5 | 6.0 | 12.9 | 5.6 | 4.1 | 2.8 | 5.1 | 8.1 | 2.2 |
| āThy1.1 | 96.4 | 3.8 | 3.3 | 99.2 | 98.0 | 97.9 | 94.9 | 94.0 | 82.3 | 85.6 | 47.5 | 96.5 | 95.5 | 96.4 |
| āThy1.2 | 6.5 | 93.2 | 98.0 | 7.1 | 6.1 | 99.1 | 95.2 | 94.5 | 10.2 | 2.3 | 2.7 | 94.9 | 97.7 | 98.0 |
| ā H-2$^b$ | 6.0 | 84.0 | 99.6 | 14.5 | 51.0 | NT[4] | NT | NT | 69.0 | 99.2 | 99.3 | 99.2 | 99.7 | NT |
| āLyt2.1 | 7.4 | NT | 4.4 | NT | NT | NT | NT | NT | NT | NT | NT | 6.3 | 6.2 | NT |
| āLyt2.2 | 5.2 | NT | 59.6 | NT | NT | NT | NT | NT | NT | NT | NT | 6.3 | 6.5 | NT |
| r-control[3] | 5.9 | NT | 3.8 | 7.6 | 5.6 | 10.3 | 12.6 | NT | 8.3 | 3.9 | 4.2 | NT | 7.0 | 10.0 |
| āLyt-2 | 6.6 | NT | 99.7 | 7.6 | 5.8 | 12.2 | 13.5 | NT | 7.2 | 2.8 | 4.3 | NT | 7.5 | 10.4 |

1) Stained cells always appeared as single populations.

2) Full details of the monoclonal antibodies used are given in the materials and methods section.

3) The background controls were adjusted against cells incubated only with the fluoroscein conjugated second antibody to read 3-10% positive according to the distribution; m-control = mouse FITC control, r-control = rat FITC control.

4) NT = not tested.

**Table 4**

Summary of the cytolytic activities and expression of TCR and Lyt-2
on BT 7.4.1, clone 96 and hybridoma clones.

| Hybridoma | Parental CTL[1] | Ag-specific[2] lytic activity | Non-specific[3] lytic activity | TCR[4] | Lyt-2[5] |
|---|---|---|---|---|---|
| BT 7.4.1 | | + | + | + | + |
| Clone 96 | | + | + | + | + |
| | | | | | |
| 15C1 | BT 7.4.1 | - | - | + | - |
| 16C3 | " | - | - | - | - |
| 22E1 | " | - | + | + | - |
| 22F7 | " | - | + | + | - |
| | | | | | |
| 19A1 | Clone 96 | - | - | - | - |
| 19A6 | " | - | - | - | - |
| 17B6 | " | - | + | + | - |
| 17E4 | " | - | + | + | - |

1) The tumour partner was always BW5147.

2) Antigen-specific lytic activity refers to the ability of the clone to lyse its specific target i.e. TNP in the context of $H-2^b$ for BT7.4.1, 15C1, 15C3, 22E1, 22F7 and P815 for clone 96, 19A1, 19A6, 17B6 and 17E4. Clones are scored + if they can induce greater than 10% chromium-51 release at 100 effectors per target.

3) Non-specific lytic activity refers to the ability of the clone to lyse P815 target cells in the presence of PHA. Clones are scored + if they can induce greater than 10% chromium-51 release at 100 effectors per target.

4) Expression of TCR was determined by 2-dimensional SDS PAGE, section 3.7.3.

5) Expression of Lyt-2 was determined by 2-dimensional SDS PAGE and flow cytometry, sections 3.7.3 and 3.6.1.

**Table 5**

The cytolytic activities of various secondary CTL hybridomas

| Hybridoma | Parental cells | ConA-SN[1] | Cytolytic activity (%)[3] | | | |
|---|---|---|---|---|---|---|
| | | | P815 | P815/PHA | RBL-5-TNP | RBL-TNP/PHA |
| 20-1.5-A6 | 17E4H1 x clone 96 | – | 0.5 | 59.3 | NT[2] | NT |
| 20-6-B2 | " | – | 2.9 | 33.8 | NT | NT |
| 20-6-D4 | " | + | 14.9 | 20.8 | NT | NT |
| 20-24-A6 | " | + | 0.0 | 16.2 | NT | NT |
| 20-24-B4 | " | + | 4.9 | 6.4 | NT | NT |
| 20-24-D5 | " | + | 0.1 | 47.7 | NT | NT |
| 23-1-C1 | 22E1C3 x BT7.4.1 | + | -3.1 | 10.1 | -5.0 | -2.0 |
| 23-1-DS | " | + | -2.5 | 29.9 | -5.0 | -3.2 |
| 23-3-C6 | " | + | -1.0 | 81.4 | -5.1 | -2.2 |
| 23-6-D4 | " | + | -0.3 | 68.2 | -3.6 | -1.8 |

1) Hybridomas were derived from cultures both with and without ConA-SN.
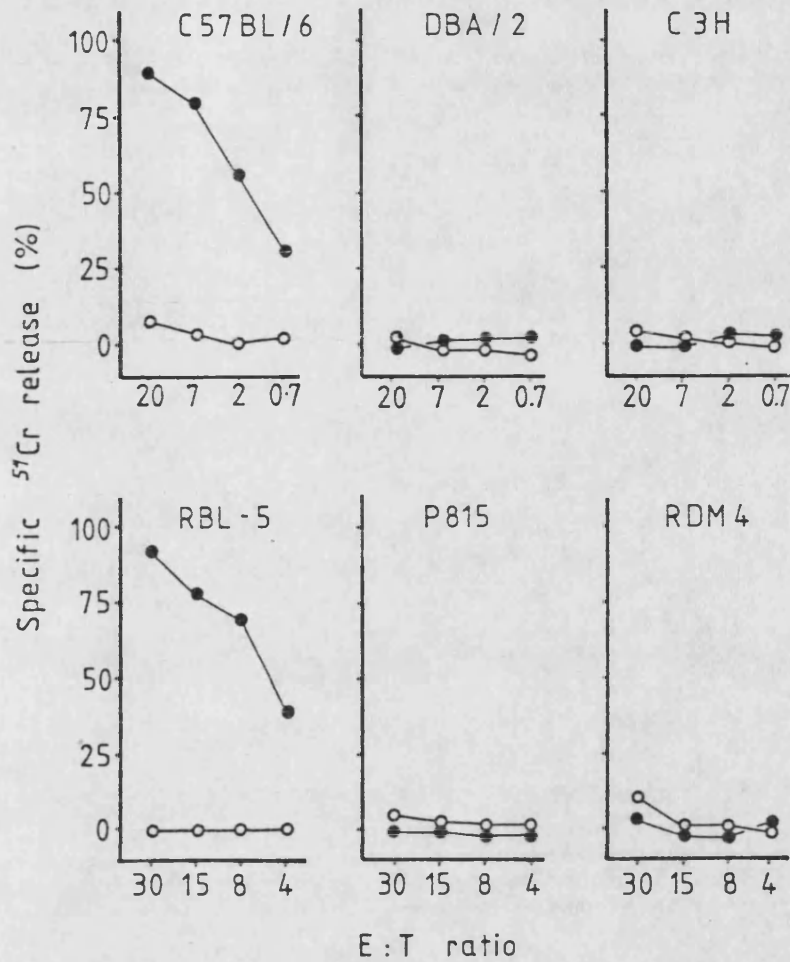2) NT = Not tested.
3) Effector to target ratios were approximately 50:1.
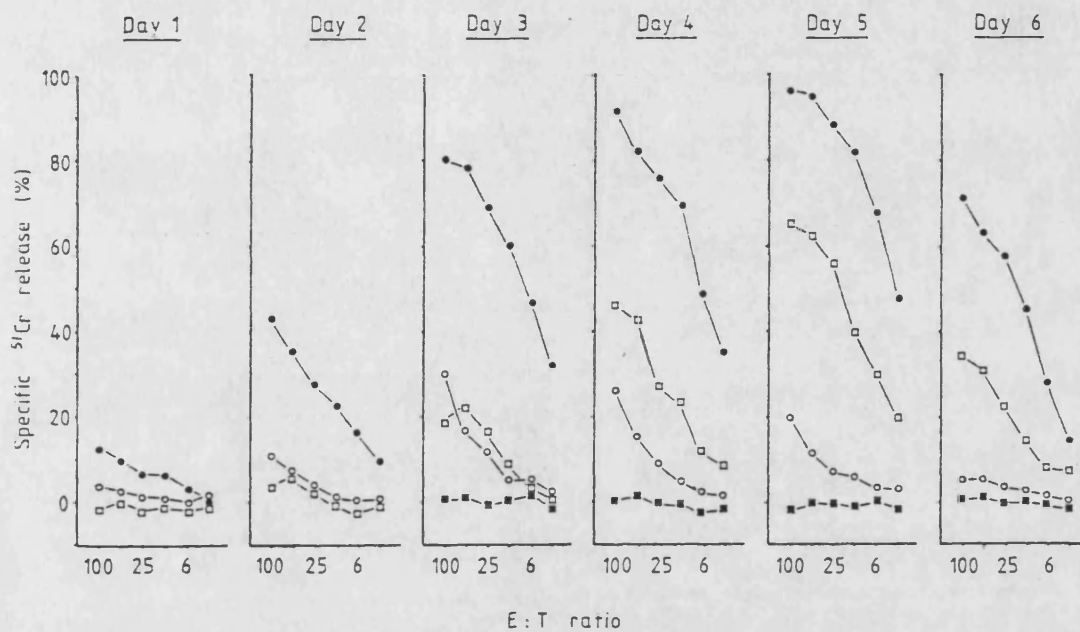
**Figure 1**
The lytic-activity of an H-Y MLC (♀C57BL/6 responder cells and irradiated ♂C57BL/6 stimulator cells) against various targets over 6 days. Cultures were initiated on day 0.
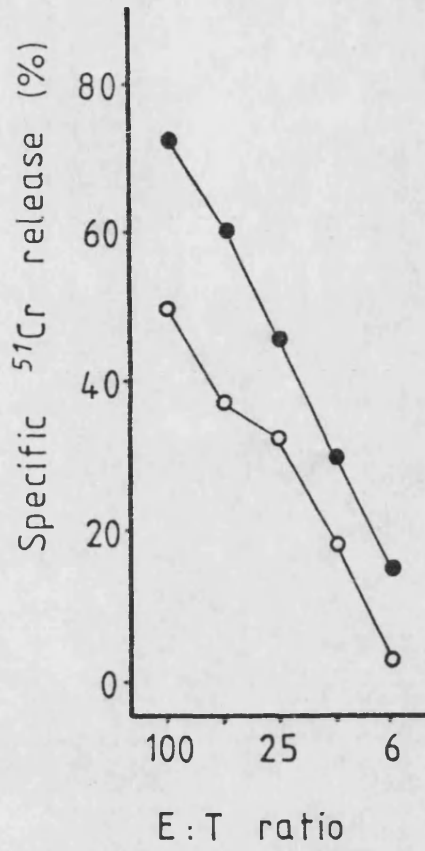
o P815
● P815/PHA
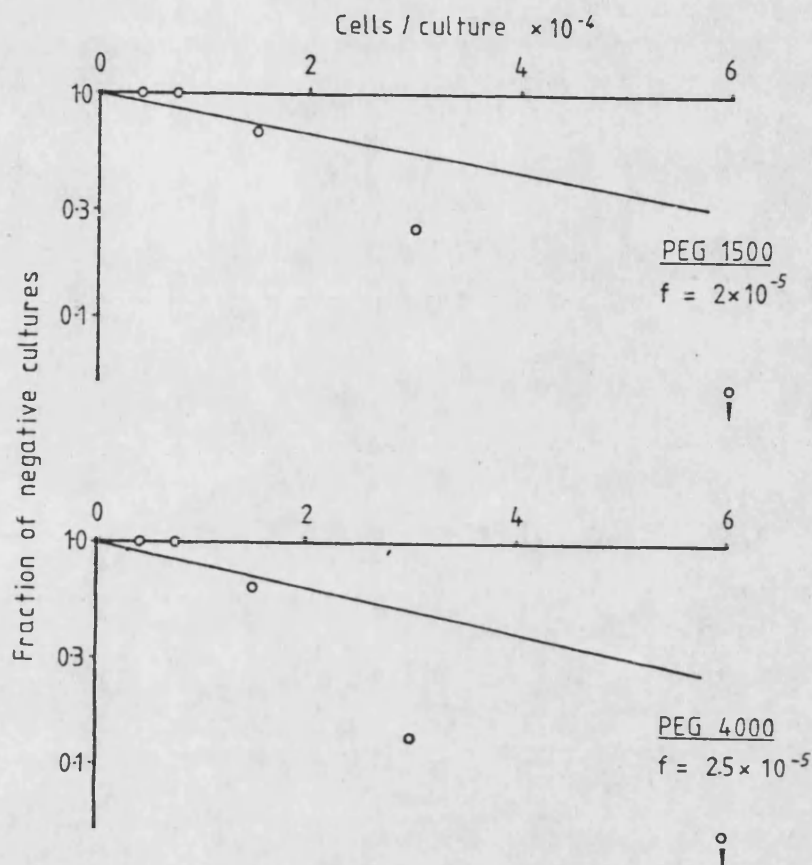□ ♂C57BL/6 ConA blasts
■ ♀C57BL/6 ConA blasts

**Figure 2**
The lytic-activity of BT 7.4.1 against ConA blasts [C57BL/6 (H-2$^b$), DBA/2 (H-2$^d$), C3H (H-2$^k$)] and tumour targets [RBL-5 (H-2$^b$), P815 (H-2$^d$), RDM-4 (H-2$^k$)]

     o  unmodified targets.
     ●  TNP-modified targets.

**Figure 3**
The lytic-activity of clone 96 against P815
o  P815
•  P815/PHA

Cells / culture × 10⁻⁶

Fraction of negative cultures

PEG 1500

$f = 2 \times 10^{-5}$

PEG 4000

$f = 2.5 \times 10^{-5}$

**Figure 4**
A comparison of the fusion efficiency of PEG1500 and PEG4000 in fusions between BW5147 and clone 96. Each point of the limiting-dilution curves represents the fraction of cultures out of 24 without growing hybridomas assessed cumulatively over 4 weeks. Arrows indicate that 24 cultures in the group were positive for growing hybridomas; these values are not included in the chi-squared analysis. The fusion frequencies (f) were determined by chi-squared analysis and the p value in both cases is <0.05.
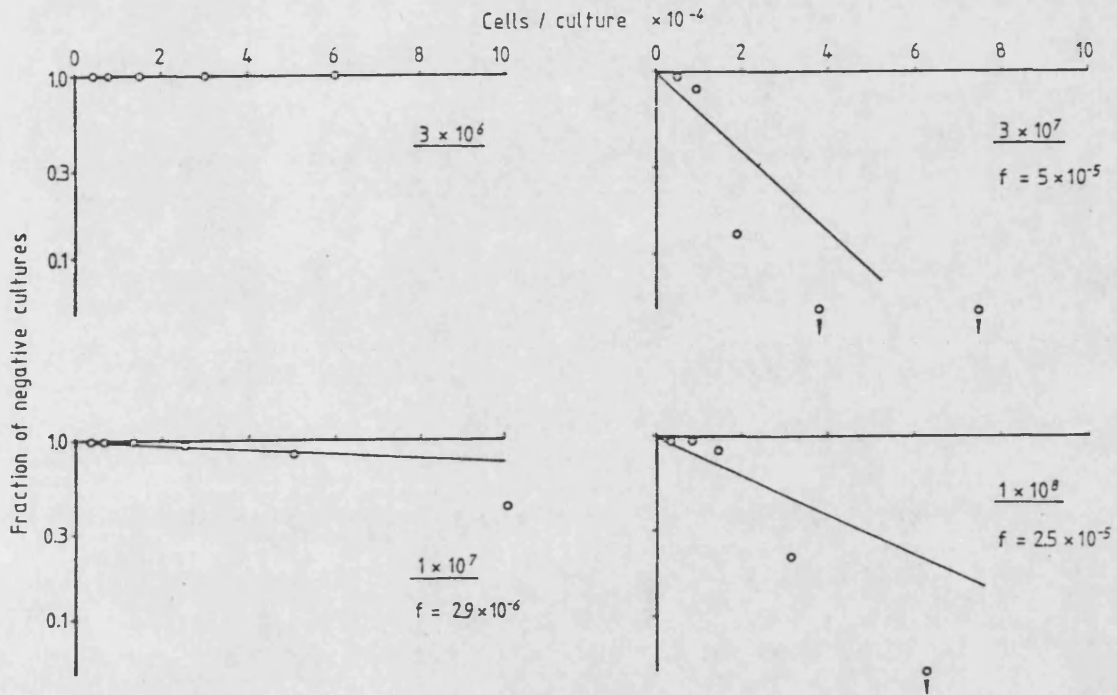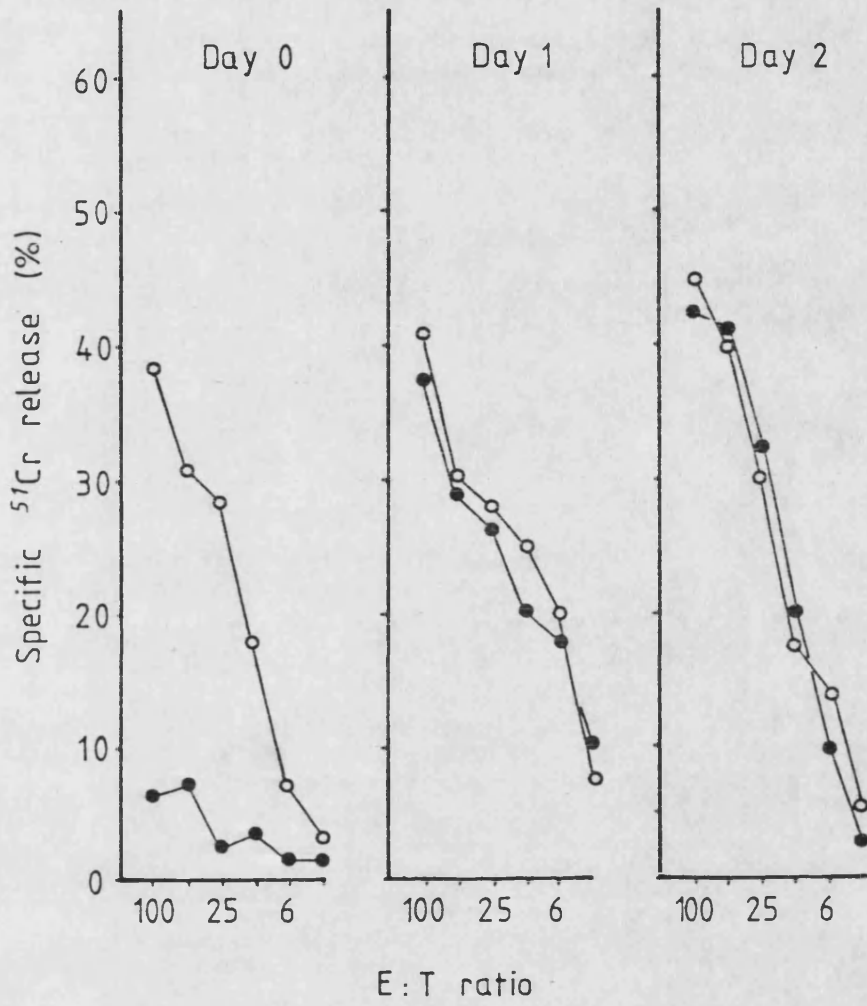
## Figure 5

A comparison of the fusion efficiencies obtained when different numbers of BW5147 and clone 96 ($1 \times 10^6$, $3 \times 10^6$, $1 \times 10^7$, $3 \times 10^7$, $1 \times 10^8$) were fused with a constant volume of PEG (0.5 ml). Each point of the limiting-dilution curves represents the fraction of cultures out of 24 without growing hybridomas assessed cumulatively over 4 weeks. Arrows indicate that all 24 cultures in the group were positive for growing hybridomas; these values are not included in the chi-squared analysis. The fusion frequencies (f) were determined by chi-squared analysis and the p values in all cases were <0.05. The results obtained from fusing $1 \times 10^6$ cells is not shown; in this case, all cultures were negative for growing hybridomas.
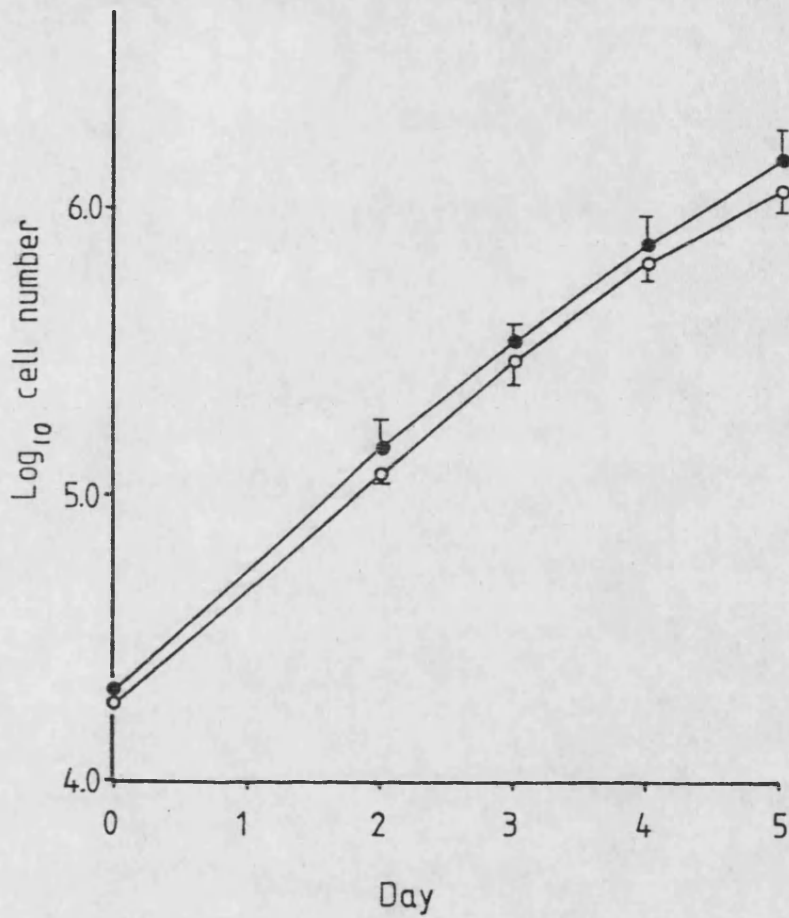
**Figure 6**
A comparison of the proliferation rates of untreated and trypsin-treated clone 96 cells. Several cultures of treated and untreated cells were initiated on day 0 with $2\times10^4$ cells in 1.0 ml. Cell numbers were assessed on successive days by trypan-blue exclusion assays. Error bars represent the standard deviations of 4 identical cultures. The rate of proliferation in both cases was approximately 1 division every 20 hours.
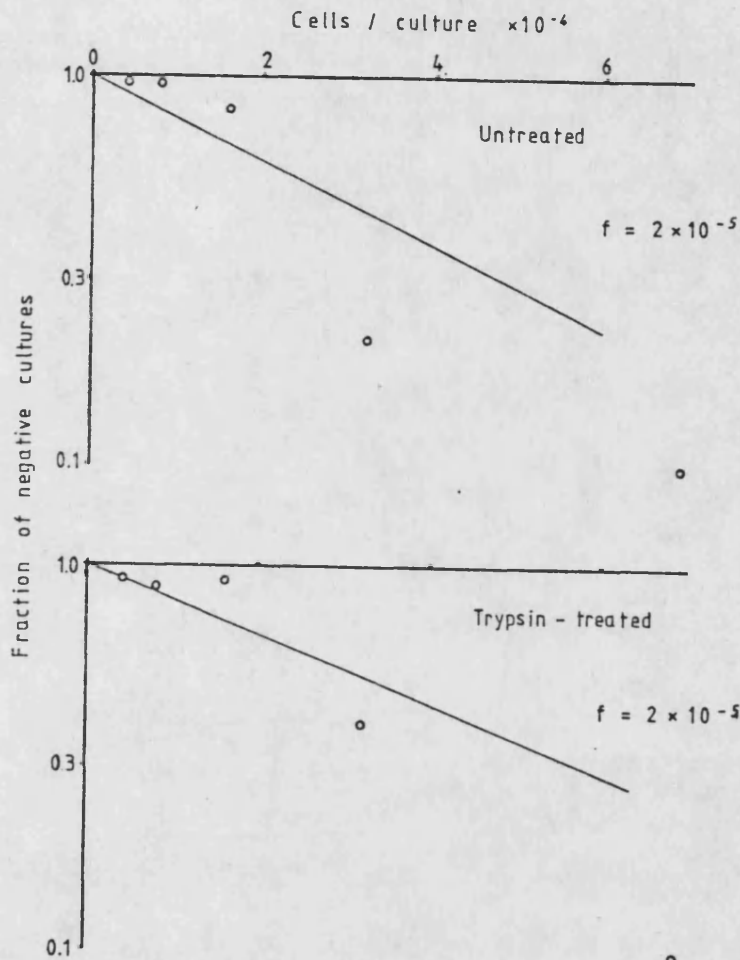
        o  untreated
        •  treated

**Figure 7**
A comparison of the lytic activity of untreated and trypsin-treated clone 96 cells against P815. Cells were treated on day 0 immediately prior to the lytic-assay. Cells were further cultured and retested for lytic-activity against P815 on days 1 and 2 after treatment.

o untreated
● treated

**Figure 8**
A comparison of the fusion efficiences of untreated and trypsin-treated clone
96 cells with BW5147. Trypsin-treatment was performed immediately prior to
fusion. Each point of the limiting-dilution curves represents the fraction of
cultures out of 24 without growing hybridomas assessed cumulatively over 4
weeks. Arrows indicate that all 24 cultures in the group were positive for
growing hybridomas; these value are not included in the chi-squared analysis.
The fusion frequencies (f) were determined by chi-squared analyisis and the p
values were <0.05 with untreated clone 96 cells and 0.08 with trypsin-treated
clone 96 cells.
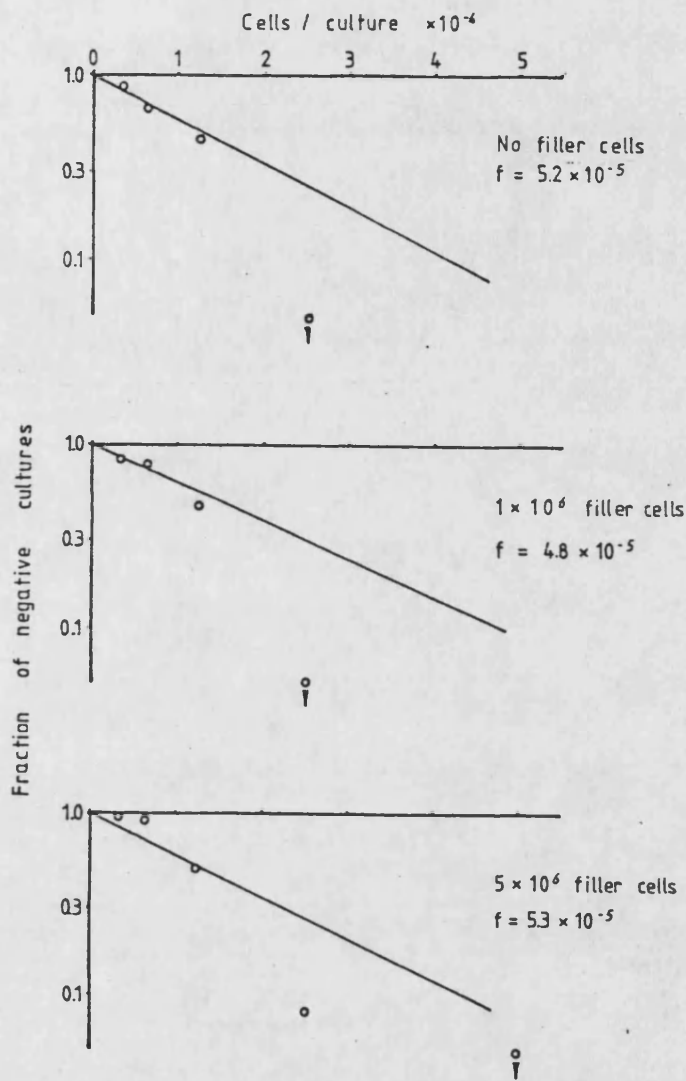
**Figure 9**

A comparison of the effect of the presence or absence of various numbers of feeder cells on the frequencies of growing hybridomas derived from a single fusion of clone 96 with BW5147 cells. Parental cells were fused and cultured under limiting-dilution conditions with either 0, $1 \times 10^6$ or $5 \times 10^6$ irradiated (C57BL/6 x AKR) $F_1$ spleen feeder cells per culture. Each point of the limiting-dilution curves represents the fraction of cultures out of 24 without growing hybridomas assessed cumulatively over 4 weeks. Arrows indicate that all 24 cultures in the group were positive for growing hybridomas; these values are not included in the chi-squared analysis. The fusion frequencies (f) were determined by chi-squared analysis and the p values were 0.73 (0 feeder cells), 0.8 ($1 \times 10^6$ feeder cells) and <0.05 ($5 \times 10^6$ feeder cells).
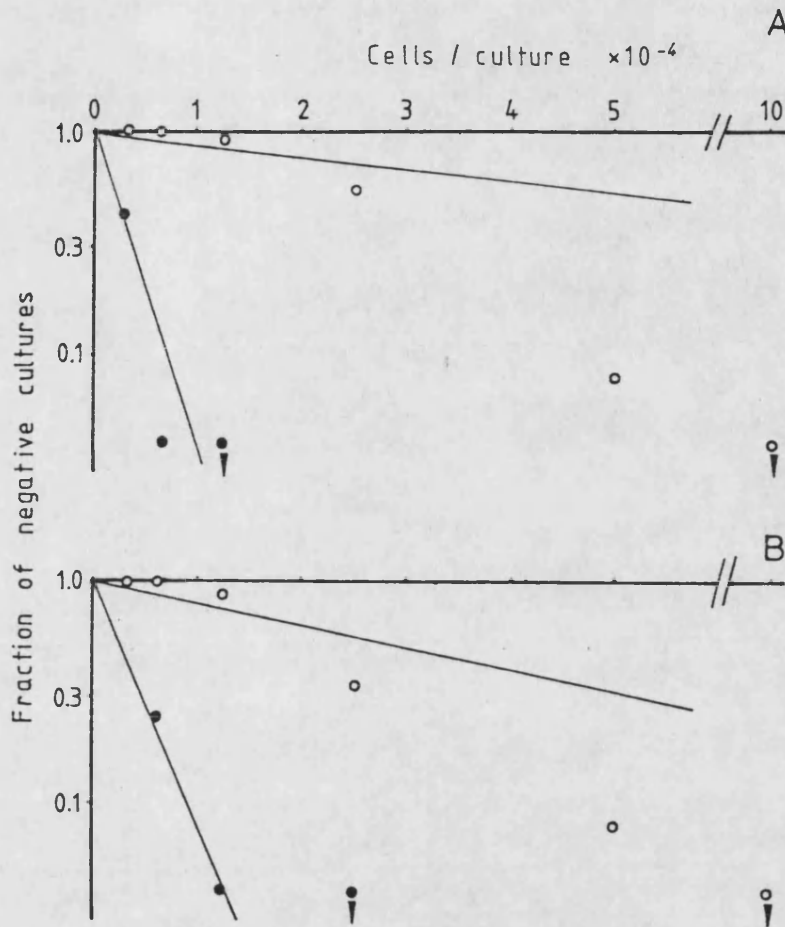
**Figure 10**

A comparison of the effect of the growth factors present in ConA-SN on the frequencies of growing hybridomas derived from single fusions of clone 96 with BW5147 cells. Parental cells were fused and cultured under limiting-dilution conditions either in the presence or absence of 10% ConA-SN and in the presence (panel B) or absence (panel A) of $5 \times 10^6$ irradiated (C57BL/6 x AKR)$F_1$ spleen feeder cells. Panels A and B represent the results of two independent fusions. Each point of the limiting-dilution curves represents the fraction of cultures out of 24 without growing hybridomas assessed cumulatively over 4 weeks. Arrows indicate that all 24 cultures in the group were positive for growing hybridomas; these values are not included in the chi-squared analysis. The fusion frequencies (f) were determined by chi-squared analysis and the p values were <0.05 in the absence of ConA-SN both with and without feeder cells, 0.01<p<0.25 in the presence of ConA-SN without feeder cells and 0.5<p<0.75 in the presence of both ConA-SN and feeder cells.

        o  without ConA-SN

        •  with ConA-SN.
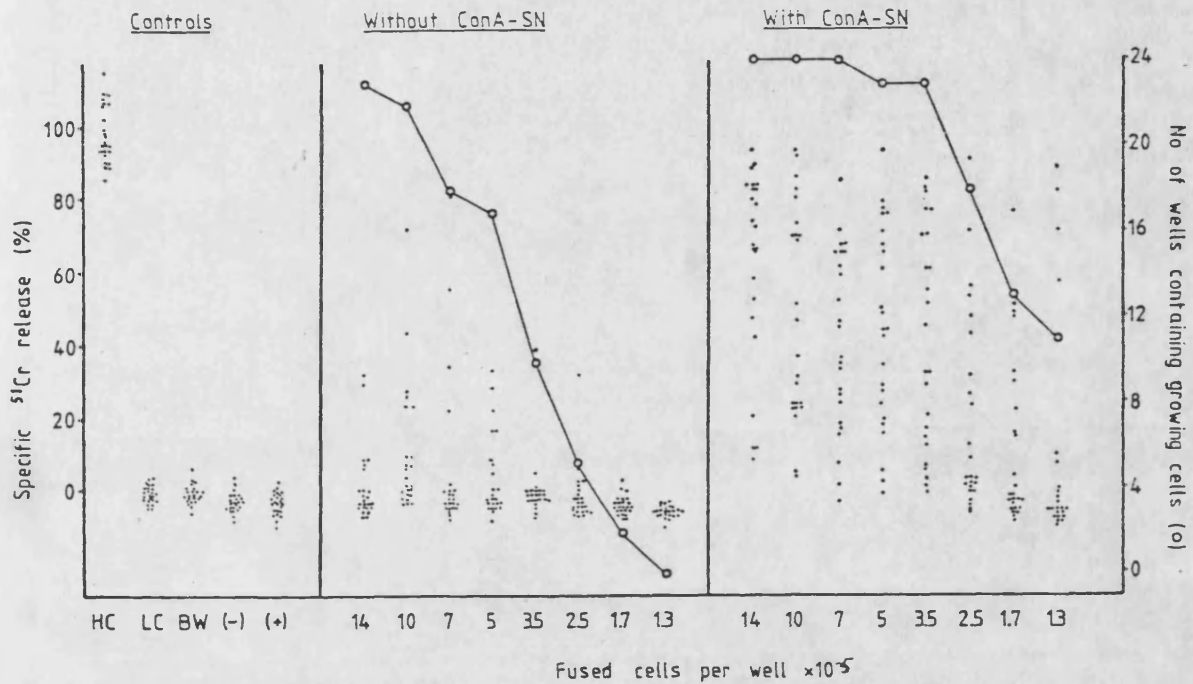
**Figure 11**

The lytic-activity and growth of hybridomas generated in a single fusion between day 4 MLC cells (reactive against H-Y) and BW5147. Parental cells were fused and cultured under limiting-dilution conditions with 24 cultures per group in the presence and absence of ConA-SN. Cultures were assayed for lytic-activity on day 13 after fusion; each dot represents the activity of each culture against P815 in the presence of PHA. The number of cultures with growing hybridomas out of each group of 24 was assessed cumulatively over 4 weeks both in the presence and absence of ConA-SN (o).

HC  High Control (total chromium-51 release)
LC  Low Control (spontaneous chromium-51 release)
BW  Lytic activity of BW5147 at an effector to target ratio of 50:1
     (24 assays)
(-)  $1\times10^5$ mock-fused parental cells cultured in the absence of
     ConA-SN (24 cultures)
(+)  $1\times10^5$ mock-fused parental cells cultured in the presence of
     ConA-SN (24 cultures)
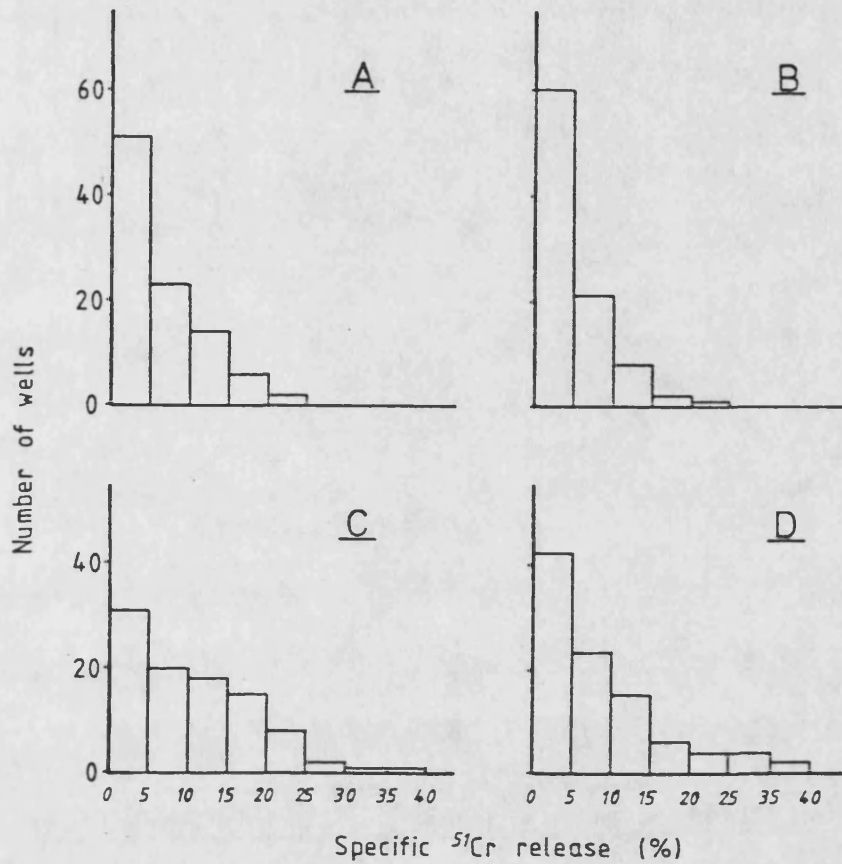
**Figure 12**
The lytic-activity of hybridomas generated in a single fusion between BT 7.4.1
and BW5147. Parental cells were fused and distributed into 96 cultures (2x10⁵
cells/culture; 1ml/culture) both in the presence (panels C and D) and absence
(panels A and B) of ConA-SN. Lytic-activity against P815 in the presence of
PHA was determined on days 12 (panels A and C) and 16 (panels B and D) after
fusion and is represented as histograms showing the number of cultures falling
within discrete ranges of lytic-activity.
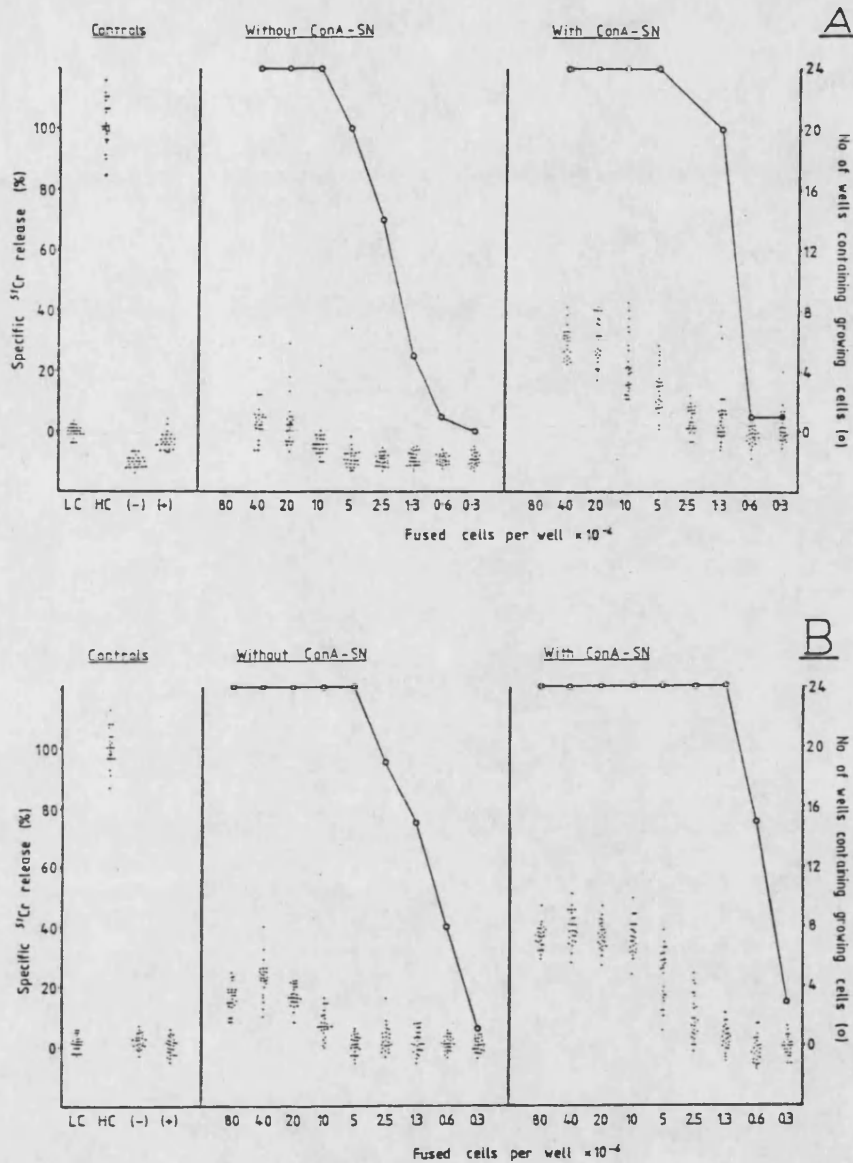
**Figure 13**

The lytic-activity and growth of hybridomas generated in fusions between clone 96 and BW5147. Parental cells were fused and cultured under limiting-dilution conditions with 24 cultures per group both in the presence and absence of ConA-SN.

Panel A shows the lytic activity of hybridomas against P815 on day 14 after fusion; each dot represents the lytic-activity of one culture.

Panel B shows the lytic-activity of hybridomas against P815 in the presence of PHA on day 10 after fusion; each dot represents the lytic-activity of one culture. The number of cultures with growing hybridomas out of each group of 24 was assessed cumulatively over 4 weeks both in the presence and absence of ConA-SN (o).
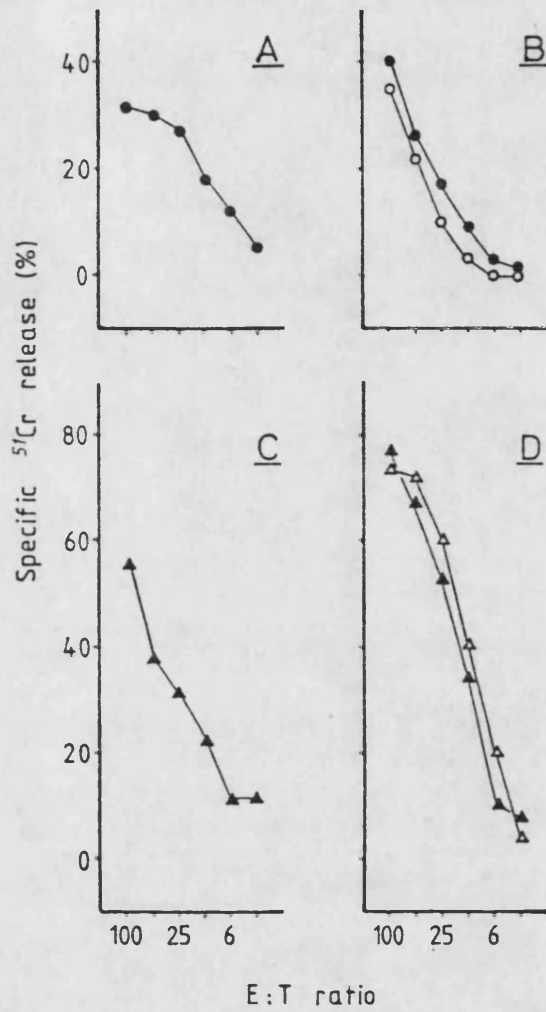
        HC  High Control (total chromium-51 release)

        LC  Low Control (spontaneous chromium-51 release)

        (-)  $1 \times 10^5$ mock fused parental cells cultured in the absence of ConA-SN (24 cultures)

        (+)  $1 \times 10^5$ mock fused parental cells cultured in the presence of ConA-SN (24 cultures)

Panels A and B represent two individual fusions.

**Figure 14**
The lectin-dependent activity of CTH clones is independent of ConA-SN.
Hybridoma clones 17B6 (panels A and B) and 22E1 (panels C and D) were tested
for lytic-activity against P815 in the presence of PHA on days 0 (panels A and
C) and 28 (panels B and D). Both clones were maintained in the presence of
ConA-SN prior to the experiment and were each cultured both in the presence
and absence of ConA-SN for the duration of the experiment.

    o    17B6  cultured without ConA-SN
    ●    17B6  cultured with ConA-SN
    △    22E1  cultured without ConA-SN
    ▲    22E1  cultured with ConA-SN
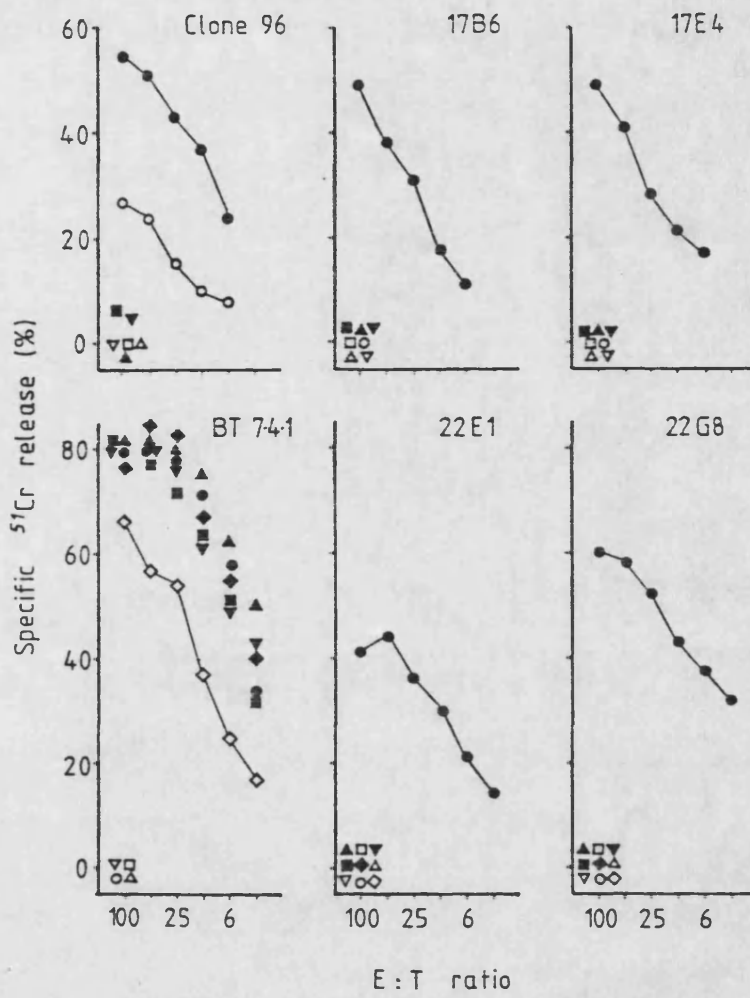
**Figure 15**
The lytic-activity of parental cells and CTH clones against various tumour-targets in the presence and absence of PHA.

□   RBL-5 (H-2$^b$)
■   RBL-5 + PHA
◇   TNP-modified RBL-5
◆   TNP-modified RBL-5 + PHA
△   EL-4 (H-2$^b$)
▲   EL-4 + PHA
○   P815 (H-2$^d$)
●   P815 + PHA
▽   RDM4 (H-2$^K$)
▼   RDM4 + PHA
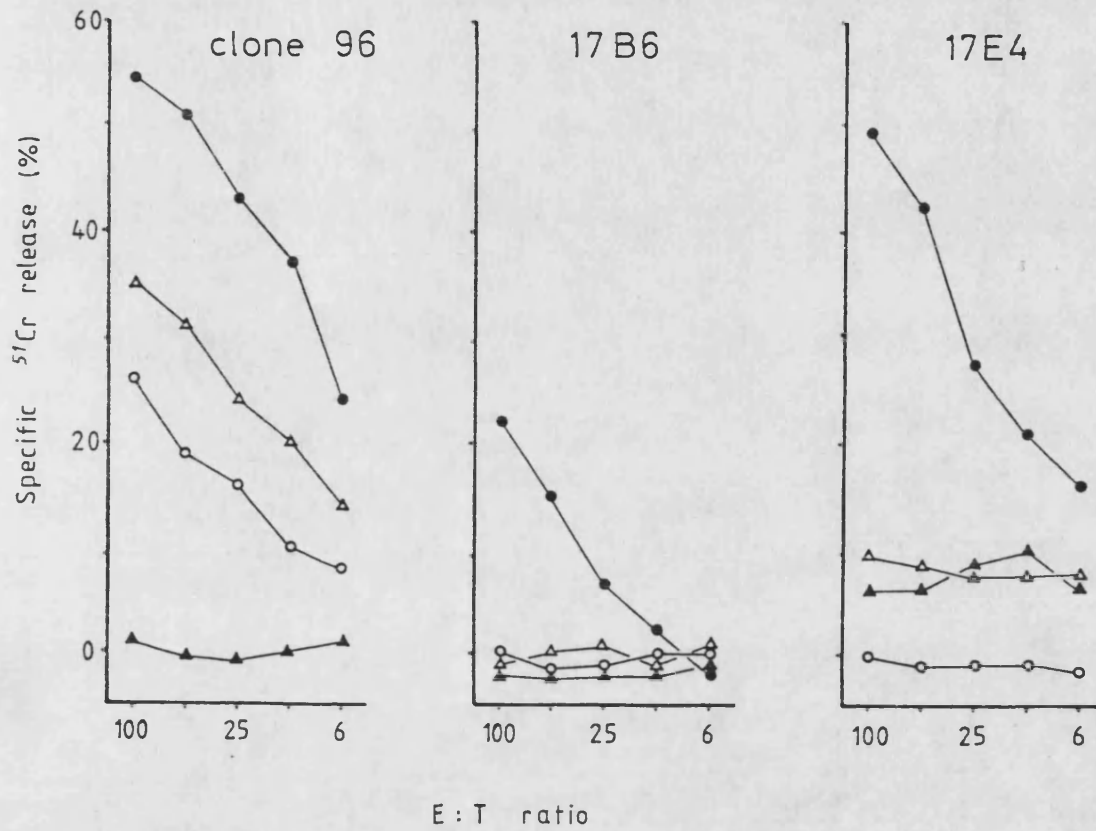
**Figure 16**
The lytic-activity of clone 96 and two CTH clones against P815 in the presence and absence of various lectins
- o  P815
- ●  P815 + PHA
- △  P815 + ConA
- ▲  P815 + SBA
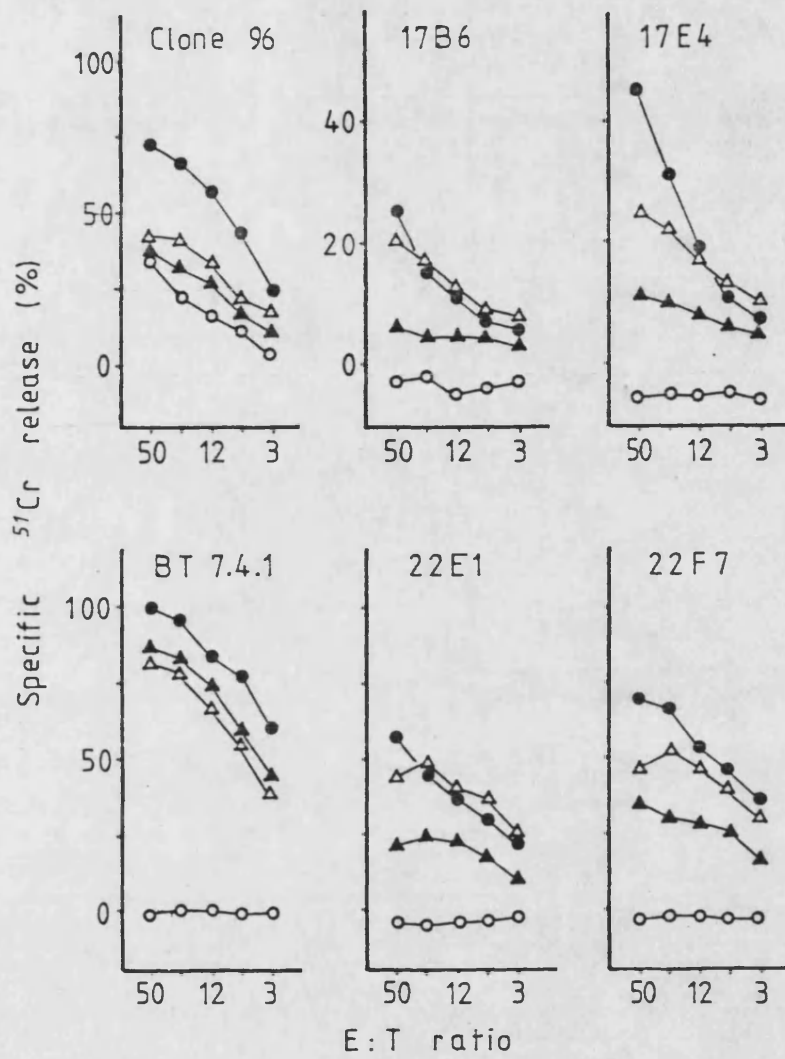
**Figure 17**
The lytic-activity of BT 7.4.1, clone 96 and various CTH clones against P815
when either effector cells, target cells, or both are incubated with PHA prior
to assay.

       o     Effectors + P815
       •     Effectors + (P815/PHA)
       △     (Effectors/PHA) + P815
       ▲     (Effectors/PHA) + (P815/PHA)
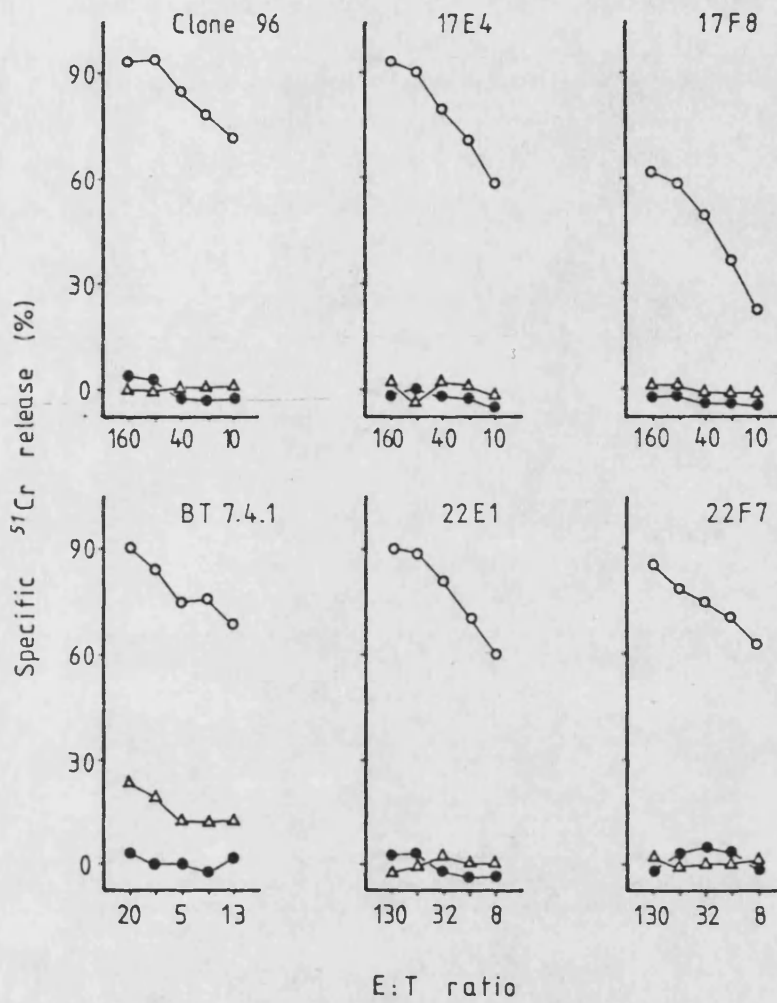
Figure 18
The effect of temperature and EDTA on the lytic-activity of BT 7.4.1, clone
96 and various CTH clones against P815 in the presence of PHA
     o    37°C
     ●    21°C
     △    37°C + EDTA
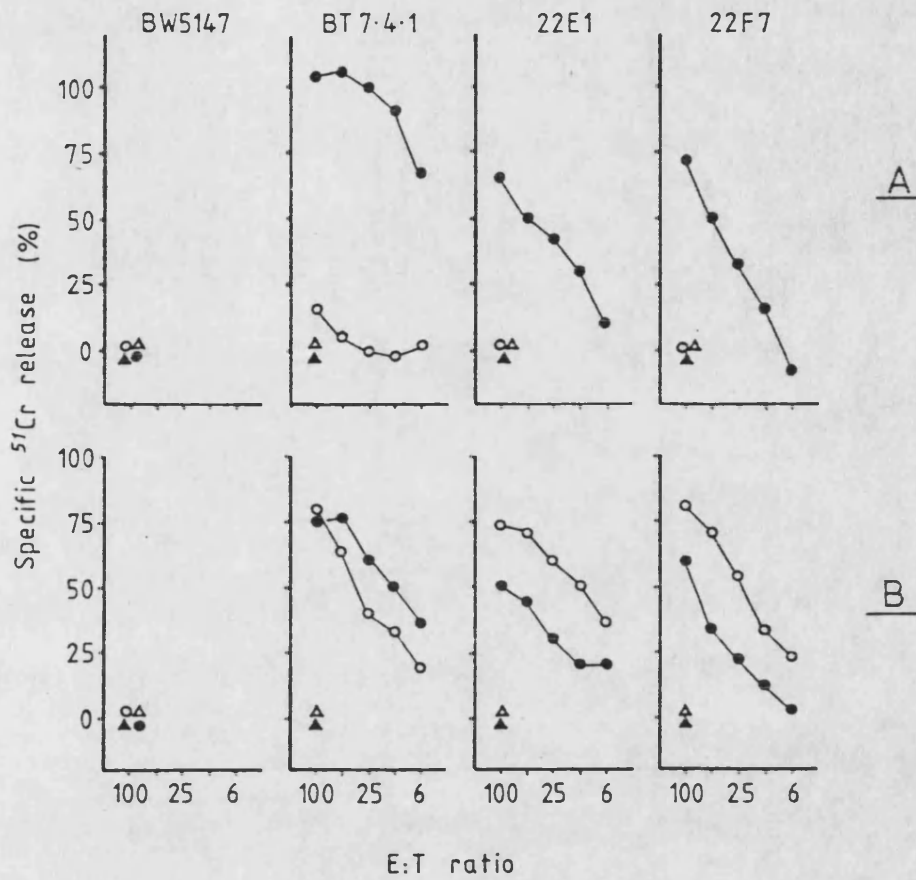
**Figure 19**
The lytic-activity of supernatants derived from BW5147, BT 7.4.1 and CTH clones against P815. Effector cells were incubated for 4 hours in the presence (panel B) or absence (panel A) of PHA. Both cells and supernatants derived therefrom were then assayed against P815 in the presence and absence of PHA.

      o    cells + P815
      ●    cells + P815/PHA
      △    supernatant + P815
      ▲    supernatant + P815/PHA
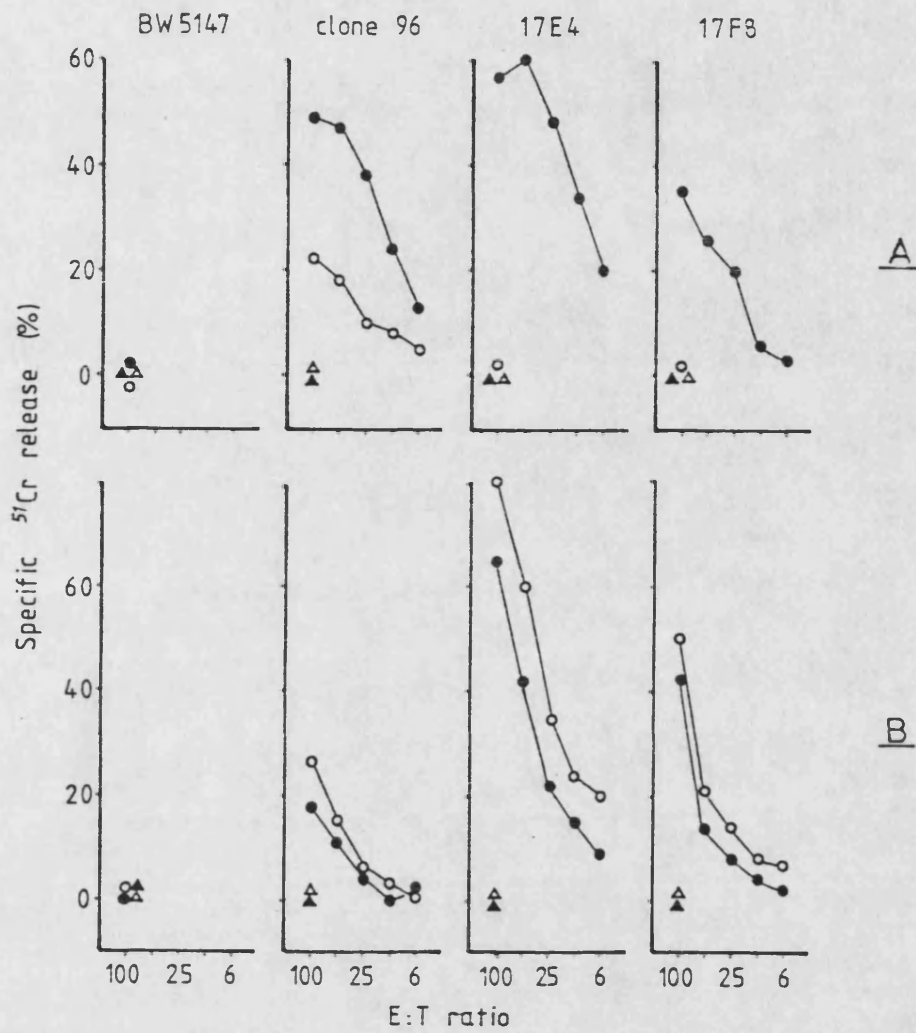
**Figure 20**
The lytic activity of supernatants derived from BW5147, clone 96 and CTH clones against P815. Effector cells were incubated for 4 hours in the presence (panel B) or absence (panel A) of PHA. Both cells and supernatants derived therefrom were then assayed against P815 in the presence and absence of PHA

    o     cells + P815
    ●     cells + P815/PHA
    △     supernatant + P815
    ▲     supernatant + P815/PHA
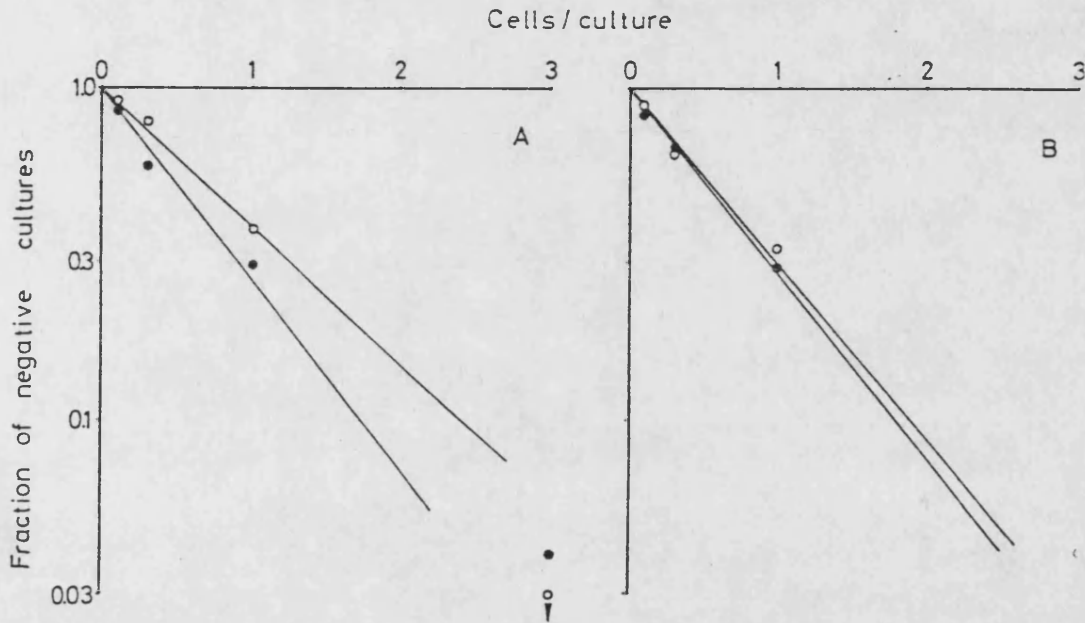
**Figure 21**

The cloning efficiency of P815 cells in the presence and absence of PHA and the presence (panel B) or absence (panel A) of irradiated DBA/2 spleen feeder cells (1.3x $10^4$ feeder cells per 200 μl culture). Each point of the limiting dilution curves represents the fraction of cultures out of 24 without growing P815 cells assessed cumulatively over 4 weeks. Arrows indicate that all 24 cultures in the group were positive for growing cells; these values are not included in the chi-squared analysis. The frequencies (determined by chi-squared analysis) are:-

| | | | | |
|---|---|---|---|---|
| Panel A | o | without PHA | f= 1.03 | p= 0.76 |
| | ● | with    PHA | f= 0.76 | p= 0.57 |
| Panel B | o | without PHA | f= 0.8 | p= 0.7 |
| | ● | with    PHA | f= 0.79 | p= 0.72 |
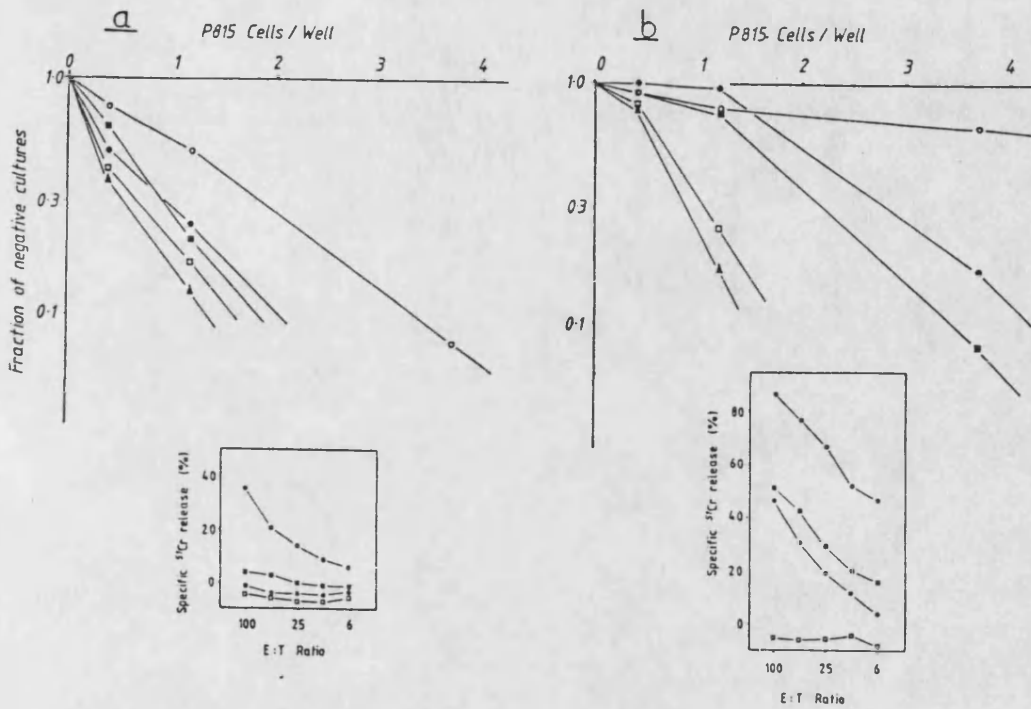
**Figure 22**

The release of chromium-51 in a 4 hr cytolytic assay of clone 96 and cytolytic hybridomas against P815 in the presence and absence of PHA correlates with a reduction in the viability of the target cell.

(i)    Chromium-51 release assay. Effector cells were irradiated to 6000 Rads and then tested in a chromium-51 release assay for activity against P815 (panel a insert) or P815 + PHA (panel b insert).

(ii)   Limiting dilution assay. Effector cells were irradiated to 6000 Rads and then incubated with P815 targets for 4 hrs. in the presence (b) or absence (a) of PHA at a ratio of 100 effectors per target. The conditions were exactly the same as for the chromium release assay except that the targets were not labelled with chromium. After incubation, the P815 cells were assayed for viability by limiting-dilution analysis. Each point of the limiting-dilution curves represents the fraction of cultures out of 24 without growing P815 cells assessed cumulatively over 4 weeks. Fusion frequencies were determined by chi-squared analysis.

| Effector cells | Symbol | Frequency of viable P815 cells | |
|---|---|---|---|
| | | without PHA (panel a) | with PHA (panel b) |
| BW5147 | □ | 1.7 (0.25<p<0.5) | 0.9 (0.5<p<0.1) |
| Clone 96 | o | 0.6 (p=0.89) | 0.14 (p=0.49) |
| 17E4 | ● | 1.4 (0.25<p<0.5) | 0.2 (p<0.05) |
| 17F8 | ■ | 1.3 (0.75<p<0.9) | 0.4 (p=0.07) |
| No effectors | ▲ | 2.0 (0.25<p<0.5) | 1.1 (0.05<p<0.1) |
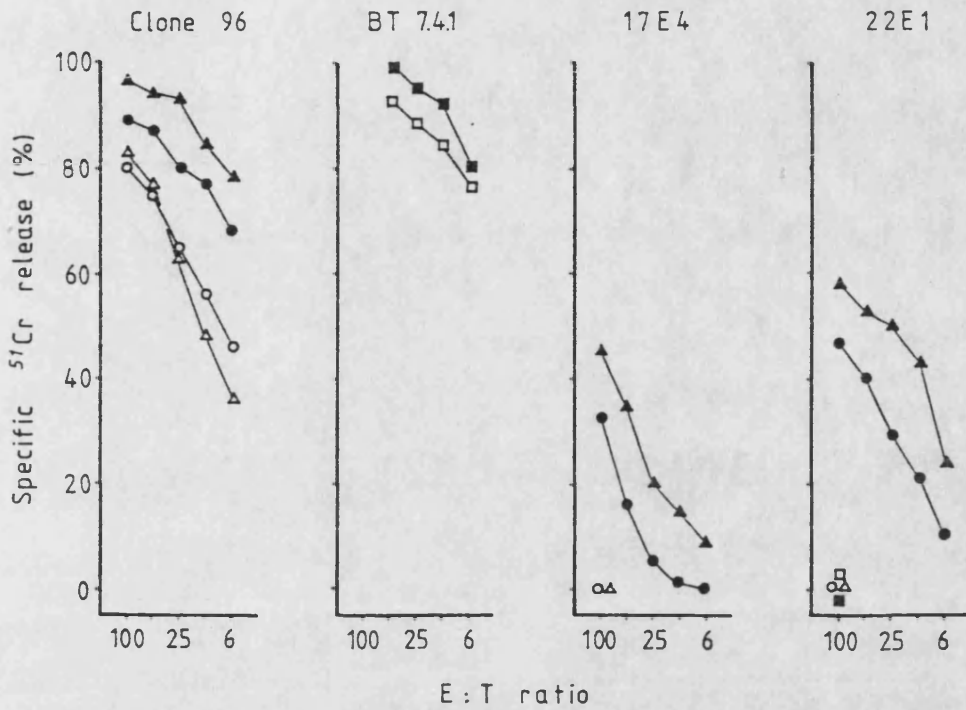
**Figure 23**
Treatment of target cells with neuraminidase does not reveal antigen-specific
(lectin-independent) lytic-activity in CTH. BT 7.4.1, clone 96 and two CTH
clones were tested for lytic-activity against untreated and neuraminidase-
treated P815 and TNP-modified RBL-5 target cells both in the presence and
absence of PHA.

        o   P815
        ●   P815 + PHA
        △   Neuraminidase treated P815
        ▲   Neuraminidase treated P815 + PHA
        □   RBL-5/TNP
        ■   Neuraminidase treated RBL-5/TNP
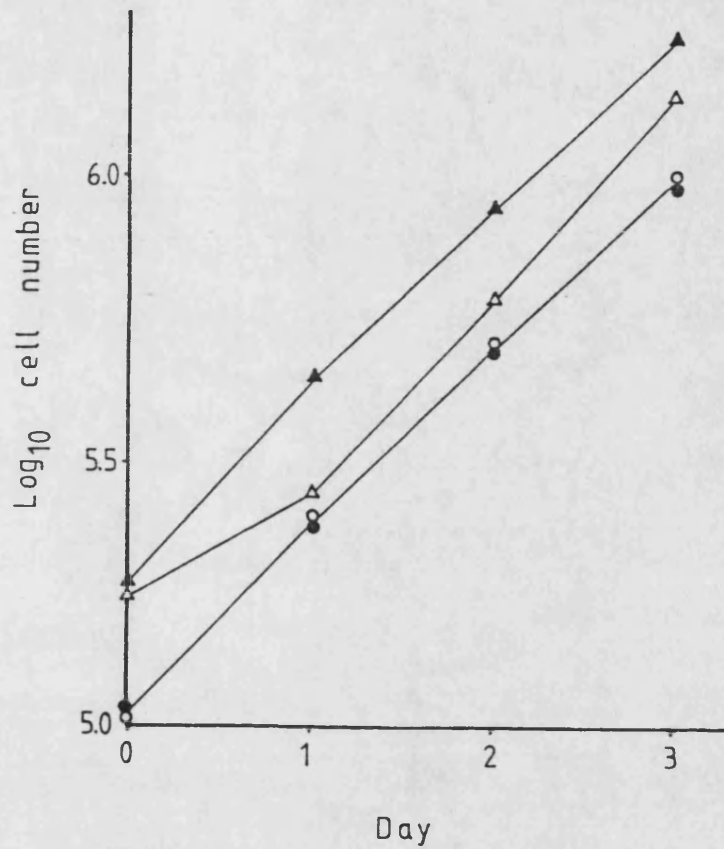
**Figure 24**

The proliferation rates of uncloned hybridomas generated between MLC cells (C57BL/6 ♀ against C57BL/6 ♂ ) and BW5147 in the presence and absence of ConA-SN. Several cultures were initiated on day 0 with either $1 \times 10^5$ or $1.8 \times 10^5$ cells in 1ml cultures. Cell numbers were assessed on successive days by trypan-blue exclusion assays.

| Symbol | Group No[1] | Development[1] | Subculture[1] |
|--------|---------|-------------|-------------|
| o | 1 | - ConA-SN | - ConA-SN |
| ● | 2 | - ConA-SN | + ConA-SN |
| △ | 3 | + ConA-SN | - ConA-SN |
| ▲ | 4 | + ConA-SN | + ConA-SN |

1) See text, section 3.7.2
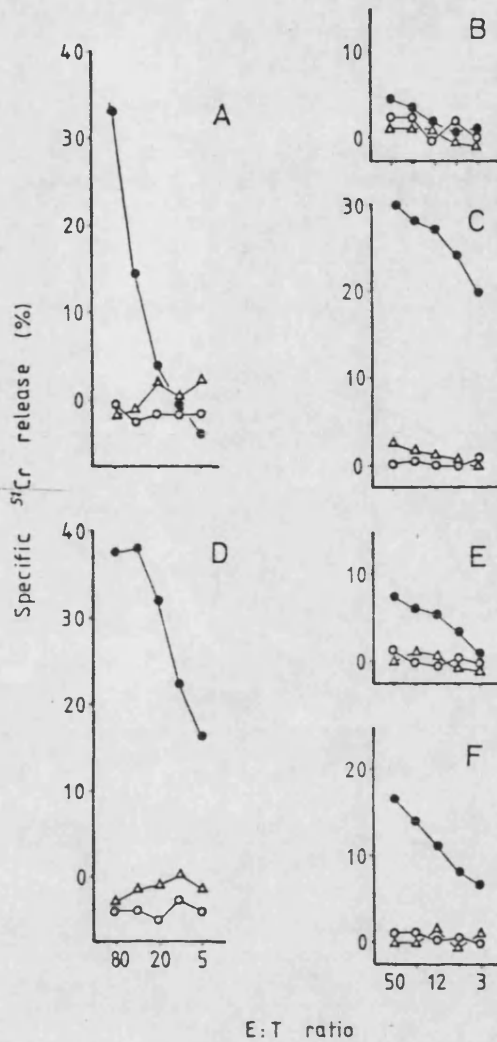
**Figure 25**
The lytic-activity of uncloned hybridoma populations generated between MLC
cells (C57BL/6 ♀ against C57BL/6 ♂) and BW5147 cultured in the presence
and absence of ConA-SN. Parental cells were fused and the hybridomas
cultured for 23 days in the presence (panel D) or absence (panel A) of ConA-SN
prior to the initial assays for lytic-activity against various targets.
Hybridomas were then transferred into new cultures both with (panels C and F)
and without (panel B and E) ConA-SN and subcultured for a further 3 weeks
before reassaying on the same target cells.

| Panel | Group No[1] | Development[1] | Subculture[1] |
|---|---|---|---|
| A | | - ConA-SN | |
| B | | + ConA-SN | |
| C | 1 | - ConA-SN | - ConA-SN |
| D | 2 | - ConA-SN | + ConA-SN |
| E | 3 | + ConA-SN | - ConA-SN |
| F | 4 | + ConA-SN | + ConA-SN |

1) See text, section 3.7.2

    o  P815
    ●  P815 + PHA
    ▲  ♂C57BL/6 ConA blasts.
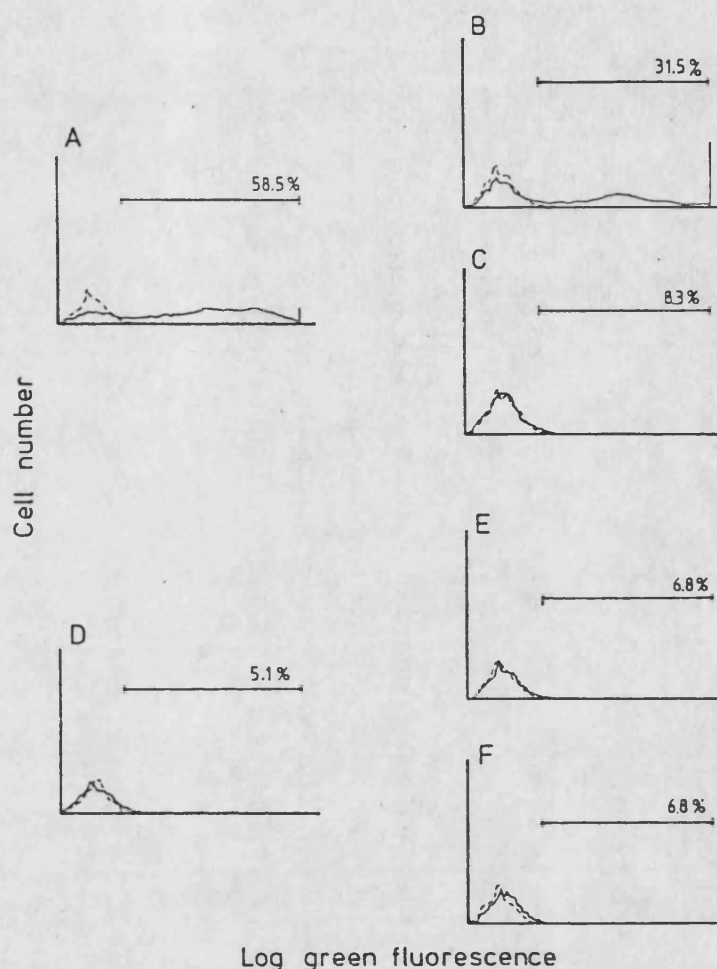
**Figure 26**
The expression of Lyt-2 on uncloned hybridoma populations generated between MLC cells (C57BL/6 ♀ against C57BL/6 ♂) and BW5147 cultured in the presence and absence of ConA-SN. Parental cells were fused and the hybridomas cultured for 23 days in the presence (panel D) or absence (panel A) of ConA-SN prior to analysis of Lyt-2 expression by flow-cytometry. Hybridomas were then transferred into new cultures both with (panels C and F) and without (panels B and E) ConA-SN and subcultured for a further 3 weeks before retesting for Lyt-2 expression. Figures refer to the percentage of cells staining positive compared to negative controls stained only with FITC-goat-anti-rat immunoglobulin i.e. falling within the region depicted by the horizontal bar.

        ----- control
        ——— Rat-anti-mouse Lyt-2

Full details of the antibodies used are given in the materials and methods (section 2.4.3)

## Figure 27

The expression of TCR and Lyt-2 on the surface of BW5147, BT 7.4.1 and both lytic (22E1) and non-lytic (16C3) CTH clones. Cells were surface-labelled with iodine-125, lysed, and the total lysate separated by 2-dimensional, reduced/non-reduced, SDS-PAGE. The origin of each gel is in the top left corner. The first dimension (non-reduced) was run horizontally and the second dimension (reduced) was run vertically

   a  BW5147

   b  BT 7.4.1

   c  16C3

   d  22E1

MW x 10⁻³
94–
67–
43–
30–
20–

MW x 10⁻³
94–
67–
43–
30–
20–
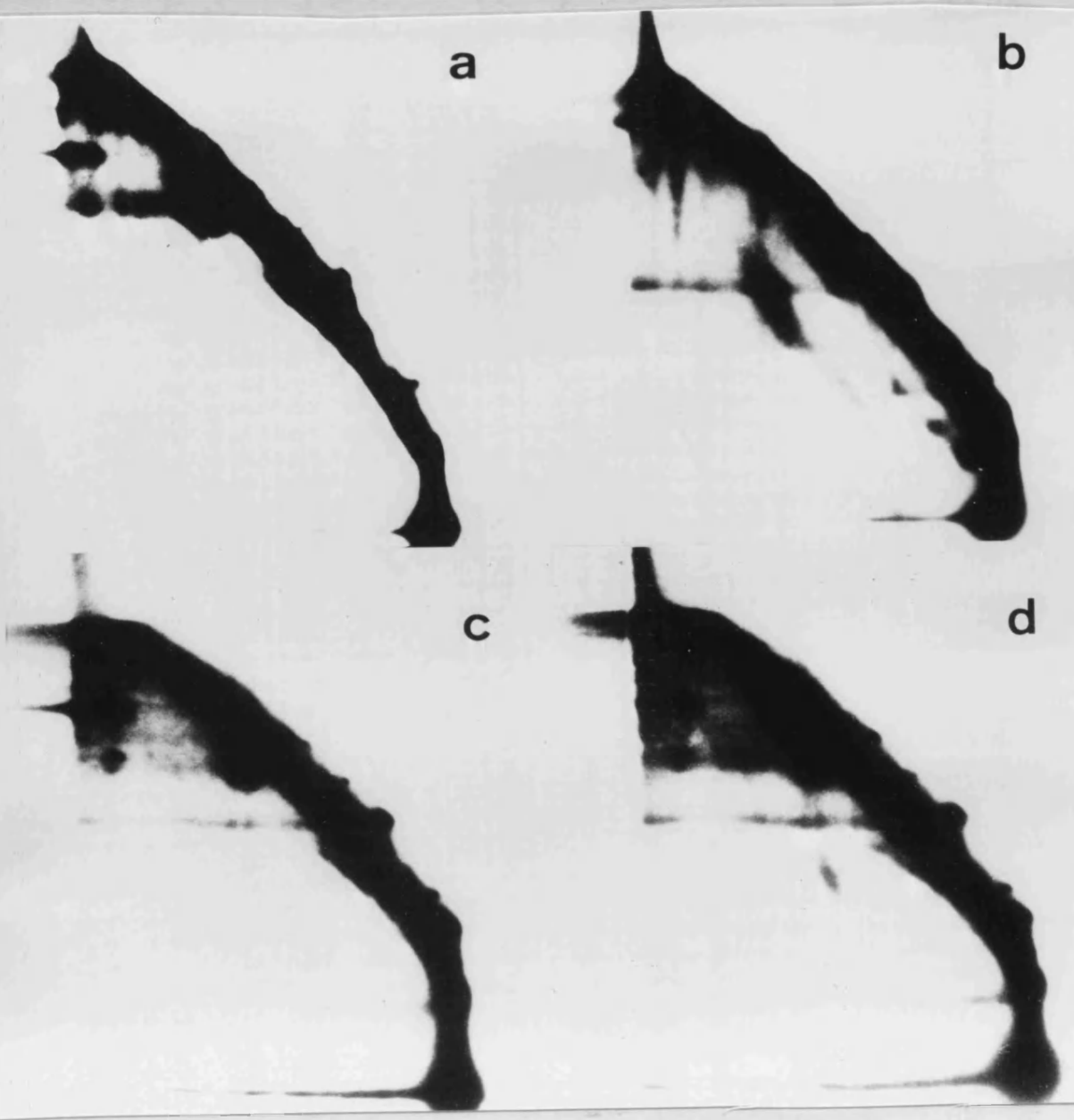
**Figure 28**

The expression of TCR and Lyt-2 on the surface of BW5147, clone 96 and both lytic (17E4) and non-lytic (19 A1) CTH clones. Cells were suface-labelled with iodine-125, lysed, and the total lysate separated by 2-dimensional, reduced/non-reduced, SDS-PAGE. The origin of each gel is in the top left corner. The first dimension (non-reduced) was run horizontally and the second dimension (reduced) was run vertically.

      a    BW5147

      b    clone 96

      c    19A1

      d    17E4
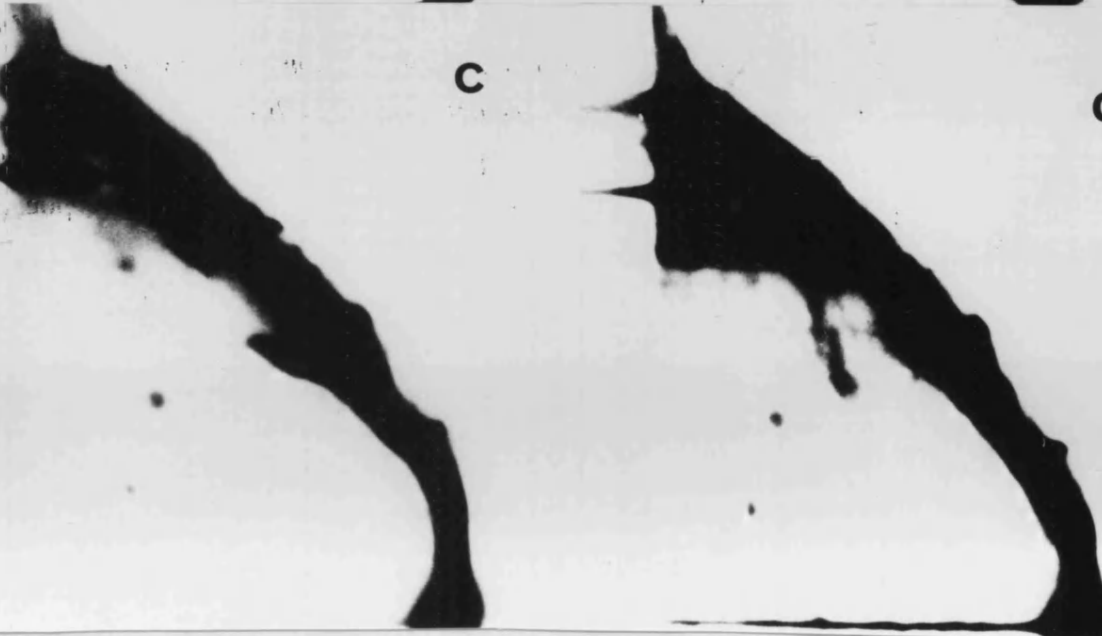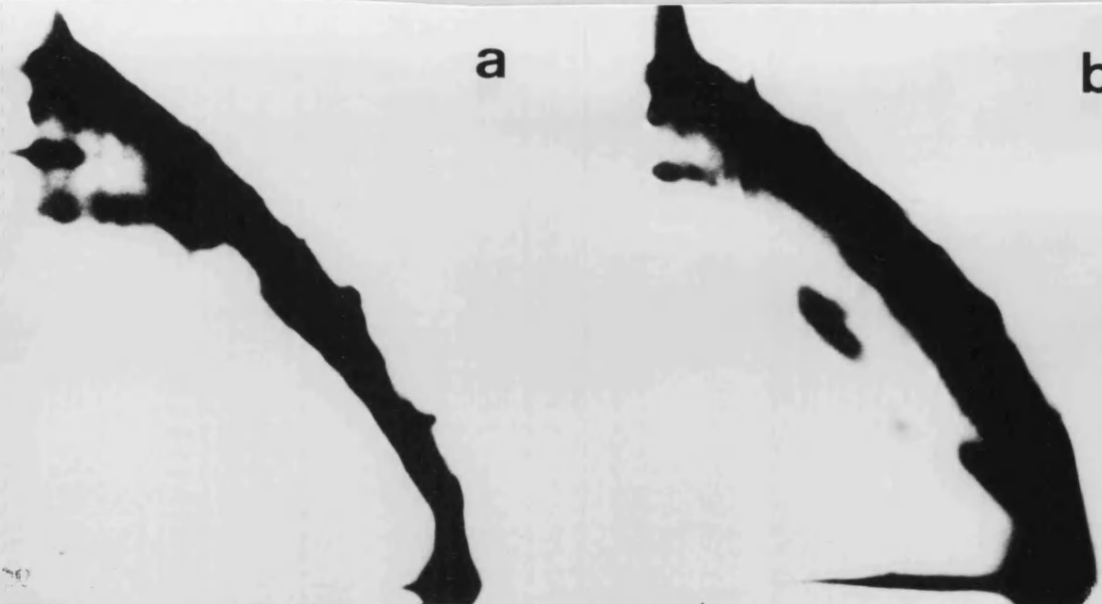
Figure 29

Northern blot hybridisation of total cellular RNA from clone 96
(lane B), 13E6K1 (lane C, a $V_{\beta AK}$ positive control cell line), 19B8
(lane D), 17E4 (lane E), 19A6 (lane F), 17B6 (lane G) and BW5147
(lane H) with $C_{\beta 1}$, $C_{\beta 2}$ and $V_{\beta AK}$ probes. Lane A contains poly A
RNA from clone 96. See materials and methods (section 2.4.5) for
practical details.

In the figure, TCR $\beta$ chain mRNA runs approximately 1.0cm below the
18S mark. Extra bands appearing at 28S and 18S result from
non-specific hybridisation of oligonucleotide probes with ribosomal
RNA. This is due to insufficient washing of the gel after
hybridisation.
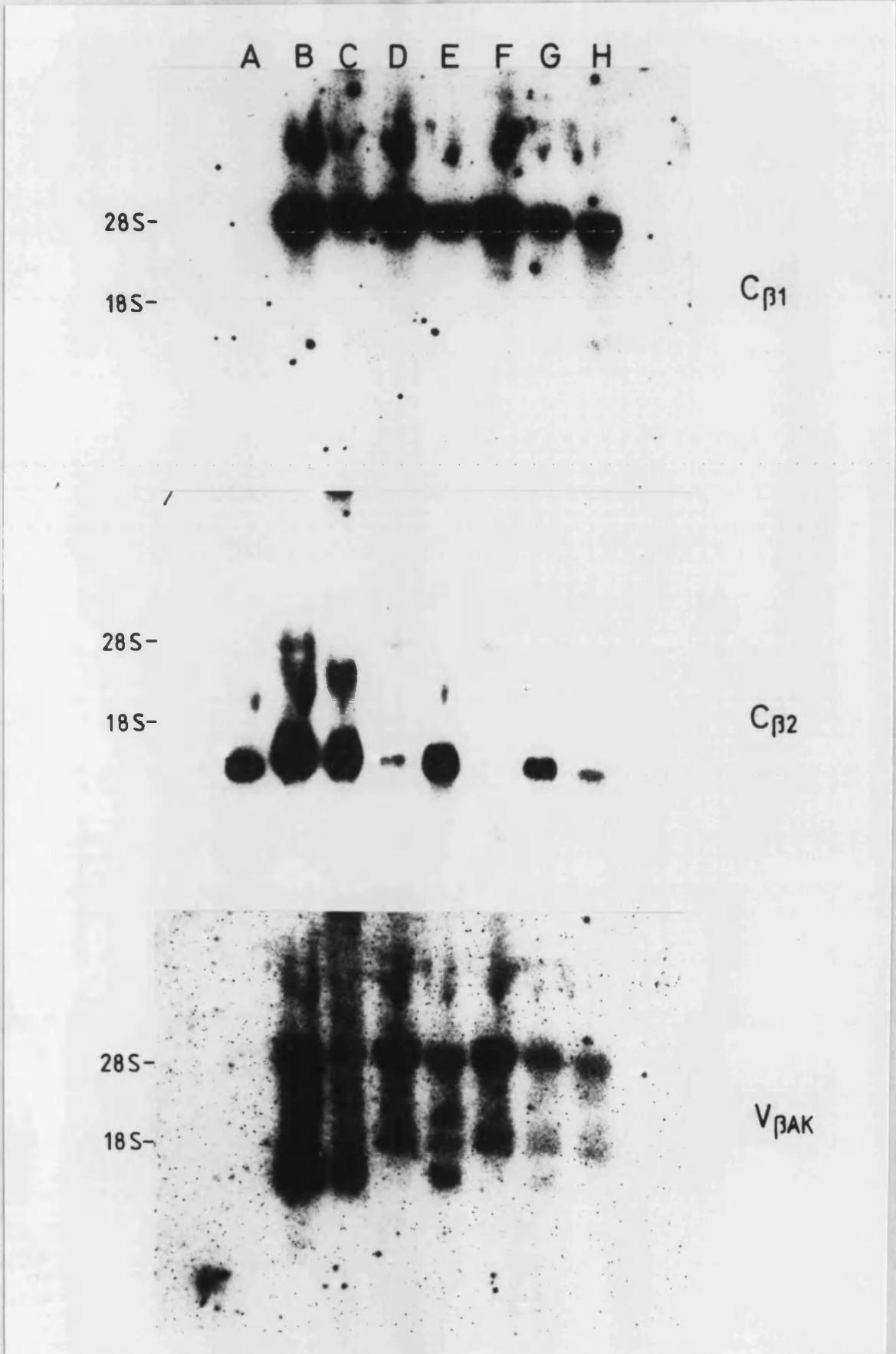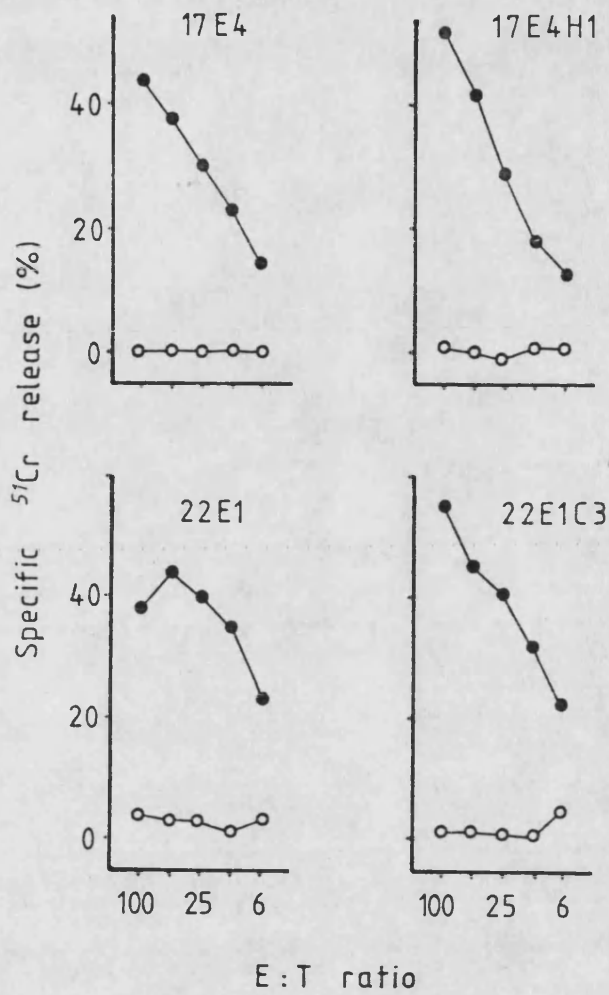
**Figure 30**

The lytic-activity of normal and HAT-sensitive CTH clones against P815. HAT-sensitive subclones are 17E4H1 (derived from 17E4) and 22E1C3 (derived from 22E1).

o    P815
●    P815 + PHA

# 4. DISCUSSION

## 4.1 Aims of the work: generation of cytolytic hybridomas with antigen-specificity

The major aim of the work presented in this thesis was the development of antigen-specific CTH which could be used for structural, functional and genetic studies. However, the difficulties encountered by other workers in this field were fully appreciated (see section 1.7 for review) and the work was therefore approached with a view to analyse why the cells generated in CTL fusions failed to lyse their specific targets. This question is of relevance because, if it could be determined exactly in which respects CTH are deficient, it may provide insights into the nature of the CTL lytic-mechanism which is, to date, poorly understood. Moreover, if the cause of dysfunction in CTH is identified, it may be possible to develop techniques for the routine generation of lytic hybridomas which would be invaluable for the study of CTL in general.

At present, most studies aimed at understanding the cytolytic-mechanism of CTL have been performed with cloned CTL. However, such cells are not optimal for such work for several reasons;

(i) Cloned CTL tend to be relatively unstable in that they rapidly lose their antigen-specificity and lytic-activity and in many cases, other activities arise such as NK or AK activity (Grim et.al., 1982; Binz et.al., 1983; Brooks, 1983; Acha-Orbea et.al., 1983; Moretta et.al., 1984; Simon et.al., 1984; Wilson and Shortman, 1985).

(ii) Cloned CTL require the continuous presence of both antigen presented on irradiated cells and of the growth factor, IL-2, for the

maintenance of antigen-specific lytic-activity, The presence of large numbers of dead cells in the system can prove problematic, particularly in biochemical studies, and necessitates that CTL are purified prior to study.

(iii)   Cloned CTL tend to grow slowly and adherently and often require culture conditions which make the production of large quantities of homogenous material for molecular and genetic studies difficult and expensive. For example, the cloned CTL used in this report, BT 7.4.1, has to be cultured in 3 ml tissue-culture wells; culture of these cells in flasks results in the rapid generation of non-lytic cells or cells with AK activity.

(iv)   Cloned CTL can thus far only be cultured after cells have been kept for several weeks as a line i.e. after a period of uncontrolled selection for unknown properties related to the adaptation to growth in tissue-culture.

(v)   The generation of large panels of cloned CTL with either widely differing or with similar and related specificities is problematic.

Theoretically at least, CTH should prove to be superior to cloned CTL for investigations into the lytic-mechanism on several grounds. Firstly, CTH would grow quickly and under convenient culture conditions to high densities allowing the rapid generation of large numbers of cells. Secondly, hybridomas would proliferate and maintain their specific lytic-activity independent of cellular antigen and/or growth factors and would thus provide a source of clean material. And finally, it would be possible to generate large panels of CTH simply by screening large numbers of fusion products as has been shown with B cells and to some extent for T helper cells (Melchers et al., 1978; von Boehmer et al., 1982)

CTH would also have some potential disadvantages for the study of the lytic-mechanism due to the presence and influence of the BW5147 genome. In addition, the fusion procedure itself may be selective in that particular subsets of cells may fuse preferentially.

## 4.2    Reasons for the loss of antigen-specificity in CTH

This work has shown that the loss of antigen-specificity is a constant feature of hybridomas generated between tumour cells and CTL but the precise reasons behind this loss remain unclear. There are several possibilities which depend on whether a total loss of lytic-activity is occuring or whether the lytic-activity is retained and only the antigen-specificity is lost. These can be summarised as follows:-

(i)    Technical reasons related to the fusion procedure:

- preferential fusion of non-lytic cells.

- lysis of the tumour partner during the fusion event.

(ii)    The loss of molecules involved in the lytic-mechanism:

- loss of activated genes due to chromosome loss (Grützmann and Hämmerling, 1978).

- suppression of activated genes by the tumour cell genome.

- reduced or lack of expression of gene products on the cell surface.    —

Such molecules would include the polyperforins, TCR, Lyt-2, LFA-1 and other molecules involved in the lytic-mechanism but not yet described.

(iii)    Alterations in the specificity of the TCR:

- mixing of α and ß chains from both parental cells.

- structural alteration of the TCR genes due to the lack of selection for specific recognition.

In order to analyse each of these points in detail, fusions were performed between BW5147 and three types of CTL; namely CTL generated in MLC cultures and two types of cloned CTL which are routinely cultured in this laboratory. Experiments were performed either on freshly prepared hybridoma populations or on hybridoma clones derived therefrom. The results of this work are discussed in the context of why CTH lose their antigen-specificity. Several possible reasons could be experimentally excluded, others are still possible.

### 4.2.1 Reasons that have been excluded for the loss of antigen-specific lytic-activity

### 4.2.1 (i) Lysis of the tumour partner by the CTL

Köhler and colleagues, 1977, first proposed that CTL non-specifically lysed the tumour partner during the intimate cell contact required for the fusion event. To circumvent this proposed difficulty, Kaufmann and colleagues, 1981a,b, transiently inactivated their CTL with trypsin prior to fusion and successfully generated hybridomas with a non-classical, but nevertheless antigen-specific lytic-activity. However, they did not comment on whether similar lytic hybridomas could be generated if the trypsin inactivation was omitted prior to fusion. The experiments presented in this report (section 3.2.4) with both MLC cells and clone 96 demonstrate that trypsinisation neither increases the fusion frequency (which would be expected if CTL could now successfully fuse) nor facilitates the generation of antigen-specific CTH. These results do not exclude the possibility that lysis of the tumour partner actually occurs during the normal fusion event but they do show that, if it occurs, it is not the primary reason for the difficulties encountered in

generating antigen-specific CTH. These results are supported by the fact that two laboratories have sucessfully generated antigen-specific CTH in the absence of a trypsin digestion of the CTL prior to fusion (Nabholz et.al., 1980; Haas et.al., 1985b,c).

## 4.2.1 (ii) Preferential fusion of non-lytic cells

Populations of activated T cells derived from MLCs contain both lytic and non-lytic cell types and it is possible that only the non-lytic populations of cells fuse to BW5147. Thus, fusions between the CTL clone BT 7.4.1 and BW5147 were performed with the intention of demonstrating that CTL are capable of undergoing fusion. It was assumed that every cell in a culture of BT 7.4.1 was lytic and that the generation of hybridomas from such a fusion must result from the fusing of a functional CTL with BW5147. But it was later demonstrated by Weltzien and Eichmann (in press) that even cloned CTL populations are heterogenous and contain non-lytic variants. Thus, the possibility remained that non-lytic variants were preferentially fusing with BW5147. However, in each of these fusions, hybridomas were recovered that were capable of a non-specific lytic-activity against P815 in the presence of the lectin PHA. This clearly demonstrates that CTL are able to fuse with BW5147.

## 4.2.1 (iii) Total or partial loss of the lytic mechanism

One possible explanation for the loss of antigen-specific lytic-activity in CTH is that there is always a total or partial loss of the lytic-mechanism.The finding that hybridomas are readily generated which retain a lectin-dependent

lytic-activity excludes the possibility that there is a total loss of the lytic-machinery. However, the fact that the lectin-dependent cytolytic-activity of the hybridomas, in contrast to that of the parental CTL, is largely restricted to P815 target cells may be interpreted as an indication that the cytolytic apparatus of the hybridomas is deficient in some way. For example, it cannot be excluded that a certain component of the polyperforin system is missing leading to an impaired lytic-activity that suffices for the lysis of highly sensitive P815 cells but not for that of other targets more resistant to lysis. Alternatively, all components of the lytic-machinery could be quantitatively diminished. In either case, one could argue that even though a cytolytic hybridoma expresses the specificity of its CTL parent, its lytic-mechanism is insufficient for the lysis of any other target but P815. Against this possibiltiy stands the finding that the specific P815 lytic-activity of MLC-derived T cells (fig.11) and of clone 96 (fig.13) is not (or only transiently) transmitted to the hybridomas. In these cases one would expect to see antigen-specific lytic-activity for P815 because the hybridomas should recognise P815 and should be capable of lysing it despite a deficient lytic-machinery. This clearly does not occur suggesting that a partial defect in the lytic-mechanism of the hybrid-omas is not solely responsible for the loss of antigen-specific lytic-activity.

## 4.2.1 (iv)   Loss of Lyt-2

It has been suggested by Silva and colleagues, 1983, that CTH often lose

their antigen-specific lytic-activity because BW5147, the most frequently used

fusion partner, does not allow the expression of the T cell antigen Lyt-2. Lyt-

2, a glycoprotein composed of subunits of 30-35 kd has been speculated to play

a role in assisting effector to target adhesion during the lytic event, perhaps

by specifically binding to class 1 MHC antigens (MacDonald et al., 1982). In

addition Janković and colleagues (in press) have positively demonstrated that

co-expression of Lyt-2 with the TCR correlates with antigen-specific lytic-

activity in AK variants. Thus, it would seem reasonable to suppose that the

loss of this surface molecule would abrogate antigen-specific lytic-activity in

CTH. Certainly, in this report, the clones generated from BT 7.4.1 and clone

96 fusions (which do not display antigen-specific lytic-activity) are shown to

be negative for the expression of Lyt-2 both by flow-cytometry (table 3) and

by two-dimensional SDS-PAGE (figures 23 and 24). However, strong Lyt-2 ex-

pression was detected in young uncloned populations of hybridomas which were

generated from MLC cells and cultured in the absence of IL-2 (section 3.7.2).

In spite of this, these hybridomas populations were not able to lyse relevant

target cells in the absence of mitogenic lectins demonstrating that, at least in

this case, the loss of Lyt-2 antigen is not the primary cause for loss of

antigen-specificity. Several hybridoma clones were selectively isolated from

this population which strongly and stably express the Lyt-2 antigen. These

clones are also unable to induce antigen-specific lysis of relevant target cells

providing further evidence that lack of Lyt-2 expression is not responsible for

the loss of antigen-specificity in CTH. It appears that the fusion of CTL

populations with BW5147 results in either Lyt-2 negative or Lyt-2 positive

hybridomas. The Lyt-2 negative hybridomas tend to proliferate more quickly than the Lyt-2 positive hybridomas, especially in the presence of ConA-SN, and quickly overgrow the cultures resulting in an apparent suppression of Lyt-2 expression by BW5147.

Other lines of evidence also suggest that loss of Lyt-2 expression is not the major cause of the loss of antigen-specific activity in CTH. For example, if the lack of Lyt-2 was the only source of dysfunction in CTH, then one would expect the hybridomas to be capable of a normal range of non-specific lysis in the presence of appropriate mitogenic lectins. However, this is clearly not the case since the non-specific lytic-activity appears to be restricted to P815. Thus, there is clearly some other dysfunction in the recognition and lytic-mechanisms of CTH generated from classical CTL.

The situation with AK cells may be some what different to that of classical CTL because hybridomas generated from clone 96 express a transient antigen-specific lytic-activity (figure 13B). Such a finding can be interpreted in two ways. Firstly, it may be the case that a transient specific-activity is present in all young CTH populations but that it usually disappears before the hybridomas have reached numbers at which they can be tested. For one reason or another this loss is slower in AK hybridomas. Or secondly, and perhaps more probably, it may be the case that the requirements of AK lytic-activity are less stringent than those of classical lytic-activity such that AK activity is more readily introduced into hybridomas. Indeed, there is much evidence for this in the literature where several groups have successfully generated stable, anti-gen-specific CTH from cells with "AK like" activities (Nabholz et al., 1980; Conzelmann et al., 1982; Berebbi et al., 1983; Foa et al., 1984; Haas and Kisielow, 1985a). If this is the case, the loss of antigen-specific lytic-activity in hybridomas generated in fusions between clone 96 and BW5147 can be rather

simply explained as due solely to the loss of Lyt-2. Janković and colleagues (in press) have shown a clear corrolation between Lyt-2 and TCR expression and lectin-dependent or lectin-independent lysis of P815 by clone 96. Co-expression of Lyt-2 and TCR appears to be required for full antigen-specific lytic-activity against P815. Loss of Lyt-2 results in the loss of this antigen-specific activity whereas the non-specific lectin-dependent activity is maintained. Loss of TCR results in abrogation of all lytic-activity. These results provide support for the theory that Lyt-2 is required as a non-specific binding agent in those CTL which have TCRs of a binding affinity too low to result in effector/target conjugations (MacDonald et al., 1982). It is intriguing to speculate that the transient antigen-specific lytic-activity observed in freshly generated clone 96 hybridomas is due to the temporary expression of Lyt-2 on some cells within the hybridoma population as has been shown within the MLC fusion system. The slow loss of antigen-specific lytic-activity would therefore be due to the growth advantage possessed by Lyt-2 negative and lectin-dependent (or non-lytic) hybridomas. This hypothesis could be tested by sorting for Lyt-2 positive hybridomas (if they are indeed present) by flow-cytometry and looking for antigen-specific activity in this population.

The difficulties encountered in isolating Lyt-2 positive and P815 specific hybridoma clones in clone 96 x BW5147 fusions may have been due to a poor plating efficiency of these cells as compared to the Lyt-2 negative hybridomas.

4.2.1 (v)  **Loss of the T cell antigen receptor**

Another possible explanation for the loss of antigen-specific lytic-activity in the hybridomas generated in this report is the total loss of TCR expression (changes in TCR specificity will be discussed in section 4.2.2). The TCR is

believed to be crucial for antigen-dependent activation of the lytic-mechanism of CTL and may also be indispensible for the activation of T cells per se; for example in the lectin-dependent lytic-activity of CTL. Certainly, in AK cells, loss of the TCR results in the complete loss of all lytic-activity whatsoever, (Janković et al, in press). A failure to express TCR could result either from the loss of appropriate chromosomes or from a suppressive effect of the BW5147 fusion partner (BW5147 itself does not express surface TCR). In contrast to this, the routine generation of antigen-specific and functional hybridomas from helper T cells suggests that the  TCR can be functionally expressed in hybridomas.

The experiments presented in section 3.7.3 exclude the possibility that the lack of TCR expression is the primary cause of dysfunction in CTH. Altogether eight BT 7.4.1 and clone 96 hybridomas (both non-specifically lytic and totally non-lytic clones) were tested by two-dimensional SDS-PAGE for the surface expression of TCR protein. All of the lytic hybridomas clearly expressed significant quantities of TCR whereas three out of four non-lytic hybridomas were negative for, or only expressed low quantities of TCR which could not be detected by this technique. These results would support the hypothesis that the presence of the TCR is absolutely necessary for the expression of any form of lytic-activity (Berke et al., 1981a, b). The correlation between non-specific lytic-activity and TCR expression is shown in Table 5. Haas and Kisielow, 1985a, have similarly shown the presence of TCR on the surface of CTH by demonstrating IFN-γ secretion in response to antigen despite the fact that the hybridomas are not capable of antigen-specific lytic-activity.

The fact that TCR is clearly expressed on hybridomas capable of non-specific lytic-activity also excludes the possibility that failure to express T3 antigen, a complex involved in the activation of T cells, is the cause of loss of antigen-specificity in CTH since T3 and TCR are mutually dependent for their co-expression (Meuer et al., 1983).

## 4.2.2   Other possible reasons for the loss of antigen-specific activity

### 4.2.2. (i) Loss of LFA-1 and/or other unknown molecules involved in the

### activation of the antigen-specific lytic-mechanism

The work presented in the report has clearly demonstrated that loss of TCR

or Lyt-2 components of the lytic-mechanism are not responsible for the loss of

antigen-specific lytic-activity in CTH. However, the possibility remains that

some other molecule involved in specific recognition has been selectively lost.

A candidate for such a molecule is LFA-1 which is a glycoprotein with subunits

of 95 and 180 kd (Springer et al., 1982) and is associated with the ability of

CTL to bind target cells. Kaufmann and collegues, 1982, have demonstrated

that their CTH, which are able to specifically lyse EL4 target cells in the

absence of mitogenic lectins express high levels of LFA-1. Moreover, the lytic

activity of these hybridomas can be inhibited by monoclonal antibodies

specific for LFA-1. Thus, the hybridomas described in this report could have

lost their specific activity due to a failure to express LFA-1. This hypothesis

could be readily tested by flow-cytometry if appropriate antibodies specific

for LFA-1 could be obtained.

Another possibility is that all components of the lytic-machinery are

expressed at lower levels, thus reducing the overall lytic-potential of the

hybridomas and not allowing the expression of antigen-specificity. Such a

hypothesis is difficult to test. Some comments on the possible role of reduced

levels of TCR expression on CTH are given in section 4.2.2 (iii).

### 4.2.2 (ii) Change in the TCR antigen-specificity

One possible explanation for the loss of antigen-specificity with the

concomitant maintenance of lytic-activity in CTH is a change in the

specificity of the TCR after the hybridisation event. It has been clearly shown

in this report (section 3.7.1) that the TCR is present on the surface of CTH, but the precise specificity of the receptor has not been demonstrated although in the case of clone 96 x BW5147 hybridomas, TCR ß-chain mRNA of the correct length and parental specificity has been demonstrated in the cytoplasm. One mechanism for a change of specificity in T cell hybridomas would be the formation of heterologous α-ß chain dimers resulting from a random mixing of the α and ß chains of both BW5147 and the parental CTL. Although TCR proteins are not expressed on the cell suface of BW5147 (section 3.7.1), there are clearly α and ß chain mRNA molecules of the correct size in the cytoplasm (Chien et al., 1984, section 3.7.1) and transcription, translation and cell surface expression may be induced as a consequence of hybridisation. In such a case, one would expect a dilution of the homologous dimers with the expected specificity (but not their total disappearance) which could result in a total loss of the original antigen-specificity if threshold concentrations of TCR were required for antigen-specific function.

The apparent induction of surface expression of BW5147 TCR molecules has been postulated to have occurred in a hybridoma described by Yagüe and colleagues, 1985. In their experiments, fusions between BW5147 and a chicken-ovalbumin specific T helper cell resulted in hybridomas which expressed α and ß chains of the TCR and responded to chicken-ovalbumin by secreting IL-2. Variants of one hybridoma which failed to express the chicken-ovalbumin specific α chain did, however, express the chicken-ovalbumin specific ß chain in combination with a new α chain. The authors think that this α chain is probably derived from BW5147 and suggest that TCR expression at the protein level can be easily induced in this cell line.

Similarly, the hybridisation event may induce the transcription, translation and expression of the rearranged TCR-γ chain gene from either parent which

may interfere with the structure of the TCR in the hybridomas. The γ chain is homologous to, and undergoes similar gene rearrangements as, the α and ß chain genes of the TCR (Hayday et al., 1985). It is strongly expressed in early thymocytes and is thought to play a role in the early development of T cells although as yet no protein products have been described. Heilig and colleagues, 1985, have reported that, although helper T cell clones contain rearranged TCR γ chain genes, the respective mRNA is not found in the cytoplasm, i.e. the gene is not transcribed. The same is true for BW5147. However, four helper T cell hybridomas (with BW5147 as the fusion partner) all expressed an identically rearranged γ chain mRNA and all data suggested that this was derived from BW5147 (Heilig et al., 1985). It appears that the fusion-event activated the transcription of this gene. This elevated expression of γ chain mRNA is reminiscent of the transcriptional activity of foetal thymocytes and indicative of de-differentiation events. Thus, it appears that the mixing of heterologous α and ß chains and perhaps even γ chains is possible, and indeed quite likely, in CTH. There are, however, several lines of evidence which in themselves do not argue against this interchain mixing, but do suggest that it may not be the primary reason for the loss of antigen-specificity. Firstly, Haas and Kiselow, 1985a, have clearly demonstrated the expression of TCR with appropriate specificity on CTH which could be induced to secrete IFN-γ in response to relevant antigen despite the fact that these cells were not lytic. Secondly, T helper hybridomas apparently maintain their antigen recognition specificity far more readily than CTH although the same mixing of receptor chains should occur.

The secondary fusions described in section 3.3 were performed with a view to examining the question of whether heterologous TCR chain mixing was occurring. The hypothesis was that fusing a CTH back with its CTL parent

would increase the proportional expression of TCR chains with the appropriate specificity in the resultant hybridomas. Although hybridomas with lytic-activity were generated in these experiments, none of them expressed stable antigen-specificity. However, these results are determined from only a few secondary hybridomas since the CTH proved to be inefficient as fusion partners.

Another possible mechanism for rapid alterations of receptor specificity is the accumulation of mutations or subsequent gene rearrangements. It is well established that cloned CTL lines rapidly lose their ability to recognise antigen when cultured without appropriate stimulator cells (Nabholz et al., 1980a). Moreover, it has been established that the maintenance of specificity in cloned CTL lines such as BT 7.4.1 is the result of continuous selection for antigen recognising cells accompanied by the continuous generation of variants which no longer recognise antigen and subsequently die - lethal variants (Weltzien and Eichmann, in press). It would seem reasonable to suggest from such data that the TCR of CTL is subject to rapid structural variation leading to alterations of its specificity. If, as is the case for T cell hybridomas, the dependence of growth on the presence of antigen is eliminated, specificity variants should have equal opportunities for growth as wild-type cells and should soon dominate the population. It was hoped to test this assumption with the fusions with clone 96 which is an AK cell line and which maintains its specificity for P815 without selection by antigen. It was reasoned that this cell has lost the ability to rapidly mutate its TCR and that this characteristic would be transmitted to the hybridomas allowing the generation of antigen-specific CTH. This seems to be the case because clone 96 hybridomas, in contrast to hybridomas made from "classical" CTL, show specific lytic activity for P815, at least for a transient period. In addition, molecular genetic

analysis demonstrates that the lytic hybridomas, although not capable of antigen-specific lytic-activity, still express the typical TCR $V_{\beta AK}$ mRNA (section 3.7.1). This result and the fact that several other laboratories have generated antigen-specific CTH from "AK like" cells (Nabholz et al., 1980; Conzelmann et al., 1982; Berebbi et al., 1983; Foa et al., 1984; Haas and Kisielow, 1985a) suggests that structural alterations in the TCR genes could be responsible for the loss of antigen-specificity in hybridomas generated from classical CTL such as BT 7.4.1. The subsequent loss of antigen-specificity in clone 96 hybridomas could be due to the loss of Lyt-2 which has been shown to be essential for the specific recognition of P815 by AK cells (Janković et al., in press).

Thus it is not possible to firmly exclude the hypothesis that changes in the structure of the TCR genes are responsible for the loss of specificity in CTH. To date, most expressed $V_\beta$ genes sequenced are germ-line genes suggesting that somatic mutational mechanisms do not occur. However, D and J sequences as well as the largely unknown $\alpha$ chain provide sufficient room for somatic alteration of TCR specificity.

## 4.2.2 (iii) Reduced expression of the TCR

It is possible that the hybridomas presented in this report lose their antigen-specificity due to a reduction in the concentration (rather than a loss) of important molecules on the cell surface. For example, although it is difficult to directly compare the two-dimensional SDS-PAGE gels in figures 26 and 27, there is a suggestion that the expression of TCR on the lytic hybridomas is reduced as compared to the parental CTL. It is intriguing to postulate that there is a requirement for a minimal concentration of TCR on the cell

membrane below which activation of the cells lytic-machinery cannot occur. Indeed, Kaufmann and colleagues, 1981a,b use the enzyme neuraminidase to increase the apparent concentration of antigen on the target cells in order to demonstrate antigen-specific lytic-activity in hybridoma clones generated from fusions between BW5147 cells and MLC cells. They interpret this effect as being due to an increase in the concentration of TCR recognising antigen at any given moment to above a certain activation threshold required for lytic-activity. In the hybridomas generated in this report, the treatment of target cells with neuraminidase did not reveal an antigen-specific lytic-activity. This does not exclude, however, that a low density of TCR is responsible for the loss of this function.

It is possible, that there are different activation requirements for CTL and helper T lymphocytes. The activation of the secretory mechanisms of helper T lymphocytes may require stimulation of only a few antigen-receptors on the cell surface whereas the cytolytic-activity of CTL may require much greater stimualtion of the TCR due to the relative complexity of this activity. Moreover, the TCR of CTL may play a greater role in the cell-cell binding involved in the generation of effector : target conjugates. This difference would then explain why it is relatively easy to generate helper hybridomas, as compared to CTH, since a reduction in TCR concentration on the hybridomas would primarily affect lytic functions but not helper-type secretory functions. The hybridomas generated by Haas and Kisielow, 1985a, are interesting in this respect. Both lytic and non-lytic hybridomas are shown to secrete IFN-γ in response to their specific antigen, or to ConA, but not to irrelevant antigen. In the case of the non-lytic hybridomas, this demonstrates the expression and stimulation of the TCR leading to IFN-γ secretion even though the lytic-machinary is not successfully activated. It would be interesting to test

whether the hybridoma cells described in this report are able to secrete IFN-γ in response to antigen or mitogenic lectin. This would demonstrate that the TCR has maintained both its specificity and functional capacity during the fusion event and would provide strong support for the hypothesis that the loss of antigen-specificity is due to a reduced concentration of TCR on the cell surface.

The role of lectin in revealing lytic-activity in CTH with low TCR expression is probably due to the strong avidity of lectins for the TCR (Chilson et al., 1984; Sitkovsky et al., 1984) and their ability to induce the formation of effector, target conjugates. However, the lower concentrations of surface TCR may mean that overall lower levels of lytic potential are released sufficing only for the lysis of highly susceptible targets such as P815. Thus, the apparent lectin-dependent specificity of the CTH for P815 would be a quantitative rather than qualitative effect.

Taken together, the results regarding TCR expression on cytolytic and non-cytolytic CTH suggest that TCR is required for cytolytic activity and that loss of antigen-specific activity may be the result of the reduced quantity of TCR on CTH as compared to parental CTL.

## 4.3    General considerations regarding BW5147 as a fusion partner

The loss of molecules involved in the lytic-mechanism has already been discussed as a reason for the loss of antigen-specificity in CTH. This loss could occur either due to the random or preferential loss of chromosomes containing critical genes (Grützmann and Hämmerling, 1978) or due to the inactivation or suppression of critical genes by the tumour cell genome. BW5147 is perhaps not an optimal choice for CTL fusions because of its helper cell origin which may make it incompatible with CTL function (Hämmerling, 1977). An example

of this type of suppression can be found in the expression of Thy-1 allo-
antigens although these molecules probably play no role in the lytic-
mechanism. Hybridomas generated between BW5147, expressing Thy1.1, and a
variety of other cells expressing Thy1.2 have been found to either express both
Thy-1 alleles, express only Thy1.1 or express no Thy-1 alleles at all (Goldsby et
al., 1977). This inability to express the Thy1.2 allele alone is not readily
explained by chromosome loss alone but must also involve a suppressive
influence of BW5147. The frequent loss of Lyt-2 in CTH could well be the
additive result of such a suppression and specific chromosome loss. Circum-
stantial evidence for this dominance of the BW5147 partner in hybridomas is
provided by the SDS-PAGE gels presented in section 3.7.3. The diagonals of
such gels contain the bulk of cell suface proteins and their shapes can be
thought of as "fingerprints" of the cells in question. In all eight hybridomas
studied by this technique, this fingerprint more closely resembles the BW5147
than the CTL parent suggesting a dominance of BW5147. Nabholz and
collegues, 1980, have noticed a similar effect in hybridomas generated
between BW5147 and a cloned CTL with "AK-like" lytic activity. The lytic
hybridomas generated in these experiments strongly resemble the parental
cloned CTL whereas the non-lytic hybridomas strongly resemble BW5147 in
their morphological and adherent properties.

A more suitable partner for CTL fusions would be a CTL tumour cell.
Theoretically, even though such a tumour might not itself be lytic, its genome
would be far more permissive to the expression of lytic-activity. Clone 96 was
a candidate for such a partner, but this cell could not be made HAT sensitive.
As a "second best" alternative, the clone 96 and BT 7.4.1 hybridomas
generated in this report were successfully made HAT sensitive and used in
secondary fusions. It was hoped that these cells would be less suppressive

towards the CTL genome but would have inherited the superb fusion charac teristics of BW5147. However, these hybridomas proved to be very poor fusion partners and the few secondary hybridomas generated were unstable and not capable of stable antigen-specific lytic-activity.

As a general principle, the use of hybridomas as fusion partners is quite feasible. Haas and collegues, 1985b,c, have successfully used hybridomas as partner cells in fusions with CTL and obtained some of the few examples in the literature of antigen-specific CTH.

A second sort of suppression could be the result of factors produced by the BW5147 parent which inhibit the lytic-activity of the hybridomas. For exam- ple, it has been reported that supernatants of BW5147 are capable of inhibiting proliferation in MLR cultures (Indrova and Bubenik, 1984; Chaoat et al., 1985). However, it is difficult to see how such a suppression would act specifically to inhibit antigen-specific but not lectin-dependent lytic-activity and is therefore unlikely to play a role in the dysfunction of CTH.


## 4.4 Conclusion


The experiments presented in this report were performed with a view to defining the reasons for the loss of antigen-specific lytic-activity in hybrid- omas generated from CTL. While a clear definitive answer has not been found, several possibilities for explaining this phenomenom have been unequivocally excluded. It is clear that a functional lytic machinery itself can be readily introduced into hybridomas. This was shown by demonstrating a non-specific lytic-activity in CTH. However, there appear to be slight alterations in the functional properties of this lytic-mechanism (i.e. the inability to lyse targets other than P815 in the presence of mitogenic lectins) which probably stem

from an overall reduced lytic capacity, perhaps due to lower expression of critical lysis-related molecules. The capacity of the hybridomas to lyse P815 is thus due to the extreme sensitivity of this cell to lysis. More general aspects of the lytic machinery such as the requirements for calcium ions and cell contact prior to lysis remain typical of classical CTL.

The loss of antigen-specificity in the absence of a concomitant loss of overall lytic-activity suggests that there is a dysfunction in the recognition apparatus or antigen-specific activation mechanisms of the CTH. However, overall losses of Lyt-2 or TCR molecules are clearly excluded as being the reasons lying behind this loss of antigen-specific activity. But the degree of expression i.e. the concentration of these molecules on the cell surface, could not be accurately detemined leaving open the possibility that their expression is too low to fulfil the perhaps stringent requirements of antigen-specific activation. This reduction in the degree of expression could occur by several mechanisms. In the case of the TCR, one can postulate a reduction in the surface concentration of relevant receptor due to the mixing of heterologous receptor chains derived in part from the tumour partner and in part from the CTL. This hypothesis is attractive because, although specific recognition would be compromised, the overall level of TCR expression would remain at levels high enough to allow cellular activation by appropriate lectins. One can explain the general finding that T helper hybridomas with antigen-specificity are readily generated despite interchain mixing by proposing that the activation requirements of T helper cells are less stringent than those of the highly complex lytic-mechanism. An alternative mechanism for the loss of specificity would be a change in antigen-specificity of the TCR either by somatic mutation or by further rearrangements of TCR genes. These possibilities cannot be excluded on the basis of the results presented in this report. In

addition, the loss of, or reduced expression of other molecules in antigen-dependent lytic-activity such as, perhaps, LFA-1 have not been excluded although the loss of mouse T3 complex has been excluded by default due to the fact that the TCR and the T3 complex are obligatorily co-expressed (Meuer et al., 1983).

Taking the data presented in this report together, it is proposed that the loss of antigen-specificity in CTH generated from classical CTL is due to a general reduction in the quantity of TCR with appropriate antigen-specificity expressed on the cell surface in conjunction with a reduction of the overall lytic-potential of hybridomas. The frequent subsequent loss of Lyt-2 expression seals the inability of CTH to lyse target cells in an antigen-specific manner.

The situation may be a little different with CTH generated from AK cells because it seems that antigen-specific lytic-activity of AK cells is more readily transfered to CTH than that of classical CTL. The reasons behind this are unclear but are probably related to the high degree of stability observed in AK cells which is somehow passed on to the hybridomas. Thus, most AK hybridomas have reduced lytic-potential, as do all CTH, but sufficient levels of the AK recognition apparatus to allow antigen-specific lytic-activity. Certainly, in the case of hybridomas generated from clone 96 cells, a specific lytic-activity against P815 was found when the hybridomas were tested early after fusion, but this later disappeared. It seems quite likely that this loss of specific-activity is correlated with the general selection of Lyt-2 negative variants under the conditions used since loss of Lyt-2 expression is known to result in the loss of specific-activity against P815 in clone 96 cells. This point can be readily tested by revising the cloning procedures employed or by specifically selecting Lyt-2 positive hybridomas by flow cytometry.

There are several lines of research open for the further investigation of the general loss of antigen-specificity in CTH. Long term projects include:-

(i) the examination of components of the recognition- and lytic-mechanism of CTH which have not been covered in this report, eg. expression of lytic-granules, polyperforins and LFA-1.

(ii) the use of more sensitive assays for antigen specificity e.g. assays for IFN-γ or IL-2 secretion.

(iii) the direct comparisons of CTH TCR specificities with those of parental CTL by molecular genetic analysis, and

(iv) the transfection of TCR genes (and perhaps Lyt-2 genes) to examine the question of whether loss of specificity is due to low TCR expression.

Finally, the question still remains as to how it may be possible to generate CTH with antigen-specificity in the future. If the hypotheses presented in this report are correct then it is clear that other fusion partners should be sought which are more suitable vehicles for the expression of antigen-specific lytic-activity. Some attempts in this direction were made by fusing CTH themselves back with CTL of the same specificity. It was hoped that this technique would result in secondary hybridomas expressing higher levels of TCR recognising relevant antigen and therefore lytic-activity of appropriate specificity. However, technical difficulties were encountered regarding the stability of these second-order hybridomas. In any case, as a general rule, the use of secondary hybridomas is not a real solution to the problem since CTH can only be back-crossed with the original parental CTL in order to generate hybridomas with higher levels of TCR recognising relevant antigen. What is required is a cell of CTL origins which is unable to express TCR chains despite fusion; hopefully, such a cell will soon be identified. Possible candidates are the hybridomas

generated in this report which do not express TCR. These hybridomas would have to be screened for their fusion efficiencies and for the stability of the hybridomas produced.

Perhaps the most promising chances of immortalising antigen-specific CTL would be to move away from standard cell-hybridisation techniques and to establish a retroviral transformation system. Such a system would eliminate all of the problems related to the influence of the tumour partner in hybridomas.

## REFERENCES

Acha-Orbea, H., Groscurth, P., Lang, R., Stitz, L. and Hengartner, H. (1983). Characterisation of cloned cytotoxic lymphocytes with NK-like activity. J. Immunol. 130, 1952.

Allison, J.P., McIntyre, B.W. and Bloch, D. (1982). Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. J. Immunol. 129, 2293.

Allison, J.P. and Lanier, L.L. (1985). Identification of antigen receptor-associated structures on murine T cells. Nature. 314, 107.

Allwood, G., Asherson, G.L., Davey, M.J. and Goodford, P.J. (1971). The early uptake of radioactive calcium by human lymphocytes treated with phyto-haemagglutinin. Immunology. 21, 509.

Augustin, A.A. and Sim, G.K. (1984). T-Cell Receptors Generated via Muta-tions Are Specific for Various Major Histocompatiblity Antigens. Cell. 39, 5.

Berebbi, M., Foa, C., Imbert, E., Fabre, I. and Lipcey, C. (1983). Cytolytically active murine T-lymphocyte/polyoma virus-transformed fibroblast hybrids. Exp. Cell. Res. 145, 357.

Berke, G. (1980). Interaction of cytotoxic T lymphocytes and target cells. Prog. Allergy. 27, 69.

Berke, G., McVey, E., Hu, V. and Clark, W.R. (1981a). T lymphocyte-mediated cytolysis. I. A common mechanism for target recognition in specific and lectin-dependent cytolysis. J. Immunol. 127, 776.

Berke, G., McVey, E., Hu, V. and Clark, W.R. (1981b). T lymphocyte-mediated cytolysis II. Role of target cell histocompatibility antigen in recognition and lysis. J. Immunol. 127, 782.

Bevan, M.J. and Cohn, M. (1975). Cytotoxic efects of antigen- and mitogen-induced T cells on various targets. J. Immunol. 14, 559.

Binz, H., Fenner, M., Frei, D. and Wigzell, H. (1983). Two independent receptors allow selective target lysis by T cell clones. J. Exp. Med. 157, 1252.

Blanckmeister, C.A., Yamamoto, K., Davis, M.M. and Hämmerling, G.J. (1985). Antigen-specific, I-A-restricted suppressor hybridomas with spontaneous cytolytic activity. Functional properties and lack of rearrangement of the T cell receptor ß chain genes. J.Exp. Med. 162, 851.

Blumenthal, R. Millard, P.J., Henkart, M.P., Reynolds, C.W. and Henkart, P.A. (1984). Liposomes as targets for granule cytolysin from cytotoxic LGL tumors. Proc. Natl. Acad. Sci. USA. 81, 5551.

Bolhuis, R.L.H. and van de Griend, R.J. (1985). Phytohaemagglutinin-Induced Proliferation and Cytolytic Activity in T3+ but not in T3- Cloned T lymphocytes Requires the Involvement of the T3 Antigen for signal Transmission. Cell. Immunol. 93, 46.

Borst, J., Prendiville, M.A. and Terhorst, C. (1983). The T3 complex on human thymus derived lymphocytes contains two different subunits of 20kd. Eur. J. Immunol. 13, 576.

Boyse, E.A., Miyazawa, M., Aoki, T. and Old, L.J. (1968). Ly-A and Ly-B: Two systems of lymphocyte isoantigens in the mouse. Proc. Roy. Soc. B. 170, 175.

Boyse, E.A., Itakura, K., Stockert, E., Iritani, C.A. and Miura, M. (1971). Ly-C: A third locus specifiying alloantigens expressed only on thymocytes and lymphocytes. Transplantation 11, 351.

Brenner, M.B., Trowbridge, I.S. and Strominger, J.L. (1985). Cross linking of human T cell receptor proteins: association between the T cell idiotype ß subunit and the T3 glycoprotien heavy subunit. Cell. 40 183.

Brondz, B.D., Snegivöra, A.E., Rassulin, Yu.A. and Shamborant, O.G. (1971). Effect of some Enzymes, Polysaccharides and Lyposome-Active Drugs on Interactions of Immune Lymphocytes with Allogenic Target Cells. In: Progress in Immunology. Amos, B. (ed.) New York, Academic, p. 447.

Brooks, C.G. (1983). Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. Nature. 305, 155.

Brunner, K.T. and Cerottini, J.C. (1971). Cytotoxic Lymphocytes as effector cells of cell mediated immunity. In: Progress in Immunology, ed. B. Amos, pp385-398. New York: Academic.

Brunner, K.T., Mauel, J., Cerottini, J.C. and Chapias, B. (1968). Quantitative assay for the lytic action of immune lymphocytes on ^{51}Cr labelled allogeneic target cells in vitro. Immunology. 14, 181.

Bykovskaja, S.N. Rylenko, A.N., Rauschenbach, M.O. and Mykovsky, A.F. (1978a). Ultrasturctural alteration of cytolytic T lymphocytes following their interaction with target cells. I. Hypertrophy and change of orientation of the Golgi apparatus. Cell. Immunol. 40, 164.

Bykovskaja, S.N., Rytenko, A.N., Rauschenbach, M.O. and Bykovsky, A.F. (1978b). Ultrasturctural alteration of cytolytic T lymphocytes following their interaction with target cells. II. Morphogenesis of secretory granules and intracellular vacuoles. Cell. Immunol. 40, 175.

Cantor, H. and Boyse, E.A. (1977). Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev. 33, 105.

Cantrell, D.A., Davies, A.A. and Crumpton, M.J. (1985). Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. Proc. Natl. Acad. Sci. USA. 82, 8158.

Carafoli, E. and Penniston, J.T. (1985). The Calcium Signal. Sci. Am. 253, 5, p. 50.

Chaoat, G., Liacopoulos-Briot, M., Couderc, J., Parlebas, J., Perrodon, Y., Briffaut, O. and Liacopoulos, P. (1985). Selection of BW5147 subclones devoid of non-specific suppressive activity for use in cell hybridization. Immunol. 54, 363.

Chien, Y., Becker, D.M., Lindsten T., Okamura, M., Cohen, D.I. and Davis, M.M. (1984). A third type of murine T-cell receptor gene. Nature. 312 31.

Chilson, O.P., Boylston, A.W. and Crumpton, M.J. (1984). Phaseolus vulgaris phytohaemagglutinin (PHA) binds to the human T lymphocyte antigen receptor. EMBO Journal 3; 3239.

Conzelmann, A., Corthesy, P., Cianfriglia, M., Silva, A. and Nabholz, M. (1982). Hybrids between a rat lymphoma and mouse T cells with inducible cytolytic activity. Nature. 298, 170.

Davignon, D., Martz, E., Reynolds, T., Kürzinger, K. and Springer, T.A. (1981). Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1). Mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. 127, 590.

Dennert, G. and Podack, E.R. (1983). Cytolysis by H-2 specific T killer cells: Assembly of tubular complexes on target membranes. J. Exp. Med. 157, 1483.

DiPauli, R. and DiPauli, G. (1978). Attempts to Produce Cytolytically Active Cell Hybrids Using EL4 and MLC Spleen. Curr. Top. Microbiol. Immunol. 81, 221.

Epplen, J.T., Wallace, R.B., Bartels, F., Becker, A., Rinaldy, A. and Simon, M.M. (in press) Change in antigen specificity of cytotoxic T lymphocytes is associated with the rearrangement and expression of a new T-cell receptor ß chain gene.

Erard, F., Corthesy, P., Smith, K.A., Fiers, W., Conzelmann, A. and Nabholz, M. (1984). Characterisation of soluble factors that induce the cytolytic

activity and the expression of T cell gowth factor receptors of a T cell hybrid. J.Exp. Med. 155, 1876.


Eshhar, Z., Waks, T., Oren, T., Berke, G. and Kaufmann, Y. (1982). Cytotoxic T Cell Hybridomas: Generation and Characterisation. Curr. Top. Microbiol. Immunol. 100, 11.


Fan, J., Ahmed, A. and Bonavida, B. (1980). Studies on the induction and expression of T cell-mediated immunity. X. Inhibition by Lyt-2,3 antisera of cytotoxic T lymphocyte mediated antigen specific and non-specific cytotoxicity: Evidence for the blocking of the binding between T lymphocytes and target cells and not the post-binding cytolytic steps. J. Immunol. 125, 2444.


FischerLindahl, K., Nordin, A.A. and Schreier, M. (1982). Lectin-Dependent Cytolytic and Cytolymic T Helper Clones and Hybridomas. Curr. Top. Microbiol. Immunol. 100, 1.


Foa, C., Lipcey, C., Simonitti, J. and Berebbi, M. (1984). Functional Characteristics of Cytotoxic T Lymphocyte Polyoma Virus-Transformed Fibroblast Hybrids. Cell. Immunol. 87, 192.


Forman, J. and Möller, G. (1973). Generation of cytotoxic lymphocytes in mixed lymphocyte reactions. J. Exp. Med. 138, 672.


Freedman, M.H. and Raff, M.C. (1975). Induction of increased calcium uptake in mouse T lymphocytes by concanavalin-A and its modulation by cyclic nucleotides. Nature. 255; 378.

Galfre, G., Howe, S.C., Milstein, C., Butcher, G.W. and Howard, J.C. (1977). Antibodies to major histocompatability antigens produced by hybrid cell lines. Nature. 266, 550.

Geiger, B., Rosen, D. and Berke, R. (1982). Spatial relationships of microtubule organising centres and the contact area of cytotoxic T lymphocytes and target cells. J. Cell. Biol. 95; 137.

Goding, J.W. and Harris, A.W. (1981). Subunit structure of cell surface proteins: Disulfide bonding in antigen receptors, Ly-2/3 antigens, and transferrin receptors of murine T and B lymphocytes. Proc. Natl. Acad. Sci. USA. 78, 4530.

Goldsby, R.A. Osborne, B.A., Simpson, E. and Herzenberg, L.A. (1977). Hybrid cell lines with T-cell characteristics. Nature. 267, 707.

Gordon, R.D., Simpson, E. and Samelson, L.E. (1975). In-vitro cell mediated immune responses to the male specific (H-Y) antigen in mice. J. Exp. Med. 142, 1108.

Green, W.R, Ballas, Z.K. and Henney, C.S. (1978). Studies on the mechanism of lymphocyte-mediated cytolysis. XI. The role of lectin in lectin dependent cell-mediated cytotoxicity. J. Immunol. 121, 1566.

Greenstein, J.L., Kappler, J., Marrack, P. and Burakoff, S.J. (1984). The role of L3T4 in recognition of Ia by a cytotoxic, H-2D$^d$-specific T cell hybridoma. J. Exp. Med. 159, 1213.

Grim, E., Mazumder, A., Zhang, H.Z. and Rosenberg, S.A. (1982). Lymphokine-activated killer cell phenomenon: Lysis of natural killer-resistant fresh solid tumour cells by interleukin 2 activated autologous human peripheral blood lymphocytes. J. Exp. Med. 155, 1823.

Grützmann, R. and Hämmerling, G.J. (1978). Characterisation and functional analysis of T cell hybrids. Curr. Top. Microbiol. Immunol. 81, 188.

Haas, W., Mathur-Rochat, J., Pohlit, H., Nabholz, M. and von Boehmer, H. (1980). Cytoltoxic T cell responses to haptenated cells III. Isolation and specificity analysis of continuously growing clones. Eur. J. Immunol. 10, 828.

Haas, W. and Kisielow, P. (1985a). Cytolytic T cell hybridomas. I. Interleukin-2 independent noncytolytic variants. Eur. J. Immunol. 15, 751.

Haas, W. and Kisielow, P. (1985b) Cytolytic T cell hybridomas. II. Interleukin 2 independent cytolytic variants. Eur. J. Immunol. 15, 755.

Haas, W., Mathur-Rochat, J., Kisielow, P. and von Boehmer, H. (1985c). III. The antigen specificity and the restriction specificity of cytolytic T cells do not phenotypically mix. Eur. J. Immunol. 15, 963.

Hämmerling, G.J. (1977). T lymphocyte tissue culture lines produced by cell hybridisation. Eur. J. Immunol. 7, 743.

Haskins, K., Kubo, R., White, J. Pigeon, M., Kappler, J. and Marrack, P. (1983). The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. J. Exp. Med. 157, 1149.

Hayday, A.C., Saito, H., Gillies, S.D., Kranz, D.M., Tanigawa, G., Eisen, M.N. and Tonegawa, S. (1985) Structure, organisation and somatic rearrangement of T cell gamma genes. Cell. 40, 259.


Hayot, B., Pierres, M. and Golstein, P. (1982). Mechanism of T cell-mediated cytolysis: An investigation of cells and stages affected by cytolysis-inhibiting monoclonal antibodies. Adv. Exp. Med. Biol. 146, 469.


Hedrick, S.M., Nielsen, E.A., Kavaler, J., Cohen, D.I and Davis, M.M. (1984). Sequence relationships between T-cell receptor polypeptides and immuno-globulins. Nature. 308, 153.


Heilig, J.S., Glimcher, L.H., Kranz, D.M., Clayton, L.K., Greenstein, J.L., Saito, H., Maxam, A.M., Burakoff, S.J., Eisen, H.N. and Tonegawa, S. (1985). Expression of the T-cell-specific γ gene is necessary in T cells recognising class II MHC determinants. Nature. 317, 68.


Henkart, P.A., Millard, P.J., Reynolds, C.W. and Henkart, M.P. (1984). Cytolytic activity of purified cytoplasmic granules from cytotoxic rat LGL tumours. J. Exp. Med. 160, 75.


Hood, L., Kronenberg, M. and Hunkapiller, T. (1985). T Cell Antigen Receptors and the Immunoglobulin Supergene Family. Cell. 40, 225.


Hubbard, A.L. and Cohn, Z.A. (1975). Externally Disposed Plasma Membrane Proteins. I. Enzymatic Iodination of Mouse L Cells. J. Cell. Biol. 64, 438.

Indrova, M. and Bubenik, J. (1984). BW5147 Supernatants Suppress ConA Stimulation of Mouse Spleen Cells. Folia biologica (Praha). 30, 390.

Jankovič, D.Lj., Woodland, D., Melchers, I., Weltzien, H.U. and Eichmann, K. (in press). Involvement of the T cell antigen receptor and of Lyt-2 in the cytotoxic function of aged killer (AK) T cells.

Kanellopoulos, J.M., Wigglesworth, N.M., Owen, M.J. and Crumpton, M.J. (1983). Biosynthesis and molecular nature of the T3 antigen on human T lymphocytes. EMBO J. 2, 1807.

Kanellopoulos, J.M., Petris, S., Leca, G. and Crumpton, M.J. (1985). The mitogenic lectin from Phaseolus vulgaris does not recognise the T3 antigen of human T lymphocytes. Eur. J. Immunol. 15, 479.

Kaufmann, Y., Berke, G. and Eshhar, Z. (1981a). Functional cytotoxic T lymphocyte hybridomas. Transpl. Proc. 13, 1171.

Kaufmann, Y., Berke, G. and Eshhar, Z. (1981b). Cytotoxic T lymphocyte hybridomas which mediate specific tumor cell lysis in vitro. Proc. Natl. Acad. Sci. USA. 78, 2502.

Kaufmann, Y., Golstein, P., Pierres, M., Springer, T.A. and Eshhar, Y. (1982). LFA-1 but not Lyt-2 is associated with killing activity of cytotoxic T lymphocyte hybridomas. Nature. 300, 357.

Kaufmann, Y. and Berke, G. (1983). Monoclonal cytotoxic T lymphocyte hybridomas capable of specific killing activity, antigenic responsiveness, and inducible interleukin secretion. J. Immunol. 131, 50.

Kavaler, J., Davis, M.M. and Chien, Y. (1984). Localisation of a T-cell receptor diversity-region element. Nature. 310, 421.

Köhler, G., Lefkovits, I., Elliot, B. and Coutinho, A. (1977). Derivation of hybrids between a thymoma line and spleen cells activated in a mixed leukocyte reaction. Eur. J. Immunol. 7, 758.

Krammer, P.H., Echtenacher, B., Gemsa, D., Hamann, U., Hültner, L., Kaltmann, B., Kees, U., Kubelka, C. and Marcucci, F. (1983). Immune-interferon (IFN-γ), Macrophage-activating Factors (MAFs), and Colony-stim-ulating Factors (CSFs) Secreted by T Cell Clones in Limiting Dilution Microcultures, Long-term Cultures, and by T Cell Hybridomas. Immunol. Rev. 61, 5.

Kürzinger, K., Reynolds, T., Germain, R.N., Davignon, D., Martz, E. and Springer, T.A. (1981). A novel lymphocyte function associated antigen (LFA-1): cellular distribution, quantitative expression, and stucture. J. Immunol. 127, 596.

Ledbetter, J.A. and Herzenberg, L.A. (1978). Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 63.

Lefkovits, I. and Waldmann, H. (1979). "Limiting Dilution Analysis of Cells in the Immune System". Cambridge Univ. Press, London and New York.

Lefrancois, L. and Bevan, M.J. (1985). Functional modifications of cytotoxic T-lymphocyte T 200 glycoprotein recognised by monoclonal antibodies. Nature 314, 449.

MacDonald, H.R., Glasebrook, A.L. and Cerottini, J.C. (1982). Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes: analysis by antibody blocking and selective trypsinsiation. J. Exp. Med. 156, 1711.

Malissen, M., Minard, K., Mjolsness, S., Kronenberg, M., Goverman, J., Hunkapiller, T., Prystowsky, M.B., Yoshikai, Y., Fitch, F., Mak, T.W. and Hood, L. (1984). Mouse T cell antigen receptor: structure and organisation of constant and joining gene segments encoding the ß polypeptide. Cell. 37, 1101.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Martz, E. and Benacerraf, B. (1976). Multiple target cell killing by the cytolytic T-lymphocyte and the mechanism of cytotoxicity. Transplantation. 21, 5.

Martz, E., Heagy, W. and Gromkowski, S.H. (1982a). The mechanism of CTL-mediated killing: Monoclonal antibody analysis of the roles of killer and target membrane proteins. Immunol. Rev. 72, 73.

Martz, E. Parker, W.L. Gately, M.K. and Tsoukas, C.D. (1982b). The role of calcium in the lethal hit of T lymphocytes mediated cytolysis. Adv. Exptl. Biol. Med. 146, 121.

Masson, D., Corthesy, P., Nabholz, M. and Tschopp, J. (1985). Appearance of cytolytic granules upon induction of cytolytic activity in CTL-hybrids. EMBO J., 4, 2533.

Matter, A. (1979). Microcinematographic and electron microscopic analysis of target cell lysis induced by cytotoxic T lymphocytes. Cell. Immunol. 40, 175.

Melchers, F., Potter, M. and Warner, N.L. (1978). "Lymphocyte Hybridomas". Curr. Top. Microbiol. Immunol. 81.

Meuer, S.C., Hussey, R.E., Hodgdon, J.C., Hercend, T. and Schlossman, S. (1982). Surface structures involved in target recognition by human cytotoxic T lymphocytes. Science, 218, 471.

Meuer, S.C., Fitzgerald, K.A., Hussey, R.E., Hodgdon, J.C., Schlossman, S.F. and Reinherz, E.L. (1983). Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. J. Exp. Med. 157, 705.

Millard, P.J., Henkart, M.P., Reynolds, C.W. and Henkart, P.A. (1984). Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumours. J. Immunol. 132, 3197.

Mitchison, N.A. (1954). Passive transfer of heterozygous tumours as material for the study of cell heredity. Proc. R. Phys. Soc. 250, 45.

Miyada, C.G. Klofelt, C., Reyes, A.A., McLaughlin-Taylor, E. and Wallace, R.B. (1985). Evidence that polymorphism in the murine major histocompatibility complex may be generated by the assortment of subgene sequences. Proc. Natl. Acad. Sci. USA. 82, 2890.

Moretta, A., Pantaleo, G., Mingari, M.C., Melioli, G., Moretta, L. and Cerottini, J.C. (1984). Assignment of human natural killer (NK)-like cells to the T cell lineage. Single allospecific T cell clones lyse specific or NK-sensitive target cells via distinct recognition structures. Eur. J. Immunol. 14, 121.

Nabholz, M., Conzelmann, A., Acuto, O., North, M., Haas, W., Pohlit, H., von Boehmer, H., Hergartner, H., Mach, J.P., Engers, H. and Johnson, J.P. (1980a). Established Murine Cytolytic T-Cell Lines as Tools for a Somatic Cell Genetic Analysis of T-Cell Functions. Immunol. Rev. 51, 125.

Nabholz, M., Cianfriglia, M., Acuto, O., Conzelmann, A., Haas, W., von Boehmer, H., McDonald, H.R., Pohlit, H. and Johnson, J.P. (1980b). Cytolytically active murine T-cell hybrids. Nature. 287, 437.

Nabholz, M. and MacDonald, H.R. (1983). Cytotoxic T Lymphocytes. Ann. Rev. Immunol. 1, 273.

Oettgen, H.C., Kappler, J., Tax, W.J.M. and Terhorst, C. (1984). Characterisation of the two heavy chains of the T3 complex on the suface of human T lymphocytes. J. Biol. Chem. 259, 12039.

Oettgen, H.C., Terhorst, C., Cantley, L.C. and Rosoff, P.M. (1985). Stimulation of the T3-T Cell receptor complex induces a membrane-potential-sensitive calcium influx. Cell. 40, 583.

Podack, E.R. and Konigsberg, P. (1984). Cytolytic T cell granules. Isolation, biochemical and functional characterisation. J. Exp. Med. 160, 695.

Podack, E.R., Konigsberg, P.J., Acha-Orbea, H., Pircher, H. and Hengertner, H. (1984). In: Mechanisms in cell mediated cytotoxicity, (Clarke, W.E. and Henkart, P.A., eds), Plenum Publishing, New York.

Podack, E.R. (1985). The molecular mechanism of lymphocyte-mediated tumor cell lysis. Immunol. Today. 6, 21.

Quan, P.C., Ishizake, T. and Bloom, B. (1982). Studies on the mechanism of NK cell lysis. J. Immunol. 128, 1786.

Reinherz, E.L., Hussey, R.E. and Schlossman, S.F. (1980). A monoclonal antibody blocking human T cell function. Eur. J. Immunol. 10, 758.

Reinherz, E.L., Meuer, S.C., Fitzgerald, K.A., Hussey, R.E., Hodgdon, J.C., Acuto, O. and Schlossman, S.F. (1983). Comparison of T3-associated 49- and 43- kilodalton cell suface molecules on individual human T-cell clones: evidence for peptide variability in T-cell receptor structures. Proc. Natl. Acad. Sci. USA. 80, 4104.

Rinaldy, A., Wallace, B.R., Simon, M., Becker, A. and Epplen, J.T. (1985). A highly homologous T cell receptor ß-chain variable region is expressed in mouse and human T cells. Immunogenetics. 21, 403.

Ruddle, N.H. (1978). T Cell Hybrids with Specificity for Individual Antigens. Curr. Top. Microbiol. Immunol. 81, 203.

Russell, J.H., Masakowski, V.R. and Dobos, C.R. (1980). Mechanisms of immune lysis. I. Physiological distinction between target cell death mediated by cytotoxic T lymphocytes and antibody plus complement. J. Immunol. 124, 1100.

Russell, J.H., Masakowski, V., Rucinsky, T. and Phillips, G. (1982). Mechanisms of immune lysis III. Characterisation of the nature and kinetics of the cytotoxic T lymphocyte induced nuclear lesion in the target. J. Immunol. 128, 2087.

Ryser, J.E., Sordat, B., Cerottini, J.C. and Brunner, K.T. (1977). Mechanism of target cell lysis by cytolytic T lymphocytes I. Characterisation of specific lymphocyte-target cell conjugates separated by velocity sedimentation. Eur. J. Immunol. 7, 11.

Saito, H., Kranz, D.M., Takagaki, Y., Hayday, A.C., Eisen, H.N. and Tonegawa, S. (1984). Complete primary structure of a heterodimeric T-cell receptor deduced from c DNA sequences. Nature. 309, 757.

Samelson, L.E., Germain, R.N. and Schwartz, R.H. (1983). Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. Proc. Natl. Acad. Sci. USA. 80, 6972.

Samelson, L.E. and Schwartz, R.H. (1984). Characterisation of the antigen specific T cell receptor from a pigeon cytochrome c-specific T cell hybrid. Immunol. Rev. 81, 131.

Samelson, L.E., Harford, J.B. and Klausner, R.D. (1985). Identification of the components of the murine T cell antigen receptor complex. Cell. 43, 223.

Sanderson, C.J. (1976). The mechanism of T cell mediated cytotoxicity. II. Morphological studies of cell death by time lapse microcinematography. Proc. R. Soc. London Ser. B. 192, 241.

Schauer, R. (1985). Sialic acids and their role as biological markers. TIBS. 10, 357.

Shapiro, D.N., Adams, B.S. and Niederhuber, J.E. (1985). Antigen-specific T cell activation results in an increase in cytoplasmic free calcium. J. Immunol. 135, 2256.

Shearer, G.M. (1974). Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. Eur. J. Immunol. 4, 527.

Shinohara, N., Taniguchi, M. and Kojima, M. (1981). Mouse alloantibodies capable of blocking T cell function III. Studies of the mechanism of blocking CML by anti-Lyt-2 antibodies. J. Immunol. 127, 1575.

Silva, A., MacDonald, H.R., Conzelmann, A., Corthesy, P. and Nabholz, M. (1983). Rat x Mouse T-cell Hybrids with Inducible Specific Cytolytic Activity. Immunol. Rev. 76, 105.

Simon, M.M., Weltzien, H.U., Bühring, H.J. and Eichmann, K. (1984). Aged murine killer T-cell clones acquire specific cytotoxicity for P815 mastocytoma cells. Nature 308, 367.

Simpson, E. (1982). The role of H-Y as a minor transplantation antigen. Immunol. Today. 3, 97.

Simpson, E., Kontianen, S., Herzenberg, L.A., Bohrer, E., Torano, A., Vogt, P., Beverley, P., Fitzpatrick, W. and Feldmann, M. (1978). T Cell Hybrids with T Cell Functions. Curr. Top. Microbiol. Immunol. 81, 195.

Sitkovsky, M.V., Pasternack, M.S., Lugo, J.P., Klein, J.R. and Eisen, H.N. (1984). Isolation and partial characterisation of concanavalin A receptors on cloned cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA. 81, 1519.

Siu, C., Kronenberg, M., Strauss, E., Haars, R., Mak, T.W. and Hood, L. (1984). The structure, rearrangement and expression of Dß gene segments of the murine T cell antigen receptor. Nature. 311, 344.

Springer, T.A., Davignon, D., Ho, M.K., Kürtzinger, K., Martz, E. and Sanschez-Madrid, F. (1982). LFA-1 and Lyt-2/3, molecules associated with T lymphocyte-mediated killing; and Mac-1, an LFA-1 homologue associated with complement receptor function. Immunol. Rev. 68, 171.

Swain, S.L. (1981a). Significance of class 1 and class 2 major histocompatibility complex antigens: help to allogeneic K and D antigens does not involve I recognition. J. Immunol. 126, 2307.

Swain, S.L. (1981b). Significance of Lyt-phenotypes: Lyt2 antibodies block activities of T cells that recognise class I major histocompatibility complex antigens regardless of their function. Proc. Natl. Acad. Sci. USA. 78, 7101.

Swain, S.L. (1983). T Cell Subsets and the Recognition of MHC class. Immunol. Rev. 74, 129.

Taswell, C. (1981). Limiting dilution assays for the determination of immuno-competent cell frequencies I. Data analysis. J. Immunol., 126, 1614.

Thomas, P. (1980). Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA. 77, 5201.

Todd, R.F. (1975). Functional characterization of membrane components of cytotoxic peritoneal exudate T lymphocytes. II. Trypsin sensitivity of the killer cell receptor. Transplantation. 20, 314.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature 302, 575.

Valentine, M.A., Tsoukas, C.D., Rhodes, G., Vaughan, J.H. and Carson, D.A. (1985). Phytohaemagglutinin binds to the 20kDa molecule of the T3 complex. Eur. J. Immunol. 15, 851.

van den Elsen, P., Shepley, B. Cho, M. and Terhorst, C. (1985). Isolation and characterisation of a cDNA clone encoding the murine homologue of the human 20K T3/T-cell receptor gylcoprotien. Nature. 314, 542.

Van Wauwe, J.P., DeMay, J.R. and Goossens, J.G. (1980). OKT3: a monoclonal anti human T lymphocyte antibody with potent mitogenic properties. J. Immunol. 124, 2708.

von Boehmer, H., Hengartner, H., Nabholz, M., Lernhardt, W., Schreir, M.H. and Haas, W. (1979). Fine specificity of a continuously growing killer cell clone specific for H-Y antigen. Eur. J. Immunol. 9, 592.

von Boehmer, H.V., Haas, W., Köhler, G., Melchers, F. and Zeuthen, J. (1982). "T Cell Hybridomas". Curr. Top. Microbiol. Immunol. 100.

Weltzien, H.U. and Eichmann, K. (in press). Somatic variation of antigen-recognition specificity in H-2$^b$-TNP-specific cytotoxic T cell clones.

Whitaker, R.B., Kauffman, S., Che, M. and Finberg, R. (1982). CT Hybridomas: Tumor cells capable of lysing virally infected target cells. J. Immunol. 129, 900.

Wilson, A. and Shortman, K. (1985). Degradation of specificity in cytolytic T lymphocyte clones: two broad specificity, H-2-independent recognition systems, one natural killer-like, develop during culture, in addition to the clonally distributed antigen-specific receptor. Eur. J. Immunol. 15, 899.

Yagüe, J., White, J., Coleclough, C., Kappler, J., Palmer, E. and Marrack, P. (1985). The T Cell Receptor: The α and ß Chains Define Idiotype, and Antigen and MHC Specificity. Cell. 42, 81.

Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S.P., Aleksander, I., Mak, T.W. (1983). A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature. 308, 145.

Yoshikai, Y., Anatoniou, D., Clark, S.P., Yanagi, Y., Sangster, R., Van den Elsen, P., Terhorst, C. and Mak, T.W. (1984). Sequence and expression of transcripts of the human T-cell receptor ß chain genes. Nature. 312, 521.

Zagury, D., Bernard, J., Thierness, N., Feldman, M. and Berke, G. (1975). Isolation and characterisation of individual functionally reactive cytotoxic T lymphocytes: Conjugation, killing and recycling at the single cell level. Eur. J. Immunol. 5, 815.

Zagury, D. (1982). susceptibility of target cells to lysis and secretion of hydrolytic enzymes by CTL. Adv. Exp. Med. Biol. 146, 149.

Zamoyska, R., Vollmer, A.C., Sizer, K.C., Liaw, C.W. and Parnes, J.R. (1985). Two Lyt-2 Polypeptides Arise from a Single Gene by Alternative Splicing Patterns of mRNA. Cell. 43, 153.

Zinkernagel, R.M. and Doherty, P.C. (1974). Restriciton of in vitro T cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature. 248, 701.