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ANALYSIS OF CLONES OF
CYTOTOXIC LYMPHOCYTES

by

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University of Auckland

Thesis submitted in fulfilment of the requirements
for the degree of Doctor of Philosophy from the
University of Auckland

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>CI</td>
<td>cellular interaction</td>
</tr>
<tr>
<td>CL.P</td>
<td>cytotoxic lymphocyte precursors</td>
</tr>
<tr>
<td>CLS</td>
<td>cytotoxic lymphocytes</td>
</tr>
<tr>
<td>CMC</td>
<td>cell-mediated cytolysis</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>Cr</td>
<td>chromium</td>
</tr>
<tr>
<td>cy</td>
<td>cyclophosphamide</td>
</tr>
<tr>
<td>DS</td>
<td>dextran sulphate</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>k</td>
<td>kilo, $10^3$</td>
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<td>l</td>
<td>litre</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide B</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>m</td>
<td>milli, $10^{-3}$</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>minor H</td>
<td>minor histocompatibility</td>
</tr>
<tr>
<td>MLC</td>
<td>mixed lymphocyte cultures</td>
</tr>
<tr>
<td>MLTC</td>
<td>mixed lymphocyte-tumour cell cultures</td>
</tr>
<tr>
<td>MSV</td>
<td>moloney sarcoma virus</td>
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</table>
N \rightarrow (3\text{-}\text{nitro}\text{-}4\text{-}\text{hydroxy}\text{-}5\text{-}\text{iodophenylacetyl})\text{-}\beta\text{-}
\text{alanylglycylglycylglycylglycyl}

Pa

pascal

PBS

phosphate buffered saline

PHA

phytohaemagglutinin

POPOP

1,4\text{-}\text{bis}\text{-}(4\text{-}\text{methyl}\text{-}5\text{-}\text{phenyloxazoly})\text{-}\text{benzene}

POP

2,5\text{-}\text{diphenyloxazole}

\mu

\text{micro}, 10^{-6}

TNBS

2,4,6\text{-}\text{trinitrobenzene sulphonic acid}

TNP

2,4,6\text{-}\text{trinitrophenyl}
SUMMARY

1. 'Spontaneously' generated cytotoxic clones were detected when normal spleen cells from CBA, DBA/2 or (CBA x DBA/2)F₁ mice were cultured in polyacrylamide cultures vessels without stimulator cells. Cytotoxicity was mediated by T cells and the highest number of clones occurred after 4 days in culture. The spontaneous cytotoxic T cell clones were detected mainly in adult spleen cell cultures. Few spontaneous clones were generated by lymph node, thymus or neonatal spleen cells.

2. The production of spontaneous clones does not increase linearly with the number of cells cultured which is in contrast with the production of 'stimulated' clones of cytotoxic lymphocytes in the polyacrylamide vessels. At the optimal cell concentration, 1.3 x 10⁷ cells per culture, 20 spontaneous clones of CLs lysing P815 mastocytoma cell targets were detected in cultures of (CBA x DBA/2)F₁ spleen cells and 14 clones were detected in cultures of CBA or DBA/2 spleen cells.

3. The specificity of the spontaneous clones was examined by dividing each clone into two halves and assaying the half clones against a pair of different target cells. A range of spontaneous CLs of different specificities was produced. Spontaneous clones lysing syngeneic and allogeneic tumour or normal spleen cell blasts, as well as hapten-modified target cells were detected.
4. Individual spontaneous clones of CLs exhibited a high degree of discrimination and were able to differentiate between many pairs of target cells which were syngeneic with respect to each other.

5. When blast cells which had been induced by various mitogens were used as the target cells, the results indicated that spontaneous CL clones could discriminate between subsets of syngeneic lymphocytes which respond to different mitogens.

6. One-way stimulated CL responses were generated using cells from mice which had been treated with cyclophosphamide. Treatment of mice with 200 mg/kg cyclophosphamide abolished the ability of the cells to generate spontaneous clones in culture without impairing their ability to stimulate the production of CLs in responder cell populations.

7. In contrast with spontaneous clones which could discriminate between different $H_2^d$ target cells, CLs produced by CBA spleen cells stimulated with $H_2^d$ alloantigens were observed not to differentiate between various $H_2^d$ target cells. The results indicated that spontaneous clones of CLs were not a representative sample of the stimulated clones of CLs.

8. When CBA spleen cells were stimulated simultaneously with $H_2^b$ and $H_2^d$ alloantigens, separate populations of CLs against the two sets of antigens were produced. Very few cross-reactive clones were detected.
9. When (CBA x C\textsubscript{57, Bl})\textsubscript{F\textsubscript{1}} spleen cells were stimulated with syngeneic F\textsubscript{1} cells modified with TNP, clones of CLs lysing TNP modified cells of the F\textsubscript{1} and the two parental strains were produced. The frequency of clones produced by F\textsubscript{1} spleen cells against F\textsubscript{1}-TNP, CBA-TNP and C\textsubscript{57, Bl}-TNP target cells was 1 per $3.3 \times 10^4$, 1 per $6.7 \times 10^4$, and 1 per $10^5$ spleen cells respectively.

10. When (CBA x C\textsubscript{57, Bl})\textsubscript{F\textsubscript{1}} cells were cultured with TNP-modified CBA cells, clones of CLs against TNP-modified cells of both the parental strains were produced. CLs which lyse CBA-TNP and CLs which lyse C\textsubscript{57, Bl}-TNP targets segregated as two distinct populations with no cross-reactivity.
CHAPTER 1

GENERAL INTRODUCTION

A striking feature of immune responses is their exquisite specificity for the immunizing antigen. This is true for both humoral responses by bone-marrow derived B lymphocytes and for cell-mediated responses mediated by thymus-derived T lymphocytes.

The specificity of humoral responses is generally attributed to a state of diversity among antibody producing B cells. There is much experimental data in support of the principles of clonal selection, which suggest that the immune system contains a large number of immunocompetent cells that differ with respect to the specificity of the antibody molecules that the cell, and all its progeny are committed to synthesize (Burnet, 1959). It is generally regarded that clonal selection and activation results from the interaction of antigen with receptor molecules on the surface of the lymphocytes, which causes the proliferation and division of each of those precursor cells to form a clone of progeny cells secreting antibodies of a single specificity (Askonas et al., 1970*). The presence of antigen-binding sites on the surface of B lymphocytes is well documented (Raff, 1970; Basten et al., 1971). With the notion that these receptors are an accurate sample of the antibody molecules that the cell is committed to synthesize (Mitchison, 1967), the nature of the receptors for antigen on B lymphocytes can be derived from the analysis of

* In the introduction, no attempt has been made to list every pertinent reference. In many cases, only a single major reference is included.
secreted antibody molecules, and much data on the biochemical structure of immunoglobulins has been accumulated (Reviewed by Vitteta and Uhr, 1975; Marchalonis, 1976).

It is still an open question whether the specificity of immune responses mediated by T lymphocytes is similarly based on a state of diversity among T cells precommitted to the expression of receptors of a single specificity, mainly because of difficulties in studying antigen receptors on T cells. While few immunologists will dispute that T lymphocytes do possess receptors, the nature of these receptors has been a matter of great controversy.

1.1 THE EXISTENCE OF A T CELL RECEPTOR

A variety of experiments such as the inactivation of T cells by heavily labelled radioactive antigen (Basten et al., 1971; Roelants and Askonas, 1971), the binding of T cells to antigen coated columns (Davie and Paul, 1970; Rutishauser and Edelman, 1972), radioautography of T cells incubated with labelled antigen (Rankhurst and Wilson, 1971) and the rosette forming test (Wilson and Feldman, 1973) have all demonstrated specific antigen-T cell contact, but the nature of the T cell antigen-binding sites remains obscure. Because of a lack of a sufficient density of receptors on the cell surface, or their rapid shedding from the T cell membrane (Ramseier, 1975), highly sensitive techniques well suited to the study of receptors on B cells have either failed or given results which have been regarded as equivocal when applied to T cells (Rabellino et al., 1971; Nossal et al., 1972). Studies on T cell receptors lack also the advantage that is obtained in similar B cell studies. B lymphocytes synthesize and secrete their antibody products which may be considered as replicas of their antigen receptors. This situation
does not occur with T cells, and replicas of T cell receptors are not found circulating in the serum to be isolated for analysis.

From considerations of economy, Mitchison (1967), reasoned that T cell receptors were antibody molecules of some kind. True to this prediction, antibody-like molecules have been found, or have been extracted from the surface of T cells (Cone et al., 1972), but the observation that T lymphocytes can bind antibodies and antigen-antibody complexes (Yosida and Andersson, 1972) has thrown doubts as to the significance of the reports, and has lead to the suggestion that T cells may mediate their function by acquiring a receptor which has been passively absorbed (Playfair et al., 1974).

Using antibodies directed against the antigen binding areas to try to analyse and isolate the antigen-binding structure of T cells, Binz and Wigzell (1975) were able to demonstrate that T and B lymphocytes of an individual with antigen-binding receptors for a given major histocompatibility antigen express shared idiotypic determinants. This indicated that antigen-binding sites of T and B receptors produced in the same individual with specificity for the same antigen were in part identical. Further analyses showed that the idiotypes shared between T and B cell receptors were present almost exclusively on the heavy chain of the immunoglobulin molecules (Binz and Wigzell, 1976).

On the other hand, a large body of evidence indicate that genes in the major histocompatibility complex (MHC) control the recognition of antigen by T lymphocytes (Katz and Benacerraf, 1975). The observation that the inability of T cells but not B cells to respond to certain antigens is often linked to the MHC locus (Benacerraf and McDevitt, 1972) has lead to the suggestion that MHC products may themselves be the basic T cell receptor.
Attempts at unifying all the groups of data have resulted in hypotheses which propose that the T cell receptor may consist of two sub-units, one of which may be immunoglobulin, while the other is a product of the MHC genes (Braun, 1976). In support of such models, Krammer and Eichman (1977) have been able to demonstrate that the expression of T cell receptor idiotypes is controlled by two unlinked genes. One of these genes is located in the heavy chain linkage group (Ig-1 complex) while the second gene is located in the MHC. Whereas all the available evidence suggests that the genes of the Ig-1 complex directly encode a heavy chain V region peptide that is part of the T cell receptor, the authors have not yet been able to determine whether the control exerted by the H2 linked genes was due to an indirect regulatory influence of H2 antigens, or to a direct coding of genes in the H2 complex for parts of the T cell receptor. However, in view of all the controversy and contradictory reports, perhaps the situation has been summarized best by Binz and Wigzell (1976) who concluded that 'attempts to construct a unified concept at the present stage of our knowledge seem somewhat premature!'

While the direct biochemical characterization of T cell receptors remains elusive, the nature of the antigen recognition unit on T cells has been studied also using less direct approaches. The specificity of the receptors has been assessed functionally as to the types of antigens which they recognize and as to their ability to discriminate between similar antigenic determinants. While some studies have concluded that the discriminatory ability of T cells is as precise as that shown by B cells (Rajewsky and Mohr, 1974; Jane-way, 1976), other investigations have suggested that T cells are more cross-reactive and have a lower degree of specificity than do B cells (Ruben et al., 1973; Hoffman and Kappler, 1973).
One of the problems encountered in assessing the specificity of T cell receptors may be due to the fact that T lymphocytes mediate a diversity of functions such as graft versus host, delayed hypersensitivity, cell-mediated cytotoxicity, as well as helper and suppressor activities for both humoral and cell-mediated responses. The work of Cantor and Boyse (1975a) has indicated that the commitment of T cells to participate in the various effector functions is a differentiation process that takes place before the cells encounter antigen. There is evidence which suggests that the individual functions are performed by subsets of T cells with different physical and biological properties and are distinguished by different Ly antigenic markers (Shiku et al., 1975). The effector cells involved in the various functions appear to respond to different types of antigens, for example, cellular antigens stimulate mainly the generation of cytotoxic lymphocytes while bacterial and fungal determinants are best at evoking delayed hypersensitivity reactions. These observations would tend to suggest that the various effector cell populations may differ in their repertoire of specificities and their mechanism of activation.

Furthermore, with the growing appreciation of the involvement of T cell - T cell interactions in the generation of many of the effector cell functions, it has become clear that in addition to possessing receptors for antigen, T cells must also possess recognition or interaction structures to enable correct cellular interactions to occur amongst the appropriate populations of cells. For the purposes of this thesis however, the specificity of antigen recognition will be considered only in relation to the generation of cytotoxic lymphocytes (CLs).
1.2 THE GENERATION OF CYTOTOXIC LYMPHOCYTES

It has been well established that T lymphocytes respond to cell-associated antigens by the production of a special class of effector cells which will specifically lyse target cells bearing the sensitizing antigens (Mauel et al., 1970). These effector cells are referred to as cytotoxic lymphocytes (CLs). The generation and characteristics of CLs have been reviewed extensively (Perlmann and Holm, 1969; Cerottini and Brunner, 1974) and will be summarized only briefly in this Introduction.

CLs are generated in vivo in allograft systems by transplanting foreign stimulator cells into an animal or in a graft versus host situation whereby live lymphoid cells are injected into an irradiated allogeneic or semi-allogeneic recipient. CLs can also be generated in vitro in mixed lymphocyte cultures (MLC) in which responder cells from one strain are cultured with stimulator cells which themselves do not react to the responder cells, or which have been inactivated by irradiation or treatment with drugs. CLs can also be produced in culture using polyclonal activators such as concanavalin A (ConA).

The presence of CLs is usually assessed by their ability to kill target cells. There are several assay systems for measuring CL activity (Bloom and Glade, eds. 1971) but the most commonly used method is the \(^{51}\text{Cr} \) release assay (Brunner et al., 1970).

MLC have provided a useful in vitro experimental model to analyze the series of events involved in the generation of CLs which are regarded as being responsible for allograft rejection and immune surveillance against abnormal cells. CLs are generated from precursor cells (CL.P) which are themselves T cells. CL.P are present in the thymus spleen and other tissues containing T cells. The precursors have been found to differ from CLs in physical properties
such as radiation sensitivity, size and buoyant density (Shortman et al., 1972; MacDonald et al., 1973).

The generation of CLs is influenced by the presence of other cell types. Macrophages have been shown to be obligatory (Wagner et al., 1972) and the addition of filler cells from nude mice will give a synergistic effect which is different from a feeder effect (Schilling et al., 1976). The magnitude of CL production is increased by helper or amplifier T cell activity (Cantor and Simpson, 1975).

Experiments using antisera directed against the Ly differentiation antigens on T cells have shown that CLs have the Ly2\(^+\)3\(^+\) phenotype, but the generation of these Ly2\(^+\)3\(^+\) effectors was amplified by T cells with the Ly1\(^+\) phenotype (Cantor and Boyse, 1975). Ly1\(^+\) cells also account for the major portion of the proliferative reaction in MLC, but these cells themselves are not part of the effector cell pool.

Other evidence has suggested that the amplifier cells are stimulated by antigens controlled by the I and S region of the MHC (LD antigens) to amplify the production of CLs which recognize antigens defined by the K and D regions of the H2 locus (SD antigens) (Schendel and Bach, 1974). However, this type of co-operation does not appear to be mandatory as cytotoxicity can be generated without an LD stimulus (Forman and Klein, 1975). Another type of helper cell, which was reported to be an absolute requirement for the generation of CLs has been described. These helpers were radio-resistant, antigen specific T cells and were able to co-operate across allogeneic barriers (Pilarski, 1977). The generation of CLs is also subject to suppressor cell activity. Suppressor cells also have the Ly2\(^+\)3\(^+\) phenotype (Feldmann et al., 1977) and both antigen specific (Wagner et al., 1976) and non-specific types of suppressor cell activity (Hirano and Nordin, 1976) have been reported to be involved in CL
production.

Anamnestic responses of CLs have been reported (MacDonald et al., 1974; Häyry and Andersson, 1975) and following the development of long term lymphocyte cultures, it has been shown that CLs generated in a primary MLC will revert with time to secondary non-lytic T cells (MacDonald et al., 1974). On antigenic restimulation these non-lytic 'memory' cells acquire antigen specific cytotoxic activity within 20-30 hours (Wagner and Röllinghoff, 1976). The antigenic requirements for the induction of a secondary response has been reported to be qualitatively different from those required for the induction of a primary response (Röllinghoff and Wagner, 1975).

The mechanism by which CLs lyse target cells is a multistep process, beginning with a specific recognition stage which involves specific receptors at the surface of the CLs recognizing antigens on the surface of the target cell (Golstein et al., 1971; Berke and Levey, 1972). The post recognition stage includes a lethal hit of an unknown nature resulting in irreversible target cell damage and finally target cell disintegration (Wagner and Röllinghoff, 1974). The continuous presence of CLs at the target cell surface is not required for target cell disintegration and the CL can go on to kill more than the one target cell (Berke and Amos, 1973). The physiological requirements and the mechanisms of target cell disintegration has been reviewed (Berke and Amos, 1973; Henney, 1973) and will not be included in this Introduction which is concerned mainly with the specific antigen recognition step.

1.3 THE SPECIFICITY OF CYTOTOXIC LYMPHOCYTES

There are two stages of antigen recognition in a cell-mediated response. There is the recognition of antigens on the stimulator
cell by CL.P during the induction phase of the response, and there is also the recognition of target cell antigens by the CLs in the lytic phase of the reaction. Many of the studies on the specificity of cell-mediated responses have been at the effector cell stage and less is known about the antigen recognition step by CL.P at the induction phase. However, if the accurate sample hypothesis (Mitchison, 1967) is also applicable to CLs, then the specificity of antigen recognition at both the induction and effector phases could be considered to be identical, but this has yet to be formally demonstrated or disproved.

Evidence that CLs have specificity was demonstrated originally by their ability to be absorbed specifically to cell monolayers (Goldstein et al., 1971). CLs were also shown to specifically lyse only target cells bearing antigens identical or crossreactive with the antigens on the sensitizing cells, while non-related third party cells were not affected (Mauel et al., 1970). Other approaches to demonstrating specificity involves the use of unlabelled 'cold targets' to competitively inhibit specific cell lysis of radioactively labelled targets. Lysis of the labelled target cells has been shown to be blocked only by the cold targets which have antigenic determinants in common with the radioactive targets. Under the right conditions, cells with unrelated determinants are not effective in inhibiting specific lysis (Ortiz de Landazuri and Herberman, 1972).

1.31 Allogeneic Responses

It has become evident that the products of the MHC play an important role both in the induction of cell-mediated cytotoxicity (CMC) and in target cell recognition by CLs. The strongest CL responses are those elicited by intra-species MHC alloantigens with the histocompatibility antigens of a single foreign haplotype being able to stimulate 1-4 per cent of the total pool of CL-P (Skinner and Marbrook, 1976; Lindahl and Wilson, 1977). Using intra H2 recombinant strains
of mice, strong lytic activity can be generated in all responder-stimulator combinations which differ at either H2D or H2K or both regions of the H2 complex (Nabholz et al., 1974). Antigenic differences derived from a single mutation in either H2K or H2D were able to stimulate CMC (Klein et al., 1975; Forman and Klein, 1975).

The specificity of CLs sensitized against a foreign H2 haplotype is primarily directed against antigens determined by the H2K and H2D regions of the MHC. This was shown most convincingly using intra H2 recombinant strains of mice (Nabholz et al., 1974). Further evidence of CL specificity for H2 antigens was demonstrated by the inhibition of CMC with alloantisera directed against H2 antigens on the target cell (Mael et al., 1970). In addition, a teratoma cell line, phenotypically H2 negative was unable to block CMC against H2 antigens of the allele of the strain from which the line was derived (Forman and Vitetta, 1975). Using cold target inhibitors (Bevan 1975a) or specific antisera against H2K or H2D specificities (Nabholz et al., 1974) it has been shown that separate populations of CLs recognize H2D and H2K determinants. Work using mice with mutations in the H2K or D regions have suggested that the response to each foreign K or D allele is multiclonal as there are more than one specificity recognized by CLs per K or D antigen (Forman and Klein, 1975; Melief et al., 1975).

When the responder and stimulator strains differ only at the I region of the H2, weak CMC can be detected against I region antigens if the target cells used are B cell blasts which express an abundant amount of Ia antigens (Wagner et al., 1975). Generally however, differences in the I region stimulate strong proliferative and helper activity which amplifies the CL response to H2K and H2D specificities. Thus, the response induced by a complete H2 complex incompatibility is much greater than the sum of H2K, H2D and I region incompatibility respectively (Wagner et al., 1975).
1.32 Xenogeneic Reactions

Cell-mediated cytotoxicity has been demonstrated for several xenogeneic combinations (Beverley and Simpson, 1972; Dennert, 1974). There is much evidence which suggests that the antigenic structures that elicit a response in xenogeneic combinations are the same as those stimulating allogeneic responses, namely the major histocompatibility antigens. CLs from rat or human lymphoid populations immunized against mouse cells are specifically cytotoxic to target cells of the same H2 type as the sensitizing strain (Burakoff et al., 1977; Carraud et al., 1977). In human anti-mouse systems, it was shown using intra H2 recombinant strains of mice that the antigens serving as targets for the xenogeneic CLs were those coded by the K and D extremities of the H2 complex (Lindahl and Bach, 1975). The LD antigens did not function as target antigens, or did so only poorly. There was no evidence of differential recognition of non-H2 antigens as no cross-reactivity was observed between target cells expressing the same non-H2 background but different H2 haplotypes. Similar observations were obtained using rat anti-mouse systems. Alloantisera directed to the products of the K and D loci of the target cells inhibited lysis, whereas antiserum to the I region gene product did not (Burakoff et al., 1977). Thus, it seems that even in xenogeneic reactions against mouse cells, the target antigens are those coded by the K and D loci of the MHC. However, the observation that a variant mouse cell line which had lost its H2 SD antigens could be lysed by xenogeneic rat lymphocytes indicated that species-specific antigens may also play a minor role in xenogeneic CMC (Dennert and Hyman, 1977).

1.33 Responses to Antigens Other Than MHC Determinants

In recent years, CL responses have been generated to syngeneic
cells which have been virally-infected or chemically-modified, as well as to tumour, minor histocompatibility and the male-specific antigens. In these situations, even though there were no differences in the H2 antigens between responder and stimulator cells, and the response was apparently against a non-H2 antigen, H2 antigens were found to be an integral part of the recognition structure on the target cell. These studies on CL responses to non-H2 antigens have contributed to our understanding of antigen recognition by CLs.

1) Responses to Virally-Infected Cells

CLs generated in mice infected with virus are specifically lytic for virus-infected target cells. As well as being specific for the infecting virus, lysis occurs only when the infected target cells and the donors of CLs are compatible for at least part of the H2 gene complex. This has been observed for CL responses to several different groups of viruses (Koszinowski and Ertl, 1975; Blanden et al., 1975; Doherty and Zinkernagel, 1975), whether the T effector cell function had been measured in vitro (Zinkernagel and Doherty, 1975) or in vivo (Zinkernagel and Welsh, 1976). Similar results were obtained with virus immune CLs generated in in vitro primary responses (Dunlop and Blanden, 1977a) or in secondary responses obtained by culturing spleen cells from mice which had been inoculated with virus with freshly infected syngeneic stimulator cells (Yap and Ada, 1977).

Studies using recombinant strains of mice have indicated that compatibility at H2K and H2D between the effectors and infected target cells was necessary for virus-specific CMC. I region compatibility was not necessary and by itself was not sufficient for cytolysis (Zinkernagel et al., 1976). In parental-F₁ interactions, the presence of a completely unshared H2 chromosome had no obvious deleterious effect (Koszinowski and Ertl, 1975) and neither did the
presence of different H2K or H2D genes in experiments using the recombinant strains (Zinkernagel et al., 1976). These observations indicated that the H2 restriction was not due to some form of allogeneic inhibition. Other evidence that H2 products were involved in CL recognition of virally-infected cells has come from the demonstration that antisera binding to H2 antigens on the target cell can block CMC by virus immune CLs (Koszinowski and Ertl, 1975). Furthermore cells which phenotypically do not express H2 antigens (Doherty et al., 1977) or which have had H2 antigens removed enzymatically (Ertl and Koszinowski, 1976) are not susceptible to virus-specific CMC, even though the cells had been infected and express viral antigens. Recombinant strains of mice used in conjunction with cold target inhibition studies have indicated that separate populations of virus immune T cells are associated with each of the H2K and H2D loci, and with each allele at these loci in F1 mice (Zinkernagel and Doherty, 1975).

The requirement for H2 compatibility was examined more closely using mice which carried mutations occurring in a single cistron at H2K. It was found that the use of responder-target cell combinations that differed only at the mutation, and shared all other genes of the H2 complex could abolish or impair CMC, even though serologically the H2 specificities of all the mutant mice remained qualitatively identical (Blander et al., 1976). No complementation of the mutant genes was observed in F1 mice which suggested that the H2K genes involved in the recognition of virus-infected cells by virus immune T cells behaved as a single element (Blander et al., 1976).

The requirement for H2 homology is not restricted to self H2 determinants. Under conditions where tolerance to H2 alloantigens is achieved, T cells of one H2 type can be conditioned to recognize virus-
infected targets of another H2 type. CMC of virus-infected cells across the H2 barrier was shown also to be H2 restricted. This was demonstrated using irradiated F1 chimaeras which had been reconstituted with bone-marrow cells from only one parent. When these mice were later infected with virus, cytotoxicity was generated against infected targets of both syngeneic and the tolerated allogeneic strains. Cytotoxicity was not detected against uninfected cells or unrelated third party infected cells (Zinkernagel, 1976). Cold target inhibition studies showed that the cytotoxicity against infected syngeneic and the tolerated allogeneic cells was mediated by different sets of CLs. These chimaera experiments demonstrated that there was no requirement for self recognition by mutually expressed H2 antigens, but T cells must recognize H2 to which they have been sensitized.

The specificity of the CLs for virus was demonstrated in that non-infected cells were not lysed and neither were H2 compatible target cells infected with viruses other than the sensitizing virus. For example, CLs from mice infected with vaccinia virus do not lyse targets infected with ectromelia virus and vice-versa (Doherty and Zinkernagel, 1976). Reciprocal exclusion of cytotoxicity has been observed for several diverse groups of viruses.

It has been shown with ectromelia and lymphocytic choriomeningitis virus that protein synthesis after infection of target cells was necessary for subsequent CMC, suggesting that the synthesis of a new protein species which appeared in the plasma membrane was necessary for recognition by CLs (Jackson et al., 1976). In the vaccinia system, using substrains of virus that differed in the expression of viral surface antigens, it was shown that DNA replication and production of late surface antigens was not a pre-requisite for cellular
anti-vaccinia virus sensitization. Induction of early vaccinia virus surface antigen was sufficient for sensitization and specific lysis (Koszinowski et al., 1976). These observations were confirmed by inhibition studies using antisera against the different vaccinia antigens. Thus the virus-specific component of the CL receptor does not appear to be recognizing antigens of the vaccinia virus particle. With Sendai virus however, viral infection or protein synthesis does not seem necessary. Responses can be generated using inactivated Sendai virus both in the immunizing step and for the modification of target cells. It was inferred that the virus-specific component was probably a preformed virion antigen absorbed or integrated into the cell membrane (Schrader and Edelman, 1977).

The fine specificity of the CLs for virus has also been studied using influenza virus. The A and B strains are serologically distinct except for a common host determinant, but within the A strain are a variety of subtypes which share internal ribonucleoprotein and matrix components but express different haemagglutinin and neuraminidase surface antigens (Choppin and Compsans, 1975). It was found that CLs from mice immunized with A strain influenza virus do not lyse target cells infected with B strain virus, and CLs from mice immunized with B strain virus do not lyse cells infected with A strain virus (Effros et al., 1977; Zweerink et al., 1977). Extensive crossreactivity was found however for lymphocyte populations from mice infected with a variety of serologically distinct influenza A viruses. A major proportion of the CLs generated were unable to discriminate between different type A substrains of influenza virus, although competitive inhibition experiments indicated that a proportion of the response was restricted to the immunizing strain of the virus. It has been suggested that the matrix proteins are the target antigens for the
virus strain specific CLs, while the specificity of the cross-reactive CLs are directed against the influenza haemagglutinin or neuraminidase determinants (Braciale, 1977).

As more information is becoming available concerning the structure of viruses, it should be possible to use the anti-viral system to define the precise interactions between cells involved in the generation of CLs and to determine the basis of the H2 compatibility requirements between CL and virus-infected targets.

ii) Responses to Hapten-Modified Cells

Spleen cells derivatized with the hapten TNP, which appears to affect plasma membrane molecules rather than internal cellular components (Forman, 1977) can sensitize T lymphocytes to develop CMC against TNP-modified targets (Dennert and Hatlen, 1975; Shearer et al., 1975). These CLs sensitized against the modified stimulator cells have specificity for the TNP determinant. This was demonstrated in that cytolysis could be inhibited by anti-TNP antisera (Forman et al., 1977a) and by the TNP coupled to chicken erythrocytes (Dennert and Hatlen, 1975). Furthermore, TNP-modified cells were much more efficient than unmodified cells as competitors in inhibition studies. CLs produced upon sensitization with TNP-modified syngeneic stimulators lysed modified syngeneic targets only and not the un-modified cells (Shearer et al., 1975). Evidence from immunoprecipitation and co-capping techniques have indicated that TNP couples directly to H2 and other proteins on the cell surface and that TNP is associated with the antigenic determinants that the CLs recognize (Forman et al., 1977a).

Involvement of H2 gene products in TNP-specific CMC was demonstrated using CLs sensitized to syngeneic TNP-modified stimulators. When spleen cells which had been sensitized in vitro by TNP-modified syngeneic cells were assayed for cytotoxicity using syngeneic, congenic, recombinant, allogeneic modified targets and also unmodified cells, it
was shown that TNP modification was necessary but was not the only requirement for CMC. H2 homology at K or D region between modified stimulator and modified targets, or between effectors and modified targets was also necessary for cytolysis (Shearer et al., 1975). Subsequent experiments using F1 mice indicated that it was H2 homology between target and stimulator, rather than homology between responder and target which was required. F1 spleen cells sensitized with TNP-modified cells of one of the parental strains developed cytotoxicity for TNP-modified cells of the strain used for sensitization and not for modified cells of the other parent (Shearer et al., 1975; Forman, 1975). As F1 effector would have the H2 haplotype of both parents, the results indicated, that rather than the specificity of CMC being controlled by a requirement for homology between responder and target, the specificity of lysis was restricted to modified targets of the same H2 type as the sensitizing strain. This concept was confirmed in studies using radiation chimaeras. The T cells from chimaeras prepared by transferring bone-marrow from one of the parental strains (P1) into an irradiated F1 recipient (P1 x P2) are almost entirely of donor P1 origin and do not manifest cytotoxicity against host determinants of the other parental strain (P2) (Sprent et al., 1975). When cells from such chimaeras were sensitized against TNP-modified histoincompatible cells of the other parent of the F1 (TNP-P2), the CLs generated lysed the modified allogeneic P2 targets but failed to lyse modified syngeneic P1 targets (von Boehmer and Haas, 1976). The data from such chimaera experiments indicated that CLs could be sensitized against modified allogeneic cells and the specificity of cytolysis was restricted to modified targets of the same H2 type as the sensitizing cells.

It has been observed that H2 homology between the cells used for
primary and secondary stimulation was required in order to restimulate a secondary response to TNP. Lymphocytes generated by primary in vitro sensitization with TNP-modified syngeneic cells could be restimulated by TNP-modified cells sharing the H2 haplotype with the primary TNP-modified immunogen, but not by TNP-modified cells allogeneic to the primary stimulators (Schmitt-Verhulst et al., 1977). Thus secondary restimulation exhibits the same H2 dependent specificity requirements as those observed in the lytic phase of the primary in vitro CMC.

Many of the observations obtained using the TNP system has been confirmed using other systems of hapten modification (Rehn et al., 1976; Starzinski-Powitz et al., 1976). With spleen cells sensitized in vitro to N-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanylglycyl-glycyl-(N) modified autologous lymphocytes, it was similarly observed that both N-modification of the targets and H2 homology between stimulator and target cells was required for lysis to occur (Rehn et al., 1976). Furthermore, CLs generated by sensitization with syngeneic spleen cells modified with either TNP or N, lysed only H2 matched targets modified with the same reagent. No cross-reactivity was detected for CMC generated against TNP-modified and N-modified syngeneic spleen cells (Rehn et al., 1976). Similarly, the lytic activity of T cells sensitized towards fluorescein isothiocyanate-(FITC) conjugated syngeneic cells was restricted to FITC-conjugated, H2 compatible target cells and did not lyse TNP-modified targets of other H2 strains (Starzinski-Powitz et al., 1976). The lack of cross-reactivity in the CMC generated against the various haptens indicated that haptenic modification created distinct new antigenic determinants which were recognized by the CLs, but the precise relationship of H2 products to the haptenic determinants recognized and the role of the H2 antigens in restricting CMC has yet to be defined.
iii) Responses to Differentiation Antigens

Very little cytotoxicity is generated in a primary MLC when the stimulator cells are not modified and share the same H2 complex as the responder cells. However, if mice are primed in vivo and then the cells are boosted in vitro in MLC, CMC against differentiation and other non-H2 associated cellular antigens can be detected. CMC against the male specific antigen has been produced in such a manner and it has been shown that the response of cells from female mice primed to the male-specific antigen was restricted to male target cells histocompatible for at least a part of the H2 complex (Gordon et al., 1975).

When cells from mice primed with stimulator cells from a strain which carries the same H2 genes, but on a different background, are boosted in vitro with the same stimulator cells, CLs which would be sensitized to minor histocompatibility (H) antigens are generated. The response to minor H antigens has also been shown to be H2 restricted, in that the CLs will lyse only targets which share the same H2 type as the immunizing strain. Targets from congenic strains which carry all the same minor H antigens but differ at both H2K and H2D regions of the MHC are not susceptible to lysis (Bevan, 1975b).

F1 cells immunized against a strain that carries one of the parental haplotypes, produced CLs which lysed target cells of the immunizing strain only. Cells congenic with the immunizing strain but carrying the H2 haplotype of the other parent of the F1 were not lysed (Bevan, 1975b). Although H2 restriction of cytolysis was almost absoluble, it was shown that H2 restriction did not apply during the in vivo priming phase of induction to minor H antigens (Bevan, 1976). It was demonstrated by Bevan (1976), that F1 cells primed in vivo with cells of a strain carrying the H2 haplotype of one of the parents,
then boosted in vitro with cells congenic with the initial priming strain carrying the H2 haplotype of the other parent of the F₁ will produce an excellent secondary response to the cells used for boosting in vitro. Thus, priming was not restricted to the H2 type of the stimulator cells but to both the H2 types of the F₁ host.

Results using radiation chimaeras also supported the concept that the H2 antigens of the host determined the responsiveness of the CLs (Bevan, 1977). Bone-marrow cells from F₁ mice were used to reconstitute lethally irradiated parental mice. The cells from these chimaeric mice were primed in vivo and boosted in vitro against minor H antigens using stimulator cells from an F₁ hybrid which carried different minor H antigens but the same H2 haplotypes as the donors of the bone-marrow cells. Following this procedure, it was shown that the cells from the chimaera responded preferentially to the minor H antigens in association with the H2 antigens of the host, although cells from normal F₁ mice similarly immunized produced CLs which responded to minor H antigens associated with each of the parental haplotypes.

iv) Response to Tumour Antigens

Cytotoxic responses to tumour antigens has been studied in several experimental tumour systems. The Moloney sarcoma virus (MSV)-induced tumour has been the most widely used. The nature of the tumour associated antigens recognized by the CLs has yet to be defined. Using an inhibition assay to study the specificity of CL activity in spleens of MSV-injected mice, it was suggested that the surface antigens involved were related to the expression of a particular endogenous C-type virus rather than to MSV-associated antigens (Herberman et al., 1974). On the other hand, partial inhibition of DNC by the addition of disrupted virus or of the major internal viral polypep-
tide has been reported (Gorczynski and Knight, 1975). Assessment of
direct lysis of various syngeneic tumour cell lines by CLs generated
in secondary mixed lymphocyte-tumour cell cultures (MLTC) indicated
that the surface antigens involved were common to MSV-induced sarcoma
cells and Rauscher virus or Graffii virus-induced lymphoma cells
(Plata et al., 1975).

Spleen cells from mice immunized with MSV exhibited strong cyto-
toxicity against MSV or Rauscher induced syngeneic tumour targets.
However, various levels of cytotoxicity were also observed against
allogeneic MSV or Rauscher tumour cells, indicating that Cls of
tumour associated antigens may not be H2 restricted as in other
responses to non-H2 antigens (Holden and Herberman, 1977). When
spleen cells from mice having rejected MSV-induced tumour were in-
cubated with syngeneic irradiated tumour cells bearing MSV-associated
antigens, the CLs generated exhibit preferential lysis of syngeneic
tumour targets compared to allogeneic tumour cells. Syngeneic
tumour targets were lysed 10-100 fold more efficiently than allogeneic
tumour targets, which suggested an MHC-linked restriction of CL
activity. More direct evidence for the involvement of MHC in lysis
of tumour targets was provided by studies using F1 mice. Stimulation
of immune spleen cells with tumour cells from one parental strain or
the other in secondary MLTC resulted in the generation of CLs which
lysed tumour targets of the same strain as the stimulating cells but
not of the other parental strain. The results suggested the presence
of two sets of CLs in F1 MSV-immune spleens, each set responding ex-
clusively to tumour antigens associated with only one of the two
parental haplotypes (Plata et al., 1976; Comard et al., 1976).
Participation of the H2 antigens of tumour cells in their lysis by
tumour-specific syngeneic CLs were also shown in in-
hibition studies using anti-H2 alloantisera. The ability of anti-H2 sera specific for the tumour target cell to block the lysis of the cells indicated that the H2 antigens on the targets were crucial to the cytotoxic interaction (Gernaia et al., 1975; Schrader and Edelman, 1976). It must be noted however, that in the tumour system, H2 incompatibility strongly decreases but does not completely abolish tumour cell lysis. Although there is evidence which suggests the involvement of H2 products in CMC of tumour cells, there is other experimental data indicating that H2 restriction may not be as rigorously applicable in the tumour system as in CMC of other non-H2 antigens.

1.4 THE CONTRIBUTION OF H2 PRODUCTS TO CELL-MEDIATED CYTOTOXICITY

Two general hypotheses have been put forward to explain the involvement of H2 antigens in CMC against non-H2 antigens. The dual recognition, or cellular interaction hypotheses grew out of studies on the interaction of helper T cells with B cells (Katz and Benacerraf, 1975) and of T cell responses to antigen-bearing macrophages (Shevach and Rosenthal, 1973). In those situations, the I region controls the cellular interaction, but the same theory can be applied to the K and D restriction of cytolytic interactions of CLs with targets. The hypothesis states that CLs have two different kinds of receptors on their surface: one is a cellular interaction (CI) structure coded in the K or D region which recognizes homologous K or D coded products on the target cell surface. The other receptor is the antigen specific receptor which recognizes a foreign structure such as viral or haptenic determinants or differentiation antigens.

An alternative model is the one receptor or altered self hypothesis which predicts that CLs have only one type of receptor on
their surface. These receptors have a high frequency of reactivity with structures which can be seen as modifications of self K or D antigens.

Presently available data does not allow the distinction between the one receptor or two receptor hypotheses. However there are several lines of evidence which does not support the dual-recognition hypothesis. Experiments using radiation chimaeras have shown that self recognition by mutually expressed H2 antigens is not necessary. Furthermore, they do not support the concept that each mouse strain has genes for only those H2 receptors that match its own H2 antigens, as cells from a chimaera could be sensitized to generate CLs capable of recognizing the tolerated foreign H2 products on virally-infected (Zinkernagel, 1976) or hapten modified cells (von Boehmer and Haas, 1976) or minor H antigens on cells of foreign H2 types (Bevan, 1977).

The dual-recognition hypothesis must also account for the apparent separation of four distinct T cell specificities in F1 mice, those associated with CI structures coded by either maternal or paternal K or D loci (Zinkernagel and Doherty, 1975). With dual-recognition, it would be necessary to postulate that only one of the CI structures would be expressed in any one T cell and some complex mechanism of receptor exclusion would be required. Furthermore, competitive inhibition studies have shown that CMC of modified target cells cannot be specifically inhibited by the addition of excess unmodified cells, or by appropriately modified cells of a different H2 haplotype (Forman, 1975; Shearer et al., 1975). If there is a separate receptor for the modifying antigen, it does not appear to bind the antigen unless the self H2 receptor also binds to the same cell.

All of the data can be more easily fitted in an altered self hypothesis. Moreover, it has been shown that TNF directly couples
to H2 antigens on the cell surface (Forman et al., 1977a) and there is a direct correlation between the extent of derivatization of H2 antigens and the ability of such derivatized cells to stimulate TNF-CMC (Forman et al., 1977b). Other evidence which tends to support the altered self hypothesis include the association of H2 antigens with budding viral particles (Bubbers and Lilly, 1977) and the physical association of H2 antigens with viral antigens (Schrader et al., 1975) and with tumour antigens (Goeling and Edidin, 1974). H2 restriction of CMC against minor H antigens cannot be so readily explained on the basis of modifications of K or D antigens, but one suggestion has been that K and D genes might code for polymorphic glycosyl transferase enzymes which modify products of the minor H genes (Bevan, 1975c).

Although many workers favour the altered self hypothesis, the data obtained so far does not allow an unequivocal choice between the two hypotheses.

In addition to determining whether CLs recognize target cells using one or two receptors, there are two other interrelated issues which are also important in the consideration of the specificity of CMC. It is evident that products of the MHC are involved in allogeneic and xenogeneic CMC as well as in CMC induced by viral antigens, haptenic determinants or minor H antigens. These observations tend to suggest that the repertoire of CL specificities may be restricted primarily to the recognition of MHC products. This raises the question as to the role or relationship of the MHC to CMC. A widely held concept is that CLs are concerned with immune surveillance against altered cells and that the MHC molecules have evolved to serve as adaptors that interact with foreign molecules to form adaptor-antigen complexes to correspond to a repertoire of T cell receptors directed against altered products of the MHC (Schrader et al., 1975).
The notion that the repertoire of CL receptors are restricted to the recognition of altered MHC antigens would account for the large number of T cells that respond to an allogeneic stimulus. Frequency determinations have shown that between 1-4 per cent of all CL.P can be stimulated by the antigens of a single foreign haplotype. This high frequency of cells which respond to alloantigens places constraints however when the specificity of the responses is being considered. There are many more than a hundred foreign H2 haplotypes (Klein, 1974). If over one per cent of the CLs will respond to any foreign H2 type, what is the degree of specificity and how is it achieved if such high numbers will respond to a particular stimulus?

It is known that the receptors on CLs have not been passively absorbed, since a population of CLs immunized simultaneously with two antigens contains separate populations directed to the two antigens (Brondz, 1972; Teh et al., 1977). Specificity, together with the high frequency of cells which will respond has prompted some investigators to propose that T cells are multi-potential (Simonsen, 1967) but monolayer adsorption experiments have suggested that CL.P are mono-specific (Lonai et al., 1972). Other suggestions have been that the antigen somehow assembles a specific receptor on T cells (Simonsen, 1974) but these ideas run counter to the fundamental selection theories of immunity. Another proposal has been that the T cell receptor is made up of a small number of subunits, and that binding of an H2 antigen requires only part of the complex receptor; in other words a restricted pluripotentiality (Ford et al., 1975). More recently a hypothesis explaining alloaggression on the basis of cross-reaction between haplotypes was put forward (Natzinger and Bevan, 1977). It was proposed that CLs do not recognize H2 antigens in isolation, but rather foreign H2 in combination with other membrane
components. By the formation of associated antigens, H2 products can be seen in combination in many different ways. Thus a cell of a foreign haplotype presents a multitude of specificities and will stimulate a large number of different CL.P. This explanation for the high frequency of alloreactive cells requires a proportion of the clones activated by a particular haplotype to cross-react on a different foreign H2 allele. Consistent with these ideas, there have been a variety of reports where cross-reactions have been shown to occur (Brondz and Snežirůva, 1971; Blomgren and Andersson, 1974). For example, a CL response by C3H lymphocytes stimulated by H2d alloantigens has been shown to contain activity against H2b targets also (Peavy and Pierce, 1975a; Lindahl and Wilson, 1977). However, the data which is available on cross-reactivity of allogeneic CMC is insufficient to either affirm or reject such a hypothesis, and furthermore, the basis for the cross-reactivity which has been detected has not been determined. It is evident that further work on the nature of T cell receptors and the specificity of cell-mediated responses is necessary to clarify the various issues related to antigen recognition by CLs.

1.5 CLONAL ANALYSIS OF CELL-MEDIATED CYTOTOXICITY

It was discussed earlier that the immune system can be described, as a large collection of lymphocytes which are individually diverse in their specificity of recognition, and that the immune response can be regarded as the selection by the immunogen of the lymphocytes with that particular specificity, and the clonal expansion of the selected cells to generate a large number of effector cells which are specific for the sensitizing antigen. It has been suggested by Watanabe and co-workers (1977) that a proper quantitation of immune responses should
necessarily include: 1) the number of selected cells driven into clonal expansion 2) the number of divisions undergone by the clones 3) the number of effector cells produced, while the qualitative description of the response would include the question of how specific the precursor and effector cells are.

The development of appropriate cloning techniques (Marbrook and Haskell, 1974) and assays for individual plaque forming cells (Jerne and Nordin, 1963) have enabled some B cell responses to be analysed to the above extent, but similar quantitative analyses on T cell functions is still lacking. The recent adaptation of culture techniques to the generation of clones of CLs have enabled the frequency of CL.P which will respond to a particular stimulus to be determined (Skinner and Marbrook, 1976), but in general, studies on CMC has been hindered by the lack of effective assays for individual CLs. There have been attempts to detect the numbers of CLs in an immune population using a plaque technique (Bonavida et al., 1976) or from the formation of CL-target cell conjugates (Berke et al., 1975), but the assays most commonly used are those which give a measure of the relative degree of killing in a population of target cells by immune lymphoid cells, and do not give a measure of the number of effector cells or the number of target cells killed.

The lack of simple assays for the activity of individual CLs has meant that usually, the specificity of CL responses has been examined on a whole population basis. Evidence for the multiclonal nature of the responses has been discussed earlier, and thus, the specificity measured in a mass population would be a composite of all the individual specificities of all the different clones which were contributing to the response, and very little information has been obtained on the specificity of individual CL receptors for antigens.
An additional complexity in the analysis of specificity using mass populations is that not only is the immune population composed of a multitude of differing specificities but also the target cell surface contains a multitude of antigenic determinants. In effect, a complex multispecific system is being assayed against another complex multideterminant system. These considerations indicate the advantage of studying single T cell clones rather than heterogeneous populations of cells in mass cultures. By analogy with antibody forming cells, the specificity of a clone of CLs might be expected to be homogeneous, and thus the analysis of single CL clones is likely to provide more detailed information on the antigen recognition potential of individual receptor sites on CLs.

In the work described in this thesis, a clonal analysis has been applied to examine cell-mediated responses generated in culture. By using a culture system which segregates the precursor cells, then taking advantage of the amplification of each of these precursors, the number and the specificity of the CL.P can be studied from the assay of the activity of the individual clones of CLs which develop.
CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.11 Chemicals

All chemicals were Analytical Reagents purchased from British Drug Houses Ltd, Poole, England with the exception of 2,5-Diphenyl-oxazole (PPO) and (1,4-bis-(4 methyl-5 phenyloxazolyl)-Benzene (POPOP) purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A. and Toluene from Shell Oil Co., N.Z.

2.12 Mitogens

Dextran sulphate (DS) was obtained as a gift from Dr J. Watson. Concanavalin A (Con A) was obtained from Calbiochem, San Diego, California, U.S.A., Lipopolysaccharide B (LPS) from Difco Laboratories Inc., Detroit, Michigan, U.S.A. and Phytohaemagglutinin (PHA) from Wellcome Research Laboratories, Beckenham, England.

2.13 Radiochemicals

Radioactive sodium chromate $^{51}$Cr$\text{Na}_2\text{Cr}_4\text{O}_7$ of specific activity 100-400 mCi per mg atom Cr was obtained as an aqueous solution from The Radiochemical Centre Ltd, Amersham, England.

2.14 Antisera

AKR antiserum to the Thyl antigen of C$^3$H mice (anti-Thyl antiserum) was obtained from Searle Laboratories, High Wycombe, England.

2.15 Complement

Rabbit complement in a lyophilised form and rabbit complement reconstitution media were obtained from Gibco, New York, U.S.A. The reconstitution media was added to the rabbit complement immediately before use.

2.16 Mice

The mice used were CBA (H$^2^k$), C$\text{57}^1\text{B}1$ (H$^2^b$), DBA/2 (H$^2^d$), and $F_1$ hybrids from (CBA x DBA/2) and C$\text{57}^1\text{B}1$ x DBA/2) and (CBA x C$\text{57}^1\text{B}1$). Unless
specified in the experiment, mice used were 12 week old males.

2.17 Tumour Cells

The DBA/2 P815-Y and the C57Bl EL-4 tumour cell lines were obtained from the Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia. They were maintained continuously in suspension cultures.

2.2 SOLUTIONS AND MEDIA

2.21 Phosphate Buffered Saline (PBS) pH 6.5

PBS was prepared by dissolving the following in 800 ml of distilled water:

- NaCl 8.0 g (0.17 M)
- KCl 0.2 g (0.0034 M)
- Na₂HPO₄ 1.15 g (0.01 M)
- KH₂PO₄ 2.0 g (0.018 M)

The solution was sterilized by filtration as described in Section 2.32 and stored at 4°C.

2.22 Culture Medium

RPMI 1640 culture medium was obtained as the dry powder from Gibco, Cleveland, Ohio, U.S.A., and dissolved in distilled water to make a twice strength solution. This solution was gassed with carbon dioxide, supplemented with penicillin (10⁵ units per litre) and streptomycin (0.1 g per litre). The solution was sterilized by filtration and stored at 4°C.

The final culture medium was prepared under sterile conditions in the following proportions:
2 x RPMI 1640: 50 ml
Sterile distilled H₂O: 26.5 ml
Sterile 7.5 x 10⁻⁵ M: 6.5 ml
2-mercaptoethanol (2-ME): 2 ml
Foetal Calf Serum (FCS): 10 ml
Sterile 0.33 M NaHCO₃: 7 ml

FCS was obtained from Australia Laboratory Services Pty Ltd, Rockdale, N.S.W., Australia and was stored at -15°C.

The final concentration of 2-ME was 5 x 10⁻⁵ M. Culture medium for use in the cytotoxicity assay contained FCS which had been heat inactivated at 56°C for 30 min and no 2-ME.

2.23 Scintillation Fluid

PPO: 7.5 g
POPOP: 0.25 g
Toluene: 2.5 litres

Radioactive samples were placed in glass vials with 5 ml scintillation fluid and counted in Packard Tricarb Scintillation Spectrometer, Model 3375.

2.3 METHODS

2.3.1 Glassware

Glassware was soaked overnight in Protosol solution, washed in tap water, rinsed in deionized water followed by a final rinse in glass distilled water.

Glassware which had contained radioactive material was soaked for several days in a solution of Decon before being washed.

2.3.2 Sterilization

Glassware and heat stable solutions used for cell culture were sterilized by autoclaving at 100 kPa for 20 min. Other solutions
were sterilized by filtration through a Millipore membrane filter of 0.45 μm pore size.

2.33 Anti-Thyl Treatment

Spleen cells from cultures (2.7 x 10^6) were incubated with anti-Thyl serum (final dilution 1 in 50) in 0.4 ml culture medium at 37°C for 30 min. Rabbit complement was added to give a final dilution of 1 in 5 and the cells incubated for a further 30 min. The cells were then washed twice with culture medium.

2.34 Cyclophosphamide Treatment

Solutions of cyclophosphamide (Endoxan Asta, kindly supplied by Bristol Myers Co. Pty Ltd, Auckland, N.Z.) were made up directly before use. Mice were injected intraperitoneally with the required dose of cyclophosphamide in 0.3 ml saline solution.

2.35 Modification of Cells with Trinitrobenzene Sulphonic Acid

Spleen cells for culture were modified with the hapten TNP after the method of Shearer and co-workers (1975) by incubating at 37°C for 10 min with 10 mM trinitrobenzene sulphonic acid (TNBS) solution in PBS, allowing 1 ml of TNBS solution per spleen. The cells were then washed twice with culture medium.

When target cells for the cytotoxicity assay were to be modified, the cells were incubated with 51Cr as described in Section 2.40, then after the appropriate labelling period, 0.5 ml TNBS solution (10 mM in PBS) was added to the cells. After 10 min at 37°C the cells were washed three times and used as targets in the cytotoxicity assay.

2.36 Preparation of Cell Suspensions

Mice were killed by cervical dislocation and the spleen, thymus or the inguinal and axillary lymph nodes were removed. The cells were teased out gently into culture medium using curved forceps. The cells were then irrigated through a 25 gauge needle and large
clumps of cells and debris were removed by allowing them to sediment into FCS. A sample of cell suspension was diluted in 5 per cent acetic acid and the number of nucleated cells counted using a haemocytometer.

2.37 Preparation of the Polyacrylamide Culture Vessels

The polyacrylamide culture vessels or 'rafts' were made in the following way:

**Solution A:**
- Acrylamide: 6.3 g
- Bis-acrylamide: 0.15 g
- Distilled water: 35 ml

**Solution B:**
- Ammonium persulphate: 52.5 mg
- Distilled water: 10 ml

**Solution C:**
- N N N'-tetramethyl-1,2-diaminoethane: 0.0375 ml
- Distilled water: 30 ml

The solutions were mixed and poured into a perspex mould containing templates for 9 polyacrylamide rafts. When the solution had polymerized, the vessels were carefully removed from the templates, soaked for 2 days in several changes of distilled water and then autoclaved in PBS and stored at 4°C. Before use, the vessels were soaked for 1 day in minimum essential medium (Eagle) at 4°C, followed by overnight incubation in serum-free RPMI culture medium at 37°C.

2.38 Cell Culture Systems

Cells were cultured at 37°C in a humidified atmosphere of 5 per cent carbon dioxide in air. Two standard techniques were employed.

i. Polyacrylamide Cultures

Polyacrylamide vessels were used for the segregation of 'clones' of effector cells after the method of Marbrook and Haskill (1974). The vessels were prepared as described in Section 2.37. Each vessel
PLATE 1

THE POLYACRYLAMIDE CULTURE VESSEL

The photograph shows a polyacrylamide culture vessel with its 64 'dimples' into which the cells settle randomly.
contains 64 small depressions or 'dimples' into which the cells settle (Plate 1). Individual vessels were placed in a 60 mm glass petri dish containing an 8 ml reservoir of culture medium. The inner well of the raft was then filled with the cell suspension, generally 3.5 ml, until there was no meniscus. After the appropriate time in culture, the cells of each dimple were removed with a fine Pasteur pipette under a binocular microscope.

**ii. Marbrook Vessels**

These vessels were used for culturing blast cells for use as target cells in the cytotoxicity assay. Vessels (Fig. 1) were prepared and used after the method of Marbrook (1967). The outer chamber contained an 8 ml reservoir of culture medium while the inner tube contained the cells in a volume of 1 ml of medium. At the termination of culture, the cells were suspended and removed with a Pasteur pipette.

**2.39 Assay of Individual Dimples of Cells for Cytotoxicity**

Cells from each dimple were removed into 0.3 ml assay medium in plastic disposable tubes. $10^4 \text{Cr}$-labelled target cells in 0.2 ml medium was added to each tube and cytotoxicity measured using the $\text{Cr}$ release assay as described in Section 2.41.

When dimples of cells were to be assayed against two types of target cells, dimples of cells were removed into 0.6 ml of medium. After gentle pipetting to disperse clumps of cells, the dimple of cells was divided into two aliquots by removing 0.3 ml of the cell suspension into another tube. $5 \times 10^3$ target cells of one type would be added to one aliquot of each dimple and $5 \times 10^3$ of another type of target cell would be added to the other aliquot. Cytotoxicity in each aliquot was measured as described in Section 2.41.

Generally each alternate dimple in the raft was harvested.
The outer chamber contains 8 ml of medium and serves as a reservoir. Cells are put into the inner chamber in 1 ml of medium and settle on to the dialysis membrane separating the two chambers.
out of 64 dimples per raft) and 2-5 replicate rafts were assayed per observation or experiment.

A sample with more than 10 per cent specific lysis was considered to contain positive amounts of cytotoxicity. This arbitrary figure was selected as being above any apparently specific lysis which was due to experimental errors or variations in the counting of radioactivity (Skinner and Marbrook, 1976).

2.40 Preparation of Target Cells for Cytotoxicity Assay

Tumour cell lines were grown in 50 ml suspension cultures in conical flasks. Usually, the cultures were seeded at 100 cells per ml and used after 5 days when the cells were at the late log phase of their growth. The cells were harvested from suspension by centrifugation (300 x g, 10 min).

Blast cells were prepared by culturing spleen cells in the presence of the appropriate mitogen in Marbrook culture vessels (4 x 10^6 spleen cells per culture). The mitogens and the concentrations used are as follows:

- **DS**: 40 μg per culture
- **LPS**: 15 μg per culture
- **ConA**: 5 μg per culture
- **PHA**: final concentration; 5 per cent

DS and LPS were added at the beginning of culture and the cells harvested after 3 days, while ConA and PHA were added after 2 days and the cells harvested after a further 3 days of culture.

Cells were labelled with ^{51}Cr by incubating at 37°C 3-6 x 10^6 cells with 100 - 200 μCi sodium[^{51}Cr]-chromate in 0.5 ml PBS.

Tumour cells were incubated for 30 min while blast cells were incubated for 90 min with ^{51}Cr. The cells were washed three times with 10 ml assay medium and then diluted to the appropriate cell concentration.
which was $5 \times 10^4$ cells per ml when the activity of a whole dimple of cells was to be assayed, or $2.5 \times 10^4$ cells per ml when the activity of 'half dimples' of cells was assayed. 0.2 ml of the suspension of labelled cells was added to each assay tube.

2.41 $^{51}$Chromium Release Assay for Cytotoxicity

The method of Brunner et al., (1968) with modifications described by Cerottini et al. (1974) was followed. Samples of lymphoid cells (0.3 ml) to be assayed for cytotoxicity were placed in plastic disposable tubes and 0.2 ml of $^{51}$Cr-labelled target cells were then added to each tube. The tubes were centrifuged ($100 \times g$, 3 min) and incubated ($37^\circ$, 4h) in 5 per cent carbon dioxide in air. After thorough mixing and centrifugation ($500 \times g$, 10 min), 0.2 ml of the supernatant of each tube was removed onto a glass fibre disc. The discs were dried at $37^\circ$, each placed in a scintillation vial containing 5 ml scintillation fluid and the radioactivity on each disc measured by liquid scintillation counting as described in Section 2.23 on the tritium channel setting.

Results were calculated in terms of specific lysis =

$$\frac{\text{Experimental } ^{51}\text{Cr release - spontaneous release}}{\text{Maximum } ^{51}\text{Cr release - spontaneous release}} \times 100$$

Spontaneous release was obtained by incubating target cells alone in medium while maximum release was obtained after three cycles of freezing and thawing. The spontaneous release as a percentage of the maximum release for tumour cells was 7-25 per cent and for the various types of blast cells 16-36 per cent. The spontaneous release for TNF-modified cells was indistinguishable from their unmodified counterparts.

2.42 Statistical Treatment of Results

i. Correction for Coincidence of More Than One Clone In a Dimple

It was assumed that cells settled into dimples in a random way,
and as the number of positive dimples increased, there was a greater probability that a positive dimple was due to the coincidence of more than one 'clone'. A correction factor was applied to calculate the number of clones from the number of positive dimples after the method of Marbrook and Haskill (1974) using the formula

\[ m = n \left(1 - \frac{1}{n}\right)^r \]

where \( m \) = number of negative dimples
\( n \) = total number of dimples
\( r \) = corrected number of 'clones'

\[ ii. \text{ Estimation of Frequency of Precursor Cells} \]

The frequency of precursor cells which responded in a lymphoid population was calculated according to the method reported by Quintáns and Lefkovits (1973). If cells settled into the dimples in a random way, then the number of precursor cells per dimple will follow a Poisson distribution. The mean number of precursors can be calculated from the observed proportion of negatives, using the Poisson formula:

\[ F(r) = \frac{u^r e^{-u}}{r!} \]

where \( F(r) \) = the probability of obtaining \( r \) precursors in a dimple when the mean number of precursors is \( u \).

If all the dimples with one or more precursors \( (r > 1) \) give a response, the expected fraction of negatives is given by

\[ F(o) = e^{-u} \]

when \( u = 1 \)

\[ F(o) = e^{-1} = 0.37 \]

This then is the fraction of negative dimples that should be expected if there is a mean of one precursor per dimple. If \( N \) responder cells cultured per raft give 37 per cent negative dimples then the frequency of clone forming cells is:

\[ 1 \text{ in } \frac{N}{64} \]
iii. The Determination of Whether Lysis of One Target Was Independent of Another Target

In experiments where individual dimples of cells were divided and assayed for cytotoxic activity against two different target cells to examine the specificity, the dimples were assigned to 4 categories according to the following criteria:

cytotoxic for target 1 (>10% lysis of target 1, <10% lysis of target 2)
cytotoxic for target 2 (<10% lysis of target 1, >10% lysis of target 2)
cytotoxic for targets 1 + 2 (>10% lysis of target 1, >10% lysis of target 2)
not cytotoxic for either target (<10% lysis of target 1, <10% lysis of target 2).

The observed frequencies were distributed in a 2 x 2 contingency table as below.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>C</td>
</tr>
</tbody>
</table>

where A = number of positive dimples against target 1
B = number of positive dimples against targets 1 + 2
C = number of positive dimples against target 2
D = number of negative dimples

The expected values (E) for each category, if lysis of target 1 was independent of target 2 was calculated according to the following:

\[
E_A = \frac{(A+B)(A+D)}{(A+B+C+D)} \\
E_B = \frac{(A+B)(B+C)}{(A+B+C+D)} \\
E_C = \frac{(B+C)(D+C)}{(A+B+C+D)} \\
E_D = \frac{(D+C)(A+D)}{(A+B+C+D)}
\]

The observed frequencies were compared with the expected frequencies by \(x^2\) test according to the formula

\[
x^2 = \frac{(\text{observed} - \text{expected})^2}{\text{expected}}
\]

or

\[
x^2 = \frac{(AC - BD)^2}{(A+B)(C+D)(A+C)(B+D)}
\]
If the probability values (P) with 1 degree of freedom for $\chi^2$ was
> 0.05, the lysis of the two target cells was considered to occur
independently.
CHAPTER 3

SPONTANEOUS CLONES OF CYTOTOXIC LYMPHOCYTES

3.1 INTRODUCTION

The development of the polyacrylamide culture system in which precursor cells become segregated and expand to form individual clones of effector cells, enabled the clonal nature of immune responses to be examined (Marbrook and Haskill, 1974). Initially, this system was used to examine antibody responses, but was later adapted for use in cell-mediated immunity studies to measure the frequency of precursor cells in lymphoid populations which will generate cytotoxic lymphocytes (Skinner and Marbrook, 1976).

The frequency of CL precursors specific for DBA/2 antigens on P815 mastocytoma target cells was measured in CBA spleen populations using (CBA x DBA/2) F₁ spleen cells as stimulators. The control experiments for that investigation led to the interesting observation that when F₁ spleen cells were cultured by themselves, a small number of clones of CLs able to lyse P815 targets was generated (Skinner and Marbrook, 1976). As no stimulator cells had been added to culture, these clones had developed in the absence of a 'specific' stimulus. To distinguish such clones from CLs generated as a result of adding stimulator cells, these clones are referred to as 'spontaneous' clones.

In this chapter, the experiments have examined various characteristics of the spontaneous generation of clones of CLs in the polyacrylamide culture system.

3.2 EXPERIMENTAL RESULTS

3.21 The Number of Spontaneous Clones on Different Days of Culture

In this first experiment, the appearance of spontaneous clones of CLs after various days in culture was examined to determine when the maximum number of clones was detectable. (CBA x DBA/2) F₁ spleen
cells were cultured in polyacrylamide rafts as described in Materials and Methods; Section 2.38 i, and assayed on days 3-6 for spontaneous clones of CLs against P815 target cells (Section 2.39). Cultures set-up at four different cell concentrations were examined and the results have been summarized in Fig. 2.

When $7 \times 10^6$ spleen cells were cultured per raft, very few clones were detected. At higher cell concentrations, $1.3 \times 10^7$, $2.6 \times 10^7$, and $4.5 \times 10^7$ cells per raft, distinct clones of CLs were detected. The maximum number of clones was observed on day 4, and by day 6, the number of clones had subsided.

The histograms of the frequency of dimples with different amounts of cytotoxicity showed that dimples with the highest levels of cytotoxicity occurred late in the response when the total number of positive dimples per culture was declining. The distribution of dimples with different amounts of cytotoxicity on days 3-6 when $4.5 \times 10^7$ cells were cultured per raft is shown in Fig. 3. Although there was a greater number of positive dimples earlier in the response, it can be seen from the results summarized in Fig. 3 that dimples with the highest levels of cytotoxicity occurred on day 6.

3.22 Treatment with Anti-Thyl Antiserum

CLs produced in response to foreign stimulator cells have been shown to be derived from the T cell population (Cerottini et al., 1970). To test whether the spontaneous clones of CLs were T lymphocytes also, the sensitivity of the spontaneous CLs to anti-Thyl antiserum was examined. $1.3 \times 10^7$ (CBA x DBA/2)$F_1$ spleen cells were cultured for 4 days, when the cells in a whole raft were harvested and combined. An aliquot of the cells was treated with anti-Thyl antiserum and rabbit complement (Section 2.33). Another aliquot was incubated with rabbit complement only, while a third aliquot was
FIGURE 2
THE NUMBER OF SPONTANEOUS CLONES OF CLS
AFTER DIFFERENT DAYS OF CULTURE

(CBA x DBA/2)F₁ spleen cells were cultured in polyacrylamide rafts
and assayed on different days for clones of CLs against P815 targets.
Each point is the mean of 2 observations (64 dimples from 2 replicate
rafts).

0 -- -- 0 7 x 10⁶ cells per culture
0---0 1.3 x 10⁷ cells per culture
0 --- 0 2.6 x 10⁷ cells per culture
0-----0 4.5 x 10⁷ cells per culture
$4.5 \times 10^7$ (CBA x DBA/2)F$_1$ spleen cells were cultured per polyacrylamide raft. Individual dimples were assayed for cytotoxicity against P815 targets. The number of dimples with different amounts of cytotoxicity was determined after various days in culture. Dimples from two rafts were assayed for each day. Dimples with less than 10 per cent specific lysis have not been plotted.
untreated. The three aliquots were then assayed for cytotoxicity against P815 targets using the $^{51}$Cr release assay described in Section 2.41. It can be seen from the results shown in Table 1 that treatment of the cells with anti-Thyl antiserum and complement abolished cytotoxicity.

3.23 Activity of Supernatants

Although it has been demonstrated that spontaneous cytotoxicity was sensitive to anti-Thyl antiserum (Section 3.22) further tests were carried out to confirm that the killing was not due to secreted factors.

First, the supernatant from cultures was tested for lytic activity. The supernatant from a day 4 culture of $1.3 \times 10^7$ F₁ spleen cells was collected and centrifuged to remove any cells which may have been present. Twenty 0.3 ml aliquots of the cell free supernatant was each added to $10^4$ $^{51}$Cr-labelled P815 cells in 0.2 ml of assay medium, and incubated for 4 hours at 37°C. The release of $^{51}$Cr from the P815 cells was then measured as described in Section 2.41 for the $^{51}$Cr release assay. None of the twenty samples contained positive amounts of specific lysis.

In addition to showing that there was no activity in the supernatant from the cultures, it was also necessary to show that cytotoxic factors were not released during the incubation with target cells. To demonstrate this, individual dimples of cells from day 4 cultures of $1.3 \times 10^7$ (CBA x DBA/2)F₁ spleen cells were taken and incubated with $10^4$ P815 cells, in a total volume of 0.5 ml medium for 4 hours at 37°C. The samples were then centrifuged to remove the cells from suspension. 0.3 ml of the supernatant was taken and tested for lytic activity by incubating with $10^4$ $^{51}$Cr-labelled P815 cells for the $^{51}$Cr release assay as described in Section 2.41.

Supernatants from 64
TABLE 1
EFFECT OF ANTI-THYL ANTISERUM ON SPONTANEOUS CLONES

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>PER CENT SPECIFIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-Thyl antisera and rabbit complement</td>
<td>0.5</td>
</tr>
<tr>
<td>rabbit complement</td>
<td>25.2</td>
</tr>
<tr>
<td>medium</td>
<td>44.6</td>
</tr>
</tbody>
</table>

1.3 x 10^7 (CBA x DBA/2)F₁ spleen cells were cultured per raft for 4 days when the cells from the whole raft was harvested and combined in 1 ml of medium. Aliquots of the cells were treated with anti-Thyl antisera and rabbit complement, rabbit complement only or with medium only before assaying for cytotoxicity against P815 target cells.
dimples of cells taken from two rafts were tested in this manner, but no activity was found in any of the samples.

3.24 Spontaneous Clones in Cultures of Cells from Mice of Different Ages

Spleen cells from young mice of different ages were tested for their ability to generate spontaneous clones of CLs in culture. 1.3 x 10^7 spleen cells from (CBA x DBA/2)F_1 mice, aged between 4-90 days were cultured for 4 days in polyacrylamide rafts and assayed for cytotoxic clones against P815 targets. The results are given in Table 2. Spontaneous CLs were not detected in cultures of spleen cells from mice less than 16 days old. A mean of 5 clones were detected in cultures of spleen cells from one month old mice compared with 22 clones in cultures of cells from 3 month old adult animals.

3.25 Generation of Spontaneous Clones by Thymus and Lymph Node Cells

The ability of cells from the thymus and lymph node to generate spontaneous clones of CLs was examined. 1.3 x 10^7 cells from the thymus or lymph node were cultured for 4 days, then assayed for cytotoxic clones lysing P815 targets. The results are shown in Table 3, and it can be seen that cells from the thymus or lymph node do not generate many spontaneous clones of CLs in culture. In some of the cultures no clones were detected, while other cultures contained one spontaneous clone lysing P815 targets.

3.26 Generation of Spontaneous Clones at Different Concentrations of F_1 Spleen Cells

In an attempt to measure the frequency of spontaneous clones, graded numbers of (CBA x DBA/2)F_1 spleen cells were cultured and assayed on day 4 for cytotoxic clones against P815 targets. The relationship between the number of cells cultured and the number of clones detected however was found to be non-linear (Fig. 4). At the
### TABLE 2

**SPONTANEOUS CLONES IN CULTURES OF CELLS FROM MICE OF DIFFERENT AGES**

<table>
<thead>
<tr>
<th>Age of spleen donor (days)</th>
<th>Clones per raft</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0, 0</td>
</tr>
<tr>
<td>9</td>
<td>0, 0</td>
</tr>
<tr>
<td>16</td>
<td>0, 4</td>
</tr>
<tr>
<td>20</td>
<td>0, 0</td>
</tr>
<tr>
<td>31</td>
<td>0, 4, 8, 8</td>
</tr>
<tr>
<td>90</td>
<td>25, 20</td>
</tr>
</tbody>
</table>

1.3 x 10^7 spleen cells from (CBA x DBA/2)F_1 mice of different ages were cultured per polyacrylamide raft for 4 days and assayed for spontaneous clones against P815 target cells.
<table>
<thead>
<tr>
<th>Cells</th>
<th>Clones per raft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>0, 1, 0</td>
</tr>
<tr>
<td>Thymus</td>
<td>1, 1, 0</td>
</tr>
</tbody>
</table>

Cells from lymph node or thymus were cultured at $1.3 \times 10^7$ cells per raft for 4 days then assayed for spontaneous clones against P815 target cells.
Different numbers of (CBA x DBA/2) F₁ spleen cells were cultured for 4 days and the rafts assayed for cytotoxic clones against P815 targets.

- - - - - clones per culture
0 - - - - 0 clones per 10⁶ cells cultured

Vertical bars indicate the standard errors of the means.
optimal concentration \((1.3 \times 10^7\) cells per raft\) a mean of 20 clones per culture was obtained. The clone numbers then declined as the cell concentration increased. At \(4.5 \times 10^7\) cells per culture, the number of clones detected per culture was half the number which was detected in cultures containing \(1.3 \times 10^7\) cells.

3.27 Spontaneous Clones in Parental Spleen Cell Cultures

The experiments described above involved the examination of cultures of cells from \(F_1\) hybrid mice. To determine whether or not the generation of spontaneous clones was a characteristic of \(F_1\) cells only, the ability of spleen cells from the pure bred parental CBA and DBA/2 mice to generate spontaneous clones was examined. Spleen cells from CBA or DBA/2 mice were cultured in polyacrylamide rafts and the cultures assayed for clones of CLs against P815 targets. Fig. 5 summarizes the results of the experiment where cultures of CBA spleen cells at different concentrations were assayed on various days for spontaneous clones lysing P815 targets. The appearance of spontaneous clones at different days in cultures of CBA spleen cells was similar to that observed in cultures of \(F_1\) spleen cells. The maximum number of clones was detected on day 4.

When CEA or DBA/2 spleen cells were cultured at different concentrations and assayed on day 4 for cytotoxic clones against P815, the dose-response relationships were found also to be non-linear (Fig. 6). For both CBA and DBA/2 cell cultures, a mean of 14 spontaneous clones lysing P815 cells was detected at the optimal concentration of \(1.3 \times 10^7\) cells per raft.

3.28 The Effect of Cell Concentration on the Generation of 'Spontaneous' and 'Stimulated' Clones

The relationship between the number of cells cultured and the number of spontaneous clones produced has been found to be non-linear
FIGURE 5

SPONTANEOUS CLONES OF CLs ON
DIFFERENT DAYS IN CULTURES OF
CBA CELLS

CBA spleen cells were cultured in polyacrylamide rafts and assayed on
different days for clones of CLs against P815 targets. Each point is
the mean of two observations (64 dimples from 2 replicate rafts).

- - - - 1.3 x 10^7 cells per culture

- - - - 2.6 x 10^7 cells per culture

- - - - 4.5 x 10^7 cells per culture
Spleen cells were cultured for 4 days in polyacrylamide rafts and assayed for cytotoxic clones against P815 targets.

A) CBA spleen cells.

B) DBA/2 spleen cells

Vertical bars represent the standard errors of the means.
(Figs. 4 and 6), in contrast with the straight line relationship obtained when graded numbers of CBA responder cells were cultured with semi-allogeneic cells in a stimulated system in the polyacrylamide rafts (Skinner and Marbrook, 1976). However, the total number of cells cultured per raft in the two systems were not similar. To determine whether the decline in the production of spontaneous clones at higher cell concentrations could be explained in terms of an 'overcrowding' of the cultures, or whether it was a feature of the spontaneous generation of CLs, the dose-response relationship at comparable cell concentrations of a spontaneous response and a stimulated response was examined.

The anti-TNP response described by Shearer and co-workers (1975) was used in this section of the work. Spleen cells from (CBA x DBA/2)F₁ mice were derivatized with TNP according to the method described in Section 2.35. 0.7 - 4.5 x 10⁷ TNP-modified F₁ spleen cells were cultured in polyacrylamide rafts for 4 days. The cultures were then assayed for clones of CLs lysing TNP-modified P815 targets (P815-TNP). For comparison, 0.7 - 4.5 x 10⁷ normal (CBA x DBA/2)F₁ cells were also cultured and assayed on day 4 for clones of CLs lysing P815-TNP targets. The dose-response relationships in cultures of normal or TNP-modified spleen cells are plotted in Fig. 7. In cultures of TNP-modified spleen cells, the number of clones of CLs detected against P815-TNP targets increased in a linear manner with the number of cells cultured (Fig. 7B). In contrast, when cultures of normal F₁ cells were assayed against the same TNP-modified P815 targets, the number of clones per culture reached a maximum when 1.3 x 10⁷ cells were cultured and then declined at higher cell concentrations (Fig. 7A).
FIGURE 7

THE EFFECT OF CELL CONCENTRATION ON THE
NUMBER OF 'STIMULATED' AND
'SPONTANEOUS' CLONES GENERATED

A) Spontaneous clones: (CBA x DBA/2)\textsuperscript{F}\textsubscript{1} spleen cells were cultured for 4 days in polyacrylamide rafts and assayed for cytotoxic clones against P815-TNP targets.

B) Stimulated clones: (CBA x DBA/2)\textsuperscript{F}\textsubscript{1} spleen cells modified by TNP were cultured in polyacrylamide rafts and assayed after 4 days for cytotoxic clones against P815-TNP targets.

Vertical bars indicate standard errors of the mean.
'Natural' or 'spontaneous' cytolytic activity of non-immunized lymphoid cells, occurring both in vivo or during the in vitro growth of cells has been reported in animal (e.g. Herberman et al., 1975; Greenberg and Playfair, 1974) and in human systems (e.g. Tagasuki et al., 1973). Different effector cells and mechanisms of lysis appear to be responsible for the cytotoxicity in the various systems. Spontaneous lysis of tumour cells by human lymphocytes can be attributed mainly to antibody-dependent-cellular cytotoxicity, or it may involve lymphotoxin-like mediators (Peter et al., 1976). Natural killers (NKs) have been shown to exist in populations of murine spleen cells. The cell type of these NKs is yet undefined. They cannot be classified as mature T, B or K cells or as monocytes (Herberman et al., 1975; Kiessling et al., 1976), but have been shown to be small lymphocytes expressing selectively an antigen recognized by C3H anti-CE antiseraum (Glimcher et al., 1977). Cytotoxicity generated spontaneously in cultures of murine spleen cells however appear to be mediated by T effector cells (Röllinghoff et al., 1975; Shustik et al., 1976).

The experiments summarized in Section 3.2 describe the generation of cytotoxic clones against P815 target cells when murine spleen cells were cultured in polyacrylamide rafts. As no antigen had been added, these clones had arisen spontaneously in the absence of any added specific stimulus.

Lysis was measured using the 51Cr release assay for cytotoxic T cell activity (Brunner et al., 1970) in complement free medium. No activity was found in supernatants, and treatment of the cells prior to assay with anti-Thyl antiseraum abolished cytotoxicity (Table 1). These observations indicated that the spontaneous cytotoxicity was
mediated by cytotoxic T effector cells and ruled against the involvement of NKs or antibody-dependent cell-mediated lysis.

The generation of the spontaneous clones of CLs appear to have been induced in vitro. Maximum number of clones occurred on day 4, and as very little activity was detected on earlier days of culture, (Fig. 2) it is unlikely to be a detection of cytolytic activity which had existed in vivo. Although there were fewer clones detected than on day 4, dimples with the highest amounts of cytotoxicity occurred on day 6 (Fig. 3). This kind of relationship between the number and the size of clones on different days of the response has been observed in humoral responses, and was attributed to an asynchronous development of clones of antibody forming cells (Warbrook and Haskell, 1974). A similar asynchronous development of spontaneous clones of CLs would also indicate that the generation of these clones had been induced in culture.

As yet, the factors which contribute to the generation of spontaneous CLs has not been established. It should be noted however, that the culture medium contained 2-ME which has been found to enhance the amount of background cell-mediated cytotoxicity in 'unstimulated' lymphocyte cultures (Bevan et al., 1974). The medium also contained FCS. It has been reported that FCS, absorbed onto the cell surface can become specifically immunogenic and induce the production of CLs directed against determinants of FCS absorbed onto target cells (Forni and Green, 1976). It has also been reported that FCS induces the development of specific CLs directed against self H2 antigens in both stimulated and non-stimulated cultures (Peck et al., 1977), but the target antigens for these anti-self CLs was shown not to involve FCS determinants. Furthermore, both 2-ME (Goodman and Weigle, 1977) and FCS (Andersson et al., 1972b) have been shown to have mitogenic
properties. The possible role of 2-ME and FCS in the induction of spontaneous CLs will be discussed later (Sections 4.3 and 6.3) in relation to the specificity of the spontaneous CLs.

The ability to generate spontaneous CLs also depended on the lymphoid cells cultures. Clones were detected mainly in cultures of spleen cells and very little activity was present in cultures of thymus and lymph node cells (Table 3). There may be several possible explanation for the difference in activity of the cells from the different organs. The generation of CLs has been shown to be regulated by suppressor and amplifier cells, and the distribution of the subsets of functional T cells in the various tissues have been shown to be different (Cantor and Boyse, 1975a; Cantor and Simpson, 1975). The lack of spontaneous CL activity in cultures of thymus and lymph node cells may be due to the absence of the precursors of spontaneous CL clones, or the ratio of the regulatory cells in those lymphoid organs does not favour CL production. Alternatively, the induction of spontaneous CL generation may require a cell which is present at a higher frequency in the spleen than in the thymus or lymph node.

In this regard, polyclonal stimulation by macrophages has been demonstrated (Opitz et al., 1976), and the 'autostimulation' observed when thymocytes were cultured with syngeneic spleen cells (Howe et al., 1970) has been attributed to a population of B cells (von Boehmer et al., 1972).

Spontaneous CLs were not detected in cultures of cells from neonatal mice (Table 2). Very few clones were observed until the age of the donors were one month old. A differential ontogeny of T cell functions has been reported. Foetal spleen cells have been found to inhibit the ability of adult cells to elicit graft-versus-host reactions (Skowron-Cendrzak and Ptak, 1976). This observation was interpreted as indicating that suppressor T cells emerged ontogenically
earlier than do helper cells. It has also been shown, that while
cells from neonatal mice will proliferate in mixed lymphocyte reactions,
specific cell-mediated lysis could not be demonstrated until 27 days
after birth (Wu et al., 1975). The absence of spontaneous cytotoxicity
in cultures of cells from young animals might reflect

i) the late ontogeny of precursors of spontaneous clones of CLs;

ii) the large amount of suppressor cell activity; or,

iii) if the stimulus for the production of spontaneous CLs is

cellular, neonatal cell populations may lack the appropriate stimul-
ator cells. It has been proposed by Lafferty and Talmage (1976)
that only mature, immunocompetent cells are capable of delivering
a 'second signal' which is required for the activation of CL
production, while von Boehmer and Adams (1973) have suggested that
the antigen responsible for the autostimulation described by Howe
and co-workers (1970) develops when the animal matures.

A feature of the spontaneous response is the non-linear relation-
ship between the number of clones produced and the number of cells
cultured, which is in contrast with the linear relationships obtained
in stimulated responses (Skinner and Marbrook, 1976; Fig. 7B). When
the number of stimulated clones of CLs against P815-TNP targets from
cultures of $F_1$-TNP cells, and the number of spontaneous clones of CLs
against P815-TNP from cultures of $F_1$ cells were measured over the
same range of cell concentration per raft, the number of stimulated
clones increased with cell number, while the number of spontaneous
clones declined at the higher cell concentrations (Fig. 7). If the
viability of the modified and unmodified $F_1$ cells were essentially
the same in culture, then the decline in the number of spontaneous
clones at high cell concentrations cannot be explained entirely in
terms of overcrowding of the cultures.
Although the nature of the stimulus which induces the production of spontaneous CLs is not clear, another explanation for the non-linear dose-response relationship might be that this stimulus becomes limiting at high cell concentrations. A further explanation might involve the effects of suppressor and amplifier cell functions on the generation of CLs. The clonal analysis is essentially a limiting dilution culture system. Not only would precursors of CLs become segregated from each other but the other sets of functional cells may also be diluted out. The complex dose-response relationships observed in the spontaneous response may reflect the differential segregation of the regulatory cells in the polyacrylamide vessels.

In the absence of a linear dose-response, the total number of precursors of spontaneous clones could not be determined, but at the optimal cell concentration, one clone per $6.5 \times 10^5$ F₁ spleen cells and one clone per $9.2 \times 10^5$ CBA or DBA/2 cells cultured was detected against P815 targets. These figures are approximately 400 fold lower than the frequency of clones detected against P815 targets in CBA spleen cell populations stimulated with H₂ d alloantigens (Skinner and Marbrook, 1976).

**Summary**

When normal spleen cells from CBA, DBA/2 or (CBA x DBA/2)F₁ mice were cultured without stimulator cells in polyacrylamide rafts, spontaneously generated cytotoxic clones against P815 targets were detected. Maximum number of clones occurred on day 4 of culture and the cytotoxicity was T cell-mediated. At the optimal cell concentration ($1.3 \times 10^7$ cells per raft) 20 clones were detected in cultures of F₁ spleen cells and 14 clones were detected in cultures of CBA or DBA/2 cells. Spontaneous clones were observed mainly in cultures of
CHAPTER 4

THE SPECIFICITY OF SPONTANEOUS CLONES

4.1 INTRODUCTION

'Natural' or 'spontaneous' cytotoxicity of lymphoid populations from normal, non-immunized donors in some of the systems reported involved specific recognition, in that only certain target cells were lysed (Herberman et al., 1973; Greenberg and Playfair, 1974), whereas in other assays, no discrimination in the lysis of targets could be demonstrated (Shustik et al., 1976; Burton et al., 1977). The experiments summarized in the previous chapter described the generation of spontaneous clones of CLs when normal spleen cells were cultured (Section 3.2), and one of the aims of the experiments described in this chapter was to determine whether the spontaneous CLs lysed target cells indiscriminately, or whether the lysis of the P815 target involved specific recognition. If the P815 cells were lysed specifically, it was of interest to examine which specificities on the P815 cell surface the spontaneous CLs recognized. As the P815 is a DBA/2 tumour cell line, spontaneous CLs from DBA/2 and (CBA x DBA/2)F_1 cells would be apparently auto-reactive if the P815 were recognized by virtue of their H2 antigens.

Whether CLs other than those which will lyse P815 targets were also produced spontaneously in culture was not known. If the spontaneous response had been induced by polyclonal stimuli, it might be expected that a range of CLs of different specificities would be produced. Alternatively maybe only CLs from a restricted part of the repertoire are produced spontaneously.

The experiments in this chapter were designed to examine both the specificity of the individual clones of spontaneous CLs, as well as the range of specificities detectable in the spontaneous response.
(CBA x DBA/2)F₁ spleen cells were cultured in polyacrylamide vessels. The specificity of the spontaneous clones of CLs which were produced was examined by dividing each clone into two halves and assaying the clone against two different target cells. Individual dimples of cells from the polyacrylamide cultures of spleen cells were removed. Each dimple of cells was divided into two aliquots, one aliquot was assayed against one type of target cell, and the other aliquot was assayed against another type of target cell. Different pairs of target cells were used in a series of experiments examining the specificity of the spontaneous CLs.

4.2 EXPERIMENTAL RESULTS

4.21 Division of Individual Dimples of Cells into Aliquots of Equal Amounts of Cytotoxicity

It was necessary to carry out initial experiments to determine whether the cells from a single dimple could be divided into two aliquots which contained equal amounts of lytic activity. In two control experiments, (CBA x DBA/2)F₁ spleen cells were cultured in polyacrylamide rafts as described in Methods, Section 2.38 i. On day 4, cells from individual dimples were removed and divided into two aliquots according to the method described in Section 2.39. In these control experiments the two aliquots of each dimple were assayed for lytic activity against the same type of target cell. Results were expressed graphically by plotting the per cent specific lysis obtained in one aliquot against the per cent specific lysis obtained in the other aliquot. In Fig. 8A, the targets used were P815 cells, while in Fig. 8B the target cells used were F₁ PHA-induced spleen blasts. The data has also been summarized in Table 4a. Of the positive dimples detected in these control experiments, those which had been divided into two aliquots, both containing positive amounts
FIGURE 8

THE ASSAY OF CYTOTOXICITY IN TWO HALVES OF THE
SAME DIMPLE OF CELLS

$1.3 \times 10^7 (CBA \times DBA/2)F_1$ spleen cells were cultured per poly-
acrylamide raft for 4 days. Individual dimples of cells were
harvested and divided into two aliquots, each of which was
assayed against the same type of target cell. The per cent
specific lysis in each of the two aliquots is compared for all
dimples assayed. Each point on the graph thus represents the
cytolytic activity found in one dimple. Dimples with less than
10 per cent specific lysis in both aliquots have not been
plotted.

A. The two aliquots of each dimple of cells were both
assayed against P815 target cells. A total of 128
dimples taken from 4 replicate cultures were assayed.

B. The two aliquots of each dimple of cells were assayed
against $(CBA \times DBA/2)F_1$ PHA-induced spleen blasts. A
total of 160 dimples from 5 replicate cultures were
assayed.
TABLE 4
THE SPECIFICITY OF SPONTANEOUS CLONES

$1.3 \times 10^7$ (CBA x DBA/2)$F_1$ spleen cells were cultured for 4 days in polyacrylamide rafts. Each dimple of cells was harvested and divided into two aliquots, each of which was assayed for cytotoxicity against two different target cells. In two control experiments (a), the 2 aliquots were similarly assayed against the same type of target cell.

*The expected number of positive dimples lysing both targets was calculated from observed frequencies distributed in a $2 \times 2$ contingency table.
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THE SPECIFICITY OF SPONTANEOUS CLONES

TABLE 4
of cytotoxicity, made up the highest proportion; 56.3% when P815 targets were used, and 48.7% when $F_1$ (PHA) blasts were used. However there were some dimples where only one of the aliquots could be classified as containing positive amounts of cytolysis (Section 2.39). These were derived mainly from dimples with low levels of activity, and thus were difficult to divide into two aliquots such that both contained sufficient amounts of cytotoxicity to be scored as being positive. Despite these, the distributions obtained in the control experiments do not conform to the calculated distributions which would be expected if the lysis in one aliquot was independent of the lysis in the other aliquot ($P < 0.001$).

4.22 Specificity of Spontaneous Clones

A subsequent series of experiments examined the specificity of the spontaneous clones of CLs. In each experiment ($CBA \times DBA/2)F_1$ spleen cells were cultured for 4 days in polyacrylamide rafts as described in Methods, Section 2.38 i ($1.3 \times 10^7$ cells per raft). Individual dimples of cells were then removed and divided into two halves (Section 2.39). Each half of a dimple of cells was assayed against a different target cell population. Different pairs of target cells were used in each experiment.

i. Discrimination Between CBA and DBA/2 Spleen Blasts

An experiment was carried out to determine whether spontaneous clones of CLs lysing the two parental CBA and DBA/2 strains of cells were produced by $F_1$ spleen cells in culture. Individual dimples of cells from polyacrylamide cultures of $F_1$ spleen cells were taken and divided into two aliquots as described previously. One aliquot of each dimple was assayed with DBA/2 PHA-induced spleen blasts and the other aliquot was assayed with CBA PHA-induced spleen blasts. The results have been plotted in Fig. 9 and are summarized in Table 4b.
FIGURE 9
LYSIS OF CBA AND DBA/2 TARGETS BY SPONTANEOUS $F_1$ CLONES

1.3 x $10^7$ spleen cells from (CBA x DBA/2)$F_1$ mice were cultured per polyacrylamide raft for 4 days. 96 dimples of cells from 3 replicate rafts were individually harvested and divided into two aliquots. An aliquot of each was assayed for cytotoxicity against CBA PHA-induced spleen blasts, while the other aliquot was assayed against DBA/2 PHA-induced spleen blasts. The per cent specific lysis obtained in one aliquot against CBA targets was plotted against the per cent specific lysis against DBA/2 targets in the other aliquot. Dimples with less than 10 per cent specific lysis have not been plotted.
Ninety-six dimples were assayed, of which 46 contained positive amounts of cytotoxicity. Nine per cent of the positive dimples contained cytotoxicity against both CBA and DBA/2 targets. The majority of the positive dimples were specific for either CBA (61%) or DBA/2 (30%). The observed distributions do not differ significantly from those expected if the lysis of one target was independent of the lysis of the other target ($P > 0.4$).

The results of this experiment have demonstrated that spontaneous clones of CLs lysing cells from the two parental strains of mice, are produced by $F_1$ spleen cells in culture. The number of positive dimples which would be expected to contain activity against both targets due to coincidence of more than one clone in the same dimple, was calculated to be 13%. As the observed number of positive dimples with activity against both the targets was only 9%, the results of the experiment indicated also that the CBA and DBA/2 target cells were lysed by separate populations of spontaneous CLs.

**ii. Discrimination Between Tumour Cells and Normal Spleen Blasts**

Experiments were carried out to determine whether the individual clones of CLs discriminated between normal and tumour cells of the same H2 haplotype. In one experiment, the results of which are shown in Fig. 10, an aliquot of each dimple of cells from cultures of $F_1$ spleen cells was assayed against P815 (H2$^d$) mastocytoma targets and the other aliquot against PHA-induced blasts from DBA/2 (H2$^d$) spleen cell populations. In another similar experiment, an aliquot of each dimple was assayed against EL4 (H2$^b$) or C$^{57}$Bl (H2$^b$) PHA-induced spleen blasts. In the first experiment, the target cells were semi-syngeneic with the effector cell population, while in the second experiment, the targets were completely allogeneic. The results of both experiments have been summarized in Table 4c. In both these experiments, the majority of the positive dimples lysed either the
FIGURE 10
LYSIS OF P815 AND DBA/2 TARGETS BY SPONTANEOUS F₁ CLONES

Spleen cells from (CBA x DBA/2)F₁ mice were cultured for 4 days in
polyacrylamide rafts (1.3 x 10⁷ cells per raft). Individual dimples
of cells were taken and divided into two aliquots. An aliquot was
assayed for cytotoxicity against P815 targets and the other aliquot
was assayed against DBA/2 PHA-induced spleen blasts. 160 dimples
from 5 replicate cultures were assayed. The per cent specific lysis
against P815 targets in one aliquot was compared with the per cent
lysis against DBA/2 cells in the other aliquot. Only dimples with
10 per cent or more specific lysis in one or both aliquots have been
plotted.
tumour or the spleen blast targets only. When F815 and DBA/2 blasts were the pair of target cells used, 7% of the positive dimples were observed to contain activity against both targets, while 2% were found to lyse both targets when EL4 and C57 Bl blast were used as the pair of targets. The expected frequency of dimples which would contain activity against both the target cell types due to coincidence in the two experiments was calculated to be 8.1 and 1.7% respectively. The observed distributions do not differ significantly from those expected if each target was lysed independently (P > 0.8 in each case), and the results indicated that spontaneous clones of CLs could discriminate between normal and tumour target cells of the same H2 haplotype.

iii. Discrimination Between Parental and F1 Target Cells

The previous experiments had shown that tumour and normal cells of the same H2 haplotype were lysed by separate populations of spontaneous CLs. In the experiment, the results of which are shown in Fig. 11, the specificity of the spontaneous clones was examined to determine whether they were able to discriminate between cells from the parental and F1 strains of mice. Dimples of cells from polyacrylamide cultures of (CBA x DBA/2)F1 spleen cells were each divided into two aliquots, and the aliquots assayed with either PHA-induced blasts from (CBA x DBA/2)F1 or DBA/2 spleen cell populations. Out of a total of 224 dimples assayed, 57 contained positive amounts of cytotoxicity. The majority of the positive dimples lysed either F1 cells (67%) or DBA/2 cells (23%). Only 10% of the positive dimples contained cytotoxicity against both targets (Table 4d). The expected frequency of dimples lysing both targets due to coincidence was 6.5% for that experiment. The observed distributions do not differ significantly from those expected if the targets were lysed independently (P > 0.2),
FIGURE 11

LYSIS OF F₁ AND PARENTAL DBA/2 TARGETS BY SPONTANEOUS F₁ CLONES

(CBA x DBA/2)F₁ spleen cells were cultured for 4 days in polyacrylamide rafts (1.3 x 10⁷ cells per raft). 224 dimples of cells from 7 replicate cultures were individually taken and divided into two aliquots. An aliquot was assayed for cytotoxicity against (CBA x DBA/2)F₁ PHA-induced spleen blasts, and the other aliquot against DBA/2 PHA-induced spleen blasts. The per cent specific lysis of F₁ cells in one aliquot was plotted against the per cent specific against DBA/2 cells in the other aliquot. Only dimples with positive levels of cytolysis have been plotted.
and indicated that spontaneous clones of CLS discriminated between the
F₁ and parental DBA/2 cells.

iv. Cross-reactivity Between TNF-modified and Unmodified Target Cells

In the above experiments described in this section (Section 4.22), the spontaneous clones of CLS have been able to discriminate between the pair of target cells used. In this experiment the aliquots of each dimple of cells from polyacrylamide cultures of F₁ spleen cells were assayed with P815 cells which had been modified with TNP (P815-TNP), or the unmodified P815 cells. These two targets would be expected to possess many antigens in common. Spontaneous clones of CLS were tested for cross-reactivity between the two targets.

F₁ spleen cells were cultured for 4 days in polyacrylamide rafts as described (Section 2.38 i) and the individual dimples of cells were removed and divided into two aliquots. One aliquot of each dimple was assayed against P815-TNP targets and the other aliquot against P815 targets. The results have been summarized in Table 4e. Of the positive dimples detected in the experiment, 47.7% lysed P815-TNP targets only, 14.8% lysed P815 cells only, while 37.5% lysed both targets. The expected frequency of dimples with cytotoxicity against both targets due to coincidence was calculated to be 20.4%.

In that experiment, the observed number of dimples lysing both targets was considerably higher than the calculated number of dimples which might be expected to lyse both targets due to coincidence of more than one clone in the same dimple. Moreover the distributions do not conform to those expected if the targets were lysed independently (P < 0.05) which indicated that there was cross-reactivity in the lysis of P815 and P815-TNP target cells by the spontaneous CLSs.
4.3 Discussion.

Cytotoxicity in day 5 cultures of normal murine spleen cells against a variety of different H2 type fibroblasts has been reported by Shustik and co-workers (1976). The results were interpreted by the authors as demonstrating 'the generation of promiscuous cytotoxic lymphocytes with unrestricted specificities'. In their analysis of specificity however, samples of a mass cell culture were assayed against different targets. As no competition inhibition experiments had been included in their investigation, it would not have been possible to decide whether

i) CLs with non-restricted specificities had been generated, or
ii) individual CLs were restricted in their specificity but a range of CLs of different specificities had been generated in culture.

This problem can be avoided using a clonal analysis where the specificity of individual clones of effector cells is examined. In the experiments described in Section 4.22 spontaneous clones of CLs were generated in polyacrylamide rafts, and the specificity was examined by dividing the 'clones' into two aliquots and assaying the activity of each 'half clone' against a different target.

The detection of a positive clone has been arbitrarily defined as any cytotoxic activity greater than 10 per cent specific lysis (Skinner and Marbrook, 1976). Although this would represent a minimal estimate, the frequency of CL precursors obtained using this approach is in agreement with other determinations (Teh et al., 1977; Lindahl and Wilson, 1977). The same approach has been adopted in scoring clones as specifically lysing one or both targets when individual clones were split and assayed against a different target cell.

In control experiments to determine whether the individual
dimples of cells could be divided into two aliquots of similar amounts of cytotoxicity, both aliquots of each dimple were assayed with the same target cell population. The results showed that the majority of the positive dimples had been divided into two aliquots with similar amounts of cytolysis (Fig. 8). According to the criterion used for defining positive dimples, there were however, some dimples where only one of the aliquots could be classified as containing positive amounts of cytotoxicity. These were derived from dimples with little more than 10 per cent specific lysis (the arbitrary cut-off point), and were difficult to divide into two aliquots such that both could be classified as containing positive amounts of cytotoxicity. Despite this source of error in the analysis of small clones, the results from control experiments are in marked contrast to the results obtained in other experiments where the aliquots of each dimple were assayed against different targets. Moreover, the frequency distributions in the control experiments (Fig. 8) do not conform to the calculated frequencies if the lysis in one aliquot was independent of the lysis in the other aliquot (P < 0.001).

Several experiments were carried out where the aliquots of individual dimples of cells were assayed against a pair of different target cells, to examine the specificity of the spontaneous clones of CLs generated in cultures of (CBA x DBA/2)F₁ spleen cells. Cytolysis was detected against all the various target cells used, and the results (Table 4) indicated that CLs from a range of different specificities were produced spontaneously in culture. Also included in Table 4 is a figure calculated from 2 x 2 contingency tables for the predicted frequency of dimples, which might be expected to contain cytotoxicity against both types of targets due to coincidence of more than one clone in a single dimple. Such coincidence of clones of a
different specificity in the same dimple would falsely indicate cross-reactivity. Taking the degree of coincidence into account, the results in Table 4 show that the spontaneous clones of CLs generated in cultures of (CBA x DBA/2)F₁ spleen cells, were able to discriminate between cells from the two parental strains and between cells from the F₁ and the parental DBA/2 mice. The spontaneous CLs also discriminated between PHA-induced blasts from normal spleen cells and tumour cells of the same H2 phenotype, whether the pair of targets were semi-syngeneic or completely allgeneic with the effector cell population. Cross-reactivity was detected however in the lysis of unmodified and the TNP-modified P815 target cells, which might be expected to possess many antigens in common. Three populations of CLs appeared to be present, those which lysed both targets as well as those specific for each of the targets. Of the three populations, CLs specific for P815-TNP was the largest group. Presumably, the specificity of these CLs were directed against the TNP determinant, or antigens which had been modified by the hapten. A small number of clones were found to lyse P815 targets only. This suggested that there were some target antigens on the P815 recognized by the CLs, which had been altered by TNP such that P815-TNP targets were not recognized by those CLs.

In situations where clones of CLs will lyse either one target or the other, the basis for the discrimination was due presumably to differences between the antigens on the surface of the targets. The ability of spontaneous CL clones to discriminate between targets which were syngeneic with respect to each other is therefore of particular interest. From a consideration of the data, it can be concluded that non-H2 antigens must be involved in the 'configuration' recognized by the spontaneous CLs. If H2 antigens are involved, they
may be contributing perhaps in the way described for the recognition of minor antigens (Bevan, 1975b), male antigens (Gordon et al., 1975), modified targets (Shearer et al., 1975) or virally-infected targets (Doherty et al., 1976).

There have been reports of responses to components of FCS which adhere to the surfaces of stimulator and target cells (Forni and Green, 1976). A number of points render this consideration as unlikely in the generation of spontaneous CLs by normal spleen cells in polyacrylamide culture vessels. All targets were grown in the same batch of FCS, and if the spontaneous CLs were directed against a serum component, there would be a lack of discrimination between targets. This is contrary to the observations obtained in the experiments described in Section 4.22 and in the next Chapter (Section 5.2). It is unlikely that the specificity of spontaneous clones is directed solely against components of FCS. If FCS is involved in the structure which is recognized by the CLs, it must be recognized in conjunction with some other antigens which are unique to the various target cell types to permit discrimination between the target cells.

The results have indicated that there was no cross-reactivity in the lysis of cells from (CBA x DBA/2)F₁ and parental DBA/2 mice. Since both targets were PHA-induced splenic blasts, they might be expected to differ only by the presence of both parental H₂ specificities on the F₁. H₂ molecules can exist at least transiently as a disulphide linked dimer on the cell surface (Henning et al., 1976). Hybridization of H₂k and H₂d molecules on F₁ cells would produce a specificity not present on parental cells. Although these hybrid molecules may be a basis for antigenic differences between F₁ and parental cells, it is unlikely that they are the explanation for the discrimination in the lysis of the targets by spontaneous CLs. If
the F₁ cells were lysed by CLs recognizing H₂<sup>k</sup>-H₂<sup>d</sup> dimers, presumably there are also spontaneous CLs against H₂<sup>d</sup>-H₂<sup>d</sup> dimers which would be formed on both F₁ and DBA/2 cell types, but cross-reactivity was not detected in the lysis of the two types of cells. An alternative explanation may be that F₁ cells possess antigens not expressed on parental cells (Pathman and Nabholz, 1977). Again, such antigens may be recognized in association with H₂ molecules in accord with current models of CL recognition of non-H₂ antigens, although the H₂ requirements in the lysis by spontaneous CLs has not been examined.

**Summary**

A variety of CLs from a range of specificities were produced in the spontaneous response. CLs which lysed allogeneic, syngeneic, tumour and TNP-modified targets were detected. Individual clones of CLs were able to discriminate between CBA and DBA/2 cells, between F₁ and DBA/2 cells, and between tumour and normal spleen cells of the same H₂ phenotype. Cross-reactivity was detected in the lysis of TNP-modified and unmodified targets only.
CHAPTER 5
THE SPECIFICITY OF SPONTANEOUS CYTOTOXIC LYMPHOCYTES: THE ABILITY
TO DISCRIMINATE BETWEEN PAIRS OF SYNGENEIC BLASTS INDUCED BY DIFFERENT
MITOGENS

5.1 INTRODUCTION

Experiments described in Section 4.22 demonstrated that individual clones of spontaneous CLs discriminated between many pairs of different targets. The basis for the discrimination was due presumably to differences in the surface antigens on the targets. When the targets are syngeneic with respect to each other, then differentiation antigens unique to each of the targets are likely to be a basis for the antigenic differences. The aim of the experiments described in this chapter, was to examine whether the spontaneous CL clones could discriminate between subsets of syngeneic lymphoid cells which may be expected to differ only with respect to differentiation antigens. Use has been made of the observation that mitogens tend to activate discrete subsets of lymphocytes (Andersson et al., 1972a; Stobo and Paul, 1973; Gronowicz and Coutinho, 1974). Spleen cell populations which had been cultured in the presence of different mitogens were used as targets. PHA, ConA, LPS and DS were selected as mitogens to produce targets, and the ability of spontaneous clones of CLs to discriminate between blast cells which had been induced by the different mitogens was examined.

5.2 EXPERIMENTAL RESULTS

Spontaneous clones of CLs were generated by culturing $1.3 \times 10^7$ spleen cells per polyacrylamide vessel (Section 2.38i). On day 4 which was the peak of the spontaneous response (Fig. 2), individual clones were examined for their ability to discriminate between two
different targets. The cells from each dimple were removed and divided into two aliquots (Section 2.39). One aliquot of each dimple was assayed for cytotoxicity against spleen blasts induced by one type of mitogen, and the other aliquot was assayed against spleen blasts of the same H2 phenotype which had been stimulated by another mitogen.

5.21 Lysis of LPS and PHA Blasts

When spontaneous clones were divided and assayed against syngeneic blasts induced by PHA or LPS, the majority of the reactive clones lysed either one or the other of the two targets. As shown in Table 5a, when spontaneous clones from cultures of CBA cells were assayed against CBA (PHA) or CBA (LPS) blasts, 35% of the responding clones were specific for the LPS blasts and 65% were specific for the PHA blasts. No clones were detected which lysed both the targets. When spontaneous clones from (CBA x DBA/2)F1 cultures were assayed for their ability to lyse CBA (PHA) or CBA (LPS) blasts, 38% lysed LPS blasts, 56% lysed the PHA blasts, and 6% lysed both targets. A similar distribution was obtained when spontaneous clones from F1 cells were split and assayed against F1 (PHA) or F1 (LPS) blasts, 30% lysed LPS blasts, 63% lysed PHA blasts and 7% lysed both. The dimples with activity against both targets could be accounted for largely by the coincidence of more than one clone in a single dimple. The expected frequency of dimples which might contain cytotoxicity against both targets due to coincidence was calculated to be 5% in the two experiments.

When results were expressed graphically by plotting the per cent specific lysis obtained in one aliquot of each dimple against the per cent specific lysis obtained in the other aliquot, the typical distribution obtained when spontaneous CL clones were assayed against LPS or PHA blasts is shown in Fig. 12. The data indicated that the spontaneous
TABLE 5

LYSIS OF DIFFERENT TYPES OF BLAST CELLS

BY SPONTANEOUS CL CLONES

Spleen cells (1.3 x 10^7 per raft) were cultured for 4 days in poly-
acrylamide rafts when individual dimples of cells were removed and
divided into two aliquots, each of which was assayed for cyto-
toxicity against two different target blast cells. In control
experiments (d) the 2 aliquots were assayed against the same type
of target cell. The spleen blast cells were induced by different
mitogens as described in Section 2.40.
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<table>
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<th>Per cent positive responders against</th>
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</thead>
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<td>Target 1</td>
<td></td>
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<table>
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<tr>
<td>Target 1 &amp; 2</td>
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<tr>
<td>Response</td>
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**Table 5**

**Table 5**

**Table 5**
1.3 x 10^7 spleen cells from (CBA x DBA/2)F₁ mice were cultured for 4 days per polyacrylamide raft. Individual dimples of cells were harvested and divided into two aliquots. An aliquot of each dimple was assayed with F₁ (LPS) blasts and the other aliquot assayed against F₁ (PHA) blasts. The per cent specific lysis obtained in one aliquot was plotted against the per cent specific lysis obtained in the other aliquot. Dimples with less than 10% lysis in both aliquots have not been plotted.
5.22 Lysis of DS and LPS Blasts

When individual dimples of cells from (CBA x DBA/2)F₁ cultures were split and the aliquots assayed against CBA (LPS) or CBA (DS) blasts, 3 populations of clones of different specificities were observed (Fig. 13). There were clones which were specific for DS blasts (47%), clones which lysed LPS blasts only (29%) and clones which lysed both LPS and DS blasts (24%) (Table 5b). The frequency of cross-reactive clones was significantly higher than the expected frequency of cross-reactivity due to coincidence of more than one clone in the same dimple, calculated to be 9%, for that experiment.

5.23 Lysis of ConA and PHA Blasts

When aliquots of individual dimples from cultures of (CBA x DBA/2)F₁ cells were assayed either with PHA or ConA induced CBA blasts, the responding clones could be divided into two main populations, those which lysed ConA blasts only (55%) and those which lysed both ConA and PHA blasts (42%) (Table 5c). The amount of cross-reactivity which can be attributed to coincidence was calculated to be 15% and is significantly lower than the observed value. Only a very small number of clones (3%) was found to be specific for PHA blasts. The distribution of the specificity of the clones is shown in Fig. 14. The results indicated that clones which lyse PHA blasts are a subset of all the clones which will lyse ConA blasts.

The results of all experiments where aliquots of each dimple were assayed against two different types of blasts were shown by $\chi^2$ tests to be significantly different from those obtained in control experiments where the aliquots of each dimple were assayed against the same target cell population ($P < 0.001$). In control experiments
FIGURE 13

LYSIS OF DS AND LPS BLASTS

Individual dimples of cells from 4 day cultures of $1.3 \times 10^7$ (CBA x DBA/2)F₁ spleen cells were divided into two aliquots. One aliquot was assayed with CBA(DS) blasts while the other was assayed with CBA(LPS) blasts. The per cent specific lysis of DS blasts in one aliquot has been plotted against the per cent specific lysis against LPS blasts obtained in the other aliquot. Dimples with less than 10% specific lysis in both aliquots have not been plotted.
Individual dimples of cells from day 4 cultures of $1.3 \times 10^7$ (CBA x DBA/2)$F_1$ spleen cells were divided into two aliquots. One aliquot was assayed with CBA(ConA) blasts and the other aliquot against CBA(PHA) blasts. Percent specific lysis obtained in one aliquot against ConA blasts has been plotted against the percent specific lysis against PHA blasts obtained in the other aliquot. Dimples with less than 10% specific lysis against both targets have not been plotted.
similar levels of cytotoxicity in the two aliquots were observed in the majority of the dimples (Table 5d). There were a few however, where cytotoxicity was observed in only one of the two aliquots. These were derived from dimples with low levels of specific lysis and were thus difficult to divide into equal amounts of cytotoxicity. The frequency distributions in control experiments do not conform to the calculated frequencies expected if the lysis in one aliquot occurred independently of the lysis in the other aliquot ($P < 0.001$).

5.3 DISCUSSION

Previous experiments in Section 4.2 had shown that spontaneous CLs discriminated between pairs of targets which had identical histocompatibility antigens on their surface (Figs. 9, 10, 11). From a consideration of these data, it was concluded that non-\(H_2\) determinants, of which differentiation antigens are likely to be contributors, must be involved in the 'recognized unit'. Use has been made of the observation that mitogens tend to activate discrete subsets of lymphocytes, and the ability of spontaneous CLs to recognize such subsets has been investigated in the experiments described in Section 5.2.

Perhaps the most clearly defined is the use of mitogens to delineate T and B lymphocytes (Andersson et al., 1972a). Antigenic differences between T and B cells are well established and the data in Fig. 12 and Table 5a suggest that the individual spontaneous CL clones lyse PHA blasts or LPS blasts with no detectable cross-reactivity. This was consistently found whether the pairs of blasts used were totally syngeneic with the CBA or F\(_1\) CL effector population, or semi-syngeneic when CLs from F\(_1\) cells were assayed against CBA blasts.
Distinct subsets of B cells appear to exist which are characterized by their responsiveness to different mitogens (Gronowicz and Coutinho, 1974; Melchers et al., 1975). DS and LPS both selectively stimulate B cells, but activation with DS results in the proliferation of cells which do not secrete antibody at a high rate, whereas activation by LPS results in end cells actively secreting immunoglobulins (Gronowicz and Coutinho, 1974). It was suggested that the mitogens activated distinct subsets of B cells and that these subsets represent successive stages along a common differentiative pathway. When individual clones of spontaneous CLs from $F_1$ cells were assayed against spleen cells which had been cultured in the presence of either DS or LPS, there were a number of clones which were able to discriminate between the two targets as well as clones which lysed both targets. The apparently cross-reactive clones are in greater numbers than can be accounted for by coincidence of more than one clone in a single dimple. The presence of these clones of CLs which lysed targets in both populations suggested that the B cells which responded to the two mitogens, expressed, in addition to antigens unique to each of the groups, shared determinants which were the target antigens recognized by the cross-reactive clones of CLs. Alternatively, the B cells which are activated by the two mitogens may be divided into three groups, one which responds to DS only, one which responds to LPS only and a third which responds to both mitogens. The cells in each group would express antigens unique to each of the groups which are the target antigens recognized by the three groups of spontaneous CLs detected in the experiment of Fig. 13.

PHA and ConA both stimulate T cells but not all T cells respond equally to both mitogens. Subsets of T cells which are stimulated by either mitogen have been shown to differ according to a variety
of criteria such as tissue distribution, function and expression of Thyl, Ia antigens and Fc receptors (Stobo and Paul, 1973; Häyry et al., 1976; Niederhuber et al., 1976; Stout and Herzenberg, 1976).

When ConA and PHA induced blasts were used as targets, the detectable clones appeared to segregate as two populations, those which lysed targets from the ConA treated spleen cells and those which lysed targets in both populations (Fig. 14). Thus spontaneous CLs which lysed targets in the PHA-treated population appear to be a subset of the total population of CLs which lyse targets in the ConA-treated population. A simple explanation of these results would be that ConA- and PHA-responsive cells share some determinants, but ConA cells have in addition, antigens not present on PHA responsive cells.

It has been shown that the population of splenic T cells which are stimulated by ConA includes the PHA-responsive T cells (Häyry et al., 1976) and it is interesting that there is an analogous segregation of the specificity of the spontaneous CL clones. To test whether the targets recognized by the different sets of spontaneous CLs corresponded to the subsets of cells delineated by their responsiveness to the mitogens, it would be interesting to remove the PHA-responsive cells using a radioactive suicide technique, restimulate the surviving cells with ConA and use these cells as one of the two target cell populations. The other target population would be cells stimulated by PHA, to see whether the spontaneous CLs detected against these two target populations would segregate as two distinct populations.

Although the experimental results summarized in Section 5.2 can be interpreted in terms of recognition by spontaneous CLs of discrete subsets of lymphocytes which are activated by individual mitogens, it has to be considered that the mitogen may be affecting a variety of cell types albeit indirectly. Further detailed analysis using 'pure'
subpopulations of blasts needs to be attempted, but it can be concluded that spontaneous clones of CLs are able to recognize subsets of syngeneic cells.

**Summary**

It was found that LPS blasts were lysed by a separate set of spontaneous CL clones from those which lysed PHA blasts of the same H2 phenotype, and the clones which lysed PHA blasts were a subset of all the clones which lysed ConA blasts. When individual clones of spontaneous CLs were assayed against LPS and DS blasts, there were clones which lysed both types of blasts as well as clones which were specific for either LPS or DS blasts. The results have been interpreted as demonstrating that spontaneous CLs can recognize and kill subsets of cells which are syngeneic with respect to each other but are stimulated by different mitogens.
CHAPTER 6

THE SPECIFICITY OF CLONES OF CYTOTOXIC LYMPHOCYTES IN STIMULATED CULTURES

6.1 INTRODUCTION

Experiments in Chapters 4 and 5 have demonstrated the potential of the clonal analysis in the investigation of the specificity of CLs. The use of this method has been extended in the experiments described in this chapter to examine the specificity of CLs from stimulated cultures. The analysis of the specificity of 'stimulated' CLs was prompted by several considerations:

i) It has been shown that a single foreign haplotype can stimulate between 1-4 per cent of all potential CL. P. (Bevan et al., 1976; Skinner and Marbrook, 1976; Teh et al., 1977; Lindahl and Wilson, 1977). With such a high frequency of cells responding to a 'single specificity' and in view of the large number of antigens capable of stimulating CL responses, the precommitment hypothesis becomes difficult to apply without evoking a high degree of cross-reactivity in the responses stimulated by the various antigens. Thus it was of interest to examine the degree of discrimination exhibited by clones of CLs produced in a stimulated response.

ii) It was of interest also to compare directly the specificity of the CLs which were produced spontaneously with the specificity of stimulated CLs. It has been shown that CLs from a range of specificities were produced spontaneously, perhaps in a polyclonal type of response. If the spontaneous response is polyclonal in nature, it might be expected that the spontaneous CLs are a representative sample of the specificities produced in a stimulated response.

iii) The data in Fig. 10 indicated that DBA/2 (H2^d) spleen blasts and the P815 (H2^d) mastocytoma cells were lysed by separate
populations of CLs with very little cross-reactivity. The P815 cells however have been used extensively as targets in the assay for CLs produced in response to DBA/2 or (CBA x DBA/2)F₁ stimulator cells. It was of practical importance therefore to determine whether H₂d stimulated CLs discriminated between the P815 and other H₂d target cells.

The experiments described in this chapter examined the specificity of individual clones of CLs produced in stimulated cultures.

6.2 EXPERIMENTAL RESULTS

6.21 Use of Cyclophosphamide-Treated Cells as Stimulators

When untreated F₁ spleen cells are used as stimulator cells in a mixed lymphocyte culture, specifically stimulated clones of CLs are generated from the responder population, but also, smaller numbers of spontaneous clones arise from the F₁ cells. In preliminary experiments it was necessary to obtain a system where specifically stimulated clones could be analysed in the absence of spontaneous clones from the stimulator cells. A variety of different types of cells such as thymocytes and mitomycin C-treated spleen cells were tried as stimulators. Although these cells did not produce spontaneous CLs, they were also inefficient in stimulating an anti-allogeneic response in polyacrylamide vessels. Finally spleen cells from cyclophosphamide-treated mice were tried. Fig. 15 shows the effect of cyclophosphamide treatment on the ability of the cells to produce spontaneous CLs. (CBA x DBA/2)F₁ mice were injected intraperitoneally with various doses of cyclophosphamide and the spleens removed 20 hours later. The spleen cells were cultured for 5 days, at which time cells from individual dimples were removed and assayed for cytotoxicity against P815 targets. As seen in Fig. 15 treatment with 200 mg/kg cyclophosphamide had completely eliminated the generation
(CBA x DBA/2)F₁ mice were injected intraperitoneally with 0-200 mg/kg cyclophosphamide and the spleens removed 20 h later. 1.3 x 10⁷ F₁ cells were cultured per polyacrylamide raft and assayed for CL clones against P815 targets on day 5. Two cultures were assayed for each dose of cyclophosphamide.

- ○ mean clones per culture
- • mean clones per spleen
of spontaneous CLs.

The ability of cells from animals which had been treated with 200 mg/kg cyclophosphamide to stimulate an allogeneic response is summarized in Table 6. Spleen cells from (CBA x DBA/2)F₁ mice which had been treated 5 hours previously with 200 mg/kg cyclophosphamide were cultured with graded numbers of CBA spleen cells in polyacrylamide rafts. The cultures were assayed on day 5 for clones of CLs against P815 targets. The number of clones increased in relation to the numbers of CBA cells, and no clones were detected when cells from cyclophosphamide-treated F₁ mice were cultured alone.

The efficiency of cyclophosphamide-treated spleen cells as stimulators was compared with untreated spleen cells. CBA cells were cultured with either 1.3 x 10⁷ untreated F₁ spleen cells, or 1.3 x 10⁷ F₁ spleen cells from mice treated 5 hours previously with 200 mg/kg cyclophosphamide. Cultures of 1.3 x 10⁷ normal or cyclophosphamide-treated F₁ cells by themselves were also set up, and all cultures were assayed on day 5 for clones of CLs against P815 targets. When the number of clones which might be expected to be due to the stimulator cells had been subtracted, it was found that stimulation by normal or cyclophosphamide-treated cells produced essentially the same number of clones (Table 7).

Thus treatment with 200 mg/kg cyclophosphamide eliminated the production of spontaneous CLs without impairing the ability of the cells to stimulate semi-allogeneic cells. This provided a system where the specifically stimulated clones of CLs could be analysed in the absence of spontaneous CLs from the stimulator cells.
### TABLE 6

**CYCLOPHOSPHAMIDE–TREATED CELLS AS STIMULATORS**

<table>
<thead>
<tr>
<th>Cells per culture</th>
<th>Mean clones per raft ± standard error</th>
</tr>
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<tbody>
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<td>$0.5 \times 10^5$ CBA + $1.3 \times 10^7$ F₁ (cy)</td>
<td>$10.7 \pm 6.5$</td>
</tr>
<tr>
<td>$1.0 \times 10^5$ CBA + $1.3 \times 10^7$ F₁ (cy)</td>
<td>$20.0 \pm 1.5$</td>
</tr>
<tr>
<td>$1.5 \times 10^5$ CBA + $1.3 \times 10^7$ F₁ (cy)</td>
<td>$43.1 \pm 2.4$</td>
</tr>
</tbody>
</table>

CBA spleen cells were cultured in polyacrylamide rafts with spleen cells from (CBA x DBA/2)F₁ mice which had been injected intraperitoneally 5 hours before with 200 mg/kg cyclophosphamide. Cultures were assayed on day 5 for CL clones against P815 targets.
<table>
<thead>
<tr>
<th>Cells per culture</th>
<th>Clones per raft ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^5) CBA + (1.3 \times 10^7) F_1</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>(1.3 \times 10^7) F_1</td>
<td>25 ± 10</td>
</tr>
<tr>
<td>(10^5) CBA + (1.3 \times 10^7) F_1 (cy)*</td>
<td>40 ± 11</td>
</tr>
<tr>
<td>(1.3 \times 10^7) F_1 (cy)*</td>
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</tr>
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</table>

Spleen cells were cultured in polyacrylamide rafts for 5 days and assayed for clones of CLs against P815 targets.

* Cyclophosphamide-treated cells were obtained from spleens of animals which had been injected intraperitoneally with 200 mg/kg cyclophosphamide 5 hours previously.
6.22 The Specificity of Clones of Cytotoxic Lymphocytes Produced in Cultures Stimulated Simultaneously by Two Foreign Haplotypes

The specificity of the CL response of CBA (H\textsuperscript{K}) spleen cells which had been stimulated simultaneously by H2\textsuperscript{b} and H2\textsuperscript{d} alloantigens has been examined. CBA spleen cells were cultured in polyacrylamide rafts with cyclophosphamide-treated stimulator cells from (C\textsubscript{57} Bl X DBA/2)F\textsubscript{1} mice which provided both the H2\textsuperscript{b} and H2\textsuperscript{d} antigenic stimuli. After 5 days in culture, the specificity of the clones of CLs were tested to determine whether separate populations of CLs had developed against the two haplotypes, and also to determine whether there was any cross-reactivity in the response to H2\textsuperscript{d} and H2\textsuperscript{b} alloantigens. Individual dimples of cells were divided into two aliquots and the aliquots assayed with either DBA/2 or C\textsubscript{57} Bl blasts. The results are shown in Fig. 16. The number of positive dimples which lysed DBA targets (59\%) was approximately three times higher than the number which lysed C\textsubscript{57} Bl targets (20\%). Twenty-one per cent of the positive dimples contained cytotoxicity against both targets, but for that experiment the expected number of dimples which might lyse both targets due to coincidence was calculated to be 17 per cent (Table 8). The results indicated that separate populations of CLs specific for either H2\textsuperscript{b} or H2\textsuperscript{d} alloantigens were produced. If cross-reactive clones against the two targets were generated, they would only have constituted a minor portion of the total response. Furthermore, the results obtained were shown by \( \chi^2 \) test not to differ significantly from those expected if the targets were lysed independently \( (P > 0.3) \).
FIGURE 16

THE SPECIFICITY OF CLONES RESPONDING TO H2^b AND H2^d ALLOANTIGENS

3.5 x 10^5 CBA spleen cells were cultured for 5 days in polyacrylamide rafts with 1.3 x 10^7 spleen stimulator cells from (CBA_{57}Bl x DBA/2)F_1 F_1 mice which had been treated with cyclophosphamide (200 mg/kg, 5 h). Individual dimples of cells were harvested and divided into two aliquots (128 dimples from 4 replicate rafts). One aliquot was assayed against DBA/2 (PHA) blasts, the other aliquot assayed against C_{57}Bl(PHA) blasts. The per cent specific lysis against DBA/2 targets in one aliquot has been plotted against the per cent specific against C_{57}Bl targets obtained in the other aliquot. Dimples with less than 10% specific lysis have not been plotted.
TABLE 8
SPECIFICITY OF CLONES STIMULATED BY ALLOANTIGENS

Responder CBA spleen cells were cultured with stimulator cells in polyacrylamide rafts. Stimulator cells were spleen cells from mice treated with 200 mg/kg cyclophosphamide. On day 5, dimples from 3-6 replicate rafts were individually harvested and divided into two aliquots. An aliquot was assayed for cytotoxicity against target 1 and the other aliquot against target 2.

* Expected number of positive dimples lysing both targets was calculated from the observed frequencies distributed in a 2 x 2 contingency table.
<table>
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<th>Expected</th>
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<th>Positive Drops</th>
<th>Drops Assayed</th>
<th>Desired Positive Drops</th>
<th>Total No. of Drops</th>
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Specificity of Copies Studied By Allocations

Table 8
6.23 The Inability of H2^d Stimulated Cytotoxic Lymphocytes to Discriminate Between Different H2^d Target Cells

It has been demonstrated that spontaneous CLs were able to discriminate between different target cells of the same H2 haplotype, for example P815 (H2^d) mastocytoma cells and DBA/2 (H2^d) spleen blasts (Fig. 10). Experiments were carried out to examine whether CLs produced upon allogeneic stimulation also exhibited a similar high degree of discrimination between different target cells of the same H2 type as the stimulating cells. Clones of CLs sensitized to H2^d alloantigens were produced by culturing CBA spleen cells in polyacrylamide vessels with stimulator cells from cyclophosphamide-treated (CBA x DBA/2)F_1 mice. After 5 days in culture, the specificity of the CL clones was analysed by dividing each dimple of cells into two aliquots and assaying the aliquots against a pair of different H2^d target cells. In the experiment summarized in Fig. 17, the aliquots from each dimple were assayed against P815 mastocytoma cells and DBA/2 (PHA) spleen blasts. Most of the positive dimples (89%) were found to contain cytotoxicity against both types of target cells (Table 8). Although, in that experiment 52 per cent of the positive dimples might be expected to contain cytotoxicity against both targets due to coincidence, the results differ significantly from those expected if the targets were lysed independently (P < 0.001).

In another similar experiment (Fig. 18) the aliquots of the individual dimples of cells were assayed against DBA/2 (PHA) and (CBA x DBA/2)F_1 (PHA) blasts. Forty-three per cent of the positive dimples were found to contain cytotoxicity against both the targets, when in that experiment only 18% might be expected to contain cytotoxicity against both targets due to coincidence. The results differ
4 x 10^5 CBA spleen cells were cultured per polyacrylamide raft with 1.3 x 10^7 spleen cells from (CBA x DBA/2)F_1 mice which had been treated with 200mg/kg of cyclophosphamide for 20 hours. On day 5, individual dimples of cells were divided into two aliquots (96 dimples from 3 replicate rafts). One aliquot was assayed for cytotoxicity against P815 targets, while the other aliquot was assayed against DBA/2 PHA-induced spleen blasts. The per cent specific lysis of P815 targets has been plotted the per cent lysis of DBA/2 blasts for positive dimples. Dimples with less than 10 per cent specific lysis against both targets have not been plotted.
FIGURE 18

LYSIS OF $F_1$ AND DBA/2 TARGETS
BY $H_2^d$-STIMULATED CLONES

$1.5 \times 10^5$ CBA spleen cells were cultured in polyacrylamide rafts with $1.3 \times 10^7$ spleen cells from (CBA x DBA/2)$F_1$ mice which had been treated with 200 mg/kg cyclophosphamide for 5 hours. On day 5, individual dimples of cells were divided into two aliquots (192 dimples from 6 replicate rafts). An aliquot was assayed with DBA/2 (PHA) blasts and the other aliquot against (CBA x DBA/2)$F_1$ (PHA) blasts. The per cent specific lysis in one aliquot against DBA/2 targets has been plotted against the per cent specific in the other aliquot against $F_1$ targets. Dimples with less than 10 per cent specific lysis have not been plotted.
significantly from those which would be expected if the parental DBA/2 and the F<sub>1</sub> target cells were lysed independently by the H<sup>d</sup><sub>2</sub> stimulated clones of CLs (P < 0.001).

The results from these experiments indicated that the majority of the clones of CLs arising from CBA spleen cells after stimulation by (CBA x DBA/2)<sub>F<sub>1</sub></sub> cells did not discriminate between different types of target cells bearing the H<sup>d</sup><sub>2</sub> antigens.

6.3 DISCUSSION

The potential of the clonal analysis for examining the specificity of spontaneously generated CLs was demonstrated in experiments described in Sections 4.2 and 5.2, and the use of the method has been extended for the analysis of the specificity of 'stimulated' CL responses.

Spleen cells for use as the stimulator population were taken from mice which had been treated with cyclophosphamide. Treatment of the stimulator cells with cyclophosphamide served two purposes. When completely allogeneic cells were used to provide antigenic stimulus, treatment with cyclophosphamide served to inactivate the cells such that they did not respond to cells used as the responder population. Even in situations where the stimulator populations were not reactive against the responder cells, as in the case when CBA cells were stimulated by (CBA x DBA/2)<sub>F<sub>1</sub></sub> cells, cyclophosphamide treatment was still necessary to eliminate the production of spontaneous CLs by the stimulator cells. It has been shown that a dose of 200 mg/kg cyclophosphamide abolishes the ability of the cells to produce spontaneous CLs (Fig. 15). Although it has been reported that treatment of cells with 250 mg/kg cyclophosphamide will abolish their ability to stimulate a mixed lymphocyte reaction (Gutman,
1974), the data summarized in Table 7 indicated that treatment of cells with 200 mg/kg cyclophosphamide did not markedly impair their ability to stimulate a CL response. Thus, the use of cyclophosphamide-treated cells as stimulators, provided a system whereby specifically stimulated CLs from the responder population can be analysed in the absence of a response from the cells used as stimulators.

Using cyclophosphamide-treated stimulator cells from (C$_{57}$Bl x DBA/2)$F_1$ mice, the response of CBA spleen cells to stimulation by two sets of alloantigens was examined. Simultaneous stimulation of CBA cells by H2$^d$ and H2$^b$ alloantigens resulted in the generation of two separate populations of clones of CLs directed against each of the haplotypes. There have been other reports where the response of lymphoid cells immunized simultaneously with two H2 antigens have been examined (Goldstein et al., 1971; Peavy and Pierce, 1975a; Teh et al., 1977). From the evidence obtained from the application of preadsorption (Brondz and Snegirova, 1971), tritium suicide (Peavy and Pierce, 1975b) or cold target inhibition techniques (Bevan, 1975a) to the analysis of a mass lymphoid population, it has been inferred that the immunized cell population contained separate populations of CLs directed against the two antigens. The results obtained from the analysis of the specificity of individual clones of CLs (Fig. 16) has provided direct confirmation of the conclusions derived from the analysis of cell populations in mass cultures. The production of separate populations of CLs against the two antigens in a lymphoid population which had been immunized simultaneously with the antigens, has been taken as evidence that CL.F are pre-committed to the expression of a single specificity (Teh et al., 1977), and that the receptors on CLs have not been passively adsorbed (Bevan, 1975a).
Very few, if any, cross-reactive clones which lysed both H2^b and H2^d targets were observed (Table 8). The results are consistent with those obtained by Teh and co-workers (1977) who concluded that CL.P in RNC (H2^k) mice against H2^b and H2^d specificities segregated as two independent populations with no cross-reactivity. Peavy and Pierce (1975a) also reported that there was no cross-reactivity in the lysis of H2^b and H2^d targets in the response of DBA/1 (H2^q) cells stimulated by either DBA/2 (H2^d) or C57Bl (H2^b) cells. However, cross-reactivity against H2^b and H2^d haplotypes was detected in responses from A (H2^a) and C3H (H2^k) mice. The reports of Brondz and Snegirëva (1971) and Blomgren and Andersson (1974), who examined the CL responses using a variety of allogenic strain combinations, showed that in some strain combinations no cross-reactive lysis of cells of a different H2 type from the stimulator cells was detected, but in other combinations, high levels of cross-reactivity could be detected, the most notable being lymphocytes sensitized against H2^k or H2^d antigens exhibited strong reactivity against H2^a cells. Recently, cross-reactivity between H2 types has been used as a basis for explaining alloaggression (Matzinger and Bevan, 1977). Although other experiments such as that described in Section 6.22 have not been carried out because of a lack of the different strains of mice available for the project, the analysis of clones of CLs generated upon stimulation by two foreign H2 types would be an ideal system for testing for cross-reactivity in CL responses.

The ability of H2^d stimulated clones of CLs to discriminate between different H2^d target cells has also been examined using the clonal analysis of the specificity of CLs. It has been shown that spontaneous CLs could discriminate between different types of target cells of the same H2 haplotype (Table 4) and it was of interest
to compare the specificity of stimulated and spontaneous CLs. \(H^2_d\) stimulated clones of CLs were produced by culturing CBA spleen cells with stimulator cells from cyclophosphamide-treated (CBA x DBA/2)\(F_1\) mice in polyacrylamide vessels. When individual dimples of cells were divided in two halves and assayed for activity against pairs of different target cells bearing the \(H^2_d\) antigens, the results indicated that CLs produced upon stimulation by \(H^2_d\) alloantigens could not discriminate between P815 and DBA/2 blasts as targets. Neither did the stimulated clones of CLs discriminate between the parental DBA/2 blasts and the blasts from (CBA x DBA/2)\(F_1\) mice, in contrast to the results obtained with spontaneous clones of CLs. A comparison of results obtained for stimulated and spontaneous CLs in the lysis of DBA/2 and P815 target cells is shown in Fig. 19. The results indicated that the clones of CLs produced in the two types of responses were different in their specificity. Spontaneous clones were generally smaller and could discriminate between the two targets, while stimulated clones lysed both targets with equal facility. Although the specificity of the clones in the two responses appear to differ, it is not known whether the discriminatory, spontaneous-type of clones are not produced in a stimulated culture. The frequency of clones detected against P815 or DBA/2 blasts in the spontaneous response is several hundred-fold lower than the frequency of clones detected in a stimulated response. If the discriminatory clones occur with the same frequency in both types of responses, they would be effectively diluted out by the high number of cross-reactive clones in stimulated cultures.

In the absence of any apparently cross-reactive spontaneous clones against various \(H^2_d\) target cells, it must be concluded that the clones which are produced spontaneously are not a representative
FIGURE 19

A. COMPARISON OF THE SPECIFICITY OF SPONTANEOUS AND

\[ H_2^d \text{ STIMULATED CLONES LYSING P815 AND DBA/2 TARGETS} \]

A. Spontaneous clones:

Data from Fig. 10

B. Stimulated clones:

Data from Fig. 17
A

per cent specific lysis DBA/2

30
25
20
15
10
5
0

per cent specific lysis P815

10
20
30
40

B

per cent specific lysis DBA/2

90
70
50
30
10
0

per cent specific lysis P815

10
20
30
40
50
60
70
80
90
sample of the total pool of CL.P which are activated by H2\textsuperscript{d} allografts. This conclusion has further implications in considering the nature of the spontaneous response.

The factors which contribute to the generation of spontaneous clones have yet to be established, but one of the suggestions was that the spontaneous response was a polyclonal type of response induced by mitogenic factors in, for example, FCS in the medium. With a polyclonal type of stimulator and the lack of selection of precursors on the basis of affinity, it might be expected that the spontaneous response would be a representative sample of the range of specificities detected in a stimulated response. The data in Fig. 19 indicate that the spontaneous clones were not representative of the total anti-H2\textsuperscript{d} response. There are reasons however, why this may be so without eliminating polyclonal activation as the source of spontaneous clone generation. According to a variety of criteria, the CL precursors consist of subsets of cells. They are heterogeneous with respect to size (MacDonald et al., 1973), density (Shortman et al., 1972), stimulatory requirements (Röllinghoff and Wagner, 1975) and Ly phenotype (Shiku et al., 1976). Similarly, lymphoid cells are heterogeneous in their response to the polyclonal stimulation by mitogens (Andersson et al., 1972a; Stobo and Paul, 1973). If the subsets of CL.P do not all have an identical repertoire, then the non-specific activation of discrete subsets would lead to the non-representative sample of specificities such as that found with the spontaneous response. The clonal analysis of the specificity of subsets of T cells may enable the significance of the spontaneous CLs within the total population of CLs to be determined.
Summary

Treatment of cells in vivo with cyclophosphamide (200 mg/kg) abolished the ability of the cells to produce spontaneous clones of CLs in culture. Cells treated with cyclophosphamide however were still capable of stimulating a CL response, and the use of these cells as the stimulator cells provided a system whereby stimulated CLs could be examined in the absence of a response from the stimulator population.

When CBA spleen cells were cultured with stimulator cells from cyclophosphamide-treated $(C_{57}Bl \times DBA/2)F_1$ mice, two separate populations of clones of CLs were generated, one which lysed $C_{57}Bl$ blasts and the other which lysed DBA/2 blasts. Few if any, cross-reactive clones were detected.

When CBA cells were stimulated with $(CBA \times DBA/2)F_1$ cells, the clones of CLs which developed did not discriminate between F815 and DBA/2 blasts, or between DBA/2 and $(CBA \times DBA/2)F_1$ blasts as targets. The results indicated that $H^2_d$ stimulated CLs do not discriminate between different target cells bearing the $H^2_d$ antigens, in contrast to the spontaneous clones of CLs which will lyse the above targets.
CHAPTER 7

THE FREQUENCY AND SPECIFICITY OF CLONES OF CYTOTOXIC LYMPHOCYTES
GENERATED IN RESPONSE TO TNP-MODIFIED SYNGENEIC CELLS

7.1 INTRODUCTION

Recently, CL responses have been generated against syngeneic stimulator cells which had been chemically- (Shearer et al., 1975) or virally-modified (Doherty and Zinkernagel, 1975) as well as to tumour or differentiation antigens (Bevan, 1975b). An important observation in all these studies was that even though there were no differences in the H2 antigens between responder and stimulator populations, and the response was stimulated by a non-H2 antigen, H2 products were still somehow found to be involved in the lysis of the target cells. CMC was found to be restricted to target cells bearing both the appropriate 'modifying' antigen and the same H2 antigens as the stimulator cells. Cells of another H2 type modified in the same way were not lysed.

In most studies, the H2 restriction appeared to be absolute, but more recently, cross-reactive lysis of TNP-syngeneic targets by CLs produced by allogeneic stimulation (Lemonier et al., 1977) and cross-reactive lysis of modified-allogeneic cells by CLs immunized by TNP-syngeneic cells were reported (Burakoff et al., 1976). It has also been observed, that during a "particularly strong response" against virus coated syngeneic cells, cytotoxic activity against cells of a different H2 type could be detected (Schrader and Edelman, 1977).

There could be two possible ways as to why there is cross-reactivity present in a response to a particular antigenic stimulus. It may be a result of cross-reactivity in the recognition of target antigens by the population of effector cells produced in a specific
response. Alternatively, immunization with a particular stimulator population may also activate low numbers of CL-precursors against other non-related specificities. It would be difficult to differentiate between these two possibilities by examining the activity of cells in mass cultures. Using a clonal analysis however, it is possible to examine the specificity of individual clones of CLs to determine whether the activity against different target cells is due to the same population of CLs, or whether the activity against the different targets resided in separate populations of CLs.

In the experiments described in this chapter, the clonal analysis has been applied to examine the frequency and specificity of clones of CLs produced upon stimulation with hapten-modified cells. (CBA x C57Bl)F1 spleen cells were cultured with TNP-modified syngeneic or semi-syngeneic cyclophosphamide-treated stimulator cells and the frequency of clones of CLs arising in such a response which will lyse various target cells was measured. By dividing individual clones and assaying an aliquot against one of a pair of targets, the specificity of the clones were analysed to determine whether the activity that was detected against the various targets were due to the production of cross-reactive clones, or whether different CLs of distinct specificities had been produced.

7.2 EXPERIMENTAL RESULTS

7.21 Development of Anti-TNP Clones on Different Lays of Culture

Before frequency determinations could be carried out it was necessary in a preliminary experiment to establish when the highest number of clones could be detected. (CBA x C57Bl)F1 spleen cells were cultured with TNP-modified cyclophosphamide-treated syngeneic cells and the number of clones against CBA-TNP targets was measured
$3 \times 10^6$ (CBA x C$_{57}$B1)$_1$ spleen cells were cultured per polyacrylamide raft with $1.3 \times 10^7$ TNP-modified spleen cells from (CBA x C$_{57}$B1)$_1$ mice which had been treated with cyclophosphamide (200 mg/kg, 5h).

After various days, cultures were assayed for clones of CLs against TNP-modified CBA target cells. Each point is the mean of 3 observations (96 dimples from 3 replicate cultures). Vertical bars represent the standard error of the means.
on days 3-7. The results have been plotted in Fig. 20 and it can be seen that the maximum number of clones occurred after 5 days in culture. In all subsequent experiments the response was examined after 5 days in culture.

7.22 Frequency of Clones Against Different Target Cells Produced in an Anti-TNP Response

The frequency of clones of CLs produced by (CBA x C57Bl)F1 spleen cells when stimulated with syngeneic, TNP-modified F1 spleen cells has been measured by two methods. In the first set of experiments, graded numbers of (CBA x C57Bl)F1 spleen cells were cultured in polyacrylamide rafts with a constant number of TNP-modified syngeneic stimulator cells from cyclophosphamide-treated mice. The cultures were assayed on day 5 for the number of dimples containing cytoxicity against a particular target. In different experiments, the response against TNP-modified F1 cells, TNP-modified parental CBA and C57Bl cells, as well as unmodified F1 cells and modified allogeneic cells was measured. The per cent negative dimples was plotted against the number of responder cells after the method of Quintáns and Lefkovits (1973) and are shown in Fig. 21. According to Poisson's distribution, the number of responder cells per dimple with which 37% of all dimples were negative contains one clone of CLs. The frequency of clones against the various targets calculated from the data in Fig. 21 has been summarized in Table 9. The frequency of clones against F1-TNP, CBA-TNP and P815-TNP was determined from the graphs in Fig. 21 from the number of responder cells per dimple with which 37% of all dimples were negative. The response against C57Bl-TNP targets was not very high. Instead of extrapolating the plotted data to find the 37% negative value, the frequency was calculated from the formula in Section 2.42 i. The frequency of clones of CLs against F1-TNP and
Graded numbers of (CBA x C57Bl)F1 spleen cells were cultures in polyacrylamide rafts with 1.3 x 10⁷ TNP-modified syngeneic spleen stimulator cells from F1 mice treated 5 hours previously with 200 mg/kg cyclophosphamide. Individual dimples of cells from the cultures were assayed for cytotoxicity against target cells. The number of responder cells per culture was plotted against the per cent negative dimples on a semi-logarithmic scale. The mean from 3 replicate rafts have been plotted. Lines were fitted by inspection.

- - - (CBA x C57Bl)F1-TNP targets
△△△△ CBA-TNP targets
♦♦♦♦ C57Bl-TNP targets
○○○○○ P815-TNP targets
▼▼▼▼ (CBA x C57Bl)F1

The P815 and PHA-induced spleen blast target cells were prepared according to the method described in Section 2.40 and modified with TNP as described in Section 2.35.
<table>
<thead>
<tr>
<th>Target</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CBA x C\textsubscript{57}BL\textsubscript{1})\textsubscript{F\textsubscript{1}}-TNP</td>
<td>1 per 3.3 x 10\textsuperscript{4}</td>
</tr>
<tr>
<td>CBA-TNP</td>
<td>1 per 6.7 x 10\textsuperscript{4}</td>
</tr>
<tr>
<td>C\textsubscript{57}BL\textsubscript{1}-TNP</td>
<td>1 per 2.9 x 10\textsuperscript{5}</td>
</tr>
<tr>
<td>P815-TNP</td>
<td>1 per 7.0 x 10\textsuperscript{4}</td>
</tr>
<tr>
<td>(CBA x C\textsubscript{57}BL\textsubscript{1})\textsubscript{F\textsubscript{1}}</td>
<td>nil</td>
</tr>
</tbody>
</table>

These frequencies were determined from the data plotted in Fig. 21.
the modified parental cells, CBA-TNP and C57Bl-TNP was 1 per 3.3 x 10^4, 1 per 6.7 x 10^4 and 1 per 2.9 x 10^5 spleen cells respectively. A relatively high frequency of clones (1 per 7 x 10^4) was detected against the modified allogeneic tumour cell targets (P815-TNP), but no clones were detected against unmodified F1 cells.

The frequencies summarized in Table 9 against each of the targets had been measured in separate experiments. As the response may vary between experiments, a further experiment was carried out to measure the frequency of clones against the various targets generated in the same set of cultures. Polyacrylamide cultures each containing 3 x 10^6 (CBA x C57Bl)F1 spleen cells and 1.3 x 10^7 TNP-modified F1 stimulator cells from cyclophosphamide-treated mice were set up. On day 5, two rafts each were assayed for activity against F1-TNP, CBA-TNP, C57Bl-TNP, P815-TNP, as well as the unmodified F1 and P615 targets. The number of clones was calculated from the number of positive dimples detected using the formula given in Section 2.42 i. The frequencies obtained have been summarized in Table 10. The frequency against the TNP-modified cells of the F1 and the parental CBA and C57Bl strains were 1 per 6.7 x 10^4, 1 per 2.3 x 10^5 and 1 per 10^6 cells respectively. In this experiment no clones lysing P815-TNP and the unmodified P815 and F1 targets were detected.

The frequencies against TNP modified F1 and parental cells in Table 10 are approximately two fold lower than the frequencies summarized in Table 9, but the ratios of the frequency of clones against the three targets in the two sets of results is similar. The frequency of clones detected against F1-TNP targets is approxim-
### TABLE 10

**FREQUENCIES OF ANTI-TNP CLONES IN (CBA x C₅₇BL)F₁ Spleen Cells**

<table>
<thead>
<tr>
<th>Target</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CBA x C₅₇BL)F₁-TNP</td>
<td>1 per 6.7 x 10⁴</td>
</tr>
<tr>
<td>CBA-TNP</td>
<td>1 per 2.3 x 10⁵</td>
</tr>
<tr>
<td>C₅₇BL-TNP</td>
<td>1 per 10⁶</td>
</tr>
<tr>
<td>P815-TNP</td>
<td>nil</td>
</tr>
<tr>
<td>P815</td>
<td>nil</td>
</tr>
<tr>
<td>(CBA x C₅₇BL)F₁</td>
<td>nil</td>
</tr>
</tbody>
</table>

3 x 10⁶ (CBA x C₅₇BL)F₁ spleen cells were cultured in polyacrylamide vessels with 1.3 x 10⁷ TNP-modified spleen cells from (CBA x C₅₇BL)F₁ mice treated 5 h previously with 200 mg/kg cyclophosphamide. On day 5, 2 cultures each were assayed for dimples of cells with cytotoxicity against the various target cells. The number of clones was calculated from the number of positive dimples using the formula in Section 2.42 i.
ately twice the frequency of clones detected against CBA-TNP cells, while the frequency of clones lysing C57Bl-TNP cells is approximately 5 fold lower than the frequency of clones against CBA-TNP cells. Although a good response against P815-TNP targets was detected in the experiment described in Fig. 21, activity against allogeneic cells was not observed in all experiments.

7.23 The Specificity of the Clones Generated in Response to TNP-Modified Syngeneic Cells

The results in the previous section have shown that when (CBA x C57Bl)F1 cells were stimulated with TNP-modified syngeneic cells, clones of CLs were detected which lysed TNP-modified cells from the F1 and the two parental strains of mice, and in some experiments the TNP-modified allogeneic P815 targets as well. To determine whether the activity against the various targets was distributed in different clones of CLs or whether it was due to cross-reactive CLs able to lyse more than the one target, the specificity of individual clones of CLs was examined. Individual dimples of cells were divided into two aliquots and each aliquot was assayed against one of a pair of target cells. Each positive dimple was scored as containing activity against either or both of the targets. The number of dimples which contained activity against both targets was compared with the expected number of dimples which might contain activity against both targets due to coincidence of more than 1 clone in the same dimple. Cross-reactive clones were considered to be present when the observed number of dimples with activity against both targets was considerably higher than the expected number of double positive dimples due to coincidence. The results of the various experiments on the specificity of clones of CLs from cultures of (CBA x C57Bl)F1 spleen cells
(CBA x C57Bl)F1 responder spleen cells were cultured with stimulator cells in polyacrylamide rafts for 5 days. The stimulator cells were TNP-modified spleen cells from mice treated 5 hours with 200 mg/kg cyclophosphamide. Individual dimples of cells from the cultures were harvested and divided into two aliquots. One aliquot was assayed for cytotoxicity against target 1, and the other aliquot against target 2.

* The expected number of positive dimples lysing both targets was calculated from the observed frequencies distributed in a 2 x 2 contingency table (Section 2.42 iii).
<table>
<thead>
<tr>
<th>Target 1</th>
<th>Target 2</th>
<th>% Positive</th>
<th>Proceeded</th>
<th>% Positive</th>
<th>Proceeded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11

SPECIFICITY OF ANTI-TRP CLONES
stimulated with TNP-\(F_1\) cells have been summarized in Table II.

Most of the clones reactive against the two modified parental cells segregated as two populations. Twenty-one per cent of the positive dimples were found to lyse both the modified parental cells when the number of double positives which might be due to coincidence was calculated to be 14% (Table IIa). The data might indicate the presence of clones with activity against both CBA-TNP and C\(_{57Bl}\)-TNP targets, but these cross-reactive clones would contribute to only a minor portion of the response against the two targets.

When the dimples of cells were each divided and assayed against CBA-TNP or CBA unmodified cells, very few clones were detected against the unmodified cells. Ninety per cent of the positive dimples detected lysed CBA-TNP targets only (Table IIb). The data indicated that some of the activity detected against the unmodified cells were due to cross-reactive clones lysing both modified and unmodified CBA cells.

In some cultures, activity against modified allogeneic targets was detected. When dimples of cells were each tested for activity against \(F_1\)-TNP and DBA/2-TNP targets, the results indicated that most of the clones detected with activity against the modified allogeneic cells were specific for that target. Cross-reactive clones would account for only part of total activity against the DBA/2-TNP cells (Table IIc).

7.24 The Response of \(F_1\) Cells Stimulated by TNP-Modified Parental Cells

When \(F_1\) cells were stimulated with modified syngeneic \(F_1\) cells, clones of CLs specific for each of the modified parental cells were produced (Table IIa). To determine whether stimulation with modified cells of one of the parental strains would activate CLs
against both the parental specificities in the F₁ population, spleen cells from (CBA x C₅₇BL)F₁ mice were cultured with CBA-TNP stimulator cells in polyacrylamide vessels for 5 days. The aliquots of individual dimples of cells were then assayed against CBA-TNP and C₅₇BL-TNP target cells. It can be seen from the results summarized in Table 11d that clones of CLs against both the parental haplotypes had been generated. The clones against the two target cells segregated as two distinct populations, and no cross-reactive clones appeared to be present. The number of dimples with cytotoxicity against CBA-TNP targets was approximately three times the number of dimples with activity against C₅₇BL-TNP targets when (CBA x C₅₇BL)F₁ responders were stimulated with CBA-TNP cells (Table 11d). The same ratio of dimples lysing the two targets was obtained also when F₁-TNP cells were used as the stimulators (Table 11a). The results are consistent with the notion that CBA-TNP and F₁-TNP stimulator cells activate the same population of responders in the F₁.

7.3 DISCUSSION

The response in F₁ spleen cell populations against TNP-modified cells has been examined. Using the clonal analysis, the frequency of clones of CLs lysing various target cells and the specificity of the clones was measured. When (CBA x C₅₇BL)F₁ cells were stimulated with TNP-modified syngeneic F₁ cells, clones of CLs lysing TNP-modified F₁ cells and modified cells of both the parental strains were detected (Fig. 21).

The clones of CLs lysing the modified cells of the parental strains segregated as two populations. Few clones which lysed both CBA-TNP and C₅₇BL-TNP cells were observed (Table 11a). Equal numbers of clones against the two parental cell types were not
detected. From the data in Table 9, the frequency of clones against TNF-modified cells of the CBA parental strain (1 per $6.7 \times 10^4$ cells) was approximately 5 fold higher than the frequency of clones detected against C$_{57}$Bl-TNP target cells (1 per $2.9 \times 10^5$ cells). The frequency of clones detected against the syngeneic F$_1$-TNP targets was one per $3.3 \times 10^4 F_1$ spleen cells cultured. These frequencies are similar to those obtained by Lindahl and Wilson (1977) for the response of DBA/2 and C$_{57}$Bl lymph node cells to stimulation by TNF-modified syngeneic cells. The frequency of clones of CLs against TNF-modified syngeneic cells is generally 100 fold lower than the frequency of clones detected in an allogeneic response (Skinner and Marbrook, 1976; Lindahl and Wilson, 1977).

The response against F$_1$-TNP target cells might be expected to be a sum of the activity against TNF-modified specificities of the two parental haplotypes. The frequency of clones detected using F$_1$-TNP target cells is higher than the sum of the frequency of clones against the two modified parental cells. As all the target cells had been prepared in an identical manner, it has been assumed that the susceptibility of the cells from the various trains to specific CMC is similar, and that the differences in the activity detected against the various target cells reflect differences in the antigenicity of the target cells. Thus, the high frequency of clones lysing F$_1$-TNP cells would indicate that there are other CLs in addition to those which lyse CBA-TNP and C$_{57}$Bl-TNP targets that will lyse F$_1$-TNP cells. Evidence has been put forward by Fathman and Nahholz (1977) that F$_1$ cells express F$_1$-specific determinants which are not on either of the parental cell types. The expression of these F$_1$-specific determinants were controlled also by the MHC. Thus, F$_1$ cells may present a greater number of specificities
than the sum of the specificites presented by the two parental cell types.

Activity against modified cells of an unrelated allogeneic strain was detected in some cultures of F₁ cells stimulated with syngeneic TNP-modified cells (Table 9 and Table 11c). However, activity against allogeneic cells was not observed in all experiments (Table 10). Similar observations have been made by other authors (Burakoff et al., 1976; Schrader and Edelman, 1977). They observed that in experiments where the specific response was particularly strong, activity against cells of a different H₂ type could be detected. Burakoff and co-workers (1976) showed that populations of C₅7BL/6 cells immunized with C₅7BL-1-TNP cells contained cytotoxicity against modified cells of H₂ᵈ, H₂ᵏ, H₂ᵦ and H₂ᵇ haplotypes in addition to the modified cells of the syngeneic H₂ᵇ haplotype. The extent of lysis of the modified allogeneic cells was found to correlate with the level of activity against the syngeneic-TNP targets. On the basis of the results from a cold target inhibition experiment, the authors concluded that the same CLs which lysed H₂ syngeneic-TNP targets were responsible for the lysis of the H₂ different-TNP targets. However, their data was not adequate to have discounted the presence of CLs which were specific for the H₂ different-TNP target cells.

Schrader and Edelman (1977) reported, that in experiments in which the cytotoxic activity against Sendai virus coated tumour cells of the same H₂ type was particularly strong, cytotoxicity against cells of a different H₂ type was observed. It was suggested that the activity against allogeneic targets probably reflected the presence of a polyclonal response stimulated perhaps by non-specific factors released during a vigorous specific response.

The results from the analysis of the specificity of the clones
in cultures of (CBA x C57Bl)F1 spleen cells stimulated with syngeneic F1-TNP cells indicated that only a small portion of the activity against H2-different DBA/2-TNP target cells cross-reacted with the syngeneic F1-TNP targets (Table 11c). A much greater portion of the activity was due to clones which lysed only the DBA/2-TNP targets. This, and together with the observation that activity against P815-TNP or DBA/2-TNP cells was not consistently found in all experiments, indicated that the activity against allogeneic targets was not an integral part of the anti-syngeneic-TNP response. The observations are more compatible with the notion that CLs of other, non-related specificities may be activated perhaps non-specifically or indirectly during a specific response.

As it was not possible to predict whether or not cultures contained activity against allogeneic targets, further analysis of the specificity of clones with activity against H2-different targets was not continued to establish whether TNP-modification of the allogeneic targets cells was necessary for lysis.

When F1 spleen cells were stimulated with TNP-modified cells of one of the parental strains, CLs against modified target cells of both the parental haplotypes were detected (Table 11d). The CLs against CBA-TNP and C57Bl-TNP segregated as two distinct populations with no detectable cross-reactivity. These results contrast with those of Forman (1975) and Shearer and co-workers (1975) who reported that an F1 cell population stimulated with TNP-modified cells of one of the parental strains, develops cytotoxicity for modified cells of the strain used for sensitization only. The results in Table 11d are perhaps more consistent with the observations of Zinkernagel (1976) on antiviral responses in chimaeric mice. F1 chimaeras reconstituted with bone-marrow cells of one of the parental strains
and immunized with virus, develops CLs against virus-infected target cells of both the parental strains. The results were explained on the basis of antigen presentation by F₁ host macrophages which would express both the parental H₂ antigens.

The difference in the observations obtained in the experiment summarized in Table 11d and those of Forman (1975) and Shearer and co-workers (1975) is not clear. Perhaps it may be attributed to differences in the procedure for detecting cytotoxicity. In their reports, the amount of cytolysis of the two parental-TNP targets by a CL populations from a mass cell culture was measured at one effector to target cell ratio and compared with each other. In contrast, in the experiments described in this thesis, cytotoxicity is determined by the detection of the activity of clones of CLs against a particular target. It has been demonstrated that an F₁ cell population may not necessarily contain equal numbers of clones of CLs against the two parental cell types. In (CBA x C₅₇Bl)F₁ spleen cell populations which have been stimulated with F₁-TNP cells, the frequency of clones generated against CBA-TNP targets was approximately 5 fold higher than the frequency of clones generated against C₅₇Bl-TNP targets (Fig. 21, Tables 9 and 10). If the clone size and the lytic ability of the CLs in the two populations are similar, the activity against C₅₇Bl-TNP in a mass cell culture would be diluted out to greater extent that the activity against CBA-TNP targets. The values for the amount of cytotoxicity against C₅₇Bl-TNP and CBA-TNP measured in a population from a mass cell culture at a particular ratio of lymphoid to target cell ratio might give the false indication that little activity had been generated against C₅₇Bl-TNP targets. The diluting effect of the rest of the cell population is avoided using the clonal analysis where the cells are
segregated in culture and the activity of isolated clones of CLs can be assayed. This is a further advantage of the clonal assay which enables relatively rare 'events' to be detected definitively.

The experiments described in Section 7.2 have indicated that stimulation with either F₁-TNP or CBA-TNP will activate, in a (CBA x C₅₇Bl)F₁ cells population, the production of CLs against modified targets of both the parental haplotypes. The ratio of the number of clones of CLs lysing CBA-TNP to the number of clones lysing C₅₇Bl-TNP is similar whether F₁-TNP or CBA-TNP were used for stimulation (Table 11a c f. lld). This observation suggests that the response stimulated by F₁-TNP or CBA-TNP cells may be the same. However, the frequency of clones of CLs against F₁-TNP and the parental-TNP targets generated by F₁ cells when stimulated with CBA-TNP has not been rigorously established. It would be worthwhile in the continuation of this project to compare the efficiency of F₁-TNP, CBA-TNP or C₅₇Bl-TNP to stimulate in F₁ cell populations the production of clones of CLs against the modified F₁ and parental target cells.

It would be of value also to demonstrate formally that the CLs lysing C₅₇Bl-TNP and CBA-TNP targets are a subset of the CLs which lyse F₁-TNP targets. Such experiments could easily be carried out using the clonal analysis of the specificity of the CLs.

**Summary**

$$(CBA \times C_{57Bl})F_1$$ spleen cells cultured with $F_1$-TNP stimulator cells generated clones of CLs lysing $F_1$-TNP and TNP-modified cells of the two parental haplotypes. The frequency of clones against $F_1$-TNP targets was 1 per $3.3 \times 10^4$ cells. The frequency of clones of CLs lysing CBA-TNP and $C_{57Bl}$-TNP were 1 per $6.7 \times 10^4$ and 1 per $2.9 \times 10^5$ spleen cells respectively. As with all limiting dilution
assays, these frequencies must be regarded as being minimal estimates. Activity against P815-TNP and DBA/2-TNP was detected in some but not all the cultures. The activity against DBA/2-TNP was due mainly to CLS which lysed DBA/2-TNP targets specifically. Only a few of the clones were cross-reactive for both DBA/2-TNP and $F_1$-TNP targets. The CLS responding against CBA-TNP and $C_{57}$Bl-TNP targets segregated as two populations with little cross-reactivity. Stimulation of $F_1$ cells with CBA-TNP will also activate CLS lysing the modified cells of both parental haplotypes.
CHAPTER 8

CONCLUDING DISCUSSION

8.1 SPONTANEOUS CLONES OF CYTOTOXIC LYMPHOCYTES

The experiments of Section 3.2 have shown that cytotoxic activity, as measured by the lysis of $^{51}$Cr-labelled target cells was generated 'spontaneously' when normal murine spleen cells were cultured without stimulator cells in polyacrylamide culture vessels. There have been reports on a variety of non-stimulated or naturally occurring types of cytotoxicity in populations of normal non-immunized cells. These may be summarized in relation to the 'spontaneous clones' described in this thesis.

i) Natural cytotoxic reactivity against syngeneic and allogeneic tumour cells, occurring in the spleens of mice was first described by Herberman and co-workers (1973). The biological relevance of these NKs has been assumed to be of importance in autoimmune reactions as well as the reaction against persistent viral infections (Greenberg and Playfair, 1974). A correlation between the amount of NK activity and the resistance of the animals to haemopoietic grafts (Kiessling et al., 1977) and resistance against syngeneic tumour cells has also been reported (Haller et al., 1977). Studies to characterize the effector cells have indicated that NKs are not mature B or T cells. Neither were they K cells which mediate antibody dependent cellular cytotoxicity, nor monocytes (Kiessling et al., 1975, 1976). The spontaneous cytotoxicity described in Section 3.2 was T cell dependent as shown by its sensitivity to anti-Thyl antiserum (Table 1). Furthermore, the spontaneous cytotoxic clones had been generated in vitro (Fig. 2), whereas NKs occur in vivo and their activity has been found to be extremely labile in culture.
ii) Spontaneous cellular cytotoxicity of normal lymphocytes but involving antibodies or lymphotoxins has also been reported in several in vitro systems (MacLennan et al., 1970; Peter et al., 1976). The effector cells responsible have been shown to be non-T cells and are most likely identical with the K cells. The spontaneous cytotoxicity generated when spleen cells were cultured in polyacrylamide rafts (Section 3.2) is entirely T cell-mediated (Table 1) and attempts to demonstrate activity in supernatants were all negative (Section 3.23).

iii) Control experiments for many studies on CMC have usually involved culturing responder or stimulator cells by themselves, or culturing responder cells with inactivated syngeneic cells. Several investigations have reported detectable levels of T cell-mediated cytotoxicity in the control cultures (Bevan et al., 1974; Röllinghoff et al., 1975; Skinner and Marbrook, 1976; Shustik et al., 1976), but usually no explanation for the levels of cytotoxicity in controls have been offered. The background levels of cytotoxicity in control experiments and the spontaneous cytotoxic response described in this thesis may be part of the same phenomenon. The 'inducer' of the spontaneous clones of CLs has not been established, but as discussed previously (Section 3.3), there may be mitogenic factors in the culture medium; or the spontaneous response may have resulted from the release of suppression due to the segregation of regulatory cells from the CL precursors under the conditions of limiting dilution in the polyacrylamide rafts.

iv) T cell responses have been generated in vitro by culturing murine spleen cells with syngeneic cells of a different tissue type
(Cohen and Wekerle, 1973; Warnatz et al., 1975) or age (von Boehmer et al., 1972). This phenomenon has been termed 'autosensitization' and is postulated as representing an 'escape' in vitro from the in vivo tolerant state. In the experiments described in Section 4.22, spontaneous CLs which lysed syngeneic target cells were detected. These spontaneous 'anti-self' CLs may bear some relation to the 'auto-
sensitized' CLs reported by other workers. Whether the spontaneous anti-self CLs play any role in in vivo autoimmune reactions has not been examined. NZB mice have a genetic disorder and develop a high incidence of autoimmunity (Howie and Helyer, 1968). It is of interest to note the spontaneous response in cultures of cells of NZB mice is no different from that observed in other strains of mice (Dr Nawa and Dr Marbrook, personal communications).

It has been shown that spontaneous CLs of different specificities were produced and the individual clones of CLs exhibited a high degree of discrimination (Table 4). Results from the specificity analyses indicated that spontaneous CLs could discriminate between subsets of syngeneic lymphoid cells which might be expected to differ only with respect to their differentiation antigens (Table 5). From a consideration of the data, it was concluded that the specificity of spontaneous CLs was not directed solely against H2 antigens. Non-H2 determinants must be involved in the 'structure' recognized by spontaneous CLs, but whether the recognition of these non-H2 determinants by spontaneous CLs also involved H2 products, as in the recognition of viral, haptenic or minor H antigens by stimulated CLs has not been established. This might be determined from whether or not antisera against H2 specificities on the target cells will block lysis by spontaneous CLs. Alternatively, it might be determined from
whether or not target cells from congenic strains of mice were lysed by the same spontaneous CLs. Cross-reactivity in the lysis of target cells from mice which were congenic might indicate that the non-H2 antigens were recognized without the involvement of MHC products.

The specificities of the spontaneous CLs do not appear to be representative of the specificities produced in a stimulated allogeneic response (Fig. 19). The implications of this on the possible existence of subsets of CLs with a differing responsiveness to polyclonal stimuli was discussed in Section 6.3. Experiments to determine the significance of the spontaneous CLs within the total CL pool would be a major consideration in the continuation of the work on spontaneous CLs. Their high discriminatory ability, and perhaps their difference in stimulatory requirements from the CLs produced in stimulated cultures might be used as a means for determining the possible role of the spontaneous CLs in the immune system.

One possibility might be that the spontaneous CLs are a subset of suppressor cells. The in vitro spontaneous generation of suppressor cell activity has been described (Burns et al., 1975; Hodes and Houghton, 1976). It was suggested by Shustik and co-workers (1976) that spontaneous CL activity and spontaneous suppressor cell activity might be different in vitro measurements of the same biological function, and reflect the dual function of a single subclass of lymphocytes. Other workers have also suggested that suppression of effector cell activity might be due to the killing of the effector cells by a population of suppressor-CLs (Goldstein, 1974; Dunlop and Blanden, 1977b). In order that such suppressor-CLs kill only the appropriate effector cells and not other cells in the population, they would be required to possess a fine ability to discriminate between subsets of syngeneic cells such as that observed for spontaneous
CLs. However, if spontaneous CLs do have a suppressor function, it might be expected that the specificity of the CLs was directed against antigens unique to the various subsets of effector lymphocytes. Although it has been demonstrated that the spontaneous CLs are able to discriminate between subsets of syngeneic cells (Table 5), the exact antigens recognized by the spontaneous CLs have yet to be determined.

Other experiments in determining the relationship of the spontaneous CLs to suppressor cells might involve the examination of the spontaneous response in cultures of cells from mice which have been thymectomized or treated with anti-thymocyte serum. It has been observed that adult thymectomy (Feldmann and Konttinen, 1976) or treatment with anti-thymocyte serum (Cantor and Simpson, 1975) alters the concentration of suppressor cells in the population of lymphoid cells. It would be of interest to examine whether such treatments have any effect on the generation of spontaneous CLs by the cells in culture.

T cells have been divided into subsets according to variety of criteria. Currently, one of the most effective techniques for identifying and separating distinct subsets of T cells has come from studies of cell-surface antigens expressed on T cell subsets (Cantor and Boyse, 1976). Using this approach it has been shown that the various T cell activities are specialized functions of distinct subclasses of T cells (Shiku et al., 1975). The cells in each of the subsets are committed before they have encountered antigen, to perform a particular function (Cantor and Boyse, 1975a). The Ly phenotype of functional subsets of T cells has been characterized, and it has been shown that suppressor cells and CLs both belong to the subclass of T cells with the ly2+3+ phenotype (Cantor and Boyse, 1976). Characterization of their Ly phenotype might be informative in
establishing the possible function of spontaneous CLs.

The cells within a particular functional subclass can be further divided into subsets according to their state of differentiation along that particular lineage. For example, the subclass of T cells which mediate CMC could be divided into precursor cells, primary CLs, non-lytic memory cells or secondary CLs. If the spontaneous CLs belong to the lineage of T cells committed to mediate CMC functions, a possibility might be that they are derived from the pool of memory CLs. Although the spleens were taken from normal non-immunized mice, they might be expected to contain memory cells from responses to environmental antigens. In the case of humoral responses, there is a progressive change in the affinity of the antibodies produced during the course of the response (Eisen and Siskind, 1964), while Cunningham (Cunningham and Pilarski, 1974) has proposed that entirely new antibody specificites are produced during a response. Although similar findings have not been demonstrated for T cell responses, it is conceivable that the range of specificities in the CLs which are recruited into the 'memory' cell pool might differ from the specificities found in the pool of virgin CL.P. Röllinghoff and Wagner (1975) have shown that the stimulatory requirements for the induction of a primary and secondary CL response are different, and differences both in the specificity and the response to stimuli of the spontaneous CLs have already been discussed (Sections 5.3 and 6.3).

Preliminary investigations have been carried out by Dr Marbrook and Dr Skinner to isolate subpopulations of CLs using buoyant density fractionations. A comparison of the properties of the spontaneous CLs with other known subsets of T cells might represent a first step towards establishing the significance of the spontaneous CLs within the population of T cells.
8.2 STIMULATED CLONES OF ALLO-REACTIVE CYTOTOXIC LYMPHOCYTES

The use of cyclophosphamide-treated stimulator cells to generate one-way CL responses, which enabled the specificity of stimulated clones of CLs to be analysed in the absence of a response from stimulator cells was described in Section 6.2.

In contrast with the spontaneous CLs, the results in Figs 17 and 18 indicated that H2d stimulated CLs do not discriminate between different H2d target cells. This might suggest that the specificity of the CLs produced in response to allogeneic cells are directed solely against H2 determinants. However, there have been proposals which suggest that even in anti-H2 responses, H2 antigens are not recognized in isolation, but in combination with other membrane components (Matzinger and Bevan, 1977). If this is so, the results of Figs 17 and 18 would indicate that P815, DBA/2 and (CBA x DBA/2)F1 spleen cell blasts have many non-H2 membrane components in common, as the cells seemed to be lysed by the same population of CLs.

The mitogen, ConA, has been used to induce polyclonal CL responses (Bevan et al., 1976; Waterfield and Waterfield, 1976). The specificities of the CLs induced by ConA are directed against H2 coded antigens, and it is considered that the ConA-activated CLs are indistinguishable from the CLs activated by alloantigens. Using the experimental system described in this thesis, it would be of interest to examine whether the discriminatory ability of ConA-activated CLs is identical to that of allo-H2 activated CLs. Alternatively, as the ConA-induced CLs are also produced polyclonally in the absence of specific selection by antigen, they may bear similarities to the spontaneous CLs.

Although the H2d stimulated CLs did not discriminate between different H2d target cells, simultaneous stimulation with H2d and H2b
alloantigens resulted in the production of two separate populations of CLs each directed against one of the haplotypes (Fig. 16). The results would be consistent with the hypothesis that CL.P are monopotent and committed in specificity (Bevan, 1975a).

Few cross-reactive clones of CLs which lysed H2b and H2d targets were produced by CBA responder cells. Most studies on the specificity of alloreactive CLs have indicated that there is very little or no cross-reactivity in the response to different haplotypes. The CLs destroyed only the target cells of the same H2 haplotype as the stimulator cells and lysis of cells of a different haplotype was not observed (Cerottini and Brunner, 1974). According to serological methods for the mapping of H2 specificities, the various H2 haplotypes have some specificities which are shared by other haplotypes (Klein, 1974). Even cells which were H2 different but shared many SD specificities with the stimulator cells were not recognized or destroyed by allosensitized CLs (Brondz and Snegirova, 1971; Blomgren and Andersson, 1974). On the other hand, Peavy and Pierce (1975a) and Lindahl and Wilson (1977) have reported lytic activity against unrelated third party targets in populations of specifically stimulated CLs. Whether the third party cells were lysed by cross-reacting CLs or heteroclitic CLs generated during a specific response, however, was not investigated. The experimental system used for the experiment in Section 6.22 would be an effective method for examining the extent of cross-reactivity, if any, in the CL recognition of various H2 haplotypes. As there is evidence which suggests that the specificity of CL recognition of H2 antigens is different from that of humoral responses, it would be of importance to establish the pattern of cross-reactivity in the CL recognition of the various haplotypes, and to compare it with the pattern of cross-reactivity determined by serological mapping.
of H2 specificities.

8.3 **TNP-SPECIFIC CLONES OF CYTOTOXIC LYMPHOCYTES**

Using the method of Shearer and co-workers (1975) for the modification of cells with TNP, the frequency and specificity of clones of CLs generated by F₁ spleen cells in response to TNP-modified stimulator cells were examined in experiments summarized in Section 7.2. The frequency of clones of CLs lysing F₁-TNP, CBA-TNP and C₅₇Bl-TNP target cells in (CBA x C₅₇Bl)F₁ spleen cell populations was measured (Fig. 21, Tables 9 and 10). The CLs lysing in the parental-TNP cells segregated as two populations with few cross-reacting clones (Table 11a). The data confirms, on a clonal basis, the conclusions of other workers using populations from mass cell cultures. From cold target inhibition experiments it was concluded that the activity against the two modified parental cells resided in separate populations of CLs (Shearer et al., 1975). With the use of intra-H2 recombinant strains of mice, the studies of Section 7.2 could be extended to show on a clonal basis, the separability of CL activity against TNP-H2K or TNP-H2D specificities, and thus, the existence of 4 main subsets of CLs in F₁ cell populations; those recognizing TNP in association with maternal or paternal K antigens, or maternal or paternal D antigens.

When (CBA x C₅₇Bl)F₁ spleen cells were cultured with parental CBA-TNP stimulator cells, clones of CLs lysing modified targets of both parental strains were detected (Section 7.24). This indicated that stimulation with CBA-TNP does not preferentially activate only anti-CBA-TNP clones in the F₁ cell population. As the same ratio of anti-CBA-TNP to anti-C₅₇Bl-TNP was obtained with either CBA-TNP or (CBA x C₅₇Bl)F₁-TNP as stimulators, this suggested that stimulation with
modified parental or F₁ cells will produce the same response in F₁ cell populations. However, this has not been rigorously established, and it would be of interest, in a continuation of the project, to measure the frequency of clones against F₁-TNP or parental-TNP targets which are produced in response to CBA-TNP or C₅₇BL-TNP stimulators. If the frequency of clones lysing the various targets using parental-TNP stimulators is similar to those obtained from the data in Fig. 21 using F₁-TNP stimulators, this would be consistent with the notion that parental or F₁-TNP stimulators activated the same set of CL.P in the F₁ responders.

The results of the experiment described in Section 7.24 however, are in contrast to those of Forman (1975) and Shearer and co-workers (1975). Their results indicated that stimulation of F₁ cells with TNP-modified cells of one of the parental strains, results in the generation of cytotoxicity against modified cells of the parental strain used for sensitization, and not against the modified cells of the other parental strain. The data was taken as indicating that the H2 restriction observed in TNP-specific CMC was determined by the H2 type of the stimulator cells. A possible explanation for the contrasting observations has been discussed (Section 7.3).

From the results of Section 7.24, it would seem that the specificity of the CL responses was determined by the H2 antigens of the F₁ cells rather than the H2 antigens of the stimulator cells. The observations would be compatible with those of Bevan (1976, 1977) on the role of H2 in the response to minor H antigens and those of Zinkernagel (1976) on anti-viral responses. Bevan (1976) found that cells with different minor H antigens but the same H2 type as one of the parents, will prime F₁ cells for a secondary response to the minor H antigens on cells of either parental H2 type. It was con-
cluded that priming was not restricted to the H2 type of the cells used for priming, but to the H2 types of the F1 host. Experiments using parental radiation chimaeras which had been reconstituted with F1 bone-marrow cells ((A x B)F1 → A recipient) also indicated that host H2 antigens influenced the specificity of the CLs (Bevan, 1977). The F1 cells which had differentiated in a parental chimaera, generated preferentially, CLs against minor H antigens in association with H2 type of the recipient. Normal F1 cells which had been similarly immunized produced CLs against the minor H antigens associated with either parental H2 types.

Zinkernagel (1976) observed that F1 chimaeras, reconstituted with bone-marrow cells from one of the parental strains, when infected with virus, will generate CLs against infected targets of both parental strains of the F1. The data indicated that the chimaeric host determined the H2 restricted specificity of the CLs. 'Adaptive differentiation', perhaps through a thymic selection process as proposed originally by Jerne (1971), was suggested by Bevan (1977) to explain the influence of the host on the specificity of T cell restriction. In support of the hypothesis, the results from thymus transplantation experiments by Zinkernagel and co-workers (1978), indicated that H2 specificity of the thymic epithelium determines what the differentiating T cells learn to recognize as self H2. This learning process was independent from the H2 type of the precursor T cells.

As an alternative explanation, it was suggested by Bevan (1977) that the preference for host haplotype seen at the level of effector CLs, did not reflect a bias in specificity at the level of CL.P, but was due to antigen presentation by host macrophages. The involvement of the host H2 antigens would be due to the recognition by CL.P of
'structures' on the macrophage determined in part by the MHC and in part by the processed antigen.

Antigen presentation by macrophages has been shown to be required for the activation of most T cell functions, for example, helper T cells involved in co-operating with B cells (Basten et al., 1975) and T cells mediating delayed hypersensitivity (Oppenheim and Seegar, 1976). MHC imposed constraints has been observed on T-B collaboration (Katz et al., 1973), transfer of delayed hypersensitivity (Miller et al., 1976) and stimulation of T cell proliferation by antigen pulsed macrophages (Shevach and Rosenthal, 1973). To explain the MHC constraints, it has been proposed that sensitization of the T cells is directed to cell surface structures on macrophages determined partly by the antigen and partly by the MHC antigens.

Although it has been shown that macrophages are essential for the induction of CL responses (Wagner et al., 1972), it has not been established whether CL.P activation requires antigen presentation by macrophages, or whether CL.P may be activated directly by the stimulator cells. If activation results from antigen presentation by macrophages, culturing an F₁ cell population with antigen would lead to the activation of two subsets of CLs, those recognizing antigen and H2 antigens of parent 1 and those recognizing antigen and H2 antigens of parent 2. On the other hand, if a population of F₁ cells, which have had their own macrophage removed, were cultured with antigen and macrophage from one of the parental strains, only CLs with specificities for the antigen associated with the H2 type of the macrophage used for sensitizing would be produced. It should be possible to adapt the polyacrylamide culture system for the examination of CL responses sensitized by different macrophage populations. Such studies would be useful in establishing the role of macrophages in
the generation of CL responses and their possible influence on the specificity of CLs which are activated.

8.4 THE CLONAL ANALYSIS OF THE SPECIFICITY OF CYTOTOXIC LYMPHOCYTES

The polyacrylamide vessels were used throughout the work described in this thesis for the generation of CL responses. The culture system is essentially one of limiting dilutions. The cell population becomes segregated in the dimples. When the dilution of responder cell cultured is such that the number of positive dimples is low and the positive dimples distributed randomly in the culture, it can be assumed that the activity contained in a dimple is due to a single clone of effector cells. By assaying individual dimples of cells, the activity of single clones can be examined. The segregation of clones permits also, the activity of clones of effectors to be assayed without the diluting effect of the rest of the population. This additional advantage of the clonal assay enables 'activities' which occur at relatively low frequencies to be detected definitively.

The system has been used for the analysis of the specificity of single clones of CLs. This was carried out by dividing each dimple of cells against a pair of different targets. Various pairs of target cells were used in different experiments. Cell-mediated lysis of a target cell depends on the recognition of specific antigens on the target cell surface and the susceptibility of the cells to be lysed. When comparing the activity of a CL population on different targets, it has been assumed in the work in this thesis that the difference in the activity observed against two targets reflect differences in the antigens on the cells only. It has not been, as part of this project, to examine the susceptibility of the various target cells to lysis, but a method for testing this might involve
measuring the degree of non-specific lysis of the various targets. The addition of ConA or PHA to the assay system can cause the agglutination of CLs to target cell and lead to non-specific lysis of the target cells (Bevan and Cohn, 1975). The 'artificial' agglutination of CL to target by ConA or PHA is considered to by-pass the binding brought about by the specific interaction of the receptor on CLs with surface target cell antigens. The degree of non-specific lysis of the various target cells might be taken as a measure of the intrinsic susceptibility to lysis of the cells.

Previous methods for investigating the clonal distribution of the cytotoxic T cell receptors have involved pre-absorption of the CL population on macrophage of fibroblast monolayers of different strains, followed by the assay of the cytotoxicity of the residual non-absorbed cells (Brondz and Snegirøva, 1971), or the use of inhibition assays using cold target competitor cells. Both these methods have severe limitations. The pre-adsorption technique is restricted to target cell types which will form monolayers, and as non-specific inhibition from steric hindrance can occur, competition studies need to include appropriate controls with adequate dose response data. The clonal analysis of the specificity of CLs described in this thesis is a much simpler method and has proved very effective in analysing such responses. In each experiment with a pair of targets, the responding CLs in the population can be divided into three subsets according to their specificity. It is possible to determine from the results whether activity against two targets resides in the same or separate populations of CLs. The experiments in this thesis have demonstrated the potential of the method for examining the basis of cross-reactivity in CL responses.

It has been suggested that CLs are responsible for immunological
surveillance and capable of detecting modifications of self-antigens on the membrane. The MHC molecules were evolved as the appropriate targets for this purpose. Alloreactive cells were thus, the inevitable but unwanted consequence of CL surveillance against altered self. Considering the specificity of CL responses in this light, it was proposed by Lemonier and co-workers (1977) that extensive cross-reactivity should be observed among CLs specific for modified self and allogeneic targets, and CLs raised against allogeneic cells should exhibit cross-reactive lysis of modified syngeneic targets.

Extensive cross-reactivity also among CLs activated by alloantigens has been suggested by Matzinger and Bevan (1977). To explain the high frequency of cell which will respond to alloantigens, it was proposed, that by the formation of combined antigens composed of an H2 product and some other membrane component, cells of a foreign H2 type present a multitude of antigenic specificities. This hypothesis predicts as a consequence, that many of the CLs activated in response to a particular foreign H2 haplotype will exhibit cross-reactivity for target cells of other H2 types. Most studies on the specificity of alloreactive CLs however have not detected cross-reactivity between different haplotypes (Cerottini and Brunner, 1974). In the few reports where lysis of third party targets in a specifically stimulated response was detected, the basis for the cross-reactive lysis, whether it was due to cross-reacting antigens on the targets being recognized by the same population of CLs, or the presence of heteroclitic CLs generated during the specific response was not examined.

From a consideration of some of these current hypotheses on the specificity of CLs, it is evident that more studies examining the degree of discrimination of cross-reactivity of CL responses is
necessary in extending our understanding the nature of CL receptors and antigen recognition by CLs. The potential of the clonal analysis of the specificity of CLs as a direct and efficient method for determining the basis for cross-reactivity in CL responses has been demonstrated in the work described in this thesis.

The use of the polyacrylamide culture system for the segregation of single clones enabled the detection of the low numbers of spontaneous clones of CLs which are produced by spleen cell populations. A comparison of the characteristics and specificity of spontaneous CLs with stimulated CLs has provided additional information on the heterogeneity of CL populations.

In studies of CL specificity by other workers, the results from absorption or cold target competition experiments using mass cell cultures have been interpreted in terms of clones, even though individual clones of CLs had not been examined. The experiments described in this thesis have confirmed, on a clonal basis, some of the conclusions on CL specificity which have been inferred from the analysis of whole populations of cells. The experiments described in this thesis, using the clonal analysis of CL specificity have provided also, additional information on the ability of individual clones of CLs to discriminate between target cells.

Although the experiments described in this thesis, do not in themselves, resolve some of the major issues in CL recognition, such as the one receptor or two receptor hypothesis, they have demonstrated the potential of the clonal assay of CL specificity for use in such analyses on CL recognition.
REFERENCES


* Denotes references obtained from the literature and not checked from original article by author.
APPENDIX

PUBLICATIONS PERTINENT TO THE INVESTIGATIONS REPORTED IN THIS THESIS

   Spontaneous clones of cytotoxic T cells in culture. I

   Spontaneous clones of cytotoxic T cells in culture. II.

   Spontaneous clones of cytotoxic T cells in culture. III.
   Discriminatory lysis of pairs of syngeneic blasts induced

   Clones of cytotoxic lymphocytes in culture: The difference
   in specificity between stimulated and non-stimulated

CLONES OF CYTOTOXIC LYMPHOCYTES IN CULTURE: THE DIFFERENCE IN SPECIFICITY BETWEEN STIMULATED AND NON-STIMULATED CYTOTOXIC LYMPHOCYTES.

By Lai-Ming Ching, Karen Zell Walker and John Marbrook.

Cell Biology Department,
University of Auckland,
New Zealand.

Running title: Specificity of Stimulated and Spontaneous Cytotoxic Lymphocytes.

Communications to: J. Marbrook,

Cell Biology Department,
University of Auckland,
Private Bag, Auckland,
New Zealand.

Abbreviations:

CLs : cytotoxic lymphocytes
PCS : fetal calf serum
ip. : intraperitoneal
MHC : major histocompatibility complex
PHA : phytohemagglutinin
SUMMARY

The specificity of individual clones of CLs which develop upon stimulation by semi-allogeneic cells have been examined and compared with the specificity of CL clones which develop spontaneously when normal spleen cells are cultured without stimulator cells. It was found that the specificity of stimulated clones was different from the specificity of 'spontaneous' clones. Specifically stimulated clones from CBA cells cultured with (CBA x DBA)$_1^1$ stimulator cells, did not discriminate between the H2$^d$ P815 and H2$^d$ DBA splenic blasts, and lysed both targets. In contrast, spontaneous CL clones from cultures of CBA or (CBA x DBA)$_1^1$ spleen cells were found to lyse either P815 or DBA blasts, but not both. The results indicated that the spontaneous CL clones were not a representative sample of the total pool of CLs.
INTRODUCTION

Cytotoxic lymphocytes which are produced during a cell-mediated immune response will lyse specifically the appropriate target cells which bear antigens identical to those on the stimulating cells (1). CLs appear to be monospecific and bear receptors which are clonally restricted and which recognise specificities determined at least in part by the K and D loci of the MHC (2). Whether stimulated by allogeneic cells (3-5) or polyclonal activators (6,7), as many as 1-4 per cent of all potential CL precursors can be activated to produce CLs of a single specificity.

The measurement of the specificity of a CL response has been made more amenable to detailed analysis by the ability to segregate individual clones of CLs in culture (3). Using this technique, it has been found that spleen cells cultured alone will generate a small number of definite clones of CLs in the absence of stimulator cells. A comparison of the specificity of individual 'spontaneous' and 'stimulated' clones has been carried out by testing the ability of both types of clones to discriminate between P815 (H2<sup>d</sup>) and splenic (H2<sup>d</sup>) blast cells.

It has been established that, by this criteria, the specificity of spontaneous clones is quite different from the specificity of stimulated clones. One of the interpretations of these data is that the spontaneous clones are not a representative sample of the total pool of precursors of CLs which are of an anti-H2<sup>d</sup> specificity. The proposal that the spontaneous clones are derived from a discrete subset of precursors is discussed.

MATERIALS AND METHODS

Mice

Mice used were 12 weeks old CBA/J (H2<sup>k</sup>), DBA/2 (H2<sup>d</sup>) and (CBA x DBA)<sub>F1</sub> males.
Cyclophosphamide Treatment

Solutions of cyclophosphamide (Endoxan Asta, kindly supplied by Bristol Myers Co. Pty Ltd, Auckland, N.Z.) were made directly before use. Mice were injected ip. with 25-100 mg/kg cyclophosphamide in 0.3 ml saline. Spleens were removed 5 or 20 hr after injection.

Target Cells

P815 (H2d) tumor cells and DBA splenic blasts induced with PHA were used as targets for the chromium release assay. They were grown and labelled with sodium 51chromate as described previously (9).

Cytotoxicity Assay

Individual dimples of cells were taken into 0.3 ml medium (NMP1640 with 10% heat inactivated FCS) in 76 x 11 mm disposable tubes, and incubated with 51Cr-labelled target cells (10^4 cells per tube) as previously described (8). 32 dimples were harvested from each raft and 2-5 duplicate rafts were assayed in each experiment.

When individual clones were to be assayed against two different targets, each dimple of cells were removed into 0.6 ml medium. After gentle pipetting to disperse clumps, 0.3 ml of the cell suspension were removed into a second tube. 51Cr-labelled DBA splenic blasts (5 x 10^3 cells/tube) were added to one aliquot and 51Cr-labelled P815 targets (5 x 10^3 cells/tube) were added to the other aliquot. 51Cr release was then assayed in the normal manner.

Results have been expressed as per cent specific lysis,

\[
\text{Results} = \left( \frac{\text{experimental } 51\text{Cr release - spontaneous release}}{\text{maximum } 51\text{Cr release - spontaneous release}} \right) \times 100
\]

Spontaneous release was estimated by incubating the target cells alone, while maximum release was obtained after 3 cycles of freezing and thawing. The spontaneous release from P815 targets was 10-20% and 17-36% of the maximum release from DBA blast targets.
The polyacrylamide culture system, statistical treatment of results and other materials and methods were as described elsewhere (3,8,9).

**RESULTS**

**Effect of Cyclophosphamide on the Spontaneous Generation of CL Clones**

When normal CBA and (CBA x DBA)F<sub>1</sub> spleen cells are mixed and cultured in polyacrylamide vessels, clones of CLs can arise from both populations. Specifically stimulated clones are generated from CBA spleen cells and smaller numbers of 'spontaneous' clones arise from the F<sub>1</sub> cells. The spontaneous clones were eliminated by using cells from cyclophosphamide-treated animals.

(CBA x DBA)F<sub>1</sub> mice were injected ip. with 0-200 mg/kg cyclophosphamide and the spleen removed 20 hours later. Spleen cells were cultured at a concentration of 1.3 x 10<sup>7</sup> cells per raft for 5 days. The cells from individual dimples were removed and assayed for cytotoxicity against P815 mastocytoma cells. As seen in the results summarized in Fig. 1 the spontaneous clones were completely abolished in cultures of cells from animals which had received 200 mg/kg cyclophosphamide.

- Fig. 1 -

**The Ability of Cyclophosphamide Treated Cells to Stimulate**

The ability of cells from animals treated with 200 mg/kg cyclophosphamide to stimulate CL production in culture was examined. Spleen cells from (CBA x DBA)F<sub>1</sub> mice which had been treated 5 hrs previously with 200 mg/kg cyclophosphamide were cultured with graded numbers of CBA spleen cells in polyacrylamide rafts. The cultures were assayed on day 5 for clones of CLs against P815 targets. The number of clones increased in relation to the numbers of CBA cells, and no clones were detected when cells from cyclophosphamide treated F<sub>1</sub> mice were cultured alone (Table 1).

- Table 1 -
Although the production of spontaneous clones had been abolished, spleen cells from mice treated with 200 mg/kg cyclophosphamide still functioned as stimulators. This provided a system where specifically stimulated clones could be analysed in the absence of spontaneous clones from the stimulator cells.

**Specificity of Stimulated Clones**

CBA spleen cells were cultured with stimulator cells from cyclophosphamide treated (CBA x DBA)F₁ mice and the specificity of the individual clones of CLs which developed after 5 days of culture was analysed. Individual dimples of cells were divided into two aliquots. One aliquot was assayed for cytotoxicity against P815 cells and the other aliquot was assayed against DNA splenic blast cells. The percent specific lysis against P815 has been plotted against the percent specific lysis of DBA blasts for individual dimples with positive levels of cytotoxicity (Fig. 2).

- Fig. 2 -

Most of the positive dimples (89.2%) contained cytotoxicity against both types of target cells. Although 52.2% of the positive dimples might be expected to contain cytotoxicity against both targets due to coincidence, the results differ significantly from that which would be expected if the targets were lysed independently (p<0.001).

The results indicated that the majority of clones of CLs arising after stimulation by H₂d alloantigens did not discriminate between the H₂d P815 and H₂d splenic blasts as targets.

**Specificity of Spontaneous Clones**

The specificity of spontaneous clones of CLs were similarly examined. Normal spleen cells from CBA or (CBA x DBA)F₁ mice were cultured in polyacrylamide rafts and spontaneous clones of CLs were assayed at the peak of the response on day 4. Individual dimples were split, one aliquot assayed with P815 targets and the other aliquot assayed with DBA splenic
blasts. The specificity of positive dimples from CBA cultures is shown in Fig. 3.

- Fig. 3 -

Of the positive dimples, the number which were specific for P815 targets (88.9%) was much higher than those specific for DBA blasts (11.1%) but none contained cytotoxicity against both targets.

The specificity of positive dimples from cultures of spleen cells from (CBA x DBA)F1 mice is shown in Fig. 4.

- Fig. 4 -

Of the positive dimples, 65% were specific for P815 cells and 28% were specific for DBA blasts. The 7% which contained cytotoxicity against both the targets can be attributed to coincidence of two clones of different specificities in the same dimple which was calculated to be 8.1% for that experiment.

The results in Fig. 3 and Fig. 4 indicated that spontaneous clones of CLs lysed either one or the other target cell, but not both, in contrast to the stimulated clones of Fig. 2.

DISCUSSION

The characteristics and specificities of the 'spontaneous' generation of CLs have been described elsewhere (8,9). The individual clones in such a response appear to be able to discriminate between many pairs of syngeneic targets (9). The frequency of spontaneous clones is approximately three hundred-fold lower than the frequency of clones in a stimulated culture, and the aim of this work was to examine the specificity of the anti-H2d clones in a stimulated response compared to spontaneous clones against the same targets, to determine whether the range of specificities were identical in the two types of responses.

The method used was to determine whether individual clones could discriminate between two target cells, both bearing H2 antigens of the
same haplotype. The particular pair of targets selected for this analysis were the DBA mastocytoma P815 cells and PHA-induced splenic blasts from DBA mice. The spontaneous clones were detected when a population of spleen cells were cultured alone, and stimulated clones were detected when CBA cells were cultured with (CBA x DBA)F1 cells. The spontaneous clones from the F1 stimulators were eliminated by using spleen cells from animals treated with 200 mg/kg cyclophosphamide. It has been demonstrated that 200 mg/kg is a dose of cyclophosphamide which eliminates the production of spontaneous clones (Fig. 1) without markedly impairing their ability to stimulate semi-allogeneic cells (Table 1).

When the individual clones were divided into two aliquots and each half assayed for cytotoxic activity against one of the two targets, the two responses appeared to produce clones of different specificity. Spontaneous clones were generally smaller and could discriminate between the two targets (Fig. 3 and Fig. 4) whereas stimulated clones lysed both targets with similar facility (Fig. 2). Although the specificity of the clones in the two responses appear to differ, it is not known whether the discriminatory, spontaneous-type of clones are not produced in a stimulated culture. The frequency of clones detected against P815 or DBA blasts in the spontaneous response is several hundred-fold lower than the frequency of clones detected in a stimulated response. If the discriminatory clones occur with the same frequency in both types of responses, they would be effectively diluted out by the high number of cross-reactive clones in stimulated cultures.

In the absence of any apparently cross-reactive spontaneous clones, it must be concluded that the clones which are produced spontaneously are not a representative sample of the total pool of CL precursors which are generated in a stimulated culture. This conclusion has further implications in considering the nature of the spontaneous response.

The factors which contribute to the generation of spontaneous
clones have yet to be established, but it is unlikely that the spontaneous
CLs have resulted from self stimulation, as anti-allogeneic clones can also
be found. There were clones, for example, produced from (CBA x DBA)F1 cells
which could discriminate between C57Bl blasts and EL-4 tumor cells (9).

There have been reports of responses to components of fetal calf
serum which adhere to the surfaces of target and stimulator cells (10).
The response against such a ubiquitous antigen would result in a loss of
the ability to discriminate between pairs of similar targets. This is
contrary to our findings (9).

Another explanation for the spontaneous response is that the culture
medium contains a mitogenic factor in, for example, fetal calf serum. If
this is so, the spontaneous response could be regarded as a polyclonal
type of response. With a polyclonal type of stimulator and the consequent
lack of selection of precursors on the basis of affinity, it might be
expected that the spontaneous response would be a representative sample of
the range of specificites detected in a stimulated response.

The data in Figs 2, 3, 4 indicate that spontaneous clones are
not a representative sample of the total anti-H2d response. There are
reasons however why this may be so without eliminating polyclonal activation
as the source of spontaneous clone generation. According to a variety of
criteria, the CL precursors consist of subsets of cells. They are hetero-
geneous with respect to size (11), density (12), stimulatory requirements
(13) and Ly phenotype (14). Similarly lymphoid cells are heterogeneous in
their response to the polyclonal activation by mitogens (15-17). If these
subsets do not all have an identical repertoire, then the non-specific
activation of discrete subsets would lead to the non-representative sample
of specificities such as that found with spontaneous clones.
Finally it should be mentioned that the clonal analysis is essentially a limiting dilution culture system, and this has further implications. Not only are CL precursors being diluted out but also other populations such as specific suppressor cells, the possible presence of which has yet to be determined. The appropriate dilution of suppressor cells could have the effect of altering the specificity range of the clones, although attempts to demonstrate this effect have been negative.

In conclusion, it has been demonstrated that the specificity of spontaneous clones is not identical to the specificity of clones of CLs in stimulated cultures. The implications of these findings have been discussed. From a consideration of the frequency of clones and other factors, it has been concluded that it is necessary to apply the clonal analysis of the specificity of subsets of T cells to determine the significance of the spontaneous clones within the total responding population. This is at present under investigation.

REFERENCES


Acknowledgements

We wish to thank Ms T. Schaafl and Ms D. Thompson for excellent technical assistance.

This work was supported by a grant from the Medical Research Council of New Zealand.
Table 1

The ability of cyclophosphamide treated cells to stimulate a)

<table>
<thead>
<tr>
<th>Cells per culture</th>
<th>mean clones per raft ± standard error</th>
</tr>
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<tbody>
<tr>
<td>$0.5 \times 10^5$ CBA + $1.3 \times 10^7$ F$_1$ (CY)</td>
<td>10.7 ± 6.5</td>
</tr>
<tr>
<td>$1.0 \times 10^5$ CBA + $1.3 \times 10^7$ F$_1$ (CY)</td>
<td>20.0 ± 1.5</td>
</tr>
<tr>
<td>$1.5 \times 10^5$ CBA + $1.3 \times 10^7$ F$_1$ (CY)</td>
<td>43.1 ± 2.4</td>
</tr>
<tr>
<td>$1.3 \times 10^7$ F$_1$ (CY)</td>
<td>0.4 ± 0.3</td>
</tr>
</tbody>
</table>

a) CBA spleen cells were cultured in polyacrylamide rats with spleen cells from (CBA x DBA)$_F$ mice which had been injected ip. 5 hours before with 200 mg/kg cyclophosphamide. Cultures were assayed on day 5 for CL clones against P815 targets.
Figure 1

Effect of cyclophosphamide on the generation of spontaneous clones of CLs in culture. (CBA × DBA) F₁ mice were injected ip. with 0-200 mg/kg cyclophosphamide and the spleens removed 20 hr later. 1.3 × 10⁷ F₁ cells were cultured in polyacrylamide rafts and assayed for CL clones against P815 targets on day 5. Two cultures were assayed for each dose of cyclophosphamide.

- - - - - - mean clones per spleen

- - - - - - - mean clones per culture
Figure 2

Specificity of stimulated clones of CLs. CBA spleen cells \(4 \times 10^5\) were cultured in polyacrylamide rafts with \(1.3 \times 10^7\) spleen cells from (CBA x DBA)\(F_1\) mice which had been treated with 200 mg/kg of cyclophosphamide for 20 hours. On day 5, individual dimples were divided into 2 aliquots. One aliquot was assayed for cytotoxicity against P815 targets, while the other was assayed against DBA PHA-induced splenic blasts. The percent specific lysis of P815 targets has been plotted against the percent specific lysis of DBA blasts for positive dimples. Dimples with less than 10% specific lysis against both targets have been omitted.
SPONTANEOUS CLONES OF CYTOTOXIC T CELLS IN CULTURE.
III DISCRIMINATORY LYSIS OF PAIRS OF SYNGENEIC BLASTS
INDUCED BY DIFFERENT MITOGENS.

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Running title: Discriminatory Lysis of Syngeneic Blasts
Induced by Different Mitogens.

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New Zealand.

Abbreviations:
CLs : cytotoxic lymphocytes
ConA : concanavalin A
DS : dextran sulphate
FCS : fetal calf serum
LPS : lipopolysaccharide
PHA : phytohemaglutinin
Summary

When normal spleen cells are cultured for 4 days in polyacrylamide vessels, individual clones of cytotoxic lymphocytes can be detected. The specificity of these 'spontaneous' CLs was investigated by assaying the cytotoxic activity of cells from individual clones against pairs of different targets. Target cells used were syngeneic blast cells induced by dextran sulphate, LPS, ConA and PHA. It was found that LPS blasts were lysed by a separate set of CL clones from those which lysed PHA blasts of the same H2 haplotype, and the clones of CLs which lysed PHA blasts were a subset of all the clones which lysed ConA blasts. When individual clones of spontaneous CLs were assayed against LPS and DS blasts, there were clones which lysed both types of blasts as well as clones which were specific for either LPS or DS blasts. These results have been interpreted as demonstrating that spontaneous CLs can recognize and kill subsets of cells which are stimulated by different mitogens.
Introduction

We have described previously the generation of clones of CLs in a polyacrylamide culture vessel (1). The use of the culture vessel allows the segregation of precursors of the effector cells so that individual clones of CLs may be assayed after a period in culture. When normal spleen cells were cultured without stimulator cells, small numbers of 'spontaneous' clones of CLs were generated (1,2). In experiments to determine the specificity of the spontaneous T cell-derived CLs, it was found that individual clones were able to discriminate between pairs of syngeneic target cells. For example, spontaneous clones of CLs from cultures of (CBA x DBA)\textsubscript{F}_1 spleen cells would lyse either F\textsubscript{1} blasts induced with PHA or F\textsubscript{1} blasts induced with LPS (3). These data suggested that such syngeneic killing may be recognizing antigenic determinants which are unique to such targets, and that differentiation antigens may be involved.

In view of the general observations that subsets of lymphoid cells respond differently to mitogens (4-7), the specificity of the spontaneous CLs has been examined in more detail and the ability of individual clones to discriminate between different blasts has been investigated.

PHA, ConA, LPS and DS have been selected as mitogens to produce targets. The degree to which spontaneous CLs can discriminate between the different targets has been interpreted as demonstrating the recognition of subsets of B and T cells to varying degrees. Just as mitogens have been used to define discrete subsets of lymphoid cells (4-7), the spontaneous clones appear to recognize the uniqueness of similar subsets.

Materials and Methods

Mice

Mice used were CBA/J, DBA/2 and (CBA x DBA)\textsubscript{F}_1 12 week old males.

Culture System

Spleen cells were prepared and cultured in polyacrylamide rafts as described previously (1,2). Each raft contains 64 small depressions or 'dimples' into which the cells settled.
randomly.

The culture medium used was RPMI (Gibco, Grand Island, N.Y.) supplemented with 10% FCS (Australian Laboratory Services). The culture medium also contained antibiotics (penicillin, 50 i.u./ml; streptomycin, 50 μg/ml) and 2-mercaptoethanol at a final concentration of 5 x 10^{-5} M.

Mitogens

Mitogens used were LPS (Difco, U.S.A.), PHA (Wellcome Lab., England), ConA in a glucose solution (Calbiochem, U.S.A.) and dextran sulphate obtained as a gift from Dr J. Watson.

Target Cells

Blast cells for use as targets in the cytotoxicity assay were prepared by culturing spleen cells in the presence of mitogen in Marbrook culture vessels (4 x 10^6 cells in 1 ml per culture).

The B cell mitogens, DS (40 μg per culture) or LPS (15 μg per culture) were added at the beginning of culture and the cells used after 3 days.

With T cell mitogens, cells were cultured for 2 days, then ConA (5 μg per culture) or PHA (final conc. 5%) was added and the cells cultured for a further 3 days.

Assay of CL Clones

Individual dimples of cells were removed into plastic disposable tubes containing 0.6 ml medium (RPMI with 10% heat inactivated FCS). The cells were pipetted gently to disperse clumps and the dimple of cells was then divided into two aliquots by removing 0.3 ml of the cell suspension into another tube. Each alternate dimple was harvested (32 dimples per raft) and 4 replicate rafts were assayed in each experiment.

Cytotoxicity was measured using the ^{51}Cr release assay after Brunner and co-workers (8). ^{51}Cr-labelled target cells (5 x 10^3 cells per tube) were added and treated as described previously (3).
Results were expressed as percent specific lysis

\[
\frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous release}} \times 100
\]

Spontaneous release was estimated by incubating target cells alone, while maximum release was obtained after 3 cycles of freezing and thawing. The spontaneous release as a percentage of the maximum release for the various blasts were as follows:

PHA 16-35%; ConA 24-32%; LPS 31-42%; DS 34-36%.

**Statistical Treatment of Results**

Aliquots of cells assayed against a particular target were considered to contain cytotoxicity if they yielded more than 10% specific lysis (1). In each experiment dimples were assigned to 4 categories according to the following criteria:

- cytotoxic for target 1 (>10% lysis of target 1, <10% lysis of target 2)
- cytotoxic for target 2 (<10% lysis of target 1, >10% lysis of target 2).
- cytotoxic for targets 1 + 2 (>10% lysis of target 1, >10% lysis of target 2)
- not cytotoxic for either target (<10% lysis of target 1, <10% lysis of target 2)

The observed frequencies were distributed in a 2 x 2 contingency table and compared by $\chi^2$ test with frequencies expected if lysis of target 1 occurred independently from the lysis of target 2. The observed frequencies from experiments where aliquots of the same dimple were assayed against two different target cells were also compared by $\chi^2$ test with the frequencies obtained in control experiments where the aliquots had been assayed against the same target cell.

The predicted frequency of dimples containing cytotoxicity against both targets due to coincidence of 2 or more clones in the same dimple was also calculated from the observed frequencies.
distributed in a 2 x 2 contingency table. The high percentage of negative dimples in most rafts assayed served to minimize the occurrence of apparent cross-reactivity due to the coincidence of more than one clone in the same dimple.

Results

Spontaneous clones of CLs were generated by culturing 1.3 \times 10^7 spleen cells in polyacrylamide vessels. On day 4, the peak of the response (2), individual clones were examined for their ability to discriminate between two different target cells. The cells from each dimple were removed and divided into 2 aliquots. An aliquot was assayed for cytotoxicity against splenic blast cells induced by one type of mitogen, and the other aliquot was assayed against splenic blasts of the same H2 haplotype which had been induced by another mitogen.

a) Lysis of LPS and PHA blasts

When spontaneous clones were divided and assayed against syngeneic blasts induced by PHA or LPS, the majority of the reactive clones lysed either one or the other of the two targets. As shown in Table 1, when spontaneous clones from cultures of CBA cells were assayed against CBA (PHA) or CBA (LPS) blasts, 35% of the responding clones were specific for the LPS blasts and 65% were specific for the PHA blasts. No clones were detected which lysed both the targets. When spontaneous clones from (CBA x DBA)F_1 cultures were assayed for their ability to lyse CBA(PHA) or CBA(LPS) blasts, 38% lysed LPS blasts, 56% lysed the PHA blasts, and 6% lysed both targets. A similar distribution was obtained when spontaneous clones from F_1 cells were split and assayed against F_1 (PHA) or F_1 (LPS) blasts, 30% lysed LPS blasts, 63% lysed PHA blasts and 7% lysed both. The dimples with activity against both targets could be accounted for largely by the coincidence of more than one clone in a single dimple. The expected frequency of dimples which might contain cytotoxicity against both targets due to coincidence was calculated to be 5% in the two experiments.
Results were also expressed graphically by plotting the percent specific lysis obtained in 1 aliquot of each dimple against the percent specific lysis obtained in the other aliquot. Fig. 1 shows the typical distribution obtained when aliquots of spontaneous clones were assayed against LPS or PHA blasts. The data indicated that the spontaneous CLs which lyse LPS blasts do not lyse PHA blasts and vice-versa.

b) **Lysis of DS and LPS blasts**

When individual dimples of cells from (CBA x DBA)F\(_1\) cultures were split and the aliquots assayed against CBA (LPS) or CBA(DS) blasts, 3 populations of clones of different specificities was observed (Fig. 2). There were clones which were specific for DS blasts (47%), clones which lysed LPS blasts on (29%) and clones which lysed both LPS and DS blasts (24%) (Table 1). The frequency of cross-reactive clones is significantly higher (p<0.02) than the expected frequency of cross-reactivity due to coincidence of more than one clone in the same dimple, calculated to be 9 percent, for that experiment.

c) **Lysis of ConA and PHA blasts**

When aliquots of individual dimples from cultures of (CBA x DBA)F\(_1\) cells were assayed either with PHA or ConA-induced CBA blasts, the responding clones could be divided into two main populations, those which lysed ConA blasts only (55%) and those which lysed both ConA and PHA blasts (42%) (Table 1). The amount of cross-reactivity which can be attributed to coincidence was calculated to be 15 percent and is significantly lower than the observed (P<0.01). Only a very small number of clones (3%) were found to be specific for PHA blasts. The distribution of the specificity of the clones is shown in Fig. 3. The results indicated that clones which lyse PHA blasts are a subset of all the clones which will lyse ConA blasts.

The results of all experiments where aliquots of each dimple were assayed against two different types of blasts were shown by \(x^2\) tests to be significantly different from those obtained in control experiments where the aliquots of each dimple were assayed
against the same target cell population (P<0.001). In control experiments, similar levels of cytotoxicity in the two aliquots were observed in the majority of the dimples (Table 1). There were a few however, where cytotoxicity was observed in only one of the two aliquots. These were derived from dimples with low levels of specific lysis and were thus difficult to divide into equal amounts of cytotoxicity. The frequency distributions in control experiments do not conform to the calculated frequencies expected of the lysis in one aliquot occurred independently of the lysis in the other aliquot (P<0.001).

**Discussion**

The detection of a positive clone has been arbitrarily defined as any cytotoxic activity greater than 10 percent specific lysis (1). Although this would represent a minimal estimate, the frequency of CL precursors obtained using this approach is in agreement with other determinations (9,10). The same approach has been adopted in scoring clones as specifically lysing one or both targets when individual clones were split and assayed against pairs of different target cells.

Previous work had shown that spontaneous clones which lysed normal PHA-induced DBA blasts did not lyse P815 mastocytoma targets (3). In situations where clones of CLs will lyse either one target or the other, the basis for the discrimination was due, presumably, to differences between surface antigens on the targets. There were several cases in which spontaneous CLs discriminated between pairs of targets which had identical histocompatibility antigens on their surface (3). In consideration of these data, it was concluded that if H2 antigens were involved in the configuration which is recognized on the target cell surface by spontaneous CLs, then other non-H2 determinants must also be contributing to the 'recognisable unit', perhaps in the way described for male antigen (11), modified targets (12), or virally-infected targets (13). When the targets are syngeneic with respect to each other, then differentiation antigens are likely to be a basis for antigenic differences.
Use has been made of the observation that mitogens tend to activate discrete subsets of lymphocytes and the ability of CLs to recognize such subsets has been investigated. Perhaps the most clearly defined is the use of mitogens to delineate T and B lymphocytes (14). Antigenic differences between T and B cells are well established and the data in Fig. 1 and Table 1 suggests that the individual clones lyse PHA blasts or LPS blasts with no detectable cross-reactivity. This was consistently found whether the pairs of blasts used were totally syngeneic with the CBA or $F_1$ CL responder population or semi-syngeneic when CLs from $F_1$ cells were assayed against CBA blasts.

Evidence has been presented, that although LPS and DS are both B cell mitogens, they do not activate identical populations of lymphocytes (5,6). From a consideration of the distribution of points in Fig. 2, when individual clones of CLs from $F_1$ cells were assayed against spleen cells which had been cultured in the presence of either DS or LPS, there are a number of clones which are able to discriminate between the two targets and a proportion which lysed targets in both populations. The apparently cross-reactive clones are in greater numbers than can be accounted for by coincidence of more than one clone in a single dimple (p<0.04).

PHA and ConA both stimulate T cells but not all T cells react equally to both mitogens. Subsets of T cells which are stimulated by either mitogen have been shown to differ according to a variety of criteria such as tissue distribution, expression of Thy-1 and Ia antigens, $F_c$ receptors and function (4,7,15,16). When ConA and PHA induced blasts were used as targets, the detectable clones appeared to segregate as two populations, those which lysed targets from the ConA treated spleen cells and those which lysed targets in both populations (Fig. 3). Thus, CLs which lysed targets in the PHA treated population appear to be a subset of the total population of CLs which lyse targets in the ConA treated cells. The ConA-responsive population of splenic T cells has been shown to include the PHA-responsive T cells (7) and it is interesting that there is an analogous segregation of the specificity of the spontaneous clones.

Although the experimental results can be interpreted in terms of discrete subsets of lymphocytes which are activated by individual mitogens, it has to be considered that the mitogen may
be affecting a variety of cell types albeit indirectly. Unique
differentiation antigens are likely to be involved in the
determinants which are recognized by the CLs, but there are other
explanations which need to be excluded. One putative contributor
to the specificity of the clones is FCS in which the cells are
grown. There have been reports of responses against a FCS component
(17). A number of points render this consideration as unlikely.
All targets were grown in the same batch of serum and if the
spontaneous CLs were directed against a serum component, there
would be a lack of fine discrimination between targets. Similarly
it would not lead to the apparent discrimination between PHA blasts
from parent and F1 cells (3).

It can be concluded that spontaneous clones of CLs can
recognize subsets of syngeneic cells, but further detailed
analysis of the specificity of the clones needs to be attempted
with 'pure' subsets of syngeneic blasts. These results have
demonstrated the potential of the clonal analysis in investigating
the specificity of CLs. An investigation of the relationship of
the spontaneous response and a stimulated response is summarized
in the accompanying paper.

References

Table 1. Lysis of target blast cells induced by different mitogens by spontaneous CL clones a).

a). Spleen cells (1.3 x 10^7 per raft) were cultured for 4 days in polyacrylamide rafts when individual dimples of cells were removed and divided into two aliquots, each of which was assayed for cytotoxicity against two different target blast cells. In control experiments the 2 aliquots were assayed against the same type of target cell.
<table>
<thead>
<tr>
<th>Target 1</th>
<th>Target 2</th>
<th>Target 1</th>
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<td>3.5</td>
<td>CBA (ConA)</td>
<td>CBA (PHA)</td>
<td>CBA (LPS)</td>
</tr>
<tr>
<td>25.0</td>
<td>28.5</td>
<td>46.5</td>
<td>CBA (LPS)</td>
<td>CBA (PHA)</td>
<td>CBA (LPS)</td>
</tr>
<tr>
<td>7.0</td>
<td>30.0</td>
<td>63.0</td>
<td>F&lt;sup&gt;T&lt;/sup&gt;(LPS)</td>
<td>F&lt;sup&gt;T&lt;/sup&gt;(PHA)</td>
<td>CBA (PHA)</td>
</tr>
<tr>
<td>6.3</td>
<td>37.5</td>
<td>66.2</td>
<td>CBA (LPS)</td>
<td>CBA (PHA)</td>
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<td>(d)</td>
<td>(e)</td>
<td>(f)</td>
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<table>
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<tr>
<th>Target 1</th>
<th>Target 2</th>
<th>Target 1</th>
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</thead>
<tbody>
<tr>
<td>Percent Positive Duplicates against responder</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 1.
Figure 1: Lysis of PHA and LPS blasts. $1.3 \times 10^7$ spleen cells from (CBAxDBA)$_1$ mice were cultured for 4 days in polyacrylamide rafts. Individual dimples of cells were harvested and divided into two aliquots. An aliquot of each dimple was assayed with $F_1$ (LPS) blasts and the other aliquot assayed against $F_1$ (PHA) blasts. The percent specific lysis obtained in one aliquot was plotted against the percent specific lysis obtained in the other aliquot. Dimples with less than 10% lysis in both aliquots have been omitted.
Figure 2: Lysis of DS and LPS blasts. Individual dimples of cells from 4 day cultures of $1.3 \times 10^7$ (CBA x DBA)$_1$ spleen cells were divided into two aliquots. Once aliquot was assayed with CBA(DS) blasts while the other was assayed with CBA(LPS) blasts. The percent specific lysis of DS blasts in one aliquot has been plotted against the percent specific lysis against LPS blasts obtained in the other aliquot. Dimples with less than 10% specific lysis in both aliquots have been omitted.
against DS blasts (% sp. lysis)

against LPS blasts (% sp. lysis)
Figure 3  Lysis of ConA and PHA blasts. Individual dimples from day 4 cultures of $1.3 \times 10^7$ (CBA x DBA)$_1$ spleen cells were divided into two aliquots. One aliquot was assayed with CBA(ConA) blasts and the other aliquot against CBA(PHA) blasts. Percent specific lysis obtained in one aliquot against ConA blasts has been plotted against the percent specific lysis against PHA blasts obtained in the other aliquot. Dimples with less than 10% specific lysis against both targets have been omitted.
Specificity of spontaneous clones of CLs from cultures of CBA spleen cells. CBA spleen cells (1.3 x 10^7 cells per raft) were cultured for 4 days in polyacrylamide rafts. Individual dimples were divided into 2 aliquots, one of which was assayed with P815 target and the other was assayed with DBA PHA-induced splenic blasts. The per cent specific lysis of P815 cells in one aliquot was plotted against percent specific lysis of DBA blasts in the other corresponding aliquot. Dimples with less than 10% specific lysis for both targets have been omitted.
Figure 4

Specificity of spontaneous clones of CLs from cultures of (CBA x DBA)F\textsubscript{1} cells. (CBA x DBA)F\textsubscript{1} spleen cells (1.3 x 10\textsuperscript{7} cells per raft) were cultured for 4 days in polyacrylamide rafts. Individual dimples were divided and assayed against P815 or DBA blasts as described for Fig. 3.
% specific lysis against P815

% specific lysis against DBA
Spontaneous Clones of Cytotoxic T Cells in Culture

I. Characteristics of the Response

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Received December 21, 1976

A culture system has been developed which allows segregation of individual clones of cytotoxic lymphocytes (CLs). When CBA, DBA, or (CBA × DBA) F₁ spleen cells from adult mice were cultured, clones of CLs able to lyse P815 target cells were generated in the absence of stimulating cells. The effector cells are sensitive to anti-Thy-1 serum and include cells with anti-self reactivity. The maximum number of CL clones was detected on Day 4, while the largest size of clones occurred 1 or 2 days later. In contrast to stimulated cultures, there was a nonlinear relationship between the number of clones and the concentration of spleen cells in the culture. The generation of spontaneous cytotoxicity is a characteristic of adult spleens and does not develop until mice are 4 weeks old.

INTRODUCTION

The polyacrylamide culture system of Marbrook and Haskill (1) has recently been adapted for assay of cell-mediated immunity (2). This system allows precursors of cytotoxic T lymphocytes to become segregated during culture, so that the responding effector cells can be harvested as separate ‘clones’ (1, 2). In a study using this technique (2), it was found incidentally that (CBA × DBA) F₁ spleen cells, cultured alone in polyacrylamide culture vessels, generate small numbers of effector cell clones which will lyse P815 mastocytoma targets. Although ‘natural’ or ‘spontaneous’ cytotoxicity against tumour cell lines or virus-induced lymphomas has now been detected in a number of in vitro systems, both in mice (3–8) and in humans (9–11) many of these systems are not dependent on T cells (3–7, 11). Nevertheless, in some instances, spontaneous cytotoxicity attributable to the action of cytotoxic T lymphocytes has been reported when normal murine spleen cells were cultured in vitro (12, 13).

In this paper, we examine further aspects of the spontaneous cytotoxicity generated during culture in polyacrylamide vessels. This work served as a preliminary to a subsequent investigation on the specificity of the effector cell clones generated in this response.

METHODS

Mice. Mice used were 12-week-old CBA/J (H-2k), DBA/2 (H-2b), and (CBA × DBA) F₁ males or neonatal animals of both sexes from (CBA × DBA) F₁ litters.

* Supported by The Medical Research Council of New Zealand.
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Culture medium. Cells were cultured in RPMI 1640 (Gibco) supplemented with with 10% fetal calf serum (FCS) (Australian Laboratory Services). It also contained 2-mercaptoethanol at a final concentration of $5 \times 10^{-3} M$ and antibiotics (penicillin, 30 µg/ml; streptomycin, 50 µg/ml).

Culture vessels. Polyacrylamide 'rafts' containing 64 small depressions or 'dimples' in which cells could settle at random were prepared from a Plexiglas (polymethyl methacrylate) mould (1). The rafts were washed twice in distilled water, autoclaved in phosphate-buffered saline (PBS), and stored at 4°C. Before use, they were washed in Eagle's minimal essential medium (Gibco) at 4°C for 24 hr and then transferred to RPMI 1640 without FCS for 12 hr. This was followed by a 3-hr preincubation at 37°C to prevent distortion of the raft when cultures were set up (2).

Cell suspensions. Mice were killed by cervical dislocation. Their spleens were removed and gently teased out into culture medium. The cell suspension was layered over 1 ml of FCS in a conical tube for 5 min and cell clumps and debris were allowed to settle into the serum.

Culture system. Each polyacrylamide raft was placed in a 5-cm petri dish containing 8 ml of culture medium and the raft was then filled with 3.5 ml of cell suspension. Cultures were maintained in an atmosphere of 5% CO₂ in air.

Target cells. P815 (H-2b) mastocytoma cells were used as targets for the cytotoxicity assay. The cell line was maintained in continuous suspension culture in RPMI containing 10% FCS and antibiotics.

Cytotoxicity assay. Cytotoxicity was assayed as described previously (2) after the method of Brunner and co-workers (14). P815 mastocytoma cells, $3 \times 10^6$ cells, in 0.1 ml of PBS were labelled with 100 µCi of sodium. $[^{51}\text{Cr}]$chromate (The Radiochemical Centre, Amersham) at 37°C for 50 min. The cells were washed three times in medium (RPMI with 10% heat-inactivated FCS) and made up to a concentration of $5 \times 10^4$ cells/ml. To harvest a raft, cells from each dimple were removed separately into 0.3 ml of medium (RPMI with 10% heat-inactivated FCS) in 76 × 11-mm disposable tubes. Generally, each alternate dimple was harvested (32 from a raft of 64).

$[^{51}\text{Cr}]$-labelled P815 target cells, $10^4$ cells, were added to each tube. The tubes were centrifuged (100g, 3 min) and incubated at 37°C for 4 hr in 5% CO₂ in air. After thorough mixing and centrifugation (500g, 10 min), 0.2 ml of supernatant was removed into 2.5-cm glass-fibre disks (Whatman). These were placed in vials containing 5 ml scintillation fluid [toluene with PPO (3 g/liter) and POPOP (0.1 g/liter.)] $[^{51}\text{Cr}]$ released from lysed target cells was then measured in a Packard Tri-Carb liquid scintillation counter (Model 3375).

Our results have been calculated in terms of percentage specific lysis:

\[
\frac{\text{Experimental }[^{51}\text{Cr}] \text{ release} - \text{spontaneous release}}{\text{maximum }[^{51}\text{Cr}] \text{ release} - \text{spontaneous release}} \times 100.
\]

Spontaneous release was estimated by incubating the target cells alone, whilst maximum release was obtained after three cycles of freezing and thawing. The spontaneous release from normal or TNP-modified P815 targets was 7–25% of the maximum release. Dimples which gave more than 10% specific lysis against a particular target were scored as positive for cytotoxicity against that target as described previously (2).
Statistical treatments of results. It is considered likely that positive dimples result from the clonal expansion of single precursor cells which settle at random, although this remains to be formally demonstrated (1, 2). The number of clones generated in a single culture was calculated from the number of positive dimples detected per raft by a method described previously (1) which uses a correction factor to take into account coincidence between clones as the number of positive dimples per culture is increased.

Anti-Thy-1 treatment. (CBA × DBA)F₁ spleen cells, $1.3 \times 10^7$ cells, were cultured for 4 days in polyacrylamide rafts. All dimples in the raft were harvested and the cells were combined in 1 ml of culture medium. Cells, $2.7 \times 10^6$ in 0.4 ml, were then treated with anti-Thy-1 antiserum (Searle) at a final dilution of 1:50 for 30 min at 37°C. Rabbit complement (Gibco) was added at a final dilution of 1:5 and the cells were incubated a further 30 min at 37°C. They were then washed twice in 2 ml of medium before assaying for cytotoxicity.

TNP modification of cells. In some experiments spleen cells modified with TNP (15) were cultured. Spleen cells were centrifuged (200g, 7 min) and resuspended in 10 mM trinitrobenzyl sulphonate (TNBS) in PBS, allowing 1 ml of TNBS per spleen. The cells were incubated for 10 min at 37°C, washed twice with medium, and then cultured in polyacrylamide rafts.

Target cells were also modified with TNP. P815 cells, $3-5 \times 10^6$ cells, in 0.1 ml of PBS were labelled with 100 μCi of sodium $[^{51}Cr]$chromate (50 min, 37°C). Five-tenths milliliter of TNBS (10mM in PBS) was added, and after a 10-min incubation at 37°C the labelled cells were washed three times in culture medium and used as target cells in the cytotoxicity assay.

RESULTS

Spontaneous Cytotoxicity against P815 Generated from Spleen Cell Cultures

Previous results in our laboratory had shown that (CBA × DBA)F₁ spleen cells cultured alone in polyacrylamide rafts generate clones of cytotoxic lymphocytes.

![Fig. 1](attachment:image.png)

Fig. 1. Numbers of clones detected against P815 mastocytoma targets from cultures of (CBA × DBA)F₁ spleen cells. Cells were cultured from 3 to 6 days in polyacrylamide vessels before assay. (○---○), $0.7 \times 10^7$ cells per raft; (○----○), $1.3 \times 10^7$ cells per raft; (●--●), $2.6 \times 10^7$ cells per raft; (●----●), $4.55 \times 10^7$ cells per raft. Each point is the mean of two observations.
able to lyse P815 mastocytoma targets (2). The time course of this response at four cell concentrations is plotted in Fig. 1. When 7 million spleen cells were cultured per raft, very few positive dimples could be detected, whereas with higher cell concentrations (1.3–4.55 × 10⁷ cells per raft) a distinct clonal response developed, which reached a maximum on Day 4, but had subsided by Day 6. An examination of the frequency of dimples with different levels of cytotoxicity showed that dimples with the highest amounts of cytotoxicity occurred late in the response when the total number of positive dimples per culture was declining. Rafts cultured with 4.55 × 10⁷ spleen cells, for example, yielded dimples with the highest levels of cytotoxicity on Day 6 (Fig. 2).

To examine the effect of cell concentration on the generation of cell clones, polyacrylamide rafts containing 0.7–4.55 × 10⁷ (CBA × DBA)F₁ spleen cells were cultured for 4 days, harvested, and assayed against P815 mastocytoma targets. The relationship between the number of spleen cells cultured and the number of

![Graph showing frequency of dimples vs. culture days.](image)

**Fig. 2.** Frequency of dimples containing different levels of cytotoxicity from rafts of (CBA × DBA)F₁ spleen cells after 3 to 6 days of culture. The cells were cultured at a concentration of 4.55 × 10⁷ cells per raft and assayed for cytotoxicity against P815 mastocytoma targets.

![Graph showing clones per culture vs. cells per culture.](image)

**Fig. 3.** The effects of cell concentration on the number of clones detected in cultures. (CBA × DBA)F₁ spleen cells were cultured for 4 days in polyacrylamide rafts and assayed for cytotoxicity against P815 mastocytoma targets. (□—□), Clones per culture; (○—○), clones per 10⁶ cultured cells. The vertical bars indicate the standard errors of the means.
clones detected was found to be nonlinear (Fig. 3). A maximum of about 20 clones per culture was detected when $1.3 \times 10^7$ spleen cells were cultured per raft, and clone numbers then declined as cell concentration increased.

The effect of cell concentration on the development of clones in cultures of parental strain spleen cells was also examined. CBA or DBA spleen cells were cultured for 4 days at concentrations of $0.7-2.6 \times 10^7$ cells per raft, harvested, and assayed against P815 mastocytoma targets. Again a nonlinear relationship was observed between the number of cells cultured and the number of clones which developed (Fig. 4). In both CBA and DBA cultures, the maximum number of clones (about 14 clones per raft) developed when $1.3 \times 10^7$ spleen cells were cultured and fewer clones were detected at higher concentrations.

**The Effect of Cell Concentration on the Generation of Clones in a Stimulated Culture System**

When the relationship between the number of cells cultured and the number of clones per culture was found to be nonlinear in unstimulated cultures, it became
TABLE 1
Effect of Age on the Development of Spontaneous Cytotoxicity against P815 Mastocytoma in Cultures of (CBA × DBA)F₁ Spleen Cells*

<table>
<thead>
<tr>
<th>Age of spleen donor (days)</th>
<th>Clones/raft</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0, 0</td>
</tr>
<tr>
<td>9</td>
<td>0, 0</td>
</tr>
<tr>
<td>16</td>
<td>3.8, 0</td>
</tr>
<tr>
<td>20</td>
<td>0, 0</td>
</tr>
<tr>
<td>31</td>
<td>3.8, 7.7, 7.7, 0</td>
</tr>
<tr>
<td>90</td>
<td>20.5, 25.6</td>
</tr>
</tbody>
</table>

* Spleen cells were cultured at 1.3 × 10⁷ cells/raft for 4 days before assay.

of interest to examine the effect of cell concentration on the generation of clones in a stimulated system. To do this we used the system of Shearer and co-workers (15) for modification of cells with TNP. Normal or TNP-modified (CBA × DBA)F₁ spleen cells, 0.7–4.55 × 10⁷ cells, were cultivated in rafts for 4 days, harvested, and assayed against TNP-modified P815 target cells. The dose–response relationships in cultures of normal or TNP-modified spleen cells were found to be quite different (Fig. 5). When TNP-modified P815 targets were cultured, the number of clones of CLs against TNP-modified P815 targets increased in a linear manner with cell concentration (Fig. 5A). In contrast, when cultures of unmodified F₁ cells were assayed on the same TNP-modified targets, the number of clones per culture reached a maximum when 1.3 × 10⁷ spleen cells were cultured, while at higher cell concentrations the number of clones detected declined.

Spontaneous Cytotoxicity in Neonatal Mice

Spleen cells from neonatal mice were cultured in polyacrylamide rafts in order to determine the age at which spleen cells become able to generate clones of CLs during culture. Spleen cells, 1.3 × 10⁷ cells, from 4- to 90-day-old mice were cultured for 4 days and assayed against P815 target cells. No clones were detected

TABLE 2
Effect of Anti-Thy-1 Antiserum on the Spontaneous Cytotoxicity of (CBA × DBA)F₁ Spleen Cells against P815 Mastocytoma Targets*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity against P815 (％ specific lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>44.6</td>
</tr>
<tr>
<td>Rabbit complement</td>
<td>25.2</td>
</tr>
<tr>
<td>Anti-Thy-1 antisera and rabbit complement</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Spleen cells were cultured at 1.3 × 10⁷ cells/raft for 4 days when all dimples in the raft were harvested and the cells were combined in 1 ml of medium. The harvested cells were then treated with anti-Thy-1 antiserum and rabbit complement or with rabbit complement alone before assaying for cytotoxicity.
before 16 days and significant numbers of clones did not develop until the mice were 31 days old (Table 1).

Effects of Treatment with Anti-Thy-1 Antiserum

Cultures were treated with anti-Thy-1 antiserum to determine whether the spontaneous cytotoxicity generated when spleen cells are cultured in polyacrylamide rafts is dependent on T cells. \((CBA \times DBA)_{F_1}\) adult spleen cells, \(1.3 \times 10^6\) cells, were cultured for 4 days when cells from each dimple were harvested and combined before treatment with the antiserum. Incubation of the cells with anti-Thy-1 antiserum and rabbit complement was found to abolish cytotoxicity against P815 target cells (Table 2).

DISCUSSION

In this paper we have examined the spontaneous cytotoxicity which develops when spleen cells are cultured in polyacrylamide rafts, a system which allows a 'clonal' analysis of the immune response (1). CBA, DBA, or \((CBA \times DBA)_{F_1}\) adult spleen cells cultured by this method generate clones of effector cells able to lyse P815 mastocytoma targets. Forms of 'natural' or 'spontaneous' cytotoxicity have been reported in other systems, but these are often antibody dependent or do not involve T cells (3-7, 11). Kiessling and co-workers, for example, recently reported spontaneous cytotoxicity in several mouse strains against Moloney virus-induced leukemia cells. This response was induced by a nonadherent effector cell of an undefined type which was neither a T cell, a B cell, nor a monocyte (5-7). In contrast, the cytotoxicity which develops during culture in polyacrylamide vessels appears to be mediated by cytotoxic T lymphocytes. It is detected by the T-cell-mediated cytotoxicity assay of Brunner and co-workers (14) and can be abolished by treatment with anti-Thy-1 antiserum and complement. Shustik and co-workers (13) recently described similar T-cell-mediated cytotoxicity generated spontaneously when normal spleen cells were cultured in vitro.

As yet there is no ready explanation for the appearance of effector cell clones in the absence of any antigenic stimulus specifically added to the culture system. Further work which has been carried out on the specificity of the response (Ching, Marbrook, and Walker, unpublished observations) has shown that clones specific for a variety of targets develop simultaneously during the culture period, which suggests that the spontaneous cytotoxicity generated in polyacrylamide culture systems may be due to a polyclonal type of response. A polyclonal response might be stimulated by mitogenic factors present in the fetal calf serum and could be enhanced by the presence of 2-mercaptoethanol in the culture medium (16). It is also of interest that the spontaneous cytotoxicity includes clones of apparently self-reactive effector cells, as seen when DBA or \((CBA \times DBA)_{F_1}\) spleen cells generate CLs able to lyse P815 targets. Similar self-reactive cytotoxic T lymphocytes have been reported recently to be temporarily present in mice undergoing an immune response to lymphocytic choriomeningitis (17) or ectromelia virus (18, 19).

Various aspects of the spontaneous response to P815 by cultured spleen cells have been investigated. It was found that the response from \((CBA \times DBA)_{F_1}\) spleen cells reached a maximum on Day 4 and then subsided. On Day 4 many positive dimples were detected but these contained relatively small amounts of cyto-
toxicity. In contrast, on Days 5 and 6, fewer positive dimples were detected, but the levels of cytotoxicity in many dimples were greater. This type of response may be due to an asynchronous triggering of clones, which would allow large clones to develop at a relatively late stage when the total number of clones is declining (1).

In the absence of linear dose-response relationships, the total number of precursors able to generate spontaneous clones cannot be determined, although at optimum cell concentrations as many as 28 clones were detected per culture. This maximum figure would correspond to one CL precursor per \(4.6 \times 10^5\) spleen cells. It is interesting that this figure is very much lower than the frequency of CL precursors recently measured when both stimulator and responder populations are present in the culture vessel (2). In these studies, dimples were considered positive for cytotoxicity above an arbitrary level of 10% specific lysis. Frequency measurements using this method yield results essentially identical to those of other workers (20; Teh, Harley, Phillips, and Miller, personal communication). However, as the results depend on the sensitivity of the assay, any estimate of frequency using clonal analysis must be a minimal estimate.

Another feature of the spontaneous cytotoxicity generated by cells cultured in polyacrylamide rafts is the nonlinear relationship exhibited between cell concentration and the number of clones which develop. For cultures of DBA, CBA, or (CBA × DBA)\(F_1\) spleen cells, a maximal response was obtained when \(1.3 \times 10^6\) spleen cells were cultured per raft and at higher cell concentrations few clones could be detected.

The factors contributing to the nonlinear dose-response relationship are currently under investigation since there seem to be a number of possible explanations for this phenomenon. The nature of the stimulus which induces the production of spontaneous clones has not been established. It is possible that this stimulus may become limiting at high cell concentrations. However, since the efficiency of the response (expressed in terms of clones per \(10^6\) spleen cells) declines at concentrations both below and above the optimum (Fig. 3), suboptimal stimulation is unlikely to be the sole factor involved. An alternative explanation might involve the effects of subpopulations of T cells since it is known that the generation of CLs from precursor cells can be influenced by both suppressor and helper T cells (21, 22). It is possible that a differential segregation of these interacting subpopulations might occur within the culture vessel, particularly at limiting cell concentrations, and this might account for the shape of the dose-response curve.

When parental strain spleen cells are cultured with \(F_1\) spleen cells in polyacrylamide rafts, a direct relationship between the number of responder cells and the number of clones is observed (2). A similar direct relationship between cell concentration and the number of clones was also observed in 'stimulated' cultures where cells were modified before culture with TNP according to the method of Shearer and co-workers (15) and assayed against TNP-modified P815 targets. In contrast, when cultures of normal unmodified cells were assayed against the same TNP-modified targets, a nonlinear dose-response relationship was obtained. This observation makes it seem unlikely that the decline in clone numbers at high cell concentrations, which characterises cultures of 'unstimulated' cells, can be explained entirely in terms of overcrowding of the culture vessel.

We also cultured spleen cells from neonatal mice to determine the age at which
spleen cells develop the capacity to generate clones of CLs during culture. Spontaneous cytotoxicity appears to be a characteristic of adult spleen cells and does not develop in culture until the mice are at least 1 month old. This may reflect the relatively late appearance of effector T cells during ontogeny (23).

In summary, we have described aspects of the spontaneous cytotoxicity which develops when adult spleen cells are cultured in polyacrylamide rafts. This has served as a preliminary study to an investigation of the specificity of cytotoxic T lymphocytes, in which the polyacrylamide culture system was used to generate discrete clones of CLs, so that cells from a single clone could be assayed against different types of targets.

ACKNOWLEDGMENTS

We are grateful to Ms. J. Booth, T. Schaaf, and L. Savu for excellent technical assistance.

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Spontaneous Clones of Cytotoxic T Cells in Culture
II. Specificity of the Response

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When normal (CBA × DBA)F₁ spleen cells are cultured for 4 days in polyacrylamide vessels, clones of cytotoxic lymphocytes (CLs) are generated. The specificity of these apparently spontaneous CL clones has been investigated by assaying cells from individual clones against pairs of different target cells. CL clones were found to discriminate between the two parental strain splenic blasts, between splenic blasts and syngeneic tumour cells, and between two F₁ splenic blasts induced with different mitogens (LPS and PHA). The CL clones generated spontaneously in culture also discriminate between semisyngeneic targets [DBA blasts and (CBA × DBA)F₁ blasts]. Significant cross-reactivity however, was detected when CL clones were assayed against normal P815 targets and TNP-modified P815 targets.

INTRODUCTION

We have recently described a culture system which segregates the precursors of cytotoxic lymphocytes (CLs) during culture and allows the responding effector cells to be harvested as separate ‘clones’ (1, 2). In a study on the frequency of CL precursors, it was shown that, when spleen cells were cultured in this system without the addition of stimulating cells, small numbers of CL clones were generated (1). Various conditions which influence this ‘spontaneous’ or ‘background’ response have been investigated (3) and we now report on the specificity of these apparently spontaneous CL clones.

Early studies on the specificity of cell-mediated lysis indicated that antigens of the major histocompatibility complex (MHC) play an important role in effector cell recognition of allogeneic or xenogeneic target cells (4, 5). More recent work with virus-infected (6), chemically modified (7), or non-H₂-histoincompatible (8, 9) target cells has shown that CLs also recognise a wide variety of other cell surface antigens, although it appears that these antigens are recognised in association with products of the MHC.

In many studies on CL specificity, the cytotoxic response in a whole cell culture has been examined against various target cells. In contrast, we have taken individual clones of CLs and assayed the cells in each clone against pairs of different target cells. By this means, we have been able to gain additional information on CL specificity which suggests that CL clones discriminate between different targets to a degree that is not normally detectable when a whole culture is assayed for cyto-

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toxicity. The results are discussed in relation to current knowledge on CL recognition of target cells.

MATERIALS AND METHODS

Mice. Mice used were 12-week-old CBA/J (H-2^b), DBA/2 (H-2^a), C57BL/10 (H-2^b), and (CBA \times DBA)F_1 males.

Culture medium. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (Australian Laboratory Services). It also contained antibiotics (penicillin, 30 \mu g/ml; streptomycin, 50 \mu g/ml) and 2-mercaptoethanol at a final concentration of 5 \times 10^{-8} M.

Culture system. Cells were prepared and cultured as described previously (3) using a polyacrylamide culture vessel or ‘raft’, which contains 64 small wells or ‘dimples’ into which cells can settle at random (1, 2).

Target cells. Target cells for the cytotoxicity assay included tumour cells and normal spleen cells stimulated either with phytohaemagglutinin (PHA) or lipopolysaccharide (LPS). Three tumour lines were used: P815 (H-2^a), EL-4 (H-2^b), and C1-18 (H-2^k). The EL-4 and C1-18 lines were kindly obtained from Dr. A. W. Harris, The Walter and Eliza Hall Institute, Melbourne. Tumour cells were maintained in culture medium without 2-mercaptoethanol.

PHA-stimulated blasts were prepared after Forman (10). Spleen cells, 4 \times 10^6 cells, in 0.8 ml of culture medium were grown for 2 days in Marbrook culture vessels. Two-tenths milliliter of medium containing PHA (Difco) to give a final concentration of 0.5% was then added. The cells were harvested for use as targets on Day 5. LPS blasts were prepared in a similar manner by culturing 4 \times 10^6 spleen cells for 3 days in culture medium containing LPS (Difco) at a concentration of 15 \mu g/ml.

Radioactivity labelled target cells for the chromium release assay were prepared by incubating 3-5 \times 10^6 tumour cells or splenic blasts with 100 \mu Ci of sodium [^{51}Cr]chromate (The Radiochemical Centre, Amersham) at 37°C for 60 or 90 min, respectively. The cells were washed three times in RPMI 1640 with 10% heat-inactivated FCS and made up to 4 \times 10^6 cells/ml.

P815 target cells were modified with TNP after radioactive labelling with ^{51}Cr as described previously (3).

Harvesting cells for assay. Cells from each individual dimple were removed into 76 x 11-mm disposable tubes containing 0.6 ml of medium (RPMI with 10% heat-inactivated FCS). The cells were pipetted carefully to disperse clumps, and 0.3 ml of the suspension was then removed to a second tube. In this manner, 32 dimples were harvested from each raft, and three to eight duplicate rafts were assayed in each experiment.

Cytotoxicity assay. ^{51}Cr-labelled target cells, 10^4 cells were added to each aliquot of harvested cells. They were then treated as described previously (3) for the cytotoxicity assay of Brunner and co-workers (11). Results have been calculated in terms of percentage specific lysis:

\[
\left( \frac{\text{Experimental } ^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous release}} \right) \times 100.
\]

Spontaneous release was estimated by incubating the target cells alone, while maximum release was obtained after three cycles of freezing and thawing. Spon-
taneous release was 7–25% of the maximum release for tumour target cells, 26–36% of the maximum release for splenic blasts, and 10–24% of the maximum release for TNP-modified P815 targets.

Statistical treatment of results. Aliquots of harvested cells assayed against one particular target were considered to contain cytotoxicity if they yielded more than 10% specific lysis. This arbitrary percentage is well above any apparent lysis due to errors in isotope counting (1). In each experiment dimples were assigned to four categories according to the following criteria: (i) cytotoxic for target 1 (>10% lysis of target 1, <10% lysis of target 2); (ii) cytotoxic for target 2 (<10% lysis of target 1, >10% lysis of target 2); (iii) cytotoxic for targets 1 and 2 (>10% lysis of target 1, >10% lysis of target 2); (iv) not cytotoxic for either target (<10% lysis of target 1, <10% lysis of target 2).

These observed frequencies, distributed in a 2 × 2 contingency table, were compared by a χ² test with the frequencies expected if lysis of target 1 occurred independently from the lysis of target 2. Where expected frequencies were small (≤ 5) the results of the χ² test were confirmed by calculation of the exact (hypergeometric) distribution. The predicted frequency of dimples containing cytotoxicity against targets due to coincidence of two or more clones in the same dipple was also calculated from the observed frequencies distributed in a 2 × 2 contingency table. The high percentage of negative dimples in most rafts assayed (52.1–90.5%, from Table 1) served to minimise coincidence.

RESULTS

Division of Dimples into Aliquots of Equivalent Cytotoxicity

(CBA × DBA)F₁ spleen cells, 1.3 × 10⁷ cells, were cultured for 4 days in polyacrylamide rafts. Individual dimples were harvested and divided into two aliquots, each of which was assayed against the same type of target cell, in one case these

TABLE 1

<table>
<thead>
<tr>
<th>Number of dimples assayed</th>
<th>Number of positives</th>
<th>Target 1</th>
<th>Target 2</th>
<th>Percentage of positive dimples against</th>
<th>Expected percentage of positive dimples against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P815</td>
<td>P815</td>
<td>Target 1</td>
<td>Target 2</td>
</tr>
<tr>
<td>128</td>
<td>48</td>
<td>P815</td>
<td>P815</td>
<td>25.0</td>
<td>18.7</td>
</tr>
<tr>
<td>160</td>
<td>41</td>
<td>(CBA × DBA)F₁</td>
<td>(CBA × DBA)F₁</td>
<td>24.6</td>
<td>26.8</td>
</tr>
<tr>
<td>96</td>
<td>46</td>
<td>PHA blasts</td>
<td>PHA blasts</td>
<td>61.0</td>
<td>30.0</td>
</tr>
<tr>
<td>256</td>
<td>42</td>
<td>CBA PHA blasts</td>
<td>CBA PHA blasts</td>
<td>63.0</td>
<td>30.0</td>
</tr>
<tr>
<td>224</td>
<td>57</td>
<td>PHA blasts</td>
<td>LPS blasts</td>
<td>67.0</td>
<td>23.0</td>
</tr>
<tr>
<td>160</td>
<td>51</td>
<td>P815</td>
<td>DBA PHA blasts</td>
<td>65.0</td>
<td>28.0</td>
</tr>
<tr>
<td>256</td>
<td>49</td>
<td>EL-4</td>
<td>C57BL PHA blasts</td>
<td>13.0</td>
<td>85.0</td>
</tr>
<tr>
<td>192</td>
<td>88</td>
<td>P815</td>
<td>P815-TNP</td>
<td>14.8</td>
<td>47.7</td>
</tr>
</tbody>
</table>

¹(CBA × DBA)F₁ spleen cells, 1.3 × 10⁷ cells, were cultured for 4 days in polyacrylamide rafts. Each dipple was harvested and the cells were divided into two aliquots, each of which was assayed for cytotoxicity against two different target cells. In two control experiments the two aliquots were similarly assayed against the same type of target cell.

²The predicted frequency of dimples containing cytotoxicity against both target 1 and target 2 due to coincidence of two or more clones in the same dipple. This was calculated from the frequencies observed when dimples were distributed according to specificity in a 2 × 2 contingency table.
Fig. 1. Division of dimples into aliquots of equivalent cytotoxicity. $1.3 \times 10^7$ (CBA × DBA)$F_1$ spleen cells were cultured for 4 days in polyacrylamide culture vessels. Individual dimples of cells were harvested and divided into two aliquots, each of which was assayed against the same type of target cell. Either P815 mastocytoma cells (A and C) or (CBA × DBA)$F_1$ PHA-induced splenic blasts (B and D) were used as target cells. In A and B, the percentage specific lysis in each of the two aliquots is compared for all dimples assayed. Each point on the graph thus represents the cytolytic activity found in one dimple. Dimples with less than 10% specific lysis in both aliquots have been omitted. The histograms in C and D represent the proportion of positive dimples with more than 10% specific lysis in both aliquots or in only one of the two aliquots.

were P815 mastocytoma cells, in another case they were PHA-induced (CBA × DBA)$F_1$ splenic blasts. The cytotoxicity in each of the two aliquots was then compared. As seen in Figs. 1A and B, the majority of dimples had been divided into aliquots of equivalent cytotoxicity, although dimples containing very small amounts of cytotoxicity were the most difficult to divide accurately. The proportion of dimples containing more than 10% specific lysis in one or both aliquots is given in Figs. 1C and D. When P815 mastocytoma cells were used as targets, 56.3% of the dimples assayed contained more than 10% specific lysis in both aliquots (Fig. 1C), whereas when (CBA × DBA)$F_1$ blasts were used as target cells, 48.7% of the dimples contained more than 10% specific lysis in both aliquots (Fig. 1D). These percentagess are substantially higher than those observed in experiments where each aliquot was assayed against a different target (Table 1). Moreover, the frequency distributions, as might be expected, do not conform to the calculated frequencies expected if two targets are lysed independently ($P < 0.001$).

**Specificity of Effector Cell Clones**

A subsequent series of experiments examined the specificity of CLs arising 'spontaneously' in individual dimples. In each of these experiments, $1.3 \times 10^7$ (CBA × DBA)$F_1$ spleen cells were grown in polyacrylamide rafts for 4 days. Individual dimples were then harvested and divided for assay against two different types of target cell.
Discrimination between allogeneic target cells. Aliquots from each dimple were assayed against CBA (H-2\(^b\)) or DBA (H-2\(^a\)) PHA-induced splenic blasts to determine whether CLs from a single dimple could discriminate between different H-2 haplotypes. Of the 96 dimples which were assayed, 46 contained significant levels of cytotoxicity. However, only 9% of these positive dimples generated cytotoxicity against both types of target cell, and the remainder appeared to be specific for either CBA (61%) or DBA (30%) targets (Table 1). The observed distributions did not differ significantly from those expected if both targets were lysed independently (\(P > 0.4\)).

Discrimination between syngeneic targets of different tissue specificity. To determine whether CLs from one clone could discriminate between syngeneic targets of different tissue specificity, aliquots from each dimple were assayed against (CBA × DBA)\(F_1\) splenic blast cell targets which had been induced to transform with either PHA (a T-cell mitogen) or LPS (a B-cell mitogen). From the 256 dimples assayed, 42 positive dimples were detected (Table 1). When cytotoxicity in each of the two aliquots per dimple was compared (Fig. 2A), it was apparent that, unlike the control experiments illustrated in Fig. 1, very few pairs of aliquots contained equivalent amounts of cytotoxicity in each aliquot. Only 7% of the positive dimples contained cytotoxicity for both types of target cell, and the remainder contained cytotoxicity only for LPS blasts (30%) or PHA blasts.

**Fig. 2.** The specificity of clones of cytotoxic lymphocytes. 1.3 \(\times\) 10\(^6\) (CBA × DBA)\(F_1\) spleen cells were cultured for 4 days in polyacrylamide culture vessels. Individual dimples of cells were harvested and divided into two aliquots, each of which was assayed against a different type of target cell. A and C present the results obtained when LPS-induced (CBA × DBA)\(F_1\) blasts (target 1) were added to one aliquot while PHA-induced (CBA × DBA)\(F_1\) blasts (target 2) were added to the second aliquot. B and D present similar results when PHA-induced (CBA × DBA)\(F_1\) blasts (target 1) and PHA-induced DBA blasts (target 2) were added to the two aliquots. A and B compare the cytotoxicity in each pair of aliquots for all positive dimples assayed (dimples with less than 10% specific lysis against both targets have been omitted). C and D indicate the proportion of positive dimples containing cytotoxicity against target 1, against target 2, or against both types of target cell.
(63%) (Fig. 2 and Table 1). These observed distributions did not differ significantly from those expected if both targets were lysed independently ($P > 0.4$).

**Discrimination between semisyngeneic targets.** To determine whether CLs from one clone discriminate between semisyngeneic targets, aliquots from each dimple were assayed against PHA-induced splenic blasts from DBA or (CBA × DBA)F$_1$ mice. When cytotoxicity in each of the two aliquots per dimple was compared, we again found very few dimples when the two aliquots contained equivalent cytotoxicity (Fig. 2B). Only 10% of the positive dimples contained cytotoxicity against both targets, and the majority lysed only (CBA × DBA)F$_1$ cells (67%) or DBA cells (23%) (Fig. 2D and Table 1). These observed distributions did not differ significantly from those expected if both targets were lysed independently ($P > 0.2$).

**Discrimination between splenic blasts and syngeneic tumour cells.** In order to determine whether CLs from the same clone discriminate between splenic blasts and syngeneic tumour cells, aliquots from each dimple were assayed against P815 mastocytoma cells and PHA-induced DBA splenic blasts. In a second similar experiment, the aliquots were assayed against EL-4 tumour cells and PHA-induced C57BL splenic blasts. As seen in Table 1, only 7% of positive dimples contained cytotoxicity against both P815 tumour cells and DBA splenic blasts, and only 2% contained cytotoxicity against both EL-4 tumour cells and C57BL splenic blasts. The majority of dimples contained cytotoxicity against only one of the two types of target cell. Again these observed distributions did not differ from those expected if each target was lysed independently ($P > 0.8$ in each case).

**Discrimination between TNP-modified and normal targets.** In a final experiment, we examined whether CLs from a single clone discriminate between normal and hapten-modified targets. Aliquots were assayed against P815 mastocytoma and P815 mastocytoma modified with TNP (P815-TNP). As seen in Table 1, a small proportion (14.8%) of the positive dimples contained cytotoxicity only against P815 targets, and many more contained cytotoxicity only against P815-TNP (47.7%), but there were also many which contained cytotoxicity against both normal and modified targets (37.5%). In this case, the observed distributions did not conform to those expected if the two targets were lysed independently ($P < 0.05$) indicating some cross-reactivity in the lysis of the two target cells.

**DISCUSSION**

There are two aspects to the specificity of a cell-mediated response. There is recognition of the stimulator cell by the precursor cell and also recognition of an appropriate target cell by the effector cell (12). It is the specificity of the effector cell which has been investigated in this paper. Studies with various types of target cell (4-9) indicate that surface antigens on the target cell surface are recognised by CLs in association with antigens of the MHC. Hypotheses which account for the specificity of CLs involve either the dual recognition of MHC and other antigens by two receptors on the CL or the recognition of some type of MHC antigen–surface antigen complex by a single receptor (13).

In many studies of CL specificity, the cytotoxic response of a whole cell culture has been examined. In contrast, we have attempted to examine CL specificity by generating what are assumed to be individual clones of CLs (1-3). As the specificity of a response is the sum of the specificities of the contributing clones,
clonal analysis of a response is likely to provide detailed information on the discriminatory ability of CLs. In the absence of simple assays for individual CLs, clonal analysis also takes advantage of the multiplication factor involved in the generation of each clone from a single precursor cell.

In this paper we have examined the specificity of CLs generated spontaneously when (CBA × DBA)F₁ spleen cells are cultured alone, without the addition of stimulating cells. This represents a first step towards measuring the range of CL specificities generated when stimulating cells are present during culture. Various other characteristics of the spontaneous CL response have already been described (3).

As outlined under Materials and Methods, cells from individual dimples were taken and divided into two halves. Each aliquot was then assayed for cytotoxicity by incubating the cells with $1 \times 10^4$ $^{51}$Cr-labelled targets. Low cell numbers did not allow the assay to be carried out at a range of killer to target ratios. A range of clone sizes was detected when CL cultures were harvested. Small clones, with little more than 10% specific lysis (the arbitrary cutoff point), were found to be the most difficult to divide into aliquots containing equal amounts of cytotoxicity, and errors thus increased in the analysis of small clones.

The data from four representative assays have been expressed graphically (Figs. 1 and 2) either by comparing percentage specific lysis in each of the two aliquots or by comparing histograms where cytotoxicity in individual dimples has been directed into three different categories of specificity. Figure 1 presents results from two experiments in which the two aliquots taken from individual dimples were each assayed against the same type of target cell. When the percentage specific lysis in the two aliquots is compared graphically (Figs. 1A and B), the majority of points indicate that, as predicted, aliquots from the same dimple contain similar levels of cytotoxicity. However, quite a few clones were detected when cytotoxicity was observed in only one of the two aliquots. As mentioned above, these were derived mainly from dimples which yielded very low levels of specific lysis and were thus difficult to divide into equal halves. Despite this source of error, the results of control experiments, where aliquots from individual dimples were assayed for cytotoxicity against the same type of target cell (Fig. 1), are in marked contrast to the results obtained in other experiments where each aliquot was assayed against different target cells (Fig. 2). Moreover, the frequency distributions in the two control experiments do not conform to the calculated frequencies expected if two targets are lysed independently ($P < 0.001$).

The results from experiments using a variety of different target cells have been summarised in Table 1. Also included in the table is a value, calculated from $2 \times 2$ contingency tables, for the predicted frequency of dimples which might be expected to contain cytotoxicity against both types of target cell due to coincidence of more than one clone in a single dimple. Such coincidence of clones of different specificity could obscure the real specificity and falsely indicate cross-reactivity. Taking the degree of coincidence into account, the results in Table 1 show that CL clones generated spontaneously in culture are able to discriminate between two allogeneic targets, between splenic blast targets and syngeneic tumor cells, and between splenic blasts induced with T-cell or B-cell mitogens. Significant cross-reactivity was only detected between normal tumour and hapten-modified tumour targets. In this case, three populations of CL clones appeared to be present: those specific for
TNP-modified P815 targets, those specific for normal P815 targets, and others which would lyse both types of target cell.

The ability of spontaneous CL clones to discriminate between syngeneic targets is of particular interest. It seems unlikely that this is attributable to differences in target cell lysis, for example, at different stages of the cell cycle (14). Presumably CLs discriminate here between antigens restricted to B cells, T cells, or tumour cells, although current models of T-cell recognition would suggest that these specific antigens are probably recognised in combination with H-2 antigens (13, 15).

One interesting finding was the ability of CLs to discriminate between (CBA × DBA)F₁ and DBA blasts. Since both of these targets were spleen cell blasts induced with PHA, they might be expected to differ only with respect to the H-2K specificities on the F₁ cells. In the absence of precise information about the antigenic determinants on the blast cells which are recognised by CLs, it is not possible to discriminate between two main alternative explanations for this event. Since H-2 molecules can exist, at least transiently as a disulphide-linked dimer on the cell surface (16), the CLs may recognise a new specificity on the F₁ cells due to the association of H-2K and H-2K molecules. Alternatively, the basis for the discrimination might be antigens expressed on the F₁ cells but not on the parental type used for comparison. Again the recognition of such antigens would be expected to occur in association with H-2 antigens (13) which would thus also contribute to detectable differences between the targets.

It is important to emphasise that, in this paper, only the specificities of CLs generated in a spontaneous response have been analysed. The spontaneous CL response appears to differ from a stimulated response, both in terms of the numbers of clones generated and in CL specificities. With CBA spleen cells, the frequency of precursors against P815 mastocytoma targets has been found to be as high as 1 in 1700 spleen cells (1), a ratio which is in general agreement with the results of other workers (17; Teh, Harley, Phillips, and Miller personal communication). In contrast, the number of clones generated in the spontaneous response is approximately 400 times lower (3). As regards specificity, it has been shown in this paper that spontaneous CL clones have a fine ability to discriminate between different syngeneic targets. However, similar work with stimulated cultures indicates that the majority of CL clones are unable to discriminate between different syngeneic targets in a similar manner (unpublished observations).

The basis for these differences has not been established. It is assumed that, in the absence of stimulating cells, only a small proportion of the total immune potential is realised as spontaneous CL clones. The generation of spontaneous clones might thus resemble a low affinity response where the ability to discriminate between closely related antigens is favoured. Alternatively, if spontaneous clones do not constitute a representative sample of those produced in a stimulated response, it is possible they may be generated from a discrete subset of T cells. For example, they may be T cells which normally act as suppressor cells but which can be detected as killer cells in isolated spontaneous clones. The possible identity of suppressor cells and CLs has recently been considered (18), and it is of interest that many spontaneous clones detected are of an apparently anti-self specificity. Further studies on the relationship between stimulated and spontaneous CLs are at present being undertaken, and these may yield further information on this question.
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REFERENCES