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# THE DETECTIONS AND IDENTIFICATION OF SUBPOPULATIONS OF CIRCULATING HUMAN LYMPHOCYTES, MONOCYTES AND NEUTROPHILS CAPABLE OF EFFECTING THE MITOGEN-INDUCED CELLULAR CYTOTOXIC REACTION TOWARDS ERYTHROCYTES OF VARIOUS SPECIES.

BY

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This thesis is submitted to the school of graduate studies of the University of Ottawa as partial fulfillment of the requirements for the degree of Master of Science in Pathology (Immunology).

## ABSTRACT

The mitogen-induced cellular cytotoxic (MICC) reaction was evaluated in terms of its capacity to identify distinct and/or unique cytotoxic cells among the normal human circulating leukocytes. Three conventional mitogens were used (phytohemagglutinin, pokeweed and concanavalin-A) as well as eight different erythrocyte target cells (chicken, guinea pig, horse, human, ox, rabbit, rat, and sheep). The effector cells investigated were mononuclear cells (monocytes and lymphocytes), monocyte-depleted mononuclear cells (lymphocytes) and polymorphonuclear leukocytes. It was found that:

1) Phytohemagglutinin (PHA) could mediate the lysis of only chicken and, to a much lesser degree, sheep erythrocytes. Concanavalin-A (CON-A) could mediate the lysis of all of the targets, except human and ox erythrocytes. Pokeweed (PWM) could mediate the lysis of rabbit and, to a much lesser degree, chicken and rat erythrocytes.
2) In the presence of PHA or CON-A, monocytes and polymorphonuclear cells, but not lymphocytes, were consistently cytotoxic in the MICC reaction irrespective of the target cell used. The cytotoxic monocytes were shown to bear surface receptors for FcG and complement. On the other hand, PWM induced cytolysis of rabbit erythrocyte targets was unequivocally carried out by lymphocytes and polymorphs. The role of monocytes in this cytotoxic reaction remains to be determined. The cytotoxic lymphocytes (in the presence of pokeweed) evaded characterization with respect to surface receptors, since all subpopulations isolated expressed cytotoxic activity.

3) The secretion of soluble cytotoxins was not apparently involved in the PHA, CON-A or PWM mediated MICC reactions.
4) The capacity of a mitogen to agglutinate certain erythrocyte target cells did not necessarily impart to the mitogen the ability to mediate lysis of these target cells by co-incubated effector leukocytes. However, those erythrocytes which were lysed in the presence of a mitogen in the MICC reaction were also invariably agglutinated by the mitogen.

These results suggest that the MICC assay can be utilized as a probe to permit the selective detection of functionally distinct subpopulations of cytotoxic monocytes, lymphocytes and polymorphs.

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## ABBREVIATIONS

| | |
|---|---|
| MICC | mitogen-induced cellular cytotoxicity |
| ADCC | antibody-dependent cellular cytotoxicity |
| NOCC | naturally-occurring cellular cytotoxicity |
| MLR | mixed leukocyte reaction |
| MICC effector cell | the cytotoxic cell in the MICC reaction |
| ADCC effector (K) cell | the cytotoxic cell in the ADCC reaction |
| NOCC effector (NK) cell | the cytotoxic cell in the NOCC reaction |
| PHA | phytohemagglutinin |
| CON-A | concanavalin-A |
| PWM | pokeweed mitogen |
| CRBC | chicken erythrocytes |
| GRBC | guinea pig erythrocytes |
| HoRBC | horse erythrocytes |
| HuRBC | human erythrocytes |
| ORBC | ox erythrocytes |
| RabRBC | rabbit erythrocytes |
| RatRBC | rat erythrocytes |
| SRBC | sheep erythrocytes |
| MC | mononuclear cells |
| MDMC | monocyte-depleted mononuclear cells |
| PC | polymorphonuclear cells |
| FcG | Fc region of the IgG antibody molecule |
| FcM | Fc region of the IgM antibody molecule |
| C'3 | complement |

ABBREVIATIONS (Continued)

| | |
|---|---|
| E | sheep erythrocytes |
| EA | antibody(IgG)-sensitized ox erythrocytes |
| EAC | antibody(IgM)-sensitized ox erythrocytes complexed with complement |
| RFC | rosette forming cell |
| $ER^+$ cells | cells bearing receptors for sheep erythrocytes |
| $FcR^+$ cells | cells bearing receptors for FcG |
| $C'3R^+$ cells | cells bearing receptors for complement |
| $SmIg^+$ cells | cells with surface-membrane immunoglobulin |
| Tγ cells | T cells bearing receptors for FcG |
| Tμ cells | T cells bearing receptors for FcM |
| E/T | effector to target cell ratio |
| M199 | Medium 199 |
| RPMI | Medium RPMI-1640 |
| CMRL | Medium CMRL-1066 |
| HBSS | Hank's balanced salt solution |
| FCS | fetal calf serum |
| F-H | Ficoll-Hypaque density gradient solution |
| $Cr^{51}$ | radioactive chromium-51 |
| cpm | counts per minute |
| CI | cytotoxic index |
| SR | spontaneous release |

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# CHAPTER 1

## INTRODUCTION

Circulating leukocytes of normal, non-sensitized humans and animals are cytotoxic to a variety of target cells provided that a mitogen (PHA, CON-A or PWM) is present in the culture medium. This reaction is termed mitogen-induced cellular cytotoxicity (MICC). It was first described by Holm, Perlman and Werner in 1964 (1), who showed that human mononuclear cells and rat leukocytes, in the presence of PHA, were able to induce the lysis of cultured human kidney cells and rat kidney cells, respectively. The reaction has since been corroborated by a large number of investigators, using PHA (2-36), CON-A (15,18,19,21,22,27,28,32,33,37,38,39) and PWM (15,40,41) as mitogens in the reaction mixture.

Initially, $Cr^{51}$-labelled chicken erythrocytes (3,5,6,7, 10,15,16,18,19,20.22,23,24,25,26,29,30,31,33,34,35) and $Cr^{51}$-labelled Chang epithelial cells (12,13,14,25,27,31,41) were employed as targets for the reaction. More recently, a number of other target cells, including various transformed cell lines (4,8,9,12,13,14,17,23,25,27,31,37,38,39,41) have also been used.

Although the MICC reaction has not yet been shown to have an in vivo counterpart (42), MICC activity may serve as a probe for the identification of circulating cells which

bear a cause effect and/or correlational relationship to a number of disease states. To this end, it is necessary to identify the cells which are involved in the reaction, a matter which is at present unsettled. T cells (2,3,4, 5,14,16,20,23,24,25,27,34,37); non-T cells (3,4,5,6,23,24,25); B cells (25,33); non-B cells (25); FcG receptor bearing cells (4,5,10,20,25,27,31,34); cells lacking FcG receptors (35,31); monocytes (14,19,23); and polymorphonuclear cells (15,25,29,30) have all been described as exhibiting MICC activity. It is possible that these conflicting data regarding the identity of the cytotoxic cells are a consequence of the use of different culture conditions, different target cells, different methods of cell purification, and different mitogens in the MICC assay.

This investigation was concerned primarily with the identification of erythrocytes which may serve as targets in the PHA, CON-A and PWM mediated MICC reactions, and with the identification and characterization of the human circulating cells which demonstrate cytotoxicity in these reactions. Furthermore, this study set out to define the mechanism by which the mitogens induce cytotoxicity amongst the circulating leukocytes.

# CHAPTER 2

# REVIEW OF THE LITERATURE

## 2.1 IN VITRO MODELS OF CELL-MEDIATED IMMUNITY

Cytotoxic lymphocytes are thought to participate in autoimmunity, allograft rejection, tumour immunology, and defense to numerous pathogenic microorganisms. Over the past twenty years a number of in vitro assays have been developed to study cell-mediated cytotoxicity. It has been shown that circulating lymphocytes from sensitized animals are capable of lysing target cells possessing the surface antigens against which the animals had been sensitized (4-47). In addition, non-sensitized leukocytes are capable of mediating the lysis of particular target cells in vitro; i) after stimulation with allogeneic leukocytes in the mixed leukocyte culture reaction (MLR) (48-55); ii) in the presence of antitarget antibodies (antibody-dependent cellular cytotoxicity or ADCC) (56-64); iii) without any apparent stimulus (naturally-occurring cellular cytotoxicity or NOCC) (65-86); and iv) in the presence of a mitogen (mitogen-induced cellular cytotoxicity or MICC) (1-36).

These in vitro reactions are thought to be manifestations of cell-mediated reactions which occur in vitro. It is generally accepted that the MLR is the in vitro counterpart

of allograft rejection. The ADCC reaction may provide a mechanism for the killing of microorganisms in the presence of antibodies in low titer, or in the presence of non-complement fixing antibodies (42). It has been suggested that the NOCC effector cells may constitute the 'immuno-surveillance' system, capable of recognizing and destroying newly emerging mutant, potentially-neoplastic cells (42,87,88). Although the role of the MICC reaction in vivo is not known, it is known that mitogenic factors are released from lymphocytes upon antigenic stimulation (89-91). It is therefore possible that these factors secreted in vivo may mediate target cell lysis in a manner that is similar to that mediated by phytohemagglutinin (PHA), concanavalin-A (CON-A) and pokeweed mitogen (PWM) in vitro.

The application of these models to the study of disease states not only requires the identification of the effector cells involved, but also the demonstration that these reactions are distinct from one another.

There are two cell types which participate in the MLR; those which become cytotoxic killer cells, and those which undergo blast transformation and proliferation (49). Each represents a distinct subpopulation of thymus-derived cells (T cells) (52,55). The cytotoxic cells are specifically directed to the alloantigens of the stimulating cells (49,54,44). With respect to the ADCC, it has been demonstrated that lymphocytes of a particular subclass possess receptors

for the Fc region of the IgG antibody molecule (FcG), and that events initiated by the interaction between the FcG receptor-bearing cells and the target-cell bound antibodies culminate in the lysis of the target cells (62,48,44). Monocytes (62,46), eosinophils (56,57) and neutrophils (44,46,58,64) have also been shown to possess these surface receptors, and have been implicated in the ADCC reaction. There are conflicting results regarding the identification of the NOCC effector cell(s). It has been reported as being a T cell with or without receptors for FcG (32,66,68,74,77,78); a non-T cell (61,69,84,86); a non-B cell (61,69,71,76); a C'3 and FcG receptor bearing cell (37); and a non-T, non-B 'Null' cell with receptors for FcG but not for C'3 (11,70,72,75,76,79,84). Although most studies have implicated an FcG receptor bearing cell (11,70,72,73,75,76,79,82,74), others have reported that a cell without receptors for FcG is involved (66,67). The identity of the MICC effector cell is also a matter of controversy. As was mentioned in the introduction, T cells, non-T cells, B cells, non-B cells, FcG receptor bearing cells, cells devoid of FcG receptors, monocytes, and polymorphs have all been described as exhibiting MICC activity.

Several investigators have provided evidence to show that some of the discrepancies regarding the identity of the ADCC (26), NOCC (65) and MICC (14,20,24,25) effector cells relate to the utilization of different target cells by different investigators. That is, effector cell-target

cell specificity apparently exists, whereby one subpopulation of leukocytes may be cytotoxic to one type of target cell and another subpopulation cytotoxic to another type of target cell. It is important, therefore, that the effector cells in these reactions be defined in relation to the target cell utilized, rather than in absolute terms. This investigation examined the capacity of different leukocyte subpopulations to participate in the PHA, CON-A and PWM mediated MICC cytolysis of eight different erythrocyte target cells.

## 2.2 THE MICC ASSAY

### 2.2.1 INTRODUCTION

In order to assay for MICC cytotoxicity, cultures are set up containing effector leukocytes, radio-labelled target cells and the mitogen. After one to forty hours of incubation at 37°C, the cells are centrifuged and the amount of cytotoxicity is expressed in terms of the amount of radioactivity released into the supernatants of the cultures.

### 2.2.2 THE MITOGENS UTILIZED IN THE MICC ASSAY

A mitogen is a substance which induces blast transformation and proliferation in cell culture. Conventionally, three types of soluble plant-derived mitogens are used in the MICC assay: i) phytohemagglutinin (PHA), which is an extract of the bean Phaseolus vulgaris; ii) concanavalin-A (CON-A), which is an extract of the Jackbean Canavalia; and (iii) pokeweed mitogen (PWM), which is an extract of the plant Phytolacca americana. PHA and CON-A stimulate blast transformation in T cells only, whereas PWM stimulates transformation of B and T cells (92). Paradoxically, and most unexpectedly, the ability of these mitogens to stimulate blastogenesis bears little or no relationship to their ability to induce cytotoxicity in the MICC reaction (2,3,6,16,21,22,25).

Several other lesser known mitogens have been utilized successfully in the MICC assay. These include lentil mitogen and Pultanea graveolus mitogen (2). Conversely, Escherichia coli lipopolysaccharide and purified protein derivative of tuberculin, both of which are potent B cell mitogens, have failed to induce cytotoxicity in the MICC assay (2,22).

### 2.2.3 THE TARGET CELLS UTILIZED IN THE MICC ASSAY

The target cells which have been utilized most often in the MICC assay are presented in Table 1. It can be seen that the target cells fall into two categories - erythrocytes and nucleated transformed cell lines grown in vitro. Most investigators utilize either chicken erythrocytes (CRBC) or Chang cells.

Generally, the cells are labelled with $Cr^{51}$ and cytotoxicity is reported as a function of the release of $Cr^{51}$ from the targets following their incubation with effector cells and the mitogen.

### 2.2.4 THE EFFECTOR CELLS UTILIZED IN THE MICC ASSAY

A large variety of leukocyte populations have been prepared and assayed for MICC activity. A list of the leukocyte preparations which have been utilized in the assay is presented in Table 2.

TABLE 1

TARGET CELLS USED IN THE MICC ASSAY

| NAME | | REFERENCES TO THE LITERATURE |
|---|---|---|
| ERYTHROCYTES | | |
| Chicken (CRBC) | | 3,5,6,7,10,15,16,18, 19,20,22,23,24,25,26, 29,30,31,33,34,35 |
| Sheep (SRBC) | | 14,29 |
| Human (HuRBC) | | 16,29 |
| Burrow | | 16 |
| CELL LINES | ORIGIN | |
| Chang cell | Human cervical carcinoma | 12,13,14,25,27,31,41 |
| L cells | Mouse fibroblasts | 8,9,17,23,39 |
| P815 | Methylcholanthrene-induced mastocytoma | 20,23,28 |
| EL-4 | Methylcholanthrene-induced lymphoma | 4,24 |
| Molt 4 | Human acute lymphocytic leukemia cells (T cell) | 38 |
| K562 | Human chronic myelogenous leukemia cell line | 37 |
| P3HR | Burkitt lymphoma cell (B cell) | 37,38 |
| RAJI | Burkitt lymphoma cell (B cell) | 38 |
| MAKU | Burkitt lymphoma cell (B cell) | 37 |

TABLE 2

EFFECTOR CELL PREPARATIONS USED IN THE MICC ASSAY

| Name of cellular prepations | Predominant cell types present |
|---|---|
| Polymorphonuclear cells | Neutrophils, basophils and eosinophils |
| Mononuclear cells (MC) | Lymphocytes and monocytes |
| Monocyte-depleted mononuclear cells | Lymphocytes |
| Plastic adhering MC | Monocytes |
| Plastic non-adhering MC | Lymphocytes |
| Nylon, glass, cotton or rayon adhering MC | Monocytes and B cells |
| Nylon, glass, cotton or rayon non-adhering MC | Non-B lymphocytes |
| E-RFC | Cells bearing receptors for SRBC ($ER^+$ cells) (T cells) |
| EA-RFC | Cells bearing receptors for FcG ($FcG^+$ cells) |
| EAC-RFC | Cells bearing receptors for complement ($C'3R^+$ cells) (B cells) |
| $T_\gamma$ cells | T cells bearing receptors for FcG |
| $T_\mu$ cells | T cells bearing receptors for FcM |
| Surface-membrane-Ig-positive ($SmIg^+$) cells | B cells |
| Surface-membrane-Ig-negative ($SmIg^-$) cells | Non-B cells |
| Animal organs | |
| Thymus | T cells |
| Bursa of Fabricius | B cells |
| Bone marrow | Mixed |
| Spleen | Mixed |
| Peritoneal cavity cells | Macrophages |

## 2.3 THE IDENTIFICATION OF THE CELLS INVOLVED IN THE MICC REACTION

### 2.3.1 INTRODUCTION

Numerous discrepancies have appeared in the literature with regard to the nature of the MICC effector cells. In part, this may be due to the failure to recognize that the choice of target cell utilized in the assay is of primary importance in influencing the leukocyte that can act as an effector cell in the reaction (14,20,24,25). For example, Hunninghake, Haynes, Parrillo, and Fauci (14) observed that cytotoxicity toward Chang cells was dependent upon T cells, whereas monocytes were required for the cytolysis of sheep erythrocytes. Likewise, Lum, Muchmore, Decker, and Blaese (20) reported that T cells bearing receptors for either FcG or FcM expressed MICC activity with P815 mastocytoma targets, while only T cells bearing receptors for FcG were able to lyse chicken erythrocytes.

It is not unlikely that the different mitogens also induce cytotoxicity amongst separate leukocyte subpopulations. Wisloff and Froland (33) observed that the depletion of nylon-adherent cells from mononuclear cell preparations resulted in an almost total loss of CON-A mediated cytotoxicity toward CRBC targets, whereas PHA mediated cytotoxicity was only partially affected. Furthermore, it appears that the different mitogens are specific with respect to the types of target cells which can be lysed in the assay (16,29).

For instance, Kirchner and Blaese (16) found that PWM treated chicken spleen cells could lyse human erythrocytes, but not chicken or burrow erythrocytes. Conversely, CON-A-treated chicken spleen cells could lyse burrow erythrocytes, but not the erythrocytes of the other two species.

### 2.3.2 THE IDENTITY OF THE MICC EFFECTOR CELL(S) WITH RESPECT TO NUCLEATED CELL LINE TARGETS

There is considerable agreement that the PHA induced lysis of transformed cell lines is mediated by thymus-derived T cells. Nelson, Bundy, Pitchon, Blaese, and Strober (25) found that with Chang cells as targets, MICC was mediated by lymphocytes and not by monocytes or polymorphs. After fractionating the lymphocytes on immunoglobulin (Ig) absorbent columns into $SmIg^+$ lymphocytes (B cells) and $SmIg^-$, E-rosette-positive lymphocytes (T cells), they found that MICC activity was confined to the T cell fraction. Similarly, Pichler, Gendelman and Nelson (27) separated human lymphocytes by means of various rosetting techniques into $T(ER^+)$, $B(ER^-, C'3R^+)$ and $L(ER^-, C'3R^-)$ subpopulations, and found that only T cells exhibited cytotoxicity toward Chang targets. Furthermore, by subfractionating the T cells on the basis of their surface receptors for FcG and FcM, these investigators were able to demonstrate the existence of two separate subpopulations of cytotoxic cells - T cells with receptors for FcG ($T\gamma$) and T cells with receptors for FcM ($T\mu$). This experiment was corroborated by Lum, Muchmore, Decker, and Blaese (20) who performed much

the same experiment, but with P815 mastocytoma targets instead of Chang cells.

Additional evidence which favors T cell involvement is provided by several studies on mice. Nelson, Bundy, West, and Strober (24), using EL-4 lymphoma targets, and Muchmore, Nelson, Kirchner, and Blaese (23), using P815 lymphoma and L-fibroblast targets, compared MICC activity of spleen and bone marrow cells taken from athymic nude and euthymic mice. They found that normal mouse spleen cells were capable of killing each of the targets, whereas neither normal bone marrow cells nor spleen or bone marrow cells from athymic mice were capable of mounting an MICC response.

Although Bonavida, Robins and Saxon (4) have reported that non-T as well as T cells are active effector cells, most investigators (23,24,25,27) agree that T cells are absolutely required for the PHA-mediated reaction. T cells have also been implicated in the CON-A (2,37,23) and PWM (2) induced cytolysis of various nucleated cell lines.

### 2.3.3 THE IDENTITY OF THE MICC EFFECTOR CELL(S) WITH RESPECT TO ERYTHROCYTE TARGETS

The results of numerous studies are in conflict or disagreement with regard to the nature of the human lymphoid cells which are capable of mediating MICC cytotoxicity towards erythrocyte targets. Nelson, Bundy, Pitchon, Blaese, and Strober (25) have reported that with CRBC targets, PHA

induced cytotoxicity is mediated by polymorphs, monocytes, $SmIg^+$ lymphocytes (B cells) and $SmIg^-$ lymphocytes. Wisloff and Froland (33) observed that the passage of leukocytes through nylon wool columns, to remove adherent B cells as well as neutrophils and monocytes, resulted in a complete loss of the capacity to mediate CON-A MICC cytotoxicity, and a partial loss in the capacity to mediate PHA MICC cytotoxicity. On the basis of the demonstration that the lymphocytes from patients with hypogammaglobulinemia exhibit depressed cytotoxicity (33), they suggested that B cells play a significant role in the CON-A mediated reaction. In a subsequent study (34), these same investigators reported that lymphoid cells depleted of E-RFC or EA-RFC cells displayed reduced cytotoxicity in the presence of PHA or CON-A. They concluded that MICC cytotoxic activity is a function of cells of thymic and of non-thymic origin.

Cordier, Samarut and Revillard (5) assayed lymphocytes that had been selectively enriched with or depleted of E, EA and EAC rosette forming cells. They observed that both $T(ER^+)$ and non-$T(ER^-)$ cells exhibited PHA induced cytotoxicity toward CRBC. Furthermore, the effector lymphocytes were shown to bear receptors for FcG ($FcGR^+$) but to lack C'3 receptors ($C'3R^-$).

Experiments with animal models have not helped to clarify the situation. In mice, PHA induced cytotoxicity has been attributed to T cells (3,23), non-T cells (3,23,24),

macrophages (23), and non-macrophages (23). In guinea pigs, polymorphs have been reported as being active effector cells (15). Cytotoxic T cells and cytotoxic plastic non-adhering cells have been described in chickens (16).

In spite of numerous references to the lymphocyte MICC effector cell, several investigators have produced evidence to suggest that this cell may be ineffective at mediating erythrocyte target cell lysis. In studies carried out by Simchowitz and Schur (29), glass bead column-purified lymphocyte preparations, which were virtually free of monocytes or neutrophils, were shown to be devoid of cytotoxic activity in the presence of PHA or CON-A, even after 18 hours in culture. Conversely, purified neutrophils expressed potent MICC cytotoxicity toward the CRBC targets after only one hour in culture. Levy, Silverman, Schmid, and Cooperband (19) prepared monocyte-enriched and monocyte-depleted mononuclear cell preparations using a free flow electrophoretic technique. They found that PHA-induced MICC activity was proportional to the number of monocytes in these preparations. In addition, depletion of phagocytic cells with carbonyl iron, or depletion of plastic-adherent cells, resulted in a marked reduction in cytotoxicity. These experiments suggest that the effector lymphocytes are monocytes and neutrophils and not lymphocytes.

Thus, PHA and CON-A mediated cytotoxicity towards erythrocyte targets has been attributed by some investigators

to monocytes and polymorphs and not to lymphocytes. The cytotoxic lymphocytes have variously been described as being T cells, non-T cells, B cells, non-B cells, and non-T, non-B cells. The identity of the human effector cell in the PWM mediated MICC reaction has thus far not been investigated.

### 2.3.4 SUMMARY

A wide variety of leukocyte cell types have been reported to participate as cytotoxic effector cells in the MICC assay. When a transformed cell line is used as a target, it is generally observed that cytotoxicity is mediated by T cells. On the other hand, there is disagreement with regard to the identity of the cells involved in the MICC lysis of erythrocyte targets. Some investigators have reported that a variety of lymphocyte subpopulations can act as effector cells in this cytotoxic reaction. Others have reported that monocytes and polymorphs, but not lymphocytes, are cytotoxic.

## 2.4 THE MECHANISM OF THE MICC CYTOTOXIC REACTION

### 2.4.1 INTRODUCTION

A number of studies have been carried out to determine the physical and biochemical mechanisms which are operating in the MICC reaction. Presumably, a knowledge of these mechanisms should facilitate our understanding of the mechanisms involved in other cell-mediated destructive reactions which occur in vivo (allograft rejection, auto-immunity, etc.).

### 2.4.2 THE RELATIONSHIP BETWEEN MITOGEN-INDUCED BLASTOGENESIS AND MITOGEN-INDUCED CELLULAR CYTOTOXICITY.

As stated previously, a mitogen is a substance which induces blastogenesis and proliferation in cell cultures. Thus, it is surprising that many investigators have found that mitogen-induced target cell lysis occurs independently of mitogen-induced blastogenesis, or any of the classical expressions of blastogenesis (RNA, DNA and protein synthesis). This has been demonstrated in a variety of ways. For example, Lundgren and Moller (21) found that mitomycin-C or actinomycin-D caused an inhibition of PHA induced DNA synthesis, RNA synthesis and blast transformation in lymphocytes, but left PHA induced cytotoxicity against fibroblasts intact. Kirchner and Blaese (16) showed that neither PHA, CON-A nor PWM could stimulate DNA synthesis in chicken bone marrow cells, yet these mitogens could

induce these cells to become cytotoxic to various erythrocyte targets. It was observed by Nelson, Bundy, Pitchon, Blaese, and Strober (25) that lymphocytes from patients with certain immunodeficiency diseases could not proliferate in response to PHA, yet they could exhibit cytotoxicity toward Chang cells. Dawkins and Zilco (6) demonstrated that blastogenesis and MICC are carried out by two separate cellular populations. They found that PHA-treated mononuclear cells exhibited a strong blastogenic response and a weak MICC response (CRBC targets), whereas non-T mononuclear cells displayed a weak blastogenic but strong MICC response.

These experiments, along with others (2,3,22), indicate that mitogen induced blastogenesis and cytotoxicity are two separate and distinct phenomena.

### 2.4.3 THE RELATIONSHIP BETWEEN MICC CYTOTOXICITY ACTIVITY AND THE ABILITY OF A MITOGEN TO AGGLUTINATE EFFECTOR AND/OR TARGET CELLS.

The ability of a substance to agglutinate effector and/or target cells, rather than the ability to induce blastogenesis, may be of more importance in rendering that substance capable of inducing MICC cytotoxicity. This matter has been investigated by Simchowitz and Schur (30) who utilized an MICC assay system involving neutrophil effector cells, CRBC targets and a variety of different plant lectins (PHA, CON-A, PWM, soy bean agglutinin,

Ricinius communuis agglutinin, Abrus precatorius agglutinin, and wheat germ agglutinin). They found that PHA, CON-A, soy bean, Ricinius, and Abrus could induce significant cytotoxicity, whereas wheat germ and PWM could not. In terms of agglutinability, all of the lectins which could mediate target cell lysis could also agglutinate the effector cells, and less consistently, the target cells. Wheat germ and PWM, which could not induce cytotoxicity, caused little or no agglutination of either the effector or the target cells. Only PHA, CON-A and PWM were effective in inducing blastogenesis of lymphocytes. These results suggest that close cell-to-cell contact is at least partially involved in the mechanism of MICC cytotoxicity. Presumably the mitogen acts as a link between the effector and the target cell.

### 2.4.4 THE ROLE OF PHAGOCYTOSIS IN THE MICC REACTION

Since polymorphs and monocytes, both capable of exhibiting phagocytic activity, are capable of mediating MICC lysis of erythrocyte targets, it has been speculated that phagocytosis is involved in the MICC reaction. However, experiments carried out by Simchowitz and Schur (29) suggest the contrary. They set up reaction mixtures containing PHA, polymorphs and unlabelled CRBC targets, and observed them under light microscopy. They noted the appearance of cytoplasmic bridges extending between the neutrophils and the CRBC targets, but could see no evidence

of erythrophagocytosis. In a separate experiment, mixtures were set up containing PHA, polymorphs and CRBC that had been labelled with $Cr^{51}$. After specific time intervals, extra-neutrophilic CRBC were lysed with $NH_4Cl$ and the lysate removed. To determine internalized CRBC, the remaining polymorphs were then counted for radioactivity. They found that only a very minimal amount of $CR^{51}$ had been taken up by the neutrophils, and offer this as evidence against a role of phagocytosis in the reaction.

Whether or not these results also apply to monocytes, and to systems involving other mitogens, has not been determined. With respect to the lysis of various transformed cell lines, MICC is mediated by T lymphocytes and it is generally accepted that lymphocytes are not capable of phagocytosis.

### 2.4.5 THE ROLE OF CYTOTOXINS IN THE MICC REACTION

It has been postulated that MICC effector leukocytes release cytotoxic factors when cultured with a mitogen, and that these factors are responsible for the lysis of the target cells which are present in the assay. In order to resolve this issue, several investigators have taken supernatants from MICC cultures and tested them for cytotoxicity toward fresh target cells. In this manner, Granger and Williams (9) and Granger and Kolb (8) discovered what they termed a 'lymphocyte cytotoxic factor'. This factor

was released from mouse and rat lymphocytes upon incubation with PHA, and was cytotoxic towards mouse L fibroblasts. Using the same target and mitogen, Kramer and Granger (17) found that an analogous factor was released from human lymphocytes. Likewise, Schwartz and Wilson (39) demonstrated that CON-A treated guinea pig lymphoid cells released a 'lymphotoxin' which lysed L cells. However, results from experiments carried out by Muchmore, Nelson, Kirchner, and Blaese (23) are in conflict with these results. They found that when supernatants from PHA and CON-A treated mouse lymphoid cells were incubated with P815 mastocytoma or L cell targets, no target cell lysis occurred.

With respect to the lysis of erythrocyte targets, Kirchner and Blaese (16) set up cultures containing chicken lymphocyte effector cells, a mitogen and erythrocyte target cells which were 'appropriate' and 'inappropriate' for the particular mitogen. They found that, while the appropriate targets were lysed, the inappropriate bystander targets were not damaged. They concluded that soluble cytotoxic factors do not play a significant role in the MICC reaction. The importance of cytotoxins in the mitogen-induced lysis of erythrocytes targets by human lymphoid effector cells has not yet been studied.

Thus, although there is some evidence (unconfirmed) that PHA-induced lysis of L fibroblasts is mediated by cytotoxins, the role of cytotoxins in other MICC assay

systems (involving other cell line and erythrocyte targets, various mitogens, and human as well as animal models) remains to be elucidated.

### 2.4.6 THE BIOCHEMICAL MECHANISMS OF THE MICC REACTION

Information regarding the biochemical processes in the MICC reaction is limited to a rather extensive study carried out by Simchowitz and Schur (30). They observed the effects of various drugs and metabolic inhibitors on MICC activity in an assay system which utilized neutrophil effector cells, PHA and CRBC targets. They found that: i) MICC cytolysis was dependent on anaerobic glycolysis, as cytotoxicity was reduced in the presence of inhibitors of this process (2-iodoacetamide, 2-deoxy-D-glucose and NaF); ii) inhibitors of respiration, oxidative phosphorylation and the pentose phosphate shunt did not affect MICC cytotoxicity; iii) MICC cytotoxicity was independent of protein or nucleic acid synthesis as inhibitors of these processes (puromycin, cycloheximide, actinomycin-D and mitomycin-C) did not alter MICC activity; iv) di-isopropylfluorophosophate (which is an inactivator of serine dependent esterases), colchinine (which disrupts microtubules), and cytochalasin B (which alters microfilaments), all caused inhibition of MICC; and v) that MICC cytolysis may be influenced by intracellular levels of cyclic AMP, as the analogue dibutyryl cyclic AMP inhibited MICC activity.

## 2.5 MICC CYTOLYTIC ACTIVITY IN DISEASED STATES

Several studies have been carried out to determine the value of the MICC assay in assessing leukocyte function in various disease states. Holm, Perlman and Johanssen (13) found that the lymphocytes from patients with chronic lymphocytic leukemia, and from patients with clinically active Hodgkins disease, exhibited reduced MICC cytotoxicity toward Chang target cells. It was reported by Zighelboim (36) that cells of patients with acute myelogenous leukemia in remission are defective with respect to MICC cytotoxic activity. This deficiency became more marked following monthly courses of chemotherapy with arabinofuranosylcytosine and thioguanine. They (36) emphasized the potential value for the MICC assay in providing a basis for the scheduling of immunotherapy programs that are used in conjunction with chemotherapy regimens. Wisloff, Froland and Engeset (35) observed that PHA-mediated cytotoxicity toward CRBC targets of cells of patients with irradiated testicular tumors was lower than in normal controls. They suggested that this may reflect a depression of leukocyte function as a result of either the disease process itself or of the radiation therapy that these patients had received. Podleski and Grimes (28) demonstrated hyperreactive lymphocytes by the MICC assay in patients with bronchial asthma. They offer this as evidence for the involvement of cell-mediated mechanisms in the pathogenesis of this disease. In addition, these investigators observed that MICC activity was depressed

in asthmatics on high-dose corticosteroid therapy, and therefore they suggest that the MICC assay may be of some value in monitoring the therapy in these patients.

These initial studies, although limited, point toward a potential role of the MICC assay as both a diagnostic tool and a means of monitoring the course and management of certain diseases.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 MATERIALS

Medium 199 (M199) with L-glutamine and Hank's balanced Salt Solution and modified with 1.4 grams of sodium bicarbonate per liter was obtained from Microbiological Associates, Bethesda, Maryland, and stored at 4°C.

Medium CMRL-1066 (CMRL) with L-glutamine was obtained from Microbiological Associates and stored at 4°C.

Medium RPMI-1640 (RPMI) with L-glutamine was obtained from Microbiological Associates and stored at 4°C.

Hank's Balanced Salt Solution (HBSS) was obtained from Microbiological Associates and stored at 4°C.

Penicillin streptomycin mixture, containing potassium penicillin (5,000 I.U./ml) and streptomycin sulphate (5,000 µg/ml) was obtained from Microbiological Associates and stored at -20°C.

Hepes buffer solution (1 molar) in saline was obtained from M.A. Bioproducts, Walkerville, Maryland, and stored at 25°C.

Fetal calf serum (Rehatuin) was obtained in a sterile filtered form from Connaught Laboratories, Willowdale, Ontario, and stored at -20°C.

Ficoll 400 was obtained from Pharmacia Fine Chemicals A.B., Uppsala, Sweden.

Hypaque sodium (50 per cent w/v, pH = 6.5-7.7) was obtained from Winthrop Laboratories, Aurora, Ontario, and stored in the dark.

Sterile water (pyrogen-free) was obtained from Abbott Laboratories Ltd., Montreal, Quebec.

Dextran T 500 was obtained from Pharmacia Fine Chemicals A.B.

Heparin sodium (1000, U.S.P. units/ml) was obtained from Organon Canada Ltd., Toronto, Ontario, and stored at 4°C.

Saline (0.9 per cent) in pyrogen-free sterile water was obtained from Abbott Laboratories Ltd.

Ammonium chloride was obtained from the British Drug Houses (Canada) Ltd., Montreal, Quebec.

Tris buffer was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey.

GAF carbonyl-iron powder grade SF was obtained from Chemical Development of Canada Ltd., Toronto, Ontario.

Sodium nitrite was obtained in crystalline form from Fisher Scientific Co.

Ethylene glycol monomethyl ether was obtained from Fisher Scientific Co.

Alpha-naphthyl acetate was obtained in anhydrous form from Sigma Chemical Co., St. Louis, Montana.

Pararosanilin was obtained in anhydrous form from Sigma Chemical Co.

Sodium phosphate, monobasic in anhydrous form was obtained from Fisher Scientific Co.

Potassium phosphate, dibasic was obtained in crystalline form from J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Methyl green was obtained from J.T. Baker Company, Co.

Sodium acetate in anhydrous form was obtained from Fisher Scientific Co.

Hydrochloric acid (HCl) was obtained from J.T. Baker Chemical Co.

Sodium hydroxide electrolytic pellets were obtained from Fisher Scientific Co.

Methanol was obtained from Fisher Scientific Co.

Phytohemagglutinin-P (PHA) was obtained from Difco Laboratories, Detroit, Michigan, and stored at $4^{\circ}C$.

Concanavalin-A (CON-A) was obtained from Calbiochemical Co., San Diego, California, and stored at $4^{\circ}C$.

Pokeweed mitogen (PWM) was obtained from Grand Island Biological Co., Grand Island, New York/Berkley, California, and stored at -20°C.

Chicken, guinea pig, rabbit, and rat blood were obtained as needed by bleeding our own laboratory animals into syringes containing 100 units of heparin per ml of blood. The blood was stored at 4°C.

Human blood was obtained by bleeding volunteers into syringes containing 50 units of heparin per ml of blood. The blood was stored at 4°C.

Horse, ox and sheep blood in a citrate buffer solution were obtained every week from Qualicum Laboratories, Ottawa, Ontario, and stored at 4°C.

Radioactive chromium-51 ($CR^{51}$) in solution as Sodium Chromate and with a specific activity of 1 mCi per ml was obtained from New England Nuclear, Boston, Massachussetts, and stored at 4°C.

The Beckman biogamma counter and bio-vials were obtained from Beckman, Toronto, Ontario.

Sterile disposable microtiter plates (12.7 x 9.5 cm, 96 conical "V" wells) were obtained from Cynatech Laboratories Incorporated, Alexandria, Virginia.

Sterile disposable Micro Test II lids (for the microtiter plates) were obtained from Falcon Plastics, Oxnard, California.

Plastic Falcon conical centrifuge tubes (50 ml) were obtained from Falcon Plastics. These were sterilized by autoclaving before use.

Plastic sterile tubes (Falcon 2006) (17 x 100 mm) were obtained from Falcon Plastics.

Plastic sterile petri dishes (Falcon 3002) were obtained from Falcon Plastics.

All glassware was obtained from Fisher Scientific Co. It was sterilized by autoclaving before use.

Syringes - sterile disposable syringes of all sizes were obtained from Becton, Dickinson Co., Canada Ltd., Mississauga, Ontario.

Hypodermic needles - sterile Luer-Lok hypodermic needles were obtained from Becton, Dickinson Co.

Pipettes - 1, 5 and 10 ml pyrex disposable serological pipettes were obtained from Corning Glass Works, Corning, New York.

Automatic MLA pipettes (100 µl and 50 µl) were obtained from Medical Laboratory Automation, Inc., Mount Vernon, New York. Pipette tips were obtained from Fisher Scientific Co.

Haemocytometer counting chambers - AO Spencer "Bright Line" type - were obtained from Canlab, Ottawa, Ontario.

Hanging drop chambers were obtained from Fisher Scientific Co.

Rabbit IgG and IgM anti-ORBC antibodies were obtained from Cappel Laboratories, Inc., Cochranville, PA., and stored at 4°C.

Nalgene filters (45 µl) were obtained from Sybron Corporation, Rochester, New York.

The Coulter Counter used was the model ZBI made by Coulter Electronics, Hialeah, Florida.

The incubator - a National Incubator was obtained from Fisher Scientific Co. This was maintained at 37°C. The interior was humidified by distilled water in a tray on the bottom shelf. The atmosphere was maintained at an air to $CO_2$ ratio of 95:5. Compressed air was passed through a Norgren filter (Littleton, Colorado) to remove suspended oil and water.

## 3.2 METHODS

### 3.2.1 PREPARATION OF REAGENTS

Medium 199, RPMI-1640, CMRL-1066, Hank's balanced salt solution. Penicillin, streptomycin and Hepes buffer were added to these solutions to final concentrations of 100 I.U. penicillin per ml, 100 µg streptomycin per ml and 25 mM Hepes buffer.

Fetal calf serum (FCS) was complement inactivated by heating in a 56°C water bath for 30 minutes.

The Ficoll-Hypaque density gradient solution (F-H) consisted of an 8 per cent solution of Ficoll in distilled water which was adjusted to a specific gravity of 1.077 at 18°C by the addition of Hypaque. The solution was sterilized by filtration through a 45 µ Nalgene filter and stored at 4°C.

The dextran solution consisted of 6 per cent Dextran T 500 in saline. The solution was sterilized by autoclaving and stored at 4°C.

Tris-buffered ammonium chloride. Tris buffer (2.059 g) was dissolved in 100 ml of distilled water and the pH of this preparation adjusted to 7.65 with HCl. Ammonium chloride (8.3 g) was dissolved in one litre of distilled water. One hundred ml of the Tris buffer solution was then mixed with 900 ml of the ammonium chloride solution and the pH of the mixture was adjusted to 7.2 with HCl. The mixture was sterilized by filtration through a 45 µ Nalgene filter and stored at 4°C.

Phosphate buffer (0.1M), pH 7.6, was prepared by mixing 2,000 ml of 0.1M monobasic sodium phosphate with 190 ml of 0.1M dibasic potassium phosphate.

The methyl green solution consisted of 1 per cent methyl green w/v and 1.4 per cent sodium acetate w/v in distilled water. It was adjusted to pH 4.2 with concentrated acetic acid.

The pararosanilin solution was made by mixing together 1g of pararosanilin, 20 ml of distilled water and 5 ml of concentrated HCl.

### 3.2.2 PREPARATION OF EFFECTOR CELLS

The various leukocyte preparations were isolated from the heparinized blood (50 units heparin per ml blood) of human volunteers.

#### 3.2.2.1 Mononuclear cells (MC)

Ten ml of blood were diluted with an equal volume of physiological saline, and layered on 10 ml of Ficoll-Hypaque (F-H) (specific gravity 1.077) in 50 ml glass conical tubes. The tubes were centrifuged at 400 g for 30 minutes at 18°C. The interface mononuclear cells were collected, pooled, washed three times in saline, and resuspended in M199.

#### 3.2.2.2 Unfractionated leukocytes

Thirty-three ml of blood were mixed with 17 ml of 6 per cent Dextran T 500. The cells were permitted to sediment in a graduated cylinder at room temperature for about 30 to 45 minutes. Once the erythrocytes had settled to the bottom, the leukocyte rich upper layer was collected and the cells were washed once in saline.

In order to lyse contaminating erythrocytes, the leukocytes were suspended in 10 ml of tris-buffered ammonium chloride and incubated for 5 minutes in a 37°C water bath. The cells were then washed three times in saline and resuspended in M199.

3.2.2.3 Polymorphonuclear cells (PC)

Unfractionated leukocytes, which had not been treated with ammonium chloride, were suspended in M199 at a concentration of between 5 and 10 x $10^6$ cells per ml. Twenty ml of this suspension were layered on 10 ml of Ficoll-Hypaque and centrifuged as described for the isolation of mononuclear cells. The pelleted polymorphs from 2 tubes were pooled and washed once in saline. Contaminating erythrocytes were lysed as described for the unfractionated leukocytes. The cells were then washed three times in saline and resuspended in M199.

3.2.2.4 Monocyte-depleted mononuclear cells (MDMC)

Monocyte-depleted mononuclear cells were isolated by the use of the carbonyl-iron magnet technique. The mononuclear cells were suspended in sterile disposable tubes (Falcon 2006) in 5 ml serum-saline (1:1) (2 x $10^6$ cell per ml) containing 2 mg carbonyl-iron filings per ml. The tubes were placed on a tissue culture rotator and the cells were incubated in 5 per cent $CO_2$ in air at 37°C for 30 minutes. The cells were then placed over a magnet and the iron containing cells were pulled to the bottom of the tube as a pellet. The suspended non-phagocytic cells were carefully pipetted off, passed over the magnet two more times, washed twice in saline, and resuspended in M199.

3.2.2.5 E, EA and EAC rosette enriched and depleted mononuclear cells

E, EA, and EAC indicator cells form rosettes with mononuclear cells which have surface membrane receptors for sheep erythrocytes ($ER^+$ cells), FcG ($FcGR^+$ cells) and complement ($C'3R^+$ cells), respectively. After centrifugation on a Ficoll-Hypaque density gradient, the rosetted cells sediment to the bottom of the tube and constitute the pelleted cells (or pellet). The non-rosetted cells remain at the interface. In this manner, mononuclear cells and monocyte-depleted mononuclear cells were separated on the basis of their surface receptors.

3.2.2.5.1 E indicator cells (SRBC)

Sheep erythrocytes (SRBC) were washed three times in saline and resuspended in HBSS at a concentration of $10^8$ cells per ml.

3.2.2.5.2 EA indicator cells (IgG sensitized ORBC)

The procedure is similar to that described by Froland, Wisloff and Michaelsen (93). Ox erythrocytes (ORBC) were washed three times in saline and resuspended in HBSS at a concentration of $5 \times 10^8$ cells per ml. This suspension was mixed with an equal volume of rabbit IgG anti-ORBC antibodies (used at a sub-agglutinating concentration in HBSS) and incubated in a 37°C water bath for 60 minutes. The mixture was centrifuged at 500 g for 10 minutes. The pelleted IgG-sensitized ORBC were washed three times in saline and then resuspended in HBSS at a concentration of $10^8$ cells per ml.

3.2.2.5.3 EAC indicator cells (IgM-sensitized ORBC complexed with C'3)

The procedure is similar to that described by Ross, Rabellino, Polley and Grey (94). ORBC were washed three times in saline and resuspended in HBSS at a concentration of $5 \times 10^8$ cells per ml. This suspension was mixed with an equal volume of rabbit IgM anti-ORBC antibodies (used at a sub-agglutinating concentration in HBSS) and incubated in a 37°C water bath for 30 minutes. The mixture was centrifuged at 500 g for 10 minutes. The pelleted IgM-sensitized ORBC were washed three times in saline and then resuspended to their original volume in HBSS. This suspension was mixed with an equal volume of human serum (used as a source of complement (C'3) at a concentration which produced minimal lysis of the IgM sensitized ORBC), and incubated in a 37°C water bath for 30 minutes. The mixture was centrifuged at 500 g for 10 minutes. The pelleted IgM-C'3-sensitized ORBC were washed three times in saline and then resuspended in HBSS at a concentration of $10^8$ cells per ml.

ORBC are used for the preparation of both EA and EAC indicator cells since ORBC, unlike SRBC, do not form spontaneous rosettes with human mononuclear cells. Only antibody and antibody-complement sensitized ORBC (EA and EAC, respectively) can form rosettes.

It should be pointed out that neither the IgM anti-ORBC antiserum, nor the human serum used as the source of complement,

in the concentrations used in the preparation of EAC, are capable of independently sensitizing the ORBC sufficiently to impart to them the ability to form rosettes. This can be taken as evidence that no EA indicator cell are formed.

3.2.2.5.4 Isolation of mononuclear cells depleted of or enriched for E, EA and EAC rosette-forming cells (RFC) ($ER^+$ cells, $FcGR^+$ cells, and C'3 $R^+$ cells, respectively)

Three ml of HBSS containing 3 x $10^7$ mononuclear effector cells were mixed with 3 ml HBSS containing $10^9$ washed indicator cells. In the cases of the EA and EAC rosetting cells, the indicator and effector cells were incubated at 37°C for 15 minutes, centrifuged at 150 g for 5 minutes, gently resuspended, layered over 3 ml Ficoll-Hypaque, and centrifuged at 400 g at 18°C for 30 minutes. In the case of the E rosetting cells, the indicator-effector cell mixture was incubated at 37°C for 15 minutes, centrifuged at 800 rpm for 5 minutes, and incubated at 4°C for 2 hours or overnight before centrifugation on the Ficoll-Hypaque density gradient was carried out. The interface cells (RFC-depleted mononuclear cells) and the pellet cells (RFC-enriched mononuclear cells) were collected. The latter cells were incubated in tris-buffered ammonium chloride for 10 minutes at 37°C in order to lyse the indicator

erythrocytes. Both cell preparations were washed three times in saline and resuspended in M199. Monocyte-depleted mononuclear effector cells were also separated into RFC-enriched and RFC-depleted cells as described above for unfractionated mononuclear cells.

3.2.2.5.5 Microscopic characterization of the RFC-enriched and RFC-depleted mononuclear cells on the basis of their surface membrane receptors

In order to determine the effectiveness of the rosetting procedures to separate distinct cell types, cells from each of the various RFC-enriched and RFC-depleted cell suspensions were analyzed microscopically with respect to their abilities to form rosettes with E, EA, and EAC indicator cells. One tenth of a ml of indicator cells was mixed with 0.1 ml of the appropriate mononuclear cell preparation (adjusted to a concentration of $3 \times 10^6$ cells per ml). The mixtures were incubated at $37^{\circ}C$ for 15 minutes and centrifuged at 150 g for 5 minutes. An additional overnight incubation at $4^{\circ}C$ (or 2 hours on ice) was carried out with the mixtures containing the E indicator cells. One drop of 3 per cent methylene blue (w/v in HBSS) was added to each tube. The cells were gently resuspended and analyzed in a haemocytometer chamber under a light microscope. The percentage of cells which formed rosettes (leukocytes surrounded by three or more tightly adherent indicator RBC) was recorded.

3.2.2.6 Dilution of the effector cells for use in the MICC assay

Prior to their use in the assay, all of the various preparations of effector cells were counted in a Coulter Counter and diluted to the desired concentration in M199. If an effector to target cell ratio of 10:1 was required, then the effector cells were diluted to $4 \times 10^6$ cells per ml; if 5:1 was required, then the effector cells were diluted to $2 \times 10^6$ cells per ml; and so on. One hundred µl aliquots of the effector cell suspensions were added to the assay wells. Since the final volume of each well was 250 µl, the cells were diluted by an additional factor or 2.5.

3.2.2.7 Non-specific esterase staining of the effector cells

In order to determine the relative proportions of polymorphs, lymphocytes and monocytes in the effector cell preparations, smears were made and stained for non-specific esterase. This technique for the identification of cells containing the esterase enzyme was developed by Yam, Li and Crosby (95). It involves treating slide preparations with the substrate alpha-napthyl acetate and the dye pararosanilin. The esterase enzyme splits the substrate producing a napthyl end product which becomes stained by the dye. The cells are then counterstained with methyl green

which allows the cytoplasm and the nuclei of the cells to be visualized.

The esterase staining solution was prepared as follows: equal volumes of pararosanilin solution and 4 per cent sodium nitrite were mixed; after exactly one minute 6 volumes of this mixture were added to 5 volumes of ethylene glycol monomethyl ether containing 20 mg of alpha-napthyl acetate per ml; 90 volumes of phosphate buffer of pH 7.6 were added to the mixture and the pH was adjusted to 6.1 with HCl; the buff-coloured precipitate which developed was removed by filtration.

Slide preparations of cells in serum were rapidly dried using cold air from a hair dryer. Each slide was covered with the esterase staining solution for 45 minutes at room temperature. The slides were then washed with water and dried and fixed in methanol for two minutes. After the methanol had evaporated, the slides were counter-stained with a 1 per cent solution of methyl green for two minutes and then washed with water.

The slides were examined under the light microscope. The cytoplasm of monocytes (esterase-positive cells) stains reddish brown, whereas the cytoplasm of lymphocytes (esterase-negative cells) stains pale yellow. Polymorpho-nuclear cells have a pale bluish-red cytoplasm and characteristic segmented nuclei.

### 3.2.3 PREPARATION OF ERYTHROCYTE TARGET CELLS

Chicken, guinea pig, horse, human, ox, rabbit, rat, and sheep erythrocytes (CRBC, GRBC, HoRBC, HuRBC, ORBC, RabRBC, RatRBC, and SRBC, respectively) were used as targets in the MICC assay. The cells in one ml of blood, obtained from each of these animals, were washed three times in saline. One tenth of a ml of the pelleted cells was mixed with 0.9 ml M199 (a 10 per cent suspension) and counted in a Coulter Counter. The erythrocytes were labelled by incubating 0.1 ml of the 10 per cent suspension with 0.1 ml of $Cr^{51}$ (sodium chromate, 1 mCi per ml) on a rotator at 37°C in 5 per cent $CO_2$ in air for one hour. The cells were washed three times in HBSS and suspended in M199 supplemented with 12.5 per cent FCS at $4 \times 10^5$ cells per ml. One hundred µl of the target cell suspensions ($4 \times 10^4$ cells) were added to each assay well. The final volume of each well was 250 µl and thus the final concentration of target cells was $1.6 \times 10^5$ cells per ml. The final concentration of FCS was 5 per cent.

### 3.2.4 PREPARATION OF MITOGENS

#### 3.2.4.1 Phytohemagglutinin-P (PHA)

The contents of a vial (containing approximately 50 mg) were dissolved in 5 ml sterile water (undiluted PHA). This solution was diluted in M199 100 fold (stock solution).

#### 3.2.4.2 Concanavalin-A (CON-A)

The contents of a vial (containing approximately 250 mg) were dissolved in 10 ml sterile water (undiluted CON-A). This solution was diluted in M199 50 fold (stock solution).

#### 3.2.4.3 Pokeweed Mitogen (PWM)

The contents of a vial (containing approximately 50 mg) were dissolved in 10 M199 (undiluted PWM or stock solution).

Fifty µl of each of the stock solutions were added to assay wells containing 200 µl of medium, effector cells and target cells. Thus the final concentrations of each of the mitogens were 20, 100 and 1000 µg per ml for PHA, CON-A and PWM, respectively.

### 3.2.5 AGGLUTINATION OF ERYTHROCYTE TARGET CELLS

Serial dilutions (0.5 ml) of the PHA, CON-A and PWM mitogens made up in M199 were carried out in 10 x 75 mm glass tubes to give the following mitogen dilutions: 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048. One ml of unlabelled erythrocyte target cells ($4 \times 10^6$ cells) and 1 ml of M199 were added to each tube. Following a 24 hour incubation at 37°C, the tubes were gently shaken and inspected for visible agglutination.

### 3.2.6 MICC CYTOTOXIC ASSAY

Cultures were set up in duplicate in the wells of microtiter plates. One hundred µl of effector cells (4, 2 and 1 x $10^5$ cells) were mixed with 100 µl of target cells (4 x $10^4$ cells) (yielding effector to target cell ratios of 10:1, 5:1 and 2.5:1) and 50 µl of the mitogen stock solutions. Controls consisted of wells containing; i) M199 (100 µl) instead of the effector cells (yielding the effector to target cell ratio of 0:1); and ii) M199 (50 µl) instead of the mitogen solution.

Unless stated otherwise, all assays employed; i) PHA at 20 µg per ml, CON-A at 100 µg per ml or PWM at 1000 µg per ml, ii) a culture time of 24 hours; and iii) M199 supplemented with FCS at a final concentration of 5 per cent. The culture plates were incubated at 37°C in 5 per cent $CO_2$.

At the end of the incubation period, the plates were centrifuged at 500 g for 10 minutes. Aliquots of the culture supernatants (100 µl) were transferred to Beckman Bio-vials and counted for 1 minute in a Beckman Biogamma Counter.

### 3.2.7 CALCULATIONS

The data were recorded as counts per minute (cpm) per culture and the amount of cytotoxicity was expressed as the cytotoxic index (CI). This was calculated as follows:

$$CI = \frac{\text{cpm (cells + mitogen)} - \text{cpm (cells alone)}}{\text{cpm (total)} - \text{cpm (cells alone)}} \times 100$$

The percentage of spontaneous release (SR) of $Cr^{51}$ from the targets was expressed as follows:

$$SR = \frac{\text{cpm (cells alone)}}{\text{cpm (total)} - \text{cpm (cells alone)}} \times 100$$

Cpm (cells + mitogen) refers to the $Cr^{51}$ detected in the supernatants of cultures containing effector cells, $Cr^{51}$-labelled target cells and mitogen solution.

Cpm (cells alone) refers to the $Cr^{51}$ detected in supernatants of cultures containing effector cells, $Cr^{51}$-labelled target cells and M199 instead of mitogen solution.

Cpm (total) refers to the $Cr^{51}$ in the resuspended contents of wells containing only target cells and culture medium.

# CHAPTER 4

# EXPERIMENTAL RESULTS

## 4.1 INVESTIGATION OF ERYTHROCYTES OF DIFFERENT SPECIES AS POTENTIAL TARGETS IN THE MICC REACTION.

### 4.1.1 OBJECTIVES

CRBC are conventionally employed as erythrocyte targets in studies of the MICC reaction using human effector cells (3,5,6,7,10,15,16,18,19,20,22,23,24,25,26,29,30,31,33,34,35). However, no systematic investigations have as yet been reported concerning the suitability of erythrocytes of other species as targets. The objectives of this study were; i) to establish which of eight different erythrocytes (CRBC, ORBC, GRBC, HoRBC, HuRBC, RabRBC, RatRBC, and SRBC) could function as suitable targets in the PHA, CON-A and PWM mediated MICC reactions; and ii) to determine whether or not these mitogens are selective with respect to the different erythrocyte targets.

### 4.1.2 PROTOCOL

The protocol for this investigation is outlined diagramatically in Figure 1. Briefly, unfractionated leukocytes in the presence of each of the three mitogens, were assayed with respect to their ability to mediate MICC cytolysis of the various erythrocytes referred to above. The leukocytes, erythrocytes and mitogens were prepared and the assays were carried out as described in Chapter 3.

The mitogen concentration, assay time and culture medium employed for these experiments were those that had been shown to provide optimal culture conditions for the assay (see Section 4.2).

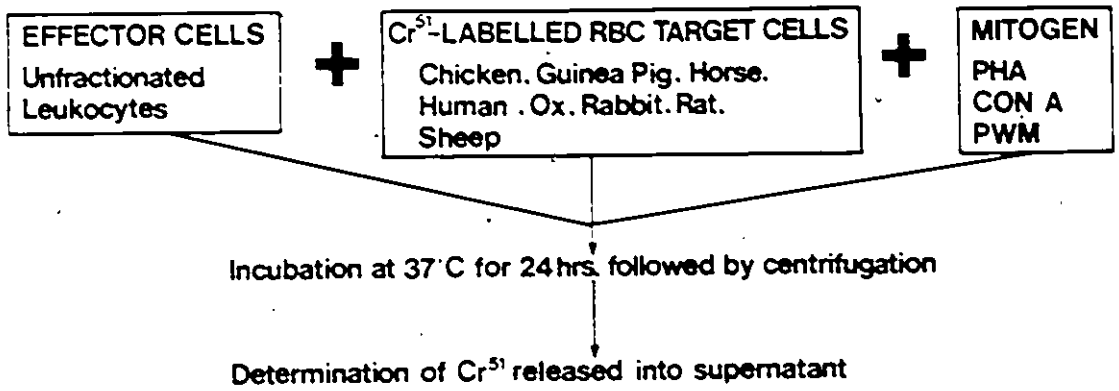


Figure 1

Protocol to determine the suitability of various types of erythrocytes as targets in the MICC assay.

## 4.1.3 RESULTS AND DISCUSSION

### 4.1.3.1 The PHA-mediated MICC reaction

As can be seen in Table 3, the unfractionated leukocytes were able to cause substantial lysis of only CRBC in the presence of PHA. SRBC, which have only occasionally been used as targets by other investigators (14,29), were lysed to a much lesser extent in comparison to CRBC and are therefore less suitable as target cells for the assay. The other erythrocytes tested (GRBC, HoRBC, HuRBC, ORBC, RabRBC, and RatRBC) were not lysed at all by the effector leukocytes and are, therefore, poor targets in this assay. Therefore, only CRBC were employed in subsequent investigations of the PHA-mediated MICC reaction.

### 4.1.3.2 The Con-A-mediated MICC reaction

In the presence of CON-A, the unfractionated leukocytes were able to lyse not only CRBC but GRBC, HoRBC, RabRBC, and RatRBC as well (Table 3). All of these erythrocytes were therefore used as targets in subsequent investigations of the CON-A mediated MICC reaction. MICC activity against the other erythrocytes was low (SRBC) or virtually absent (HuRBC and ORBC).

4.1.3.3 The PWM-mediated MICC reaction

As can be seen in Table 3, the unfractionated leukocytes were able to lyse RabRBC and to a much lesser extent CRBC and RatRBC in the presence of PWM. GRBC, HoRBC, HuRBC, ORBC, and SRBC were not lysed. Although CRBC have been used as targets in the PWM-mediated reaction using animal effector cells (16), the results presented in Table 3 indicate that RabRBC are better suited as target cells for the assay using human effector cells. In view of these results, only RabRBC were employed as targets in all subsequent investigations of the PWM-mediated MICC reaction. The other erythrocytes (CRBC, GRBC, HoRBC, HuRBC, ORBC, RatRBC, and SRBC) were considered to be unsuitable as targets for the assay.

TABLE 3

ERYTHROCYTE TARGET CELL SPECIFICITY IN MITOGEN (PHA, PWM AND CON-A) INDUCED CELLULAR CYTOTOXICITY (MICC)

| Erythrocyte target cell | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of unfractionated leukocytes* | | | | | |
|---|---|---|---|---|---|---|---|
| | | Experiment no. 1 | | | Experiment no. 2 | | |
| | | PHA | CON-A | PWM | PHA | CON-A | PWM |
| Chicken (CRBC) | 20:1 | 78 | 61 | 22 | 82 | 73 | 26 |
| | 10:1 | 76 | 54 | 27 | 82 | 75 | 19 |
| | 5:1 | 69 | 47 | 32 | 78 | 65 | 14 |
| | 0:1 | -17 | 3 | -1 | 0 | 0 | 0 |
| Guinea Pig (GRBC) | 20:1 | 0 | 51 | 0 | 2 | 58 | 1 |
| | 10:1 | 0 | 45 | 1 | 0 | 45 | 1 |
| | 5:1 | 0 | 38 | 0 | 0 | 31 | 0 |
| Horse (HoRBC) | 20:1 | 4 | 83 | 0 | 4 | 89 | 0 |
| | 10:1 | 5 | 76 | 1 | 4 | 80 | 0 |
| | 5:1 | 5 | 64 | 0 | 3 | 68 | 0 |
| | 0:1 | 2 | 2 | 1 | 4 | 1 | 1 |
| Human (HuRBC) | 20:1 | 1 | 2 | 2 | 0 | 9 | 3 |
| | 10:1 | 1 | 2 | 1 | 0 | 8 | 2 |
| | 5:1 | 0 | 1 | 0 | 0 | 6 | 0 |
| | 0:1 | 0 | 0 | 1 | 0 | 0 | 0 |
| Ox (ORBC) | 2:1 | 0 | 0 | 0 | 1 | 2 | 0 |
| | 10:1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 5:1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0:1 | 0 | 0 | 1 | -4 | -3 | -3 |
| Rabbit (RabRBC) | 20:1 | 0 | 83 | 61 | 2 | 83 | 59 |
| | 10:1 | 0 | 80 | 64 | 0 | 79 | 64 |
| | 5:1 | 0 | 60 | 61 | 2 | 75 | 74 |
| | 0:1 | -3 | -3 | -5 | 0 | -5 | -5 |
| Rat (RatRBC) | 20:1 | 3 | 56 | 18 | 1 | 42 | 11 |
| | 10:1 | 1 | 43 | 12 | 0 | 37 | 7 |
| | 5:1 | 1 | 35 | 5 | 1 | 31 | 3 |
| Sheep (SRBC) | 20:1 | 9 | 13 | 0 | 17 | 24 | 5 |
| | 10:1 | 9 | 6 | 0 | 22 | 36 | 0 |
| | 5:1 | 8 | 1 | 0 | 20 | 17 | 0 |
| | 0:1 | 0 | -1 | 0 | -1 | -3 | 0 |

*PHA, CON-A and PWM were used at the concentrations of 20, 100 and 1000 ug per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours.

### 4.1.4 SUMMARY AND CONCLUSIONS

Unfractionated human leukocytes were assayed for PHA, CON-A and PWM mediated MICC activity against eight different erythrocyte target cells (CRBC, GRBC, HoRBC, HuRBC, ORBC, RabRBC, RatRBC and SRBC). It was found that; i) PHA could mediate the lysis of CRBC and to a much lesser extent SRBC; ii) CON-A could mediate the lysis of CRBC, GRBC, HoRBC, RabRBC, and RatRBC and to a much lesser extent SRBC; and iii) PWM could mediate the lysis of RabRBC and to a much lesser extent CRBC and RatRBC.

## 4.2. THE ESTABLISHMENT OF OPTIMAL CULTURE CONDITIONS FOR THE MICC ASSAY

### 4.2.1 OBJECTIVE

The purpose of this investigation was to establish the optimal culture conditions for the PHA, CON-A and PWM mediated MICC assays.

### 4.2.2 PROTOCOL

MICC assays were carried out and compared using culture conditions which varied with respect to the mitogen concentration, effector to target cell ratio, culture time, culture medium, and the concentration of FCS in the culture medium.

Unfractionated leukocytes were used as effector cells in this series of experiments. The erythrocytes that had previously been shown to be most suitable targets for the MICC assay (Section 4.1) were employed as targets. CRBC were used as the target cells in the PHA-mediated MICC assay; CRBC, HoRBC and RabRBC were used as the target cells in the CON-A-mediated MICC assay; and RabRBC were used as target cells in the PWM-mediated MICC assay.

The effector cells, target cells and mitogens were prepared and the assays were carried out as outlined in Chapter 3.

## 4.2.3 RESULTS AND DISCUSSION

### 4.2.3.1 The PHA-mediated MICC reaction

Table 4 shows the results of MICC assays carried out using various concentrations of PHA (2 to 100 µg per ml) at various effector to target cell ratios (0.3:1 to 80:1). Significant CRBC target cell lysis occurred over the whole range of PHA concentrations and effector to target cell ratios. However, cytolysis was optimal when PHA was used at 8, 20 and 40 µg per ml, and when the effector to target cell ratios were 10:1 and 20:1.

Results of time course experiments indicate that cytotoxicity is detected by 2 hours and is maximum at 18 to 24 hours (Table 5). Cytotoxicity appears to diminish over longer periods in culture, but this is a technicality due to the large spontaneous release (SR) which takes place over periods of time longer than 24 hours.

Assays were set up and compared using three different media (M199, RPMI and CMRL). As can be seen in Table 6, all three were similar in their capacity to provide favourable conditions for the MICC reaction. However, M199 is the preferred medium since the target cells were stable in this medium in the absence of the leukocyte effector cells whereas they were unstable in media RPMI and CMRL.

Table 7 shows the effect of varying the concentration of FCS in the assay. Cytotoxicity was similar in assays containing M199 supplemented with 0, 1, 2.5, 5, and 10 per cent FCS, and was reduced when FCS was used at higher concentrations (20 and 30 per cent FCS). With 0, 1 and 2.5 per cent FCS in the medium, considerable target cell lysis occurred in the control cultures in which effector cells were excluded, and this lysis was higher in the presence than in the absence of PHA. On the other hand, this effector cell-independent lysis did not occur when the concentration of FCS in the assay was adjusted to 5 to 10 per cent. Since the primary objective of the investigation was to define the degree of lysis of target cells effected by leukocytes in the presence of the mitogen, and not that induced non-specifically by the mitogen itself, the concentration of the FCS in all subsequent experiments was adjusted to 5 per cent.

Thus, assays employing; i) PHA at 20 µg per ml; ii) an effector to target cell ratio of at least 10:1; iii) a culture time of 24 hours; and iv) M199 supplemented with FCS to a final concentration of 5 per cent, provide optimal, or at least very favourable conditions for assays of the cytotoxic activity of unfractionated leukocytes. These culture conditions were employed in all subsequent experiments.

4.2.3.2 The CON-A-mediated MICC reaction

Table 8 shows the results of MICC assays of unfractionated leukocytes which were carried out using various concentrations of CON-A and FCS. CRBC, HoRBC and RabRBC were employed as target cells. Cultures containing 75 to 200 µg per ml of CON-A and 5 per cent FCS were found to be most suitable for the CON-A-mediated MICC assay with respect to these three target cells. These conditions provided for optimal target cell lysis in the presence of effector cells, as well as minimal cytolysis of the target cells in the absence of effector cells or mitogen. In view of these results, subsequent assays employed 100 µg per ml of CON-A and 5 per cent FCS.

4.2.3.3 The PMW-mediated MICC reaction

Assays of unfractionated leukocytes were carried out using various concentrations of PWM and FCS. RabRBC were employed as target cells (Table 9). Assays containing 1000 µg per ml of PWM and 5 per cent FCS were found to be most suitable in terms of providing conditions for optimal target cell lysis in the presence of effector cells, and minimal cytolysis of the target cells in the absence of effector cells or mitogens. In view of these results, cultures for the PWM-mediated MICC assay employed 1000 µg per ml of PWM and 5 per cent FCS.

TABLE 4

THE EFFECTS OF VARYING THE CONCENTRATION OF PHA AND EFFECTOR CELLS ON THE PHA MEDIATED MICC REACTION OF HUMAN CIRCULATING UNFRACTIONATED LEUKOCYTES (CHICKEN ERYTHROCYTE TARGETS)

| Exp. No. | Conc. of PHA (µg per ml) | The MICC Cytotoxic Index (CI) of circulating unfractionated leukocytes which have been cultured with CRBC targets at the following effector to target cell ratios.* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 80:1 | 40:1 | 20:1 | 10:1 | 5:1 | 2.5:1 | 1.2:1 | 0.6:1 | 0.3:1 | 0:1 |
| 1 | 2 | 4 | 18 | 36 | 43 | 47 | 32 | 18 | 9 | 4 | 1 |
| | 4 | 17 | 35 | 52 | 58 | 52 | 47 | 30 | 17 | 8 | 1 |
| | 8 | 28 | 52 | 62 | 69 | 70 | 55 | 40 | 22 | 11 | 0 |
| | 20 | 38 | 57 | 67 | 75 | 68 | 55 | 40 | 24 | 13 | 1 |
| | 40 | 38 | 57 | 66 | 69 | 63 | 52 | 37 | 25 | 11 | 1 |
| | 100 | 42 | 57 | 65 | 60 | 49 | 40 | 26 | 15 | 8 | 0 |
| | SR | 7 | 11 | 13 | 13 | 11 | 9 | 6 | 6 | 5 | 1 |
| 2 | 2 | 2 | 7 | 22 | 36 | 37 | 25 | 12 | 4 | 4 | 1 |
| | 4 | 7 | 19 | 39 | 48 | 41 | 28 | 14 | 4 | 3 | 0 |
| | 8 | 14 | 29 | 59 | 59 | 58 | 45 | 27 | 13 | 10 | 1 |
| | 14 | 13 | 39 | 58 | 68 | 65 | 52 | 34 | 17 | 9 | 1 |
| | 20 | 16 | - | 57 | 73 | 61 | 49 | 33 | 18 | 13 | 1 |
| | 40 | 15 | 43 | 68 | 73 | 63 | 54 | 36 | 20 | 14 | 1 |
| | 100 | 32 | 63 | 70 | 65 | 52 | 41 | 26 | 13 | 7 | 1 |
| | SR | 15 | 18 | 20 | 18 | 16 | 14 | 12 | 12 | 8 | 2 |
| 3 | 2 | 2 | 14 | 32 | 40 | 37 | 22 | 13 | 8 | 3 | 0 |
| | 4 | 9 | 26 | 38 | 45 | 39 | 27 | 17 | 11 | 6 | 1 |
| | 8 | 23 | 46 | 61 | 63 | 58 | 43 | 29 | 16 | 9 | -1 |
| | 14 | 24 | 47 | 61 | 63 | 61 | 44 | 28 | 21 | 10 | 0 |
| | 20 | 19 | 38 | 53 | 67 | 61 | 45 | 34 | 20 | 12 | 0 |
| | 40 | 29 | 56 | 67 | 80 | 66 | 53 | 34 | 21 | 12 | 0 |
| | 100 | 46 | 67 | 75 | 75 | 54 | 40 | 27 | 16 | 9 | 1 |
| | SR | 2 | 3 | 3 | 2 | 3 | 3 | 2 | 2 | 3 | 2 |

* The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours.
** S.R. refers to Spontaneous Release.

TABLE 5

THE EFFECT OF TIME ON THE PHA MEDIATED MICC REACTION

OF HUMAN CIRCULATING UNFRACTIONATED LEUKOCYTES

(CHICKEN ERYTHROCYTE TARGETS)

| Culture time (hours) | The MICC Cytotoxic Index (CI) of the unfractionated leukocytes cultured with CRBC targets* | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| 2 | 47(5)** | 12(5) |
| 6 | 74(7) | 62(7) |
| 18 | 81(20) | 72(16) |
| 24 | 75(34) | 83(27) |
| 48 | 69(55) | 42(48) |

* The concentration of PHA was 20 µg per ml. The culture medium was M199 supplemented with 5% FCS. The E/T was 10:1.

** The numbers in parenthesis represent the Spontaneous Release (SR).

TABLE 6

THE EFFECT OF DIFFERENT MEDIA ON THE PHA MEDIATED MICC REACTION OF HUMAN CIRCULATING UNFRACTIONATED LEUKOCYTES (CHICKEN ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the circulating unfractionated leukocytes cultured with CRBC targets in the following media.* | | |
|---|---|---|---|---|
| | | M199 | RPMI-1640 | CMRL-1066 |
| 1 | 10:1 | 54(29)** | 52(32) | 50(30) |
| | 5:1 | 53(25) | 58(27) | 51(27) |
| | 2.5:1 | 59(22) | 58(19) | 51(21) |
| | 1.2:1 | 39(17) | 43(15) | 41(16) |
| | 0.6:1 | 26(13) | 24(13) | 21(15) |
| | 0.3:1 | 11(9) | 17(12) | 13(10) |
| | 0:1 | 1(3) | 25(48) | 41(10) |
| 2 | 10:1 | 54(26) | 48(28) | 43(29) |
| | 5:1 | 60(21) | 50(25) | 38(30) |
| | 2.5:1 | 35(16) | 43(18) | 22(23) |
| | 1.2:1 | 14(15) | 18(16) | 7(17) |
| | 0.6:1 | 3(11) | 12(9) | 4(9) |
| | 0.3:1 | 2(8) | 6(8) | 1(8) |
| | 0:1 | 1(3) | 25(48) | 41(10) |
| 3 | 10:1 | 60(39) | 46(41) | 42(35) |
| | 5:1 | 62(38) | 54(38) | 42(38) |
| | 2.5:1 | 53(33) | 51(32) | 39(33) |
| | 1.2:1 | 31(26) | 35(26) | 23(26) |
| | 0.6:1 | 13(17) | 22(14) | 14(18) |
| | 0.3:1 | 6(11) | 12(17) | 6(12) |
| | 0:1 | 1(3) | 25(48) | 41(10) |

* The concentration of PHA was 20 µg per ml. The media were supplemented with 5% FCS. The culture time was 24 hours.

** The numbers in parenthesis represent the Spontaneous Release (SR).

TABLE 7

THE EFFECTS OF DIFFERENT CONCENTRATIONS OF FETAL CALF SERUM ON THE PHA MEDIATED MICC REACTION OF HUMAN CIRCULATING UNFRACTIONATED LEUKOCYTES (CHICKEN ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell | The MICC Cytotoxic Index (CI) of circulating unfractionated leukocytes cultured with CRBC targets in M199 supplemented with fetal calf serum.* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Fetal calf serum (%) | | | | | | |
| | | 0 | 1 | 2.5 | 5 | 10 | 20 | 30 |
| 1 | 10:1 | 47(2) | 48(5)** | 48(13) | 52(12) | 47(12) | | |
| | 5:1 | 45(2) | 41(4) | 44(9) | 46(16) | 46(25) | | |
| | 2.5:1 | 27(2) | 25(3) | 26(3) | 26(7) | 26(14) | | |
| | 1.25:1 | 16(2) | 16(3) | 14(3) | 15(5) | 12(7) | | |
| | 0:1 | 35(36) | 47(20) | 20(7) | 2(3) | 0(3) | | |
| 2 | 10:1 | 48(2) | 44(3) | 38(3) | 40(2) | 39(3) | | |
| | 5:1 | 45(8) | 36(3) | 35(3) | 34(3) | 36(3) | | |
| | 2.5:1 | 22(3) | 20(3) | 19(2) | 19(2) | 16(2) | | |
| | 1.25:1 | 16(2) | 14(2) | 12(2) | 13(2) | 12(3) | | |
| | 0:1 | 30(36) | 40(25) | 28(9) | 8(3) | 3(2) | | |
| 3 | 10:1 | 64(6) | | | 55(31) | 45(30) | 32(31) | 17(33) |
| | 5:1 | 61(5) | | | 45(33) | 40(35) | 35(39) | 25(38) |
| | 2.5:1 | 28(4) | | | 24(21) | 10(32) | 11(35) | 11(34) |
| | 1.2:1 | 14(3) | | | 17(12) | 11(17) | 5(2) | 4(24) |
| | 0:1 | 48(20) | | | -1(4) | 2(3) | 1(4) | 0(4) |

* The concentration of PHA was 20 µg per ml. The culture time was 24 hours.

** The numbers in parenthesis represent the Spontaneous Release (SR).

TABLE 8

THE EFFECTS OF VARYING THE CONCENTRATIONS OF CON-A AND FETAL CALF SERUM ON THE CON-A MEDIATED MICC REACTION OF HUMAN CIRCULATING UNFRACTIONATED LEUKOCYTES (CHICKEN, HORSE AND RABBIT ERYTHROCYTE TARGETS)

| Erythrocyte target cell | Concentration of CON-A (µg per ml) | The MICC Cytotoxic Index (CI) of the unfractionated leukocytes* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Effector to target cell ratio | 10:1 | | | 5:1 | | | 2.5:1 | | | 0:1 | |
| | | Fetal calf serum (%) | 0 | 1 | 5 | 0 | 1 | 5 | 0 | 1 | 5 | 0 | 1 | 5 |
| Chicken (CRBC) | 1 | | 6 | 5 | 1 | 1 | 5 | 1 | -1 | 2 | 0 | 7 | 2 | 0 |
| | 5 | | 30 | 24 | 21 | 12 | 7 | 19 | 4 | 1 | 10 | 20 | 0 | 1 |
| | 20 | | 42 | 63 | 64 | 20 | 50 | 48 | 20 | 31 | 20 | 23 | 4 | 0 |
| | 75 | | 55 | 67 | 72 | 55 | 53 | 52 | 73 | 26 | 29 | 23 | 6 | 0 |
| | 200 | | 45 | 48 | 66 | 56 | 48 | 48 | 43 | 43 | 23 | 25 | 5 | 0 |
| | 500 | | 56 | 49 | 52 | 40 | 47 | 31 | 41 | 22 | 28 | 23 | 9 | 1 |
| | Spontaneous Release (SR) | | 15 | 19 | 6 | 16 | 8 | 6 | 7 | 14 | 10 | 20 | 32 | 3 |
| Horse (HoRBC) | 1 | | 2 | 0 | 0 | 4 | 0 | 1 | 18 | 0 | 3 | -1 | 0 | 0 |
| | 5 | | 29 | 14 | 7 | 65 | 30 | 6 | 77 | 46 | 6 | 2 | -1 | 1 |
| | 20 | | 81 | 81 | 49 | 78 | 84 | 56 | 65 | 65 | 57 | 2 | 0 | 0 |
| | 75 | | 81 | 87 | 91 | 72 | 75 | 74 | 58 | 63 | 56 | 1 | 0 | -2 |
| | 200 | | 76 | 76 | 89 | 74 | 66 | 70 | 52 | 56 | 51 | -1 | 0 | -2 |
| | 500 | | 73 | 73 | 61 | 65 | 62 | 53 | 47 | 48 | 33 | 0 | 0 | 0 |
| | Spontaneous Release (SR) | | 4 | 5 | 5 | 6 | 5 | 4 | 9 | 5 | 4 | 6 | 8 | 2 |
| Rabbit (RabRBC) | 5 | | 52 | 43 | 42 | 51 | 40 | 37 | 36 | 25 | 20 | -1 | -3 | -2 |
| | 20 | | 62 | 57 | 55 | 52 | 45 | 45 | 39 | 29 | 27 | 4 | 0 | 3 |
| | 75 | | 58 | 53 | 55 | 53 | 45 | 45 | 40 | 29 | 23 | 33 | 1 | -1 |
| | 200 | | 53 | 51 | 52 | 47 | 41 | 43 | 40 | 28 | 24 | 48 | -1 | 2 |
| | 500 | | 54 | 50 | 46 | 47 | 38 | 36 | 34 | 23 | 19 | 61 | -1 | 4 |
| | Spontaneous Release (SR) | | 14 | 11 | 11 | 13 | 11 | 10 | 13 | 10 | 11 | 21 | 18 | 6 |

* The medium was M199. The culture time was 24 hours.

TABLE 9

THE EFFECTS OF VARYING THE CONCENTRATIONS OF PWM AND FETAL CALF SERUM ON THE PWM MEDIATED MICC REACTION OF HUMAN CIRCULATING UNFRACTIONATED LEUKOCYTES (RABBIT ERYTHROCYTE TARGETS)

| Concentration of PWM (µg per ml) | The MICC Cytotoxic Index (CI) of the unfractionated leukocytes cultured with RabRBC targets* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Effector to target cell ratio | 10:1 | | | 5:1 | | | 2.5:1 | | | 0:1 | | |
| | Fetal calf serum (%) | 0 | 1 | 5 | 0 | 1 | 5 | 0 | 1 | 5 | 0 | 1 | 5 |
| 50 | | -1 | -3 | -2 | -1 | -1 | -1 | 1 | 1 | 0 | 47 | 1 | 0 |
| 500 | | 25 | 12 | 14 | 25 | 16 | 20 | 18 | 21 | 12 | 64 | 18 | -1 |
| 1000 | | 30 | 37 | 22 | 34 | 28 | 27 | 23 | 25 | 10 | 64 | 23 | -1 |
| Spontaneous Release (SR) | | 13 | 21 | 12 | 11 | 11 | 11 | 11 | 10 | 11 | 18 | 11 | 2 |

*The medium was M199. The culture time was 24 hours.

### 4.2.4 SUMMARY AND CONCLUSIONS

The MICC assay was investigated in terms of the culture conditions which provided for optimal cytolysis of the target cells. It was found that assays employing; i) PHA at 20 µg per ml, CON-A at 100 µg per ml and PWM at 1000 µg per ml; ii) a culture time of 24 hours, iii) an effector to target cell ratio of at least 10:1; and iv) M199 supplemented with FCS to a final concentration of 5 per cent, provided optimal conditions for the MICC reaction. These conditions were employed in all of the subsequent MICC assays.

## 4.3 THE IDENTIFICATION AND CHARACTERIZATION OF THE CELLS INVOLVED IN THE MICC REACTION

### 4.3.1 OBJECTIVES

The objectives of this investigation were; i) to determine whether polymorphonuclear cells, lymphocytes and monocytes are active in the MICC assay; ii) to characterize the cytotoxic cells on the basis of their surface membrane receptors for SRBC(E), FcG and complement (C'3); and iii) to determine whether the identity of the MICC effector cell(s) varies as a function of the mitogen (PHA, CON-A, PWM), or the erythrocyte target cell employed in the reaction.

### 4.3.2 PROTOCOL

The protocol for this investigation is outlined diagramatically in Figures 2 to 5. Briefly, polymorphonuclear cells, mononuclear cells (lymphocytes and monocytes) and monocyte-depleted mononuclear cells (lymphocytes) were separated and assayed for MICC activity (Figure 2). In order to characterize the cells on the basis of their surface receptors for E, FcG and C'3, mononuclear cells (untreated or monocyte-depleted) were separated into E, EA and EAC rosetting and non-rosetting cell populations (Figures 3 and 4). In addition, untreated mononuclear cells were assayed (in the PWM-mediated MICC) following their fractionation into adherent and non-adherent cell populations (Figure 5).

The erythrocytes which had previously been shown (Section 4.1) to be most easily lysed in the various MICC assays were employed as targets - CRBC with PHA; CRBC, GRBC, HoRBC and RabRBC with CON-A; and RabRBC with PWM.

The effector cells, target cells and mitogens were prepared and the assays were carried out as outlined in Chapter 3.

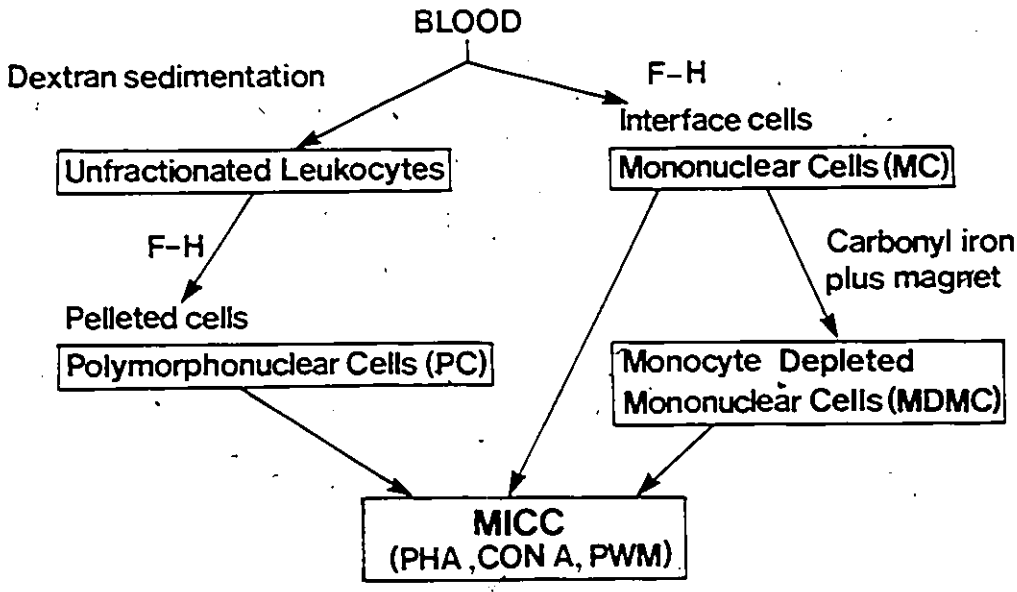


Figure 2

Protocol for the Isolation of Mononuclear cells, Monocyte - depleted Mononuclear cells and Polymorphonuclear cells.

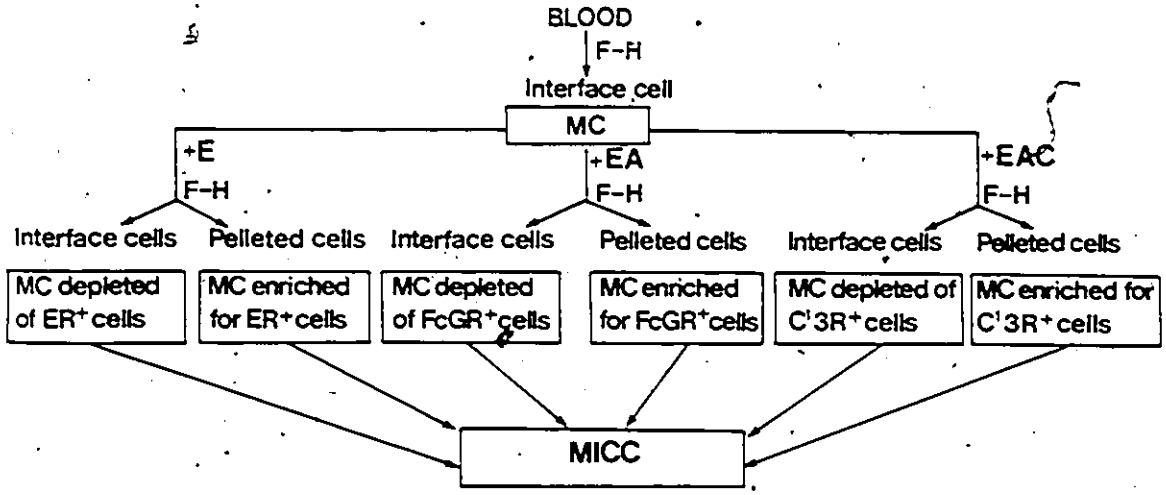


Figure 3

Protocol for the determination of the surface membrane receptors on the mononuclear MICC effector cell.

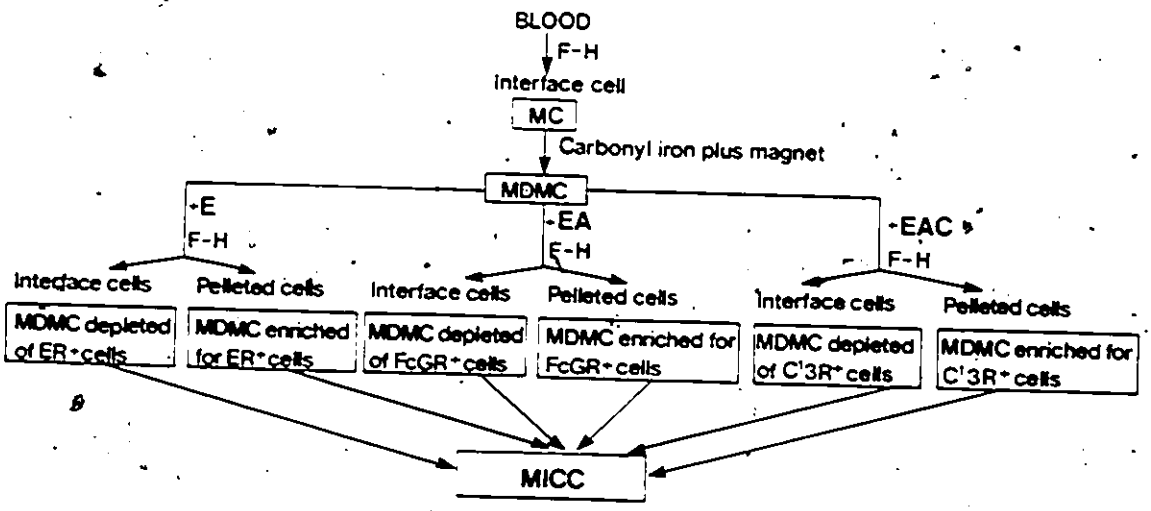


Figure 4

Protocol for the determination of the surface membrane receptors on the lymphocyte MICC effector cell.

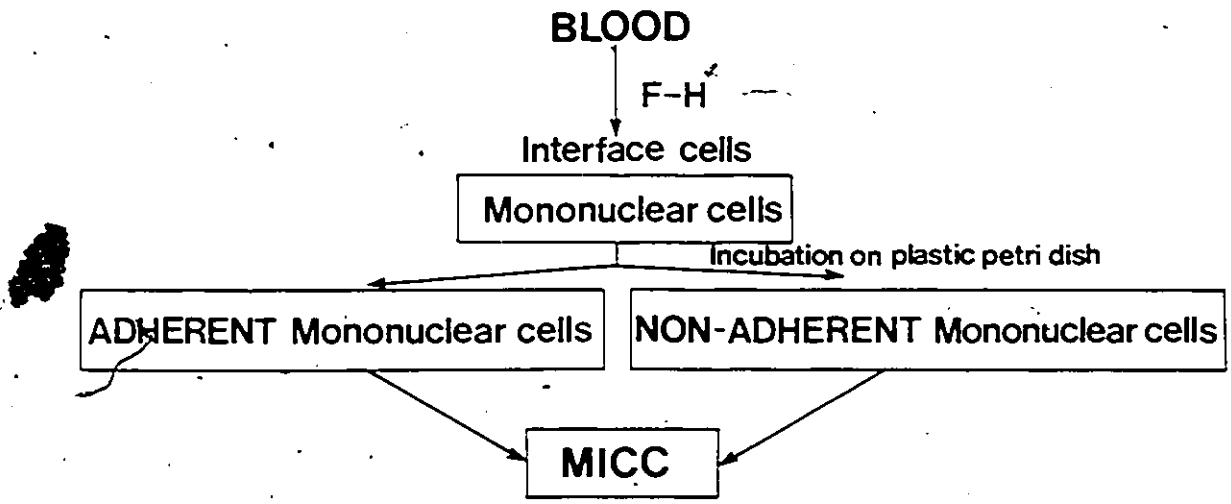


Figure 5

Protocol for the determination of the role of the monocyte in the MICC reaction.

## 4.3.3 RESULTS AND DISCUSSION

### 4.3.3.1 The PHA-mediated MICC reaction.

#### 4.3.3.1.1 The PHA-mediated MICC activity of polymorphonuclear cells, mononuclear cells and monocyte-depleted mononuclear cells.

As can be seen in Tables 10, 11 and 12, polymorphonuclear cells and mononuclear cells consistently displayed PHA-mediated MICC activity toward the CRBC targets. Monocyte-depleted mononuclear cells were almost devoid of activity.

The polymorphs were more potent as effector cells compared with the mononuclear cells, both with respect to effector to target cell ratios (Table 10), and with respect to time in culture (Table 11). For example, at the effector to target cell ratio of 1.2 to 1, the cytotoxic indices (CIs) ranged from 67 to 74 for the polymorphs, and only 12 to 27 for the mononuclear cells. At the effector to target cell ratio of 1 to 3, the CI was as high as 58 for the polymorphs, and very low or negligible for the mononuclear cells. With respect to time in culture, polymorph cytotoxic activity was apparent by 2 hours, and maximum by 18 hours. In contrast, mononuclear cell activity was demonstrable only after 6 hours in culture, and was maximum by 24 hours. Activity of the monocyte-depleted mononuclear cells was very low, even at effector to target cell ratios of 10 to 1 and when assayed over prolonged periods of time (72 hours).

Since the mononuclear cell preparations were invariably contaminated with small numbers of polymorphs (1 to 3 per cent), it was considered that the cytotoxicity exhibited by the mononuclear cell preparations could possibly be due to the contaminating polymorphs and not to the monocytes. In order to rule out any cytotoxic role for contaminating polymorphs in the mononuclear cell suspensions, the activity of mononuclear cells was compared with that of monocyte-depleted mononuclear cells which had been artificially enriched with varying numbers of polymorphs. As can be seen in Table 12, monocyte-depleted mononuclear cells which had been enriched with polymorphs to a final concentration of 1 to 3 per cent produced considerably less activity than untreated mononuclear cells. Furthermore, enrichment of the monocyte-depleted mononuclear cells with polymorphs to at least 10 per cent was required in order to simulate the activity of untreated mononuclear cells. Since polymorphs are detected only rarely at concentrations above 1 to 3 per cent in the mononuclear cell suspensions, the activity of these cells cannot be attributed to polymorphs. In addition, the time course of the cytotoxic activity of the polymorph-enriched monocyte-depleted mononuclear cells was different than that of the untreated mononuclear cells. The monocyte must therefore be considered to be *the* mononuclear cytotoxic cell in the PHA-mediated MICC cytolysis of CRBC targets.

It was concluded on the basis of these results, that polymorphonuclear cells and monocytes, but not lymphocytes, are the effector cells involved in the PHA-mediated MICC cytolysis of CRBC targets.

4.3.3.1.2 The surface membrane receptors of the cytotoxic mononuclear cells in the PHA-mediated MICC reaction.

The cytotoxic monocyte in the mononuclear cell preparation was characterized on the basis of its surface receptors (Table 13). Mononuclear cells which had been depleted of E rosette-forming cells, or enriched for EA or EAC rosette-forming cells, exhibited PHA-mediated cytotoxicity against the CRBC targets. On the other hand, mononuclear cells which had been enriched for E rosette-forming cells, or depleted of EA or EAC rosette-forming cells, exhibited little or no cytotoxicity. Thus, the effector monocyte in this assay has receptors for both FcG and complement but, not for SRBC.

4.3.3.2 The CON-A mediated MICC reaction

4.3.3.2.1 The CON-A-mediated MICC activity of polymorphonuclear cells, mononuclear cells and monocyte-depleted mononuclear cell.

As can be seen in Table 14, polymorphonuclear cells and mononuclear cells exhibited CON-A-mediated cytotoxicity against all four of the erythrocytes (CRBC, GRBC, HoRBC, and RabRBC) employed as targets in this assay. The monocyte-

depleted mononuclear cells were essentially inactive. Polymorphs were more potent as effectors of the reaction in comparison to the mononuclear cells in that fewer polymorphs were required in order to elicit any given degree of activity. These results are similar to those obtained from studies of the PHA-mediated MICC reaction. That is to say, polymorphonuclear cells and monocytes, but not lymphocytes, are the cytotoxic cells in the presence of either PHA or CON-A.

4.3.3.2.2 The surface membrane receptors of the cytotoxic mononuclear cells in the CON-A-mediated MICC reaction.

The cytotoxic mononuclear cell (monocyte) was characterized on the basis of its surface receptors. CRBC were employed as targets for the assay. As can be seen in Table 15, mononuclear cells which had been depleted of E rosette-forming cells, or enriched for EA or EAC rosette-forming cells, exhibited CON-A mediated cytotoxicity. Conversely, mononuclear cells which had been enriched for E rosette-forming cells, or depleted of EA or EAC rosette-forming cells, were inactive. Thus the effector monocyte has receptors for both FcG and complement but not for SRBC. These results are similar to those observed in the PHA-mediated MICC reaction.

4.3.3.3 The PWM-mediated MICC reaction.

4.3.3.3.1 The PWM-mediated MICC activity of polymorphonuclear cells, mononuclear cells and monocyte-depleted mononuclear cells.

The results obtained with PWM were quite distinct from those obtained with PHA and CON-A. As can be seen in Table 16, monocyte-depleted mononuclear cells, as well as untreated mononuclear cells and polymorphonuclear cells, exhibited MICC activity using the RabRBC target cells. Furthermore, mononuclear cells and monocyte-depleted mononuclear cells were more cytotoxic than the polymorphs, unlike the situation observed with either PHA or CON-A. Thus, lymphocytes and polymorphs are active in the PWM-mediated MICC reaction.

On the basis of these experiments no definite conclusions can be drawn with regard to the PWM-mediated cytotoxic activity of the monocyte. Although activity of the mononuclear cells was not diminished following the elimination of the monocytes, this does not necessarily imply that monocytes are inactive.

4.3.3.3.2 The PWM-mediated MICC activity of plastic adherent and non-adherent mononuclear cell populations

In order to determine the role of the monocyte in this cytotoxic reaction, the activity of plastic-adherent mononuclear cells was compared with that of non-adherent mononuclar cells (Table 17). The adherent cell fraction contained approximately 50 per cent lymphocytes and

50 per cent monocytes, while the non-adherent fraction consisted of greater than 95 per cent lymphocytes and less than 5 per cent monocytes. The MICC activity of the adherent cells was as great as, if not greater than, that of the non-adherent cells, and thus it is probable that monocytes, in addition to lymphocytes and polymorphs, are active in the PWM-mediated reaction.

In order to establish with absolute certainly whether monocytes are active in the presence of PWM, it would be necessary to test preparations of these cells which are not contaminated with lymphocytes. Unfortunately, none of the current procedures which are utilized for the isolation of monocytes result in a contamination with lymphocytes of less than 20 to 50 per cent. Thus, any activity of those 'purified monocytes' could be attributed to the lymphocyte contaminants.

4.3.3.3.3 The surface membrane receptors on the cytotoxic lymphocytes in the PWM-mediated MICC reaction

Monocyte-depleted mononuclear cells were depleted of or enriched for E, EA, or EAC rosette forming cells, and then assayed for PWM-mediated MICC activity against RabRBC targets. As can be seen in Table 18, all of the lymphocyte sub-populations exhibited varying degrees of cytotoxicity. Thus the cytotoxic lymphocyte in the PWM-mediated MICC reaction could not be characterized on the basis of its surface receptors for SRBC, FcG or complement.

TABLE 10

THE PHA MEDIATED MICC REACTION OF POLYMORPHONUCLEAR CELLS, MONONUCLEAR CELLS AND MONOCYTE-DEPLETED MONONUCLEAR CELLS (CHICKEN ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the following populations of cells cultured with CRBC targets* | | |
|---|---|---|---|---|
| | | Polymorphonuclear cells | Mononuclear cells | Monocyte-depleted mononuclear cells |
| | | (P:L:M = 95:5:0)** | (P:L:M = 3:90:7) | (P:L:M = 0:98:2) |
| 1 | 20:1 | 85 | 69 | 19 |
| | 10:1 | 75 | 66 | 14 |
| | 5:1 | 77 | 58 | 10 |
| | 2.5:1 | 74 | 45 | 8 |
| | 1.2:1 | 74 | 27 | 5 |
| | 0.6:1 | 69 | 15 | 1 |
| | 1:3 | 58 | 7 | 0 |
| | 0:1 | -2 | -2 | -2 |
| | | (P:L:M = 94:5:1) | (P:L:M = 1:84:15) | (P:L:M = 0:99:1) |
| 2 | 10:1 | 80 | 51 | 8 |
| | 5:1 | 75 | 37 | 6 |
| | 2.5:1 | 75 | 21 | 4 |
| | 1.2:1 | 73 | 12 | 1 |
| | 0.6:1 | 68 | 8 | 5 |
| | 1:3 | 58 | 4 | 1 |
| | 1:6 | 40 | 4 | 3 |
| | 0:1 | 7 | 7 | 7 |
| | | (P:L:M = 98:2:0) | (P:L:M = 3:72:27) | (P:L:M = 0:98:2) |
| 3 | 10:1 | 88 | 43 | 7 |
| | 5:1 | 82 | 38 | 5 |
| | 2.5:1 | 74 | 38 | 3 |
| | 1.2:1 | 80 | 17 | 2 |
| | 0.6:1 | 67 | 12 | 0 |
| | 1:3 | 37 | 1 | 0 |
| | 1:6 | 22 | 0 | 0 |
| | 0:1 | 0 | 0 | 0 |

* The concentration of PHA was 20 µg per ml. The culture medium was M199 supplemented with 5% FCS. Culture time was 24 hours.

** P:L:M = polymorphonuclear cells: lymphocytes: monocytes.

TABLE 11

THE EFFECT OF TIME ON THE PHA MEDIATED MICC REACTION OF HUMAN CIRCULATING POLYMORPHONUCLEAR CELLS, MONONUCLEAR CELLS AND MONOCYTE-DEPLETED MONONUCLEAR CELLS (CHICKEN ERYTHROCYTE TARGETS)

| Exp. no. | Culture time (hours) | The MICC Cytotoxic Index (CI) of the following populations of cells cultured with CRBC targets* | | |
|---|---|---|---|---|
| | | Polymorphonuclear cells | Mononuclear cells | Monocyte-depleted mononuclear cells |
| | | (P:L:M = 94:5:1)** | (P:L:M = 1:84:15) | (P:L:M = 0:99:1) |
| 1 | 2 | 20 | 5 | 0 |
| | 6 | 62 | 17 | 1 |
| | 18 | 82 | 33 | 5 |
| | 24 | 80 | 51 | 8 |
| | 48 | 91 | 61 | 12 |
| | 72 | 74 | 61 | 17 |
| | | (P:L:M = 98:2:0) | (P:L:M = 3:72:25) | (P:L:M = 0:98:2) |
| 2 | 2 | 19 | 2 | 2 |
| | 6 | 63 | 9 | 2 |
| | 18 | 81 | 23 | 4 |
| | 24 | 82 | 38 | 7 |
| | 48 | 64 | 48 | 6 |
| | 72 | 38 | 61 | 9 |
| | | (P:L:M = 91:9:0) | (P:L:M = 1:88:11) | (P:L:M = 0:98:2) |
| 3 | 2 | 53 | 11 | 2 |
| | 6 | 79 | 29 | 6 |
| | 18 | 87 | 58 | 10 |
| | 24 | 85 | 67 | 17 |
| | 48 | 87 | 86 | 35 |
| | 72 | 81 | 78 | 44 |

* The concentration of PHA was 20 µg per ml. The culture medium was M199 supplemented with 5% FCS. The effector to target cell ratio was 10:1.

** P:L:M = polymorphonuclear cells: lymphocytes: monocytes.

TABLE 12

THE PHA MEDIATED MICC REACTION OF HUMAN CIRCULATING MONOCYTE-DEPLETED MONONUCLEAR CELLS WHICH HAVE BEEN ENRICHED WITH POLYMORPHONUCLEAR CELLS (CHICKEN ERYTHROCYTE TARGETS)

| Exp. no. | Effector to Target Cell Ratio | The MICC Cytotoxic Index (C I) of the various leukocyte populations.* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Polymorphonuclear cells | Mononuclear cells | Monocyte-depleted mononuclear cells | Monocyte-depleted mononuclear cells to which the following per centages of polymorphonuclear cells have been added | | | | | |
| | | | | | 1 | 3 | 5 | 7 | 10 | 15 |
| | | (P:L:M = 94:5:1)** | (P:L:M = 2:78:20) | (P:L:M = 0:98:2) | | | | | | |
| 1 | 10:1 | 80 | 63 | 11 | 16 | 27 | 37 | - | - | - |
| | 5:1 | 75 | 49 | 7 | 13 | 19 | 27 | - | - | - |
| | 2.5:1 | 70 | 35 | 5 | 6 | 11 | 16 | - | - | - |
| | 1.2:1 | 60 | 28 | 2 | 4 | 7 | 8 | - | - | - |
| | 0.6:1 | 44 | 13 | 3 | 2 | 4 | 5 | - | - | - |
| | | (P:L:M = 96:4:0) | (P:L:M = 3:80:17) | (P:L:M = 0:98:2) | | | | | | |
| 2 | 10:1 | 77 | 51 | 18 | 22 | 33 | 39 | - | 52 | - |
| | 5:1 | 77 | 43 | 11 | 15 | 20 | 27 | - | 36 | - |
| | 2.5:1 | 74 | 27 | 6 | 8 | 11 | 17 | - | 23 | - |
| | 1.2:1 | 57 | 13 | 3 | 4 | 5 | 8 | - | 13 | - |
| | 0.6:1 | 36 | 9 | 2 | 1 | 3 | 6 | - | 7 | - |
| | | (P:L:M = 96:4:0) | (P:L:M = 1:84:15) | (P:L:M = 0:99:1) | | | | | | |
| 3 | 20:1 | 72 | 54 | 9 | 14 | 33 | - | 51 | 59 | 72 |
| | 10:1 | 73 | 44 | 5 | 10 | 21 | - | 41 | 45 | 57 |
| | 5:1 | 63 | 37 | 4 | 8 | 16 | - | 29 | 39 | 45 |
| | 2.5:1 | 58 | 25 | 4 | 4 | 11 | - | 20 | 31 | |
| | 1.2:1 | 44 | 14 | 4 | 2 | 7 | - | 10 | 11 | 17 |

* The concentration of PHA was 20 µg per ml. The culture medium was M199 supplemented with 5% FCS. Culture time was 24 hours.

** P:L:M = Polymorphonuclear cells: lymphocytes: monocytes.

TABLE 13

THE PHA MEDIATED MICC REACTION OF SUBPOPULATIONS OF HUMAN CIRCULATING MONONUCLEAR CELLS (CHICKEN ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the different subpopulations of mononuclear cells cultured with CRBC targets.* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Unfractionated mononuclear cells (MC) | MC enriched for E-RFC | MC depleted of E-RFC | MC enriched for EA-RFC | MC depleted of EA-RFC | MC enriched for EAC-RFC | MC depleted of EAC-RFC |
| | | ** E: 72<br>EA: 18<br>EAC:12 | E: 86<br>EA: 4<br>EAC: 4 | E: 6<br>EA: 48<br>EAC:69 | E: 27<br>EA: 42<br>EAC:25 | E: 79<br>EA: 1<br>EAC:20 | E: 15<br>EA: 24<br>EAC:44 | E: 84<br>EA: 2<br>EAC: 4 |
| 1 | 5:1 | 39 | 2 | 52 | 30 | 2 | 51 | 2 |
| | 2.5:1 | 34 | 1 | 48 | 21 | 2 | 38 | 1 |
| | 1.25:1 | 14 | 0 | 31 | 17 | 0 | 22 | 0 |
| | 0.6:1 | 7 | 0 | 18 | 7 | 0 | 13 | 1 |
| | | E: 57<br>EA: 25<br>EAC:14 | E: 78<br>EA: 2<br>EAC: 3 | E: 19<br>EA: 29<br>EAC:26 | E: 8<br>EA: 46<br>EAC:11 | E: 55<br>EA: 8<br>EAC:37 | E: 16<br>EA: 7<br>EAC:43 | E: 65<br>EA: 38<br>EAC: 2 |
| 2 | 5:1 | 63 | 3 | 88 | 70 | 12 | 66 | 15 |
| | 2.5:1 | 54 | 1 | 76 | 58 | 10 | 52 | 10 |
| | 1.25:1 | 44 | 2 | 60 | 48 | 4 | 44 | 5 |
| | 0.6:1 | 30 | 0 | 52 | 30 | 3 | 32 | 3 |
| | | E: 57<br>EA: 25<br>EAC:14 | E: 74<br>EA: 6<br>EAC: 2 | E: 4<br>EA: 39<br>EAC:49 | E: 20<br>EA: 28<br>EAC:24 | E: 58<br>EA: 2<br>EAC:15 | E: 17<br>EA: 16<br>EAC:36 | E: 53<br>EA: 4<br>EAC:10 |
| 3 | 5:1 | 43 | 5 | 54 | 38 | 4 | 48 | 7 |
| | 2.5:1 | 31 | 4 | 40 | 30 | 5 | 28 | 5 |
| | 1.25:1 | 18 | 2 | 31 | 18 | 2 | 15 | 3 |
| | 0.6:1 | 12 | 1 | 22 | 15 | 1 | 11 | 0 |

* The concentration of PHA was 20 µg per ml. The culture medium was M199 supplemented with 5% FCS. Culture time was 24 hours. RFC refers to rosette forming cells.

** Refers to the per centage of E, EA and EAC RFC in the preparation.

TABLE 14

THE CON-A MEDIATED MICC REACTION OF HUMAN CIRCULATING POLYMORPHONUCLEAR CELLS, MONONUCLEAR CELLS AND MONOCYTE-DEPLETED MONONUCLEAR CELLS (CHICKEN, GUINEA PIG, HORSE AND RABBIT ERYTHROCYTE TARGETS)

| Erythrocyte Target Cell | Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the following populations of cells* | | |
|---|---|---|---|---|---|
| | | | Polymorphonuclear cells | Mononuclear cells | Monocyte-depleted mononuclear cells |
| Chicken (CRBC) | 1 | 10:1 | 57 | 42 | 2 |
| | | 5:1 | 52 | 29 | 0 |
| | | 2.5:1 | 32 | 11 | 0 |
| | 2 | 10:1 | 66 | 55 | 5 |
| | | 5:1 | 60 | 26 | 3 |
| | | 2.5:1 | 52 | 10 | 0 |
| | 3 | 10:1 | 23 | 18 | 0 |
| | | 5:1 | 18 | 12 | 0 |
| | | 2.5:1 | 11 | 6 | 0 |
| Guinea Pig (GRBC) | 1 | 10:1 | 58 | 47 | 5 |
| | | 5:1 | 50 | 41 | 2 |
| | | 2.5:1 | 41 | 35 | 0 |
| Horse (HoRBC) | 1 | 10:1 | 43 | 26 | 4 |
| | | 5:1 | 39 | 21 | 0 |
| | | 2.5:1 | 35 | 15 | 0 |
| | 2 | 10:1 | 71 | 42 | 11 |
| | | 5:1 | 60 | 32 | 6 |
| | | 2.5:1 | 61 | 28 | 2 |
| | 3 | 10:1 | 100 | 46 | 2 |
| | | 5:1 | 85 | 39 | 5 |
| | | 2.5:1 | 70 | 25 | 0 |
| Rabbit (RabRBC) | 1 | 10:1 | 74 | 44 | 17 |
| | | 5:1 | 60 | 29 | 8 |
| | | 2.5:1 | 37 | 21 | 5 |
| | 2 | 10:1 | 56 | 41 | 8 |
| | | 5:1 | 39 | 28 | 7 |
| | | 2.5:1 | 23 | 12 | 2 |

* The concentration of CON-A was 100 µg per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours.

TABLE 15

THE CON-A MEDIATED MICC REACTION OF SUBPOPULATIONS OF HUMAN CIRCULATING MONONUCLEAR CELLS (CHICKEN ERYTHROCYTE TARGETS)

| Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the different subpopulations of mononuclear cells cultured with CRBC targets* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Unfractionated mononuclear cells (MC) | MC enriched for E-RFC | MC depleted of E-RFC | MC enriched for EA-RFC | MC depleted of EA-RFC | MC enriched for EAC-RFC | MC depleted of EAC-RFC |
| 10:1 | 51 | 1 | 42 | 41 | -1 | 26 | 2 |
| 5:1 | 24 | 2 | 22 | 21 | 0 | 21 | 1 |
| 2.5:1 | 7 | -2 | 5 | 11 | -2 | 11 | 2 |
| 0:1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |

* The concentration of CON-A was 100 µg per ml. The culture medium was M199 supplemented with 5% FCS. Culture time was 24 hours. RFC refers to rosette forming cells.

TABLE 16

THE PWM MEDIATED MICC REACTION OF HUMAN CIRCULATING POLYMORPHONUCLEAR CELLS, MONONUCLEAR CELLS AND MONOCYTE-DEPLETED MONONUCLEAR CELLS (RABBIT ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the following populations of cells cultured with RabRBC targets* | | |
|---|---|---|---|---|
| | | Polymorphonuclear leukocytes | Mononuclear cells | Monocyte-depleted mononuclear cells |
| | | (P:L:M = 96:4:0)** | (P:L:M = 1:85:14) | (P:L:M = 0:99:1) |
| 1 | 10:1 | 51 | 80 | 85 |
| | 5:1 | 48 | 78 | 72 |
| | 2.5:1 | 35 | 65 | 48 |
| | | (P:L:M = 96:4:0) | (P:L:M = 0:88:12) | (P:L:M = 0:100:0) |
| 2 | 10:1 | 21 | 64 | 64 |
| | 5:1 | 24 | 71 | 46 |
| | 2.5:1 | 20 | 53 | 30 |
| | 1.25:1 | 14 | 31 | 12 |
| | | (P:L:M = 95:4:1) | (P:L:M = 1:85:14) | (P:L:M = 0:99:1) |
| 3 | 10:1 | 34 | 70 | 85 |
| | 5:1 | 30 | 72 | 66 |
| | 2.5:1 | 18 | 56 | 38 |
| | 1.25:1 | 17 | 33 | 28 |

* PWM was used at a concentration of 1000 µg per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours.

** P:L:M = polymorphonuclear cells: lymphocytes: monocytes

TABLE 17

THE PWM MEDIATED MICC REACTION OF PLASTIC ADHERENT AND NON-ADHERENT MONONUCLEAR CELLS (RABBIT ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the following populations of cells incubated with RabRBC targets* | |
|---|---|---|---|
| | | Adherent mononuclear cells | Non-adherent mononuclear cells |
| | | **(P:L:M = 0.46:54) | (P:L:M = 0:99:1) |
| 1 | 5:1 | 53 | 45 |
| | 2.5:1 | 49 | 31 |
| | 1.2:1 | 30 | 19 |
| | 0.6:1 | 16 | 12 |
| | 0.3:1 | 12 | 3 |
| | 0:1 | 0 | 0 |
| | | (P:L:M = 0:56:44) | (P:L:M = 0:97:3) |
| 2 | 10:1 | 58 | 68 |
| | 5:1 | 58 | 55 |
| | 2.5:1 | 48 | 38 |
| | 1.2:1 | 34 | 19 |
| | 0:1 | 0 | 0 |

* The concentration of PWM was 1000 µg per ml. The culture medium was M199 supplemented with 5% FCS. Culture time was 24 hours.

** P:L:M = polymorphonuclear cells: lymphocytes: monocytes

TABLE 18

THE PWM MEDIATED MICC REACTION OF SUBPOPULATIONS OF HUMAN CIRCULATING LYMPHOCYTES (RABBIT ERYTHROCYTE TARGETS)

| Exp. no. | Effector to target cell ratio | The MICC Cytotoxic Index (CI) of the different subpopulations of mononuclear cells cultured with RabRBC* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Monocyte-depleted mononuclear cells | MDMC enriched for E-RFC | MDMC depleted of E-RFC | MDMC enriched for EA-RFC | MDMC depleted of EA-RFC | MDMC enriched for EAC-RFC | MDMC depleted of EAC-RFC |
| | | ** E: 65<br>EA: 24<br>EAC: 5 | E: 69<br>EA: 14<br>EAC: 4 | E: 6<br>EA: 53<br>EAC:22 | E: 41<br>EA: 29<br>EAC: 8 | E: 77<br>EA: 2<br>EAC:12 | E: 36<br>EA: 19<br>EAC:15 | E: 61<br>EA: 21<br>EAC: 2 |
| 1 | 10:1 | 45 | 74 | 74 | 11 | 15 | 60 | 59 |
| | 5:1 | 38 | 66 | 41 | 12 | 15 | 22 | 35 |
| | 2.5:1 | 32 | 35 | 16 | 3 | 17 | 18 | 27 |
| | 0:1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 2 | 10:1 | 69 | 48 | 52 | 19 | 21 | 50 | 43 |
| | 5:1 | 63 | 37 | 45 | 19 | 26 | 37 | 40 |
| | 2.5:1 | 39 | 18 | 35 | 31 | 40 | 36 | 30 |
| | 0:1 | -3 | -3 | -3 | -3 | -3 | -3 | -3 |

* The concentration of PWM was 1000 µg per ml. The culture medium was M199 supplemented with 5% FCS. Culture time was 24 hours. RFC refers to rosette forming cells.

** Refers to the per centage of E, EA and EAC RFC in the preparation. This data was not available for exp. no. 2.

### 4.3.4 SUMMARY AND CONCLUSIONS

In the presence of PHA or CON-A, polymorphonuclear cells and monocytes but not lymphocytes were cytotoxic in the MICC reaction, irrespective of the erythrocyte target cells used. The cytotoxic monocyte in both of these reactions was shown to bear surface receptors for FcG and complement. On the other hand, RabRBC were lysed by lymphocytes and by polymorphs in the presence of PWM. The cytotoxic lymphocyte in this reaction evaded characterization on the basis of its surface receptors.

## 4.4 THE MECHANISM OF THE MICC CYTOTOXIC REACTION

### 4.4.1 OBJECTIVES

In order to elucidate the mechanism by which mitogens (PHA, CON-A and PWM) impart cytotoxic activity to circulating leukocytes, this investigation set out; i) to determine whether or not the cellular secretion of cytotoxic factors is involved; ii) to microscopically visualize the cellular interactions in the MICC reaction; and iii) to determine whether any correlation exists between the capacity of a mitogen to effect target cell lysis, and its capacity to agglutinate target cells.

### 4.4.2 PROTOCOL

#### 4.4.2.1 The role of secreted soluble cytotoxins in the MICC reaction.

The protocol to determine whether or not mitogen-induced erythrocyte lysis is mediated by soluble cytotoxic factors is outlined diagramatically in Figure 6. Briefly, MICC cultures were set up in the same manner as described in Chapter 3 except that the erythrocyte target cells were not labelled with $Cr^{51}$. The effector cells used in all the cultures were unfractionated leukocytes. CRBC were the target cells cultured with PHA; HoRBC and RabRBC with CON-A; and RabRBC with PWM. The controls for the experiments were set up as is illustrated in Figure 6. After a 24 hour incubation at 37°C, the cultures were centrifuged, and the cell-free supernatants removed and incubated for

another 24 hours with fresh $Cr^{51}$-labelled isogeneic erythrocyte target cells. The $Cr^{51}$ released from these cells was then measured.

4.4.2.2 The microscopic visualization of the cellular interactions in the MICC reaction

MICC cultures were set up in the same manner as described in the previous paragraph. After every hour of incubation, hanging drop mounts of the MICC cultures were prepared and analyzed at 37°C under the light microscope.

4.4.2.3 The agglutination of erythrocyte targets

Unlabelled erythrocyte target cells were mixed with serially diluted mitogen solutions as described in Chapter 3. After a 24 hour incubation period the cells were inspected for the presence of agglutination.

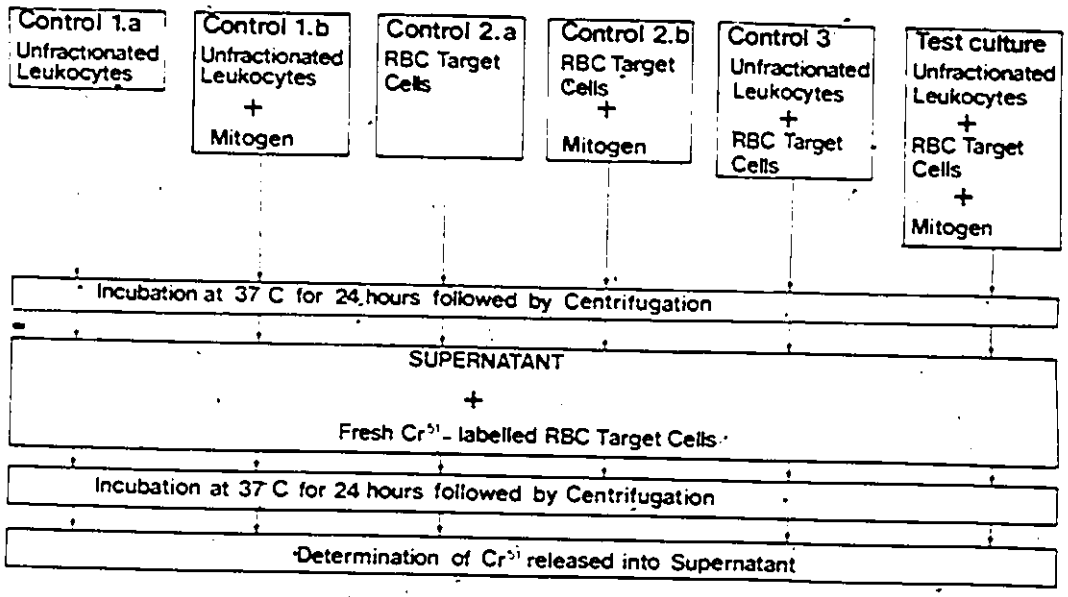


Figure 6

Protocol to determine whether or not MICC cytolysis is mediated by soluble cytotoxic substances released from target and/or effector cells.

## 4.4.3 RESULTS AND DISCUSSION

### 4.4.3.1 The role of secreted soluble cytotoxins in the MICC reaction

As can be seen in Tables 19 to 22, cell-free supernatants from 24 hour MICC cultures (containing effector cells, unlabelled erythrocyte target and a mitogen) were not cytotoxic when incubated with $Cr^{51}$-labelled isogeneic erythrocyte targets. No significant target cell lysis occurred irrespective of the mitogen or the type of erythrocyte target used.

### 4.4.3.2 The microscopic visualization of the cellular interaction in the MICC reaction

Unfortunately this aspect of the investigation was non-contributory in terms of facilitating an understanding of the mechanism of the MICC reaction due to the clumping of both target and effector cells individually in the presence of the mitogen. Because of this, no distinction was discerned between the aggregates of target cells incubated in the presence of the mitogen and the aggregates of target cells incubated in the presence of the mitogen and effector cells.

### 4.4.3.3 The agglutination of erythrocyte targets

As can be seen in Table 23, all the target cells which were lysed in the presence of a mitogen were agglutinated by the mitogens. However, erythrocytes which did not serve as target cells were also agglutinated by the mitogens. For example, GRBC, RabRBC and RatRBC did not function as target cells in PHA-mediated MICC lysis, but they were markedly agglutinated by PHA. Similarly, HuRBC were agglutinated by CON-A but were not lysed in the CON-A mediated MICC assay.

TABLE 19

THE EFFECT OF MICC (PHA) CULTURE SUPERNATANTS ON CHICKEN ERYTHROCYTE TARGETS

| Origin of the supernatants* | PHA | Control no. | The per centage of the total $Cr^{51}$ released from the $Cr^{51}$-labelled CRBC targets following a 24 hour incubation with the various supernatants |
|---|---|---|---|
| Unfractionated leukocytes | absent | 1.a | 4 |
| | present | 1.b | 5 |
| Unlabelled CRBC targets | absent | 2.a | 5 |
| | present | 2.b | 5 |
| Unfractionated leukocytes Unlabelled CRBC targets | absent | 3 | 4 |
| | present | test culture | 5 |

* The concentration of PHA was 20 µg per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours. The E/T was 10:1. A CI of 59% was obtained when the unfractionated leukocytes were cultured with $Cr^{51}$-labelled CRBC at the E/T of 10:1.

TABLE 20

THE EFFECT OF MICC (CON-A) CULTURE SUPERNATANTS ON HORSE ERYTHROCYTE TARGETS

| Origin of the supernatants* | CON-A | Control no. | The per centage of the total $Cr^{51}$ released from the $Cr^{51}$-labelled HoRBC targets following a 24 hour incubation with the various supernatants |
|---|---|---|---|
| Unfractionated leukocytes | absent | 1.a | 15 |
| | present | 1.b | 9 |
| Unlabelled HoRBC targets | absent | 2.a | 6 |
| | present | 2.b | 12 |
| Unfractionated leukocytes | absent | 3 | 12 |
| Unlabelled HoRBC targets | present | test culture | 8 |

* The concentration of CON-A was 100 µg per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours. The E/T was 10:1. A CI of 55 % was obtained when the unfractionated leukocytes were cultured with $Cr^{51}$-labelled HoRBC at the E/T of 10:1.

TABLE 21

THE EFFECT OF MICC (CON-A) CULTURE SUPERNATANTS ON RABBIT ERYTHROCYTE TARGETS

| Origin of the supernatants* | | | The per centage of the total $Cr^{51}$ released from the $Cr^{51}$-labelled RabRBC targets following a 24 hour incubation with the various supernatants |
|---|---|---|---|
| | CON-A | Control no. | |
| Unfractionated leukocytes | absent | 1.a | 7 |
| | present | 1.b | 6 |
| Unlabelled RabRBC targets | absent | 2.a | 9 |
| | present | 2.b | 10 |
| Unfractionated leukocytes | absent | 3 | 5 |
| Unlabelled RabRBC targets | present | test culture | 7 |

* The concentration of CON-A was 100 µg per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours. The E/T was 10:1. A CI of 79% was obtained when the unfractionated leukocytes were cultured with $Cr^{51}$-labelled RabRBC at the E/T of 10:1.

TABLE 22

THE EFFECT OF MICC (PWM) CULTURE SUPERNATANTS ON RABBIT ERYTHROCYTE TARGETS

| Origin of the supernatants* | | | The per centage of the total $Cr^{51}$ released from the $Cr^{51}$-labelled RabRBC targets following a 24 hour incubation with the various supernatants |
|---|---|---|---|
| | PWM | Control no. | |
| Unfractionated leukocytes | absent | 1.a | 9 |
| | present | 1.b | 10 |
| Unlabelled RabRBC targets | absent | 2.a | 7 |
| | present | 2.b | 7 |
| Unfractionated leukocytes + Unlabelled RabRBC targets | absent | 3 | 6 |
| | present | test culture | 9 |

* The concentration of PWM was 1000 µg per ml. The culture medium was M199 supplemented with 5% FCS. The culture time was 24 hours. The E/T was 10:1. A CI of 61% was obtained when the unfractionated leukocytes were cultured with $Cr^{51}$-labelled RabRBC at the E/T of 10:1.

TABLE 23

THE AGGLUTINATION OF ERYTHROCYTES OF DIFFERENT SPECIES BY PHA, CON-A AND PWM

| Mitogen evaluated | Agglutination titre of the mitogen with respect to the following erythrocytes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CRBC | GRBC | HoRBC | HuRBC | ORBC | SRBC | RabRBC | RatRBC |
| PHA | 512 | >2048 | >2048 | 64 | 1 | 128 | >2048 | >2048 |
| CON-A | 256 | >2048 | >2048 | 512 | 1 | 2 | >2048 | >2048 |
| PWM | 2 | 2 | 4 | 2 | 2 | 4 | >2048 | 16 |

### 4.4.4 SUMMARY AND CONCLUSIONS

When $Cr^{51}$-labelled erythrocytes were incubated with cell-free supernatants obtained from conventional MICC cultures, no significant target cell lysis occurred. These results suggest that soluble cytotoxic factors do not play a role in the MICC lysis of erythrocytes.

In order to determine whether any correlation exists between the capacity of the mitogen to effect target cell lysis and its capacity to agglutinate target cells, the different erythrocyte targets were assessed for their agglutinability by the three mitogens. Although no specific correlation was demonstrated, it was observed that target cells lysed in the presence of a mitogen were invariably agglutinated by the mitogen. However, various erythrocytes which do not serve as target cells were also agglutinated by the mitogens. Therefore, it must be concluded that the capacity to agglutinate the target cells does not in itself confer to the mitogen the capacity to mediate target cell lysis by the circulating leukocyte.

Due to non-specific clumping of effector and target cells by the mitogens, microscopic analyses of the MICC reaction were non-contributory to an understanding of the MICC reaction.

# CHAPTER 5

## GENERAL DISCUSSION

By conventional terminology, an immune reaction observed with the cells and/or antibodies of an individual and an antigen, implies prior sensitization of the host to the antigen as a result of active immunization. Up to the 1960s, it had been assumed that a bacteria or virus or any other antigen (i.e. an inanimate non-replicating erythrocyte) could only be lysed or degraded by antibodies and complement as a result of the host having previously been exposed to that antigen. However, in the mid-1960s, it was demonstrated that cells exist in the circulation of the normal human and animal capable of lysing erythrocytes or pathogenic microorganisms in the presence of a very limited concentration of antibodies, too little to facilitate complement-mediated lysis (56-64). This reaction is referred to as the antibody-dependent cellular cytotoxic, or ADCC, reaction. It is initiated as a result of the cytotoxic cell interacting via its receptor for FcG with the Fc region of the IgG antibody, following the attachment of the antibody to the target antigen. Although this reaction is dependent upon a minimum, almost immunologically-undetectable, amount of antibodies, the effector cytotoxic cell is a naturally circulating non-immune cell which possesses receptors for FcG.

In the early 1970s it was demonstrated that a second type of cell exists normally in the circulation capable of lysing unselected target cells (erythrocytes, malignant cell lines, fetal cells, and fibroblasts) *in the total absence of antibody molecules.* This reaction is referred to as the naturally-occurring cellular cytotoxic, or NOCC reaction (65-86). The effector cells in the ADCC and NOCC reactions are referred to as killer (K) and natural killer (NK) cells, respectively. In addition, it has also been demonstrated that still other cells normally exist in the circulation capable of lysing certain target cells (without prior exposure to them) in the presence of the mitogens PHA, CON-A or PWM. This reaction is referred to as the mitogen-induced cellular cytotoxic, or MICC reaction. Since these three cytotoxic reactions are carried out by cells of unimmunized humans or animals, they are referred to as para-immunologic reactions to distinguish them from the conventional or classical immunologic reactions carried out by antibodies or sensitized cells of specifically immunized animals or humans.

It is the intention of this investigator to determine whether patients with a number of disease states (i.e. malignant disease, autoimmune disease, or immunodeficiency disease) possess excessive numbers of, or a deficiency of, the circulating leukocytes which participate in the ADCC, NOCC and MICC reactions, in order, i) to establish a cause-effect or a correlational relationship between these cells and the

particular disease states, and ii) to determine whether the presence of excessive numbers or deficiencies in these cells may have predictive value with respect to remissions or exacerbations of these disease states. In order to carry out such an investigation, it is necessary to first define the cell types which participate in these cytotoxic reactions and to demonstrate whether the cells which carry out the cytotoxic reactions in the ADCC, NOCC and MICC assays are unique, distinct cell populations. Thus far, this objective has been attained with certainty only with respect to the ADCC reaction and partly with the NOCC reaction. However, the identity of the effector cells in the MICC reaction has not been firmly established, nor have the target cells most appropriate for this reaction been identified. The purpose of this investigation, therefore, was to define target cells for the MICC reaction using human circulating leukocytes as effector cells, and to identify the cytotoxic cells.

The immediate objectives of this investigation were; i) to establish which of eight different erythrocytes could act as suitable targets in the MICC reaction with human circulating effector cells; ii) to determine whether the three conventional mitogens (PHA, CON-A and PWM) are selective with respect to the erythrocyte target cells; iii) to identify unequivocally the effector cells capable of mediating MICC activity; iv) to establish whether there

is mitogen and/or target cell specificity with respect to the effector leukocytes involved; and v) to elucidate the mechanism by which mitogens impart cytotoxic activity to the circulating leukocytes.

In this investigation erythrocytes of eight species of animals were systematically analysed as potential targets in the MICC reaction, using the three common mitogens PHA, CON-A and PWM. It was consistently observed that the mitogens were selective in terms of their ability to impart cytotoxic activity to normal human circulating cells with respect to the erythrocyte targets. Thus, PHA was able to facilitate lysis of CRBC; CON-A was able to mediate lysis of CRBC, GRBC, HoRBC, RabRBC, and RatRBC; and PWM could mediate significant lysis of only the RabRBC.

The mitogens all displayed dose-dependent cytotoxicity. When experiments were carried out using the mitogens in various concentrations, PHA gave an optimal MICC response at 20 to 40 µg per ml; CON-A at 75 to 100 µg per ml; and PWM at 1000 µg per ml.

Although the nature and composition of the various culture media studied did not appear to play a significant or definitive role in facilitating the MICC reaction, they were of major importance in providing stability to the target cells in the assay. When assays for PHA-mediated MICC cytotoxicity were set up using three basic media (M199, RPMI and CMRL) fortified with FCS to a concentration

of 5 per cent, it was found that all the media facilitated almost equal lysis of the target cells. However, in the control cultures from which effector cells were omitted, significant spontaneous target cell lysis occurred with RPMI and CMRL, but not with M199. In view of its stabilizing properties with respect to the target cells, M199 was the only one used in all subsequent experiments.

Experiments were also carried out utilizing five mitogen-target cell combinations, M199 and various concentrations of FCS in the culture medium. It was observed that substantial spontaneous target cell lysis occurred in medium unfortified with FCS, whereas the targets were stable in medium supplemented with 5 per cent FCS. Therefore, in order to minimize any superimposition of spontaneous target cell lysis on true mitogen-mediated target cell lysis by circulating effector cells, it was decided to henceforth use M199 supplemented with 5 per cent FCS in the MICC assay.

The identity of the effector cells in human peripheral blood capable of mediating MICC cytotoxicity was investigated, using effector cell populations consisting of mononuclear cells, lymphocytes (monocyte-depleted mononuclear cells) and polymorphonuclear cells. Lymphocytes and mononuclear cells were sub-fractionated by various rosetting techniques on the basis of their surface membrane receptors for SRBC, FcG and complement, and assayed for MICC activity. It was

found that in the presence of PHA or CON-A, neutrophils and monocytes but not lymphocytes were cytotoxic irrespective of the erythrocyte target cell used. The cytotoxic monocyte was shown to be a cell with receptors for both FcG and complement. On the other hand, RabRBC were lysed by lymphocytes and neutrophils in the presence of PWM. Whether or not the monocytes are also cytotoxic in the presence of PWM could not be unequivocally established, due to the failure of current techniques to facilitate the isolation of even relatively pure (80 to 90 per cent) preparations of monocytes. Most preparations of monocytes are heavily contaminated with lymphocytes and therefore any cytotoxic activity exhibited by these preparations could be attributed to contaminating lymphocytes. The cytotoxic lymphocyte, in the presence of PWM, evaded characterization based on surface membrane receptors since all subpopulations isolated expressed cytotoxic activity. Thus it is possible to detect cytotoxic subpopulations of monocytes, lymphocytes and neutrophils by utilizing the appropriate mitogen and target cell in the MICC reaction.

The polymorphonuclear cell, by comparison to the monocyte, was an extremely potent effector cell in the PHA and CON-A MICC assay systems. With PHA, cytotoxic indices (CIs) of up to 53 were obtained after as few as two hours in culture. At the effector to target cell ratio of only one to six, CIs of up to 40 were recorded.

In order to account for this amount of cytotoxicity, each polymorph would have had to have killed more than two erythrocyte target cells. In contrast, lymphocytes (monocyte-depleted mononuclear cells) were substantially more potent than polymorphs in the PWM MICC assay. At comparable effector to target cell ratios, these lymphocytes exhibited CIs that were usually double those of the polymorphonuclear cells.

Other investigators have implicated a wide variety of lymphoid cell types as participants in the PHA and CON-A mediated MICC reactions. In a manner as yet not understood, the identity of the effector cell(s) is somehow influenced by the nature or type of target cell utilized. When transformed cell lines are used, cytotoxicity is mediated by T cells (2,4,14,20,23,24,25,27,37). However, when erythrocytes are used as target cells, B, non-B, T and non-T lymphocytes, monocytes, and polymorphonuclear cells have all been reported as being active cytotoxic effector cells (3,5,6,7,10,14,15, 16,19,20,23,24,25,29,30,33,34).

The results of this study, which employed eight different erythrocyte targets, suggest that differences in the type of mitogen utilized also influences the identity of the effector cells. With PHA and CON-A, monocytes and polymorphs were found to be active, whereas lymphocytes and polymorphs were active using PWM.

These results corroborate the work of others who attribute PHA and CON-A mediated cytotoxicity towards CRBC to monocytes and polymorphs, and who question any significant role of lymphocytes in the reaction (14,15,19,29,30). Conversely, the results of this study are in conflict with those of other investigators who, using the same target cells and mitogens, have reported that a large number of lymphocyte subpopulations are involved (3,5,6,7,10,16,20,23,24,25,33,34). The possible explanations for this discrepancy may relate to differences in the choice of MICC assay conditions, in the methods of cell purification, and/or in the interpretation of results by different investigators. Consider the following examples:

i) Nelson, Bundy, Pitchon, Blaese, and Strober (25) claim that B, non-B and T lymphocytes are involved in the PHA-mediated lysis of CRBC. They centrifuged their cultures prior to incubation, a manoeuvre which probably increases cellular contact, and they incubated their cultures for a considerably longer period of time (forty hours as compared to twenty-four hours used in this and most other studies). At the effector to target cell ratio of five to one, the CIs for their preparations ranged from approximately 10 for B cells to 50 for T cells. With respect to the culture conditions, it may be that these lymphocyte subpopulations are active in the MICC reaction, but only after manipulation of the culture conditions so as to bring the lymphocytes into close contact with the target cells and/or after

prolonged periods of time. Furthermore, if the cells are active then they are very much less active than polymorphonuclear cells. In the present study, at comparable effector to target cell ratios, CIs for polymorphs of between 20 and 50 were noted after only two hours in culture.

ii) Cordier, Samerut and Revillard (5) have reported that the effector cells involved in the PHA-mediated lysis of CRBC are both T and non-T lymphocytes. In eighteen hour cultures, they found that E-rosetting (E-RFC) and non-rosetting cells exhibited CIs of approximately 10 and 18, respectively, at the effector to target cell ratio of ten to one. Their cell preparations were contaminated with up to 1 per cent monocytes and 0.5 per cent polymorphs. In the present study, however, lymphocytes artificially contaminated with 1 per cent polymorphs exhibited CIs of 14 to 22 (24 hour cultures), and it can thus be argued that all or a great deal of the cytotoxicity observed by Cordier et al (5) was due to contaminating polymorphs and/or monocytes, rather than to the lymphocytes.

ii) Wisloff, Froland and Michaelsen (34) also concluded that cytotoxicity requires the presence of T lymphocytes after observing that the depletion of E-RFC from mononuclear cells resulted in a 25 per cent loss of MICC cytotoxicity. They did not, however, control for any deleterious effect that the E-RFC depletion technique itself might have upon

leukocyte function in the MICC assay; nor did they assay pure E-RFC cells for activity. In our study, E-RFC were essentially devoid of MICC activity in the presence of PHA or CON-A.

The preceding examples emphasize the possible significance of MICC assay conditions in influencing the identity of the cells that can act as effectors in the MICC reaction. Under the conditions described in the present study, PHA and CON-A-mediated cytotoxicity directed towards a variety of erythrocyte target cells was indisputably a reflection of monocyte and polymorph function, and not of lymphocyte function. Furthermore, the results of these studies suggest that until such time that laboratory methods allow the isolation of absolutely pure lymphocyte populations, the activity of any contaminating polymorphs, which are extremely potent effectors in MICC cytotoxicity, must be considered before cytotoxicity is attributed to any lymphocyte preparation.

This was the first study to evaluate the cytotoxic activity of human circulating leukocytes in the presence of PWM. Lymphocytes and polymorphs were cytotoxic towards RabRBC target cells in the presence of this mitogen. The lymphocyte involved evaded characterization on the basis of surface membrane receptors since all rosetting and non-rosetting cells displayed cytotoxic activity to varying

degrees. It may therefore be that MICC lysis of RabRBC target cells induced by PWM is an activity shared by all lymphocytes and may most aptly reflect that activity which has been referred to as 'non-specific cytotoxic activity'. As stated previously, the failure to isolate monocytes in a relatively pure form precludes their assessment as cytotoxic cells in the presence of PWM.

The mechanism of MICC cytotoxicity has not yet been clearly elucidated. It has been suggested by some investigators that a soluble factor ('lymphotoxin') released from mitogen-stimulated lymphocytes is capable of inducing lysis of nucleated cell line targets (8,9,33). In the present study, which utilized erythrocyte targets, such a soluble cytotoxic factor could not be demonstrated. When cell-free supernatants from MICC cultures were incubated with $Cr^{51}$-labelled erythrocyte targets, no significant target cell lysis occurred, irrespective of the mitogen used. As nucleated cell line targets and erythrocyte targets appear to induce cytotoxicity amongst separate leukocyte subpopulations (lymphocytes as opposed to neutrophils and monocytes, respectively), it is not unlikely that this discrepancy is a reflection of differences in the mechanism by which these separate leukocyte subpopulations mediate MICC lysis.

It has been suggested that those mitogens which can induce cytotoxicity also cause agglutination of effector

and/or target cells (30). The data presented in this thesis lends support to this assumption, since it was observed that all of the erythrocytes lysed in the presence of a mitogen were also agglutinated by that mitogen. It therefore appears that very close effector-target cell contact is a prerequisite for erythrocyte lysis in the MICC reaction. Presumably the mitogen acts as a bridge between the two reactants. As phagocytosis does not appear to play a role in this reaction (29), target cell lysis proceeds extracellularly in a manner as yet not fully understood or appreciated.

As was stated in the introduction, the relevance of the MICC reaction may not lie so much in the unmasking of an in vivo counterpart system, but rather in its role as a probe in the identification of circulating cells that bear a cause-effect and/or correlational relationship to disease states. The assay may prove to be valuable not only as a diagnostic tool, but also in the monitoring of the course of a disease and in the scheduling of therapy. In this regard it has been observed that the MICC cytotoxic activity of circulating leukocytes is significantly reduced in patients with chronic lymphocytic leukemia (13), Hodgkin's disease (13), acute myelogenous leukemia in remission (36), and irradiated testicular tumors (75). It has also been observed that MICC activity of lymphocytes is reduced in patients receiving immuno-

suppressive chemotherapy (36), radiotherapy (35) or corticosteroid therapy (28).

Besides the MICC reaction, two other in vitro models of cell mediated cytotoxicity involving non-immune lymphoid cells have been described - the antibody dependent cell mediated cytotoxic reaction (ADCC), and the naturally occurring cell mediated reaction (NOCC). The effector cells in these two assays have been referred to as killer (K) and natural killer (NK) cells. It has been demonstrated that in the ADCC reaction, the monocyte induces the lysis of HuRBC coated with allogeneic (human) antibody (59,63), whereas lymphocytes, neutrophils and monocytes induce the lysis of CRBC coated with rabbit antibodies (60). Similarly, in the NOCC reaction, only lymphocytes are cytotoxic with respect to the K-562 target cell (32,80, 83,85), whereas neutrophils and monocytes but not lymphocytes are capable of spontaneously lysing the RabRBC target cells (42,85). The results of this investigation indicate that a selective lysis of target cells by human cytotoxic cells can be realized in the MICC reaction as well. PHA and CON-A both facilitate the cytotoxic activity of polymorphs and monocytes, but not lymphocytes, irrespective of the target cells utilized. On the other hand, PWM facilitates the cytotoxic activity of lymphocytes utilizing the RabRBC target cell. Thus it may be possible to define distinct subclasses of immunocompetent normally-circulating leukocytes

on a functional basis, using the ADCC, NOCC and MICC assays.

It is tempting to investigate whether the presence or absence of NOCC, ADCC and MICC cytotoxic cells in patients with cancer, autoimmune disease, immunodeficiency disease, or an allograft rejection reaction, correlates with the clinical picture. However, the results would be most meaningful once it has been established that the different cytotoxic reactions (NOCC, ADCC and MICC) are mediated by distinct, as opposed to identical or overlapping cell populations, and the precise identities of these effector cells have been unequivocally defined.

# CHAPTER 6

## CONTRIBUTIONS TO KNOWLEDGE

1. The candidate has determined the optimal culture conditions for the assay of the mitogen (PHA, CON-A and PWM) induced cytotoxic reaction of human circulating leukocytes.

2. The candidate has determined that erythrocytes from a variety of species can function as target cells in the MICC assay. Chicken erythrocytes are suitable targets with PHA; chicken, guinea pig, horse, rabbit, and rat erythrocytes are lysed in the presence of CON-A; and rabbit erythrocytes are the only erythrocytes lysed to any significant degree in the presence of PWM.

3. The candidate has determined that in the presence of PHA or CON-A, monocytes and polymorphs but not lymphocytes are cytotoxic in the MICC reaction. The cytotoxic monocyte was shown to bear surface receptors for FcG and complement.

4. The candidate has determined that in the presence of PWM, lymphocytes and polymorphs are cytotoxic in the MICC reaction. The cytotoxic lymphocyte eluded characterization on the basis of its surface membrane receptors.

5. The candidate has determined that the secretion of soluble cytotoxins is not involved in the mitogen-induced lysis of erythrocyte target cells in the MICC reaction.

6. The candidate has determined that the capacity of a mitogen to agglutinate erythrocyte target cells does not necessarily confer to the mitogen the capacity to mediate target cell lysis in the MICC reaction. However, it was determined that those erythrocytes which are lysed in the presence of a mitogen in the MICC reaction are also invariably agglutinated by the mitogen.

## CHAPTER 7

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