THE ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXIC (ADCC) REACTION WITH RABBIT LYMPHOID AND CIRCULATING CELLS: THE FUNCTIONAL HETEROGENEITY OF RABBIT ADCC CYTOTOXIC EFFECTOR CELLS

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ABBREVIATIONS AND TERMS USED IN TEXT

ADCC:	antibody-dependent cell-mediated cytotoxicity
BCG:	bacillus Calmette-Guérin
BSA:	bovine serum albumin
C:	cytotoxicity
C':	complement
C'1-9:	complement components 1 through 9
CA:	cytochalasin A
CB:	cytochalasin B
CFA:	complete Freund's adjuvant
$C\gamma2(C_H2)$:	the second domain in the constant portion of the heavy chain (γ) in IgG
$C\gamma3$ (C_H3):	the third domain in the constant portion of the heavy chain (γ) in IgG
ChRBC:	chicken erythrocytes
C.I.:	cytotoxic index
CMRL 1066-PS:	medium CMRL 1066 supplemented with 100 IU of potassium penicillin G per ml and 100 μg per ml of streptomycin sulfate

Con A:	concanavalin A
^{51}Cr:	radioactive sodium chromate solution
^{51}Cr-ChRBC:	^{51}chromium-labelled chicken erythrocytes
C-terminal:	carboxyl terminal portion of the antibody molecule
CTL:	cytotoxic T lymphocyte
Culture medium:	medium 199 supplemented with 100 IU of potassium penicillin G per ml and 100 μg per ml of streptomycin sulfate and fortified with heat-inactivated (56°C for 30 minutes) fetal bovine serum to a final concentration of 6 percent
2-DG:	2-deoxyglucose
E:	effector cell
E rosette:	formation of a cluster of erythrocytes around a central lymphocyte
EA rosette:	formation of a cluster of erythrocytes sensitized with antibody around a central lymphocyte
EAC rosette:	formation of a cluster of erythrocytes sensitized with antibody and complement around a central lymphocyte
EDTA:	ethylenediaminetetra-acetate

E/ml:	effector cell concentration per ml
E:T:	effector to target cell ratio
Fab':	digestion of an IgG molecule by the enzyme papain produces 2 Fab' (antigen-binding) fragments and 1 Fc (crystallizable) fragment
$F(ab')_2$:	digestion of an IgG molecule by the enzyme pepsin produces 1 $F(ab')_2$ molecule and small peptides
FBS:	fetal bovine serum
Fc cell surface receptor:	cell surface receptor for the Fc fragment of the antibody molecule
Fc fragment:	digestion of an IgG molecule by the enzyme papain produces 1 Fc (fragment crystallizable) fragment and 2 Fab (antigen-binding) fragments
FH:	ficoll-hypaque
FHI:	cells recovered at the blood ficoll-hypaque density gradient interface
FHP:	cells recovered in the pellet of the ficoll-hypaque density gradient
GALT:	gut-associated lymphoid tissues
hr(s):	hour(s)
HBSS:	Hank's balanced salt solution
HBSS-PS:	Hank's balanced salt solution supplemented with 100 IU of potassium

	penicillin G per ml and 100 μg per ml of streptomycin sulfate
Heat inactivation:	the heating of serum for 30 minutes at 56°C in a water bath
HRBC:	horse erythrocytes
HuRBC:	human erythrocytes
Ig:	immunoglobulin
IgA:	immunoglobulin A (sedimentation coefficients of 7S, 9S, 11S)
IgD:	immunoglobulin D (sedimentation coefficient of 7S)
IgE:	immunoglobulin E (sedimentation coefficient of 8S)
IgG:	immunoglobulin G (sedimentation coefficient of 7S)
IgM:	immunoglobulin M (sedimentation coefficient of 19S)
%Inh:	percent inhibition
IRS:	immune rabbit serum
IU:	international units
k:	kappa
K cell:	killer cell
Lagomorpha:	Order of the animal kingdom to which the rabbit belongs
LPS:	lipopolysaccharide
M199-PS:	medium 199 supplemented with 100 IU

	of potassium penicillin G per ml and 100 g per ml of streptomycin sulfate
MBP:	major basic protein
M.C.:	mononuclear cells
MICC:	mitogen-induced cell-mediated cytotoxicity
MLN:	mesenteric lymph nodes
MLR:	mixed leukocyte reaction
MTV:	mammary tumour virus
NOCC:	naturally-occurring cell-mediated cytotoxicity
NRS:	normal rabbit serum
N-terminal:	amino terminal portion of the antibody molecule
P:	probability
PBS:	phosphate buffered saline
PBL:	peripheral blood leukocytes
PEC:	peritoneal exudate cells
PHA:	phytohaemagglutinin
PLN:	popliteal lymph nodes
PMN:	polymorphonuclear leukocytes
PPD:	purified protein derivative of tuberculin
PS:	potassium penicillin G-streptomycin sulfate mixture
RACE:	rabbit anti-chicken erythrocyte antiserum

RAGG:	soluble aggregates of rabbit gamma-globulin
RBC:	red blood cells (erythrocytes)
RPMI 1640-PS:	medium RPMI 1640 supplemented with 100 IU of potassium penicillin G per ml and 100 μg per ml of streptomycin sulfate
SIg:	surface immunoglobulin
SNF:	supernatant fluid
SPF:	specific pathogen-free
SR:	spontaneous release
SRBC:	sheep erythrocytes
T:	target cells
T/ml:	target cell concentration per ml
U.S.P.:	United States Pharmacopoea
WBC:	white blood cells

CHAPTER I

INTRODUCTION AND OBJECTIVES

The past decade has been witness to a revolution in the consideration of the role of the lymphocyte. Even as recently as the early 1960's, mature lymphocytes were still considered to be end stage cells, identical in terms of morphology and function and the progeny of a single cell lineage ontogenically. By the mid 1970's, mature lymphocytes were considered to be the most pluripotent cells in the body and were classified into numerous categories on the basis of properties such as life span (short or long), origin (central or peripheral), antigenicity (thymus, bone marrow and appendix-derived lymphocytes bear unique surface antigens), immunologic role (antibody-forming cell, helper cell, cytotoxic cell, potentiating cell, antigen-driven proliferating cell, suppressor cell) and cell surface receptors.

Recent studies into the immunologic role of lymphocytes have disclosed the existence of lymphocytes uniquely capable of inducing the destruction and lysis of lymphoid and non-lymphoid target cells possessing surface antigens to which these effector lymphocytes

had been sensitized in vivo, or in vitro during the mixed leukocyte culture reaction (MLR). In a parallel system, mononuclear leukocytes were also seen to acquire cytotoxic activity following incubation with, and subsequent activation by, plant mitogens. Concurrent with these studies, investigations have revealed the existence of three types of cell surface receptors--one for a configuration on the sheep erythrocyte, one for the Fc region of the immunoglobulin G (IgG) antibody molecule subsequent to its interaction with antigen, and one for the antigen-antibody-fixed third component of complement (C'3). The identification of these cell-surface receptors allowed the classification of lymphocytes into three categories, notably E (E = erythrocyte), Fc and C'3-bearing lymphocytes. The identification of an expanded functional role for the lymphocyte together with a system of morphologic classification provided a necessary impetus in the direction of correlation of lymphocyte function and morphology.

In the continuing investigation of lymphocyte cytotoxic reactions in vitro, it was reported that unsensitized lymphocytes from normal donors were capable of lysing target cells in the absence of complement, provided that they were coated with appropriate anti-target cell antibodies. This reaction is currently known as the antibody-dependent cell-mediated

cytotoxic (ADCC) reaction. A variety of target cells such as chicken, duck, sheep, burro and human erythrocytes, Chang liver cells, human lymphocytes and lymphoblasts and various tumour cells have been utilized to describe the ADCC reaction. To date, ADCC activity has been generally attributed to mononuclear cells (lymphocytes and macrophages) of human and animal (rat, mouse, guinea pig, rabbit, monkey, duck, chicken) origin (figures 1, 2 and 3).

A common denominator characteristic of ADCC effector cells is the presence of a cell surface receptor for the Fc portion of the IgG and in some cases IgM molecule (Fc-receptor bearing cell). It is today generally accepted that the cytotoxic reaction is initiated as a consequence of the interaction of the Fc receptor on the cytotoxic effector cell with the Fc region of the cell-bound IgG (or IgM) antibody and that this interaction can be blocked by soluble aggregates of gamma-globulin and by immune complexes. Only the Fc receptor-bearing cell is implicated in this reaction as removal of T cells or B cells (defined by convention as possessing theta antigens and SRBC receptors, and surface immunoglobulins, respectively) or adherent (phagocytic?) cells does not affect the ADCC activity of freshly drawn lymphocytes. The ADCC effector cell has consequently been regarded as a "null" cell.

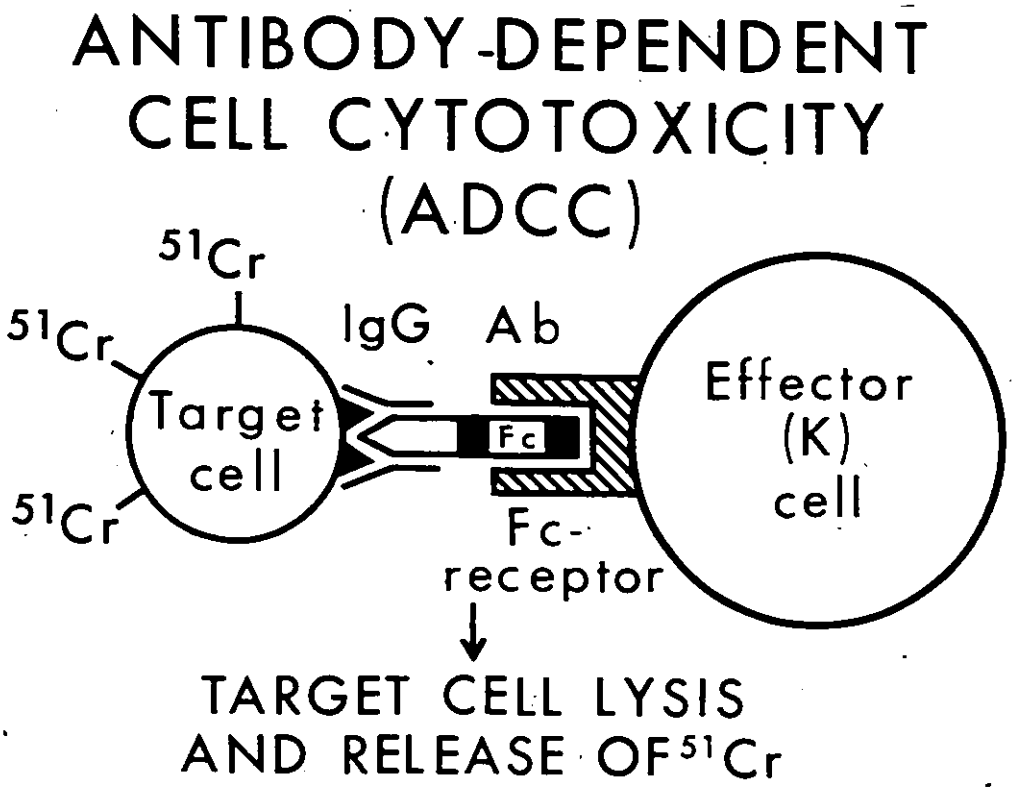

Figure 1:-- Antibody-dependent Cell-Mediated Cytotoxicity (ADCC).

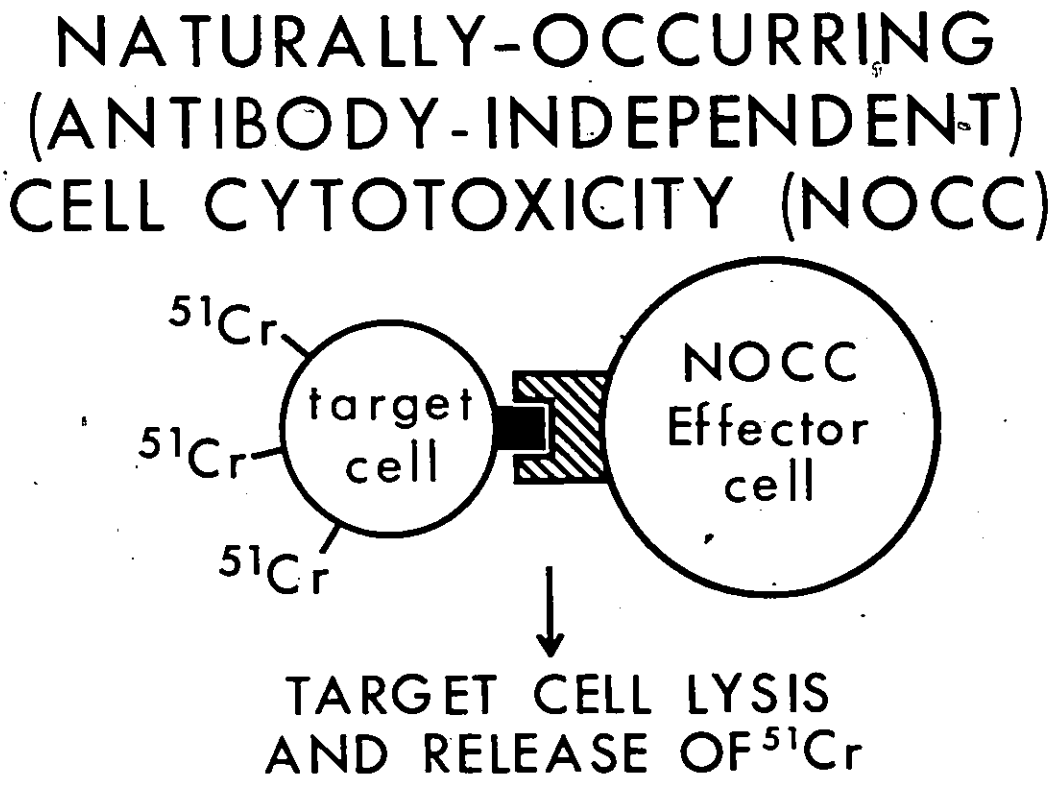

Figure 2:-- Naturally-occurring (Antibody-independent) Cell-mediated Cytotoxicity (NOCC).

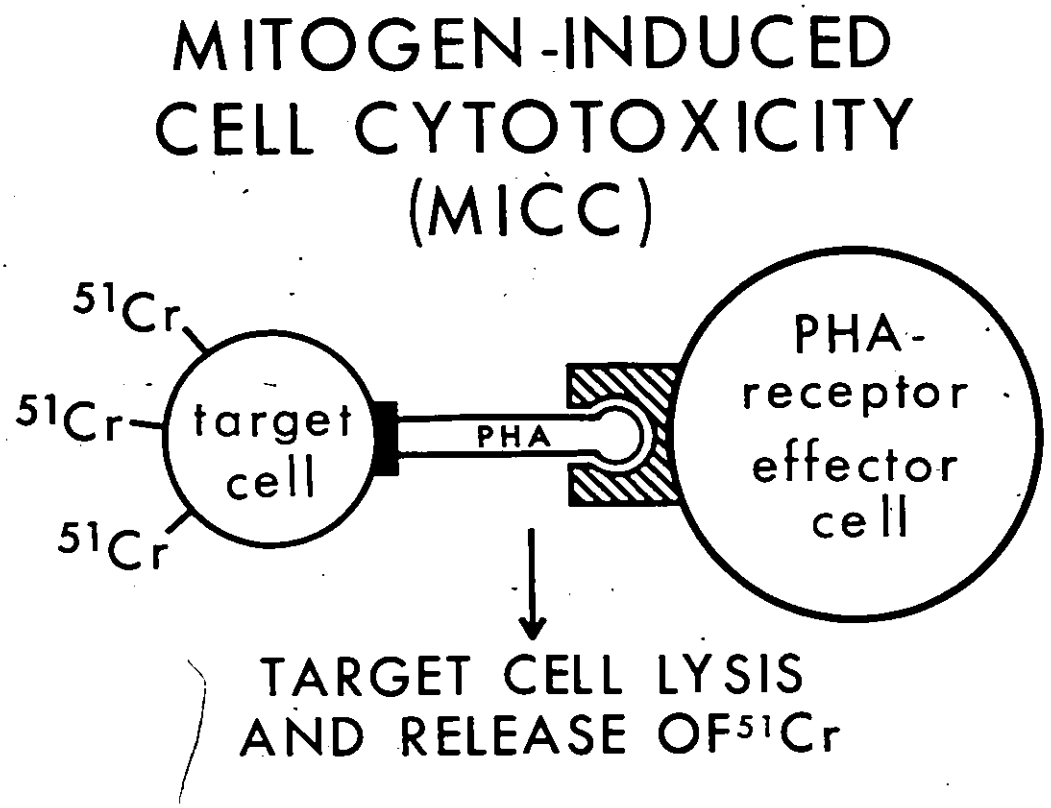

Figure 3:-- Mitogen-induced Cell-Mediated Cytotoxicity (MICC).

ADCC cytotoxic activity has also been variously attributed to eosinophils and neutrophils. The confusion regarding the precise nature of the cell mediating ADCC activity may be attributed to findings which indicate that although the reaction is dependent on target cell-fixed IgG and in some cases IgM antibodies, nevertheless the identity of the effector cell varies with the type of target cell used in the assay system.

In the assessment of ADCC cytotoxic activity in man, it is invariably only circulating cells that are evaluated. It is generally assumed, but not yet proven, that circulating ADCC cytotoxic effector cells in man are representative of similar cells present or generated in other lymphoid tissues and organs. The question must therefore be asked, whether in fact ADCC cytotoxic effector cells exist in parenchymal lymphoid organs which are similar in activity to circulating ADCC cytotoxic effector cells and if so, in which organs are they confined?

The resolution of this problem can be realized in the experimental animal in which one can simultaneously investigate circulating WBC and cells of the different lymphoid tissues for ADCC cytotoxic activity. In this way, one can demonstrate whether or not the circulating ADCC cytotoxic effector cells are identical to their functional counterparts within the lymphoid

tissues. It is also important to know which lymphoid tissues possess ADCC cytotoxic effector cells.

These considerations constituted the rationale for this investigation.

The outbred New Zealand White rabbit was selected for this study because of:

(i) its size and ease of handling;

(ii) the accessibility of lymphoid tissues and the ease of retrieval of large numbers of cells;

(iii) its outbred nature (akin to that of man);

(iv) the approximation of some of its physiological anatomy to that of man, a point in fact being the hematopoietic nature of bone marrow in rabbit and man. Both rabbit and man stand in contrast to the rodent by the normal absence of hematopoiesis in spleen and lymph node.

To date, ADCC cytotoxic effector cell function in the rabbit has been ascribed to cells prepared from thymus, mesenteric lymph nodes (MLN), peritoneal exudate cells (PEC), WBC, spleen and bone marrow. However, surprisingly, cells of the gut-associated lymphoid tissues (GALT: appendix, sacculus rotundus and Peyer's patches) have not so far been investigated for ADCC cytotoxic activity.

The objectives of this investigation were therefore:

(i) to determine the optimal culture conditions for ADCC cytotoxicity with rabbit cells;

(ii) to define precisely the tissue distribution of ADCC cytotoxic effector cells in the rabbit;

(iii) to define the functional and morphological homogeneity or heterogeneity of the ADCC cytotoxic effector cell in the rabbit; and

(iv) to define the role played by these cells in humoral immunity, cell-mediated immunity and in local immune responses.

The tissues investigated for ADCC cytotoxicity in the rabbit were: bone marrow, WBC, spleen, appendix, sacculus rotundus, Peyer's patches, mesenteric and popliteal lymph nodes and thymus.

It is hoped that the simultaneous investigation of numerous rabbit lymphoid tissues will address those questions outlined in the rationale for this investigation.

CHAPTER II

A REVIEW OF THE LITERATURE ON ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY

2.1 Antibody-Dependent Cell-Mediated Cytotoxicity: An Introduction

"Cellular cytotoxicity" is a phrase in the Immunological Literature used in part to describe a group of cytotoxic reactions effected by unsensitized lymphocytes-leukocytes. These reactions are known and further defined by nomenclature as antibody-dependent cellular cytotoxicity (ADCC), mitogen-induced cellular cytotoxicity (MICC) and naturally-occurring cellular cytotoxicity (NOCC), respectively. Antibody-dependent cellular cytotoxicity is the reaction of principal interest in this work. A brief review of the experimental data by which ADCC has become known and characterized is therefore in order.

Immunologically specific cell damage mediated by lymphocytes was first described by Govaerts in 1960. He reported that canine lymphocytes obtained through thoracic duct drainage and sensitized to renal tissue by means of renal allografts were capable of aggregating to, and subsequently destroying, these same

renal cells within 24 to 48 hours of tissue culture. He also noted that lymphoid cells similarly obtained from normal dogs or cats sensitized to a third party allograft were ineffective in destroying the renal allograft target cells in culture. However, anti-target cell antibody was not described by Govaerts (1960) as being of critical importance to the cytotoxic reaction in these experiments. This, however, was not the case in the work published by Möller in 1965.

Möller (1965) found that non-immune murine lymph node cells were capable of killing allogeneic and semi-allogeneic (F1 hybrid) sarcoma cells in the presence of heterologous heat-inactivated antiserum directed against the H-2 antigens of the murine target sarcoma cell. Non-immune lymph node cells syngeneic to the target cells were inactive even in the presence of the antiserum. Although ADCC is not conclusively described in this study, a non-immune cell(s) (murine lymph node cells) is nevertheless described as being capable of effecting the destruction of an antibody (murine anti-H-2 antibody)--coated target cell (murine sarcoma cells). The first experiments, however, to convincingly demonstrate antibody-dependent target cell lysis by non-immune lymphocytes were published by Perlmann and Holm in 1968 and further documented by MacLennan and Loewi later in the same year.

Perlmann and Holm (1968) found that antiserum from guinea pigs immunized with Bacillus Calmette-Guerin (BCG) or killed tubercle bacillus was capable of facilitating the lysis of PPD (purified protein derivative of tuberculin)-coated chicken erythrocytes by normal guinea pig spleen cells or circulating lymphocytes. The degree of lysis observed was comparable to that seen with immune guinea pig spleen cells. This suggested the existence of a very efficient mechanism whereby target cell destruction could be effected by normal lymphocytes in the presence of anti-target cell antibodies or antibodies directed against target cell-associated antigens. There was, nevertheless, some question about the validity of the conclusions drawn from these experiments (Möller, 1965; MacLennan and Loewi, 1968; Perlmann and Holm, 1968) as the concentration of antiserum used in these early studies was in fact capable of causing complement-mediated target cell lysis. It has subsequently been determined that the concentration of antiserum which need be used to effect antibody-dependent cell-mediated target cell destruction by normal, unsensitized effector cells is below that concentration which will induce complement-mediated cytolysis of the target cells (MacLennan, 1972a, 1973; Cerottini and Brunner, 1974).

Since the initial observation of antibody-dependent cell-mediated cytotoxicity (ADCC) of appropriately antibody-sensitized target cells by non-immune effector cells (Möller, 1965; Perlmann and Holm, 1968; MacLennan and Loewi, 1968; Perlmann and Holm, 1969; MacLennan, 1972a, 1973; Perlmann et al, 1972; Cerottini and Brunner, 1974; Perlmann, 1976, Lovchik and Hong, 1977, Pearson, 1978), various experimental models have been utilized to investigate and measure ADCC cytotoxic activity. ADCC cytotoxicity has been demonstrated using nucleated and non-nucleated target cells such as fowl erythrocytes (chicken [MacLennan and Loewi, 1968; Perlmann and Holm, 1969; Bubenik et al, 1970; Perlmann and Perlmann, 1970; Perlmann et al, 1972; Dennert and Lennox, 1973; Trinchieri et al, 1973; Wisloff et al, 1974a; Kovithavongs et al, 1975]; duck [Bubenik et al, 1970]), Chang liver cells (MacLennan, 1972a and 1973); human lymphocytes (MacLennan, 1972a) and lymphoblasts (Trinchieri et al, 1973, 1975a; Kovithavongs et al, 1975; Suthanthiran et al, 1979); fibroblasts (duck [Trinchieri et al, 1975b]; mouse [Möller and Svehag, 1972]; human [Lundgren and Möller, 1969; Timonen, 1979; Trofatter and Daniels, 1979]), tumour cell lines (P815 [Greenberg et al, 1975a; Lovchik and Hong, 1977], Moloney virus-induced tumours [Forman and Möller, 1973]), virus-infected cells (herpes simplex [Kohl et al, 1978, 1979; Trofatter

and Daniels, 1979]; respiratory syncytial virus [Meguro et al, 1979]; equine infectious anemia virus [Fujimiya et al, 1979]), parasites (schistosomula of Schistosoma mansoni [Butterworth et al, 1977; Capron et al, 1978a, 1978b; Glauert et al, 1978; Kassis et al, 1979]; Trypanosoma cruzi [Madeira et al, 1979; Olabuenaga et al, 1979]; microfilariae of Dipetalonema viteae [Weiss and Tanner, 1979]) and mammalian erythrocytes (sheep [Van Boxel et al, 1972; Perlmann, 1976]; burro [Kovithavongs et al, 1975]; human [Kovithavongs et al, 1975; Perlmann, 1976; Lovchik and Hong, 1977; Shaw et al, 1978b]).

In addition, ADCC cytotoxic activity has been ascribed to various leukocyte populations allogeneic (MacLennan et al, 1969; Gelfand et al, 1972; Resch et al, 1974; Lovchik and Hong, 1977; Imir et al, 1977) xenogeneic (MacLennan et al, 1970; Lovchik and Hong, 1977), or syngeneic (McConnachie and Dossetor, 1973; Lovchik and Hong, 1977) to the sensitizing anti-target cell antibodies and/or target cells.

The precise nature of the cell-mediating ADCC cytotoxic activity is not yet clearly defined. The confusion regarding its identity may be largely accounted for by the differences in the test systems utilized. However, there is some evidence which would suggest that the identity and the distribution

of ADCC cytotoxic effector cells is species dependent. Inter-species variation in ADCC has been noted both at the effector cell and/or antibody level (Zighelboim and Gale, 1974; Ormerod and Jolley, 1976; Imir et al, 1977). To date, ADCC cytotoxic activity has been generally attributed to the mononuclear cells (lymphocytes, macrophages/monocytes) of human (Perlmann and Holm, 1969; Möller and Svehag, 1972; Holm and Hammarstrom, 1973; Kovithavongs et al, 1975; Larsson and Ohlander, 1975; MacDonald et al, 1975; Perlmann and Wahlin, 1976; Shaw et al, 1978a, 1978b; 1979; Wasik, 1978; Hafeman and Lucas, 1979; Perussia et al, 1979; Serio et al, 1979; Shen et al, 1979; Waytz and Douglas, 1979), animal (monkey, rat, mouse, guinea pig, rabbit, swine [MacLennan and Harding, 1970; MacLennan et al, 1969; Gelfand et al, 1972; Larsson and Perlmann, 1972; Greenberg et al, 1973a, 1973b, 1975a, 1975b; Kedar et al, 1974; Lovchik and Hong, 1977] and fowl (duck, chicken [Bubenik et al, 1970; Calder et al, 1974a; Imir et al, 1977]) origins. However, ADCC cytotoxic activity has also been variously attributed to eosinophils (Butterworth et al, 1975, 1979; Capron et al, 1978a, 1978b; Glauert et al, 1978; Kassis et al, 1979) and neutrophils (Möller and Svehag, 1972; Gale and Zighelboim, 1974; MacDonald et al, 1975; Zighelboim et al, 1976; Shaw et al,

1978b; Olabuenaga et al, 1979) similarly of human and animal origin.

The controversy regarding the precise nature of the cell which mediates ADCC cytotoxic activity may be a reflection of current findings which indicate that although the cytotoxic reaction cannot occur in the absence of anti-target cell antibodies, nevertheless the identity of the effector cell appears to vary with the type of target cell used in the assay system (Perlmann and Holm, 1969; MacLennan, 1973; Perlmann, 1976; Lovchik and Hong, 1977; Pearson, 1978). This dilemma has yet to be resolved.

2.2 The Role of T Cells in ADCC Cytotoxicity

The results of early investigations into the nature of the ADCC cytotoxic effector cell indicated that thymus-derived lymphocytes (conventionally defined and herein defined as cells possessing theta antigens and/or SRBC receptors) were not effective in mediating antibody-dependent cellular cytolysis of antibody-sensitized target cells.

Van Boxel et al (1972) and Britton et al (1973) demonstrated in the mouse that ADCC cytotoxic effector cells were not thymus derived. Murine spleen cells (Van Boxel et al, 1972) or purified murine spleen

lymphocytes (Britton et al, 1973) treated with anti-theta antiserum and complement (which effectively destroys those cells carrying the murine T cell theta antigenic marker) were found to retain their ADCC cytotoxic activity towards antibody-sensitized target cells. Likewise, MacLennan (1973) found that splenic lymphocytes from athymic (nu/nu) mice were cytotoxic towards antibody-sensitized target cells, thus further substantiating the conclusions of Van Boxel et al (1972) and Britton et al (1973) that the ADCC cytotoxic effector cell in the mouse is not thymus derived in the conventional ADCC cytotoxicity assay.

However, ADCC cytotoxic effector cell status has been ascribed to murine thymocytes under special circumstances. Dennert and Lennox (1973) were the first workers to report that murine T cells (thymocytes) were capable of acting in ADCC to destroy IgM-sensitized target cells (ChRBC). This data was later confirmed by Lamon et al (1975a; 1975b; 1975c) using a murine-Maloney sarcoma virus system. It would appear then, that at least in the mouse, the assignation of ADCC cytotoxic effector cell status to the thymocyte or T cell necessitates the specification of the experimental system utilized.

Harding et al (1971) likewise demonstrated, through the use of irradiation and thymectomy, that T cells

were not responsible for ADCC cytotoxicity in the rat. In this model it was shown that the ADCC cytotoxicity of spleen cells taken from thymectomized (at 6 weeks of age) and irradiated (a total of 1,000 rads in 5 equal doses) rats was unaltered following these manipulations whereas the capacity of these spleen cells to mount a blastogenic response when cultured with the T cell mitogen phytohaemagglutinin (PHA) (8 weeks following the last dose of irradiation) was almost totally absent (1 percent response). This failure to respond to the T cell mitogen PHA suggested that the spleen cell preparation was devoid of PHA-reactive T cells. The spleen nevertheless did indeed possess ADCC cytotoxic effector cells.

Lovchik and Hong (1977) have also found rat thymic lymphocytes to be lacking in ADCC cytotoxic effector cell activity. This is in agreement with the earlier observations of MacLennan and Harding (1970) and MacLennan (1972a) with rat thymocytes.

Studies of ADCC cytotoxicity in man conducted with circulating lymphocytes of patients with thymic deficiencies revealed that these lymphocytes were capable of effecting the ADCC cytotoxic destruction of antibody-coated target cells (sheep and horse erythrocyte as well as P815 murine mastocytoma target cells), indirectly suggesting that in man, as in the rodent,

the ADCC cytotoxic effector cells were not thymus-derived (Lovchik and Hong, 1977).

Early studies of human circulating T-derived lymphocytes obtained through rosetting techniques (Perlmann et al, 1972, 1975; Jondal et al, 1972; Perlmann, 1976) or by passage through Ig-anti-Ig columns (Perlmann et al, 1972, 1975; Perlman, 1976) revealed that these cells were inactive in the conventional ADCC assay as were similarly derived T cells in other experimental models (Harding et al, 1971; Van Boxel et al, 1972; Britton et al, 1973). The data of Lovchik and Hong (1977) also concurred with the observations of the above workers although they noted additionally that human thymocytes directly assayed for ADCC cytotoxic activity showed no or low levels of activity depending on donor age.

The data presented by Gelfand et al (1972), Resch et al (1974) and Bona et al (1975; 1976) in the Lagomorph indicated that rabbit thymic cells were significantly active in the ADCC assay. This was in marked contrast to the findings of workers using rodent (rat, mouse) and human experimental models in the conventional ADCC cytotoxic assay. However, it was supportive of the data of Dennert and Lennox (1973) and Lamon et al (1975a; 1975b; 1975c) in the mouse described in a non-conventional IgM-mediated ADCC cytotoxicity assay. The apparent

contradictions in this matter may be a reflection of interspecies differences and/or variations in the testing procedures utilized, a point in fact being the data available on T cell ADCC activity in the mouse.

It has been recently shown that a proportion of human T cells, previously thought to be devoid of immunoglobulin Fc receptors, reveal these receptors upon incubation under the appropriate tissue culture conditions (Lamon et al, 1975a, 1975b, 1975c; Moretta et al, 1975; Ferrarini et al, 1976; MacConnell and Hurd, 1976; Wahlin et al, 1976; Fuson and Lamon, 1977; Smeraldi et al, 1977; Fuson et al, 1978). For example, normal T cells (Lamon et al, 1975a, 1975b, 1975c; Moretta et al, 1975, 1976; MacConnell and Hurd, 1976; Wahlin et al, 1976; Fuson and Lamon, 1977; Fuson et al, 1978) display receptors for the Fc regions of IgM and to a much lesser extent IgG following overnight incubation at 37°C with FBS. Samarut et al (1976) observed that the Fc receptor could be detected on 7 percent of T cells. Smeraldi et al (1977) identified receptors for Fc and C'3 on more than 50 percent of T cells following stimulation of the T cells with PHA for 16 hours. They suggested that receptors for Fc and C'3 are not constant characteristics of normal resting cells, but can be expressed when required. It has in fact been determined that 5 to 15 percent of human lymphocytes possess both T cell markers

(E-RFC or E rosette-forming cells) and Fc receptors for IgG and that these cells are indeed distinct from B cells (SIg-bearing cells) and are in fact thymus-derived (Van Oers et al, 1977). It has been conclusively demonstrated, using various preparations of antiglobulin antibodies, soluble aggregates of gamma-globulin and soluble immune complexes (Basten et al, 1972a, 1972b; Dickler and Kunkel, 1972; MacLennan, 1972a; Perlmann et al, 1972; Van Boxel et al, 1972; Dickmeiss, 1974a, 1974b; Trinchieri et al, 1975a, 1975b) that the establishment of contact between the Fc portion of the immunoglobulin molecule and the complementary Fc cell surface receptor on the ADCC cytotoxic effector cell is critical for the demonstration of ADCC cytotoxic activity. The discovery that human T cells may bear Fc cell-surface receptors leads to speculation as to their ability to participate in ADCC as they possess this fundamental requirement for ADCC cytotoxic potential. This has indeed been proven to be the case. These Fc cell-surface receptor-bearing T cells have been shown to demonstrate ADCC cytotoxic activity by a plaque assay (Wahlin et al, 1976) and have been found to be distinct from cytotoxic T lymphocytes (CTL) which are IgG independent and do not possess Fc receptors.

Although the early evidence would tend to suggest that T cells are not the principal effectors of ADCC

cytotoxicity, earlier experimental evidence and recent evidence based on analysis and fractionation of lymphocytes according to their cell surface receptors would suggest that a population(s) of Fc cell-surface receptor-bearing T cells in several experimental models (man: Lamon et al, 1975a, 1975b, 1975c; Moretta et al, 1975, 1976; mouse: Dennert and Lennox, 1973; rabbit: Gelfand et al, 1972; Resch et al, 1974; Bona et al, 1975, 1976) is capable of mediating ADCC cytotoxicity. It would also appear that this T cell-mediated ADCC cytotoxic activity is distinct from that of cytotoxic T lymphocytes (CTL) which are IgG independent and do not possess Fc receptors.

2.3 The Role of B Cells in ADCC Cytotoxicity

Although various investigators have successfully demonstrated in several animal species (mouse [Van Boxel et al, 1972; Britton et al, 1973; MacLennan et al, 1973]; rat [Harding et al, 1971; Lovchik and Hong, 1977]) and in man (Perlmann et al, 1972, 1975; Jondal et al, 1972; Perlmann, 1976; Lovchik and Hong, 1977) that ADCC cytotoxicity is generally not thymus-dependent (with the notable exception of rabbit thymic lymphocytes [Gelfand et al, 1972; Resch et al, 1974; Bona et al, 1975, 1976] and lymphocytes bearing both Fc receptors and T cell surface markers [mouse: Dennert and Lennox,

1973; rabbit: Gelfand et al, 1972; Resch et al, 1974; Bona et al, 1975, 1976; man: Lamon et al, 1975a, 1975b, 1975c; Moretta et al, 1975; Ferrarini et al, 1976; MacConnell and Hurd, 1976; Samarut et al, 1976; Wahlin et al, 1976; Fuson and Lamon, 1977; Smeraldi et al, 1977; Van Oers et al, 1977; Fuson et al, 1978]) the evidence for the participation of a cytotoxic B cell (conventionally defined and herein defined as a cell possessing surface immunoglobulins) in the destruction of IgG antibody-coated target cells is far from certain. However, the well-documented presence of Fc receptors on mature circulating B cells and on B cells in the peripheral lymphoid tissues indicate that some mature B cells may have ADCC cytotoxic potential (Perlmann, 1976).

Experiments conducted by Van Boxel et al (1972) to identify the nature of the murine ADCC cytotoxic effector cell demonstrated that cytolysis of SIg-bearing murine spleen cells through treatment with anti-k antiserum and complement diminished the capacity of the remaining spleen cells to participate in ADCC cytotoxicity to an extent commensurate with the level of destruction of SIg-bearing cells. This suggested that the ADCC cytotoxic effector cell in the murine spleen was indeed B cell-derived. However, further experiments by the same workers showed that similar treatment of murine spleen cells with anti-k antiserum in the absence of

complement, while causing no lysis of B cells, was quite effective in reducing the ADCC cytotoxicity of the treated spleen cell preparations, presumably through an Fc cell surface receptor blocking mechanism. These data strongly imply that the B cells express ADCC cytotoxic activity in the mouse.

Forman and Möller (1973) similarly observed suppression of ADCC cytotoxicity by pretreatment of murine spleen cells with anti-immunoglobin antiserum and complement followed by trypsinization to remove surface-bound antigen-antibody complexes. These treated preparations showed a lowered response to mitogenic stimulation with LPS (lipopolysaccharide of E. coli OSS:BS) but not to concanavalin A (Con A) suggesting that B cells were selectively destroyed by this treatment (LPS non-specifically activates mouse B cells as measured by increased DNA synthesis and induction of polyclonal antibody synthesis [Andersson et al, 1972]). However, the effect of anti-immunoglobin antiserum in the absence of complement was not assessed.

Perlmann et al (1972) found that highly purified human peripheral blood lymphocytes were retained on a column of immunoglobin-anti-immunoglobin-coated glass beads. The lymphocytes in the column effluent showed little ADCC cytotoxic activity, were significantly depleted of cells bearing SIg and were enriched in T

cells as determined by their ability to rosette spontaneously with SRBC. This suggested initially that the human ADCC cytotoxic effector lymphocyte did in fact bear SIg and therefore could be considered to be B cell-derived. However, the low level of ADCC cytotoxic activity found in the Ig-anti-Ig column effluent was later concluded by Perlmann et al (1974) and Perlmann (1976) to be due to the retention of cells bearing Fc receptors as well as SIg on the Ig-anti-Ig columns. Perlmann demonstrated using HGG ([human gamma-globulin]/rabbit $F[ab']_2$)-anti-HGG columns that lymphocytes in the column effluent were ADCC reactive although less than 1 percent of these cells were found to possess SIg. These data suggested that human ADCC cytotoxic activity was a property of lymphocytes with Fc receptors but not of those with SIg.

Although some of the above data may allow speculation as to the B cell identity of the ADCC cytotoxic effector cell, the overwhelming evidence does not, in fact, support this interpretation.

The complete absence of detectable ADCC cytotoxic activity by rat thoracic duct cells is extremely strong evidence against the B cell identity of ADCC cytotoxic effector cells in the rat as thoracic duct lymph is an excellent source of antibody-forming cell precursors, i.e., B cells (Gowans and McGregor, 1963; Ellis et al,

1969; MacLennan, 1972a, 1973; Cerottini and Brunner, 1974). Opposite results, however, have been reported by Fakhri and Hobbs (1972).

Other evidence which does not favour the participation of antibody-producing cells in ADCC cytotoxicity is that presented by Harding and MacLennan (1972). They observed that rat lymph nodes draining a site inoculated with Chang cells, although rich in anti-Chang cell antibody-forming cells, were a poor source of ADCC cytotoxic effector cells. Similarly, Pudifin et al (1971) observed that rats subjected to 850 r whole body irradiation were unable to mount an antibody response to Chang cells at a time when the ADCC cytotoxic activity of spleen cells had returned to within 10 percent of normal, further suggesting that the ADCC cytotoxic effector cell in the rat was not B cell-derived.

Calder et al (1974a) investigated ADCC cytotoxicity in untreated and bursectomized chickens and observed that the ADCC cytotoxic activity of circulating chicken lymphocytes was unaffected by bursectomy in ova at 17 days of incubation. At the time of analysis for ADCC cytotoxic activity, 7 of the 13 bursectomized chickens were agammaglobulinemic and the remainder were hypogammaglobulinemic. This would indicate that, in the chicken, the ADCC cytotoxic effector lymphocytes are not bursa-processed.

The bulk of human clinical evidence, derived from studies of ADCC cytotoxicity in patients with severe hypogammaglobulinemia and subnormal B lymphocyte numbers, indicates that these patients did have normal ADCC cytotoxic function and would suggest further that the ADCC cytotoxic effector cell is not a B cell (MacLennan, 1972a, 1973; Wisloff and Froland, 1973; Lovchik and Hong, 1977). However, Rachelefsky et al (1975) associated deficient ADCC cytotoxic activity in immuno-deficient patients with decreased peripheral B cell numbers thereby postulating a primary role for the B lymphocyte in ADCC cytotoxicity. However, they also described deficient ADCC cytotoxic activity in two patients with normal B cell numbers. Similarly Campbell et al (1972) described a patient with macroglobulinemia who was grossly deficient in ADCC cytotoxic effector cells. T cell function was normal in this patient.

There is, therefore, no clear cut evidence that SIg-bearing or Ig synthesizing lymphocytes are ADCC cytotoxic effector cells in either rodent, human or avian experimental models. Nevertheless, the possibility still exists that a precursor lymphocyte destined to become a fully differentiated B cell and bearing only Fc cell surface receptors constitutes an ADCC cytotoxic effector cell population. MacDermott et al (1975) observed that a high proportion of "null" cells bearing only Fc receptors and

considered to be ADCC cytotoxic effector cells can convert to SIg-bearing cells (B cells) after several days in culture. They (Chess et al, 1975) suggested that null cells or a subpopulation of null cells constituted a subset of immature B cells thereby inferring that ADCC effector cells belong to the B cell lymphocytic lineage.

2.4 The Role of Adherent Cells, Monocytes and Macrophages in ADCC Cytotoxicity

The minimum requirement for any cell to be potentially active in ADCC is the possession of the Fc cell surface receptor for the Fc region of the IgG, and in some cases the IgM, antibody molecule (Dennert and Lennox, 1973; Lamon et al, 1975a, 1975b, 1975c; Moretta et al, 1975, 1976). Fc receptors have been documented to be present on lymphocytes, polymorphonuclear leukocytes (PMN), macrophages, monocytes, mast cells, platelets and on some tumour cells (Kerbel and Davies, 1974). Although the Fc receptor is essential for the expression of ADCC, the cytotoxicity observed ultimately depends on the relationship between the effector cells, antibody molecules and target cells (Lovchik and Hong, 1977) as well as on the subtleties of the interplay between these components. The assay system must therefore be clearly defined before drawing conclusions as to the nature of the ADCC cytotoxic effector cell.

This is worthy of particular mention in this discussion of the role of adherent cells, monocytes and macrophages in ADCC, as erythrocytes (avian and mammalian) are routinely used as target cells in ADCC assays and moreover macrophages and monocytes are well known to phagocytose and destroy opsonized erythrocytes. This activity must, therefore, be differentiated from an ADCC mode of target cell destruction which is presumably distinct from the above (MacLennan, 1972a, 1972b; Lovchik and Hong, 1977).

In the mouse, ADCC cytotoxic effector activity has been found in both adherent and non-adherent spleen cell fractions. Greenberg et al (1973a; 1973b) demonstrated that murine spleen cells, depleted of phagocytic cells and of SIg-bearing cells by passage through a column of purified rabbit anti-mouse Fab-conjugated Sephadex, retained their ADCC activity against antibody-sensitized ChRBC. Further depletion of the T cell component of the column effluent by treatment with anti-T cell antiserum and complement did not alter the degree of ADCC cytotoxicity exhibited by the remaining cells. This led these workers to conclude that the ADCC cytotoxic effector cell in the mouse spleen was a non-T, non-SIg-bearing cell. Additional work by these investigators showed that the cell effecting ADCC was in fact an adherent cell whose properties

(cellular size, capacity to be inhibited by certain subclasses of immunoglobulin G) corresponded to those of the murine monocyte. This led them to conclude that the ADCC cytotoxic effector cell in the murine spleen was indeed a monocyte. This cell was described as a "myeloid K cell" (Greenberg et al, 1973a; 1973b). These findings were confirmed by Pross et al (1974).

Greenberg et al (1975a; 1975b) went on to demonstrate the existence of a population of non-adherent lymphocytes capable of mediating ADCC against antibody-coated ChRBC. These were termed "non-adherent lymphocytic K cells" to distinguish them from the previously described murine "myeloid K cell" (Greenberg et al, 1975a, 1975b; Roitt et al, 1976).

These findings were in agreement with those of Zighelboim et al (1976) who showed that EL-4 target cells sensitized with rabbit antibody were destroyed by two groups of cells, notably adherent murine spleen and peritoneal cells and non-adherent murine spleen cells. It would also appear that in this study, the adherent-poor cell preparations were less effective than the adherent-rich effector populations. A cultured cell line of murine peritoneal macrophages has also been shown by Walker and Demus (1975) to be capable of effecting lysis of antibody-coated ChRBC target cells.

It would therefore appear that in the mouse there are two categories of cells that are capable of effecting ADCC:

(i) a non-adherent "K cell" of uncertain lineage, and

(ii) an adherent "myeloid K cell" with or without phagocytic properties.

This latter cell type may be represented by immature granulocytes, polymorphonuclear leukocytes, monocytes and/or macrophages (Pearson, 1978).

(iii) Under certain circumstances, the murine T cell has also been demonstrated to possess ADCC cytotoxic activity (Dennert and Lennox, 1973).

Several investigators (MacLennan and Harding, 1970; Maclennan, 1972a; Cerottini and Brunner, 1974) have shown that in the rat, ADCC cytotoxic activity can be found in the following tissues in order of their effectiveness in ADCC:

(i) peritoneal exudate (markedly active);

(ii) spleen (moderately active);

(iii) blood lymphocytes (mildly active);

(iv) lymph node (poorly or not active);

(v) thymic or thoracic duct lymph (not active).

These workers have excluded the participation of macrophages in this system on the basis of the observation

that the removal of phagocytic or adherent cells from the peritoneal cell population had no effect on cytotoxicity. These data were generated from experiments in which the target cells utilized in the ADCC assay were Chang liver cells.

Garovoy et al (1976) on the other hand, demonstrated in the rat that two populations of cells were capable of destroying antibody-sensitized rat thymocyte target cells. An adherent and a non-adherent effector cell population was identified. It was also noted that the only constant surface marker of the rat K cell in this study was an Fc cell surface receptor. Sanderson et al (1975) have similarly noted that there are two populations of ADCC cytotoxic effector cells in rodent spleens capable of destroying antibody-coated chicken erythrocyte target cells. However, non-adherent cells alone were capable of effecting the destruction of antibody-coated target cells derived from two tissue culture lines.

These data contrast with those of MacLennan (1972a) and MacLennan and Harding (1970) and emphasize the role of the target cell in specifying the identity of the ADCC cytotoxic effector cell.

In the rabbit, Gelfand et al (1972), Gelfand and Resch (1973) and Resch et al (1974) demonstrated that two cell populations were capable of effecting ADCC

cytolysis of rabbit antibody-sensitized chicken erythrocyte target cells:

(i) an adherent, and,

(ii) a non-adherent ADCC cytotoxic effector cell population.

Both cell types were found to be present in the spleen whereas thymus was noted only to contain the non-adherent effector cell group. These two populations were differentiated on the basis of time course studies and activity in the presence of anti-immunoglobulin.

On the basis of the evidence presented by a number of investigators (MacLennan, 1972a; MacDonald et al, 1975; Trinchieri et al, 1975a, 1975b; Nelson et al, 1976; Lovchik and Hong, 1977), it would appear that in man, the ADCC destruction of target cells derived from tissue culture cell lines on the one hand is not effected by adherent and/or phagocytic cells, but rather by cells that are presumably of lymphocytic lineage (non-T, non-B, SIg-negative lymphocytes). On the other hand, it would appear that numerous cell types of the human peripheral blood leukocyte pool are capable of effecting the ADCC cytolysis of antibody-sensitized chicken erythrocyte target cells. Human adherent cells (MacDonald et al, 1975; Simchowitz and Schur, 1976), monocytes (Perlmann et al, 1975), macrophages (Nelson et al, 1976; Lovchik and Hong, 1977) and lymphocytes

(MacDonald et al, 1975; Perlmann et al, 1975; Cordier et al, 1976; Nelson et al, 1976; Simchowitz and Schur, 1976; Lovchik and Hong, 1977) have all been implicated as effector cells in the ADCC cytotoxic destruction of ChRBC target cells.

The diversity of peripheral blood leukocytes capable of effecting ADCC-induced cytolysis of ChRBC target cells is not observed when erythrocytes of human origin are substituted for avian or other erythrocytes as the target cells in the experimental system. The human erythrocyte is noted to be destroyed principally by cells of presumed myeloid origin (monocytes, macrophages). Several authors (Holm, 1972; Hinz and Chikosky, 1972; Holm and Hammarstrom, 1973; MacDonald et al, 1975; Lovchik and Hong, 1977; Norris et al, 1979) are in agreement with this observation. However, two of the papers cited in this report do state that some erythrolysis, albeit of a much smaller degree, is observed to be effected by lymphocytes when the cells are maintained in culture for a prolonged period of time (Holm and Hammarstrom, 1972; Hinz and Chikosky, 1972). This is not surprising as several investigators have reported that lymphocytes are more slowly active in the ADCC-mediated destruction of target cells than are non-lymphoid cytotoxic effector cells.

Investigation of the kinetics of the ADCC reaction by Perlmann and Holm (1969) and Perlmann and Perlmann (1970) lead these workers to conclude that greater than a 5 percent monocytic contamination of a lymphocyte preparation was found to significantly increase the initial rate of ChRBC cytolysis. Gelfand et al (1972) also reported that a 0.5 percent contamination of lymphocytes by PEC (70 to 80 percent macrophages, 20 to 30 percent granulocytes and lymphocytes) hastened the onset of the ADCC cytolysis of ChRBC target cells. Lundgren and Möller (1969) similarly noted that more than 1 percent contamination of a lymphocyte preparation with PMN and monocytes could lead to non-specific detachment of skin fibroblasts from monolayer cultures.

The data of Lundgren and Möller (1969) with non-erythroid target cells contradict those of MacLennan (1972a) with Chang target cells and those of a number of other workers who also used non-erythroid target cells (MacDonald et al, 1975; Trinchieri et al, 1975a, 1975b; Nelson et al, 1976; Lovchik and Hong, 1977). This contradiction may well be a manifestation of the relative sensitivities of the different assay systems used to study ADCC cytotoxicity.

One would conclude on the basis of these data that monocytes/macrophages and other non-lymphoid cells are rapidly acting in ADCC whereas lymphocytes are

more slowly acting. This would of necessity hold true only for those target cells susceptible to destruction by non-lymphoid cells as well as by lymphocytes. When target cells are susceptible to destruction by lymphocytes only, ADCC cytotoxicity initially proceeds linearly as a function of time and is seen to level off during several days in culture (Perlmann and Perlmann, 1970; Gelfand et al, 1972; Cerottini and Brunner, 1974; Lovchik and Hong, 1977; Pearson, 1978).

Monocytes and macrophages have been demonstrated to play an important role in ADCC, a role whose significance depends on the target cell chosen for the ADCC assay as well as on the animal origin of the proposed ADCC cytotoxic effector cells. Depending on the animal species and on the experimental parameters outlined for a given investigation, a worker may selectively choose to monitor the function of one or several of the effector cell types implicated in ADCC. To date, ADCC cytotoxic activity suggesting adherent cell, monocyte/macrophage activity has been ascribed to cells of rodent (mouse, rat), rabbit and human origin. Non-myeloid cells have also been shown to display ADCC cytotoxic activity in these animals. A variety of target cells have been utilized to elucidate effector cell populations and mechanisms of ADCC cytotoxicity in these experimental models. It is particularly

apparent, in the human system, that the choice of target cell in turn specifies the effector cell implicated in ADCC cytotoxicity. This of necessity dictates careful selection of the target cell so as to minimize the confusion, in an already chaotic situation, concerning the identity of the ADCC cytotoxic effector cell in man. This may also extend to animals and fowl. The possibility does, however, exist that the susceptibility of the erythrocyte target cell to monocyte/macrophage ADCC destruction is a reflection of the inability of erythrocytes to repair cell membrane damage in a manner as rapid and efficient as their tissue culture-derived counterparts.

Monocytes have been shown to be derived principally from bone marrow stem cells and to migrate into tissues and body cavities to become "fixed" or "free" macrophages following a period of time spent in circulation. The monocyte-macrophage system has been documented (Schalm et al, 1975) to function in the defense of the organism against microbial agents as well as in the removal of effete or damaged cells. Monocytes/macrophages are found at sites of acute inflammation but are particularly prominent at sites undergoing chronic granulomatous inflammation where they may take the form of monocytes, macrophages, epithelioid cells or giant cells. Monocytes have also been demonstrated

to function intimately in the immune response. They are thought to process antigen and to present it to lymphocytes in a highly immunogenic form and thereby interact with lymphocytes in the generation of a humoral immune response. The more recent identification of the presence of Fc cell surface receptors on monocytes/macrophages and the demonstration of the participation of these cells in ADCC may suggest yet another specific immunologic role for monocytes and macrophages and add to their already considerable and varied list of activities and functions in the living organism.

2.5 The Role of Neutrophils, Eosinophils and Platelets in ADCC Cytotoxicity

Fc receptors have been demonstrated to be present on the polymorphonuclear leukocytes (PMN) of several mammalian species (Henson, 1969) including those of man (Henson, 1969; Messner and Jelinek, 1970). Knowing that Fc receptors are essential for the demonstration of ADCC cytotoxic activity by lymphoid K cells and are also important in the ADCC cytotoxicity effected by monocytes/macrophages, the postulation that neutrophils play a role in ADCC is not unreasonable. In fact, as early as 1970, Perlmann and Perlmann suggested that human leukocytes were capable, as were lymphocytes,

of mediating the ADCC cytolysis of chicken erythrocytes sensitized with rabbit antibody. The leukocyte preparations utilized in these early investigations consisted of 80 to 90 percent PMN and 10 to 20 percent mononuclear cells (lymphocytes and monocytes). The study showed that leukocytes were rapidly acting in the ADCC assay but that target cell destruction after 24 hours of culture appeared to be largely non-specific. The opposite was true for lymphocytes. Erythrophagocytosis by PMN and monocytes was prominent and it was suggested to be an important feature of leukocyte-mediated ADCC destruction of chicken erythrocytes sensitized with rabbit antibody.

Holm (1972) reported that human erythrocytes sensitized by isoantiserum against A, B or Rh blood group factors, were lysed by human leukocyte preparations (80 to 90 percent PMN). Concurrent investigation of lymphocytic activity showed that pure lymphocyte suspensions (greater than 99 percent) were not cytotoxic toward isoantiserum-sensitized human erythrocyte target cells. Rather, it was noted that hemolysis appeared to increase with the number of non-lymphocytic (PMN and monocytes) cells added to the target cells. Again, erythrophagocytosis by PMN and monocytes was frequently observed and it was suggested to be one of the mechanisms of target cell destruction. Soluble hemolytic factors

were not detectable nor were killed leukocytes or leukocyte extracts capable of inducing cytolysis of the target cells. This led to the suggestion that direct contact between effector cell and target cell was required in order that cytolysis be effected. Perlmann and Perlmann had similarly proposed in 1970 that the lytic steps of the ADCC reaction required close contact between effector cells and target cells.

Whether or not the ADCC cytolysis observed in these early studies was the direct result of contamination of the leukocyte preparation with mononuclear cells (leukocytes and/or monocytes) or was in fact due to neutrophilic ADCC cytotoxic activity is unclear. Conclusive demonstration of human PMN ADCC cytotoxic activity utilizing pure neutrophil preparations (greater or equal to 98 percent) was made by Gale et al in 1974 and elaborated in subsequent studies (Gale and Zighelboim, 1974, 1975; Zighelboim et al, 1976). The target cells utilized in these studies were derived from the cell lines EL-4 (a chemical carcinogen-induced leukemia syngeneic to C57BL/6 mice) and (C58NT)D (a Gross virus-induced leukemia syngeneic to Wistar/Furth rats). The corresponding antisera were raised in rabbits and rats, respectively. It was found that PMN-induced cytolysis of antibody-sensitized target cells was Complement independent, rapid, detectable at low PMN

to target cell ratios and exceeded that of lymphocyte-mediated ADCC cytotoxicity particularly at the higher effector to target cell ratios. On this basis it was deemed unlikely that the ADCC cytotoxic activity of the PMN could result from mononuclear cell contamination as the PMN cell suspensions were 98 percent pure and had greater or at least comparable ADCC activity to that of the lymphocytes at all of the effector-target cell ratios tested. This was quite unlike the situation noted in the early studies where the ADCC cytotoxicity attributed to PMN activity could well have been due to mononuclear cell contamination owing to the large component of such cells (up to 20 percent) in some of the PMN cell preparations (Perlmann and Perlmann, 1970; Holm, 1972).

Gale and Zighelboim (1975) also undertook serial light microscopic studies to find out whether or not phagocytosis played a key role in the EL-4 target cell destruction by PMN. This was found not to be the case and is in marked contrast with the early reports in which erythrocytes were utilized as target cells for analysis of the ADCC reaction (Perlmann and Perlmann, 1970; rabbit antibody-sensitized chicken erythrocyte target cells; Holm, 1972: isoantiserum-sensitized human erythrocyte target cells). Gale and Zighelboim (1975) noted that rosette formation between PMN and rabbit

antibody-sensitized EL-4 target cells was a feature of the test cultures, whereas random distribution of effector and target cells was seen in the control cultures. In each of the rosettes observed, 3 to 8 PMN were seen to be closely associated with the cell membrane of a central EL-4 target cell. This is in agreement with the suggestions of Perlmann and Perlmann (1970) and Holm (1972) that the lytic steps of the ADCC reaction require close contact between effector cells and target cells.

These workers additionally concluded that the ADCC cytotoxicity attributed to the PMN was also immunologically specific. This conclusion was based principally on the results of experiments utilizing a criss-cross matching of absorbed antiserum and target cells. The demonstration of competitive inhibition by the addition of unlabelled target cells to cultures already containing radioactively labelled targets provided further evidence as to the immunologic specificity of the reaction. Inhibition of ADCC cytotoxicity could also be achieved by using aggregated gamma-globulin. This is also a feature of the ADCC cytotoxic activity effected by lymphocytes-K cells (Basten et al; 1972a, 1972b; Dickler and Kunkel, 1972; MacLennan, 1972a; Perlmann et al, 1972). These data and those derived from preincubation experiments which demonstrated that

PMN preincubated with rabbit anti-target cell antiserum were ineffective in mediating ADCC unless additional antiserum was added to the culture medium whereas similarly treated target cells were as susceptible to lysis as the control cells, lead Gale and Zighelboim (1975) to conclude that the ADCC cytotoxicity observed throughout their investigation was indeed due to PMN activity. It was also suggested that PMN-mediated ADCC activity involved a PMN population with demonstrable Fc cell surface receptors.

This was confirmed by Zighelboim et al (1976) in the following year by means of inhibition studies using aggregates of IgG, fractionation studies using EA monolayers and enzymatic studies using papain, neuraminidase and phospholipase-C as well as combinations of the above procedures. Fc receptors on human PMN have been previously identified by the ability of antibody-coated erythrocytes to bind to PMN (Henson, 1969; Messner and Jelinek, 1970) and it was concluded that a PMN Fc cell surface receptor was also required for the binding of the human PMN ADCC cytotoxic effector cell to the antibody-coated target cell in order to effect target cell destruction.

MacDonald et al (1975) investigated ADCC cytotoxicity by using various purified populations of cells derived from the human peripheral blood leukocyte-lymphocyte

pool. These were simultaneously assayed on three different target cells, notably chicken and human erythrocytes and P-815-X2 murine mastocytoma cells, a tissue culture cell line. They demonstrated that human PMN (more than 95 percent pure) were capable of lysing the erythrocyte target cells but were incapable of destroying the mastocytoma target cells. These findings were corroborated by those of Lovchik and Hong (1977). Likewise, Nelson et al (1976) found that ChRBC were susceptible to cytolysis by human PMN (greater than 95 percent pure) whereas Chang liver cells, another tissue culture cell line, were not. Trinchieri et al (1975a) found human lymphoblastoid target cells to be resistant to human PMN-mediated ADCC cytolysis.

It would appear then that target cell identity is as critical to the detection of PMN ADCC cytotoxic activity as it is to the detection of lymphocyte and/or macrophage-mediated ADCC cytotoxicity. Furthermore, it would also appear that tissue culture-derived target cells are less susceptible to lysis by human PMN as compared to erythrocyte target cells, a case reminiscent of that seen with human macrophage-mediated ADCC cytotoxicity.

Mantovani (1975) investigated the different roles of IgG and complement receptors in the phagocytosis of immune complexes by murine PMN. (The complexes

were: EA: sheep erythrocytes coated with murine anti-SRBC antiserum; EAC: sheep erythrocytes covered with murine anti-SRBC antiserum and murine complement). The granulocytes used in this study were obtained by peritoneal lavage following intraperitoneal injections of glycogen solution. Although the principal goal of this study was the analysis of cell surface receptors, it was noted that non-immune murine PMN were capable of binding to and of destroying isoantiserum-coated sheep erythrocytes in a manner reminiscent of ADCC-mediated target cell destruction. Penfold et al (1976) likewise implicated murine PMN and immature granulocytes, along with macrophages and lymphocytes, as being cytotoxic for antibody-coated chicken erythrocyte target cells. All of the effector cells utilized in this study were obtained from non-immune mouse spleens. It was additionally noted that eosinophils and basophils were observed not to exhibit any ADCC cytotoxicity whatsoever in this experimental system.

Eosinophils have, however, been implicated in the destruction of antibody-sensitized parasitic organisms. In point of fact, Butterworth et al (1974; 1975) were the first workers to ascribe a definitive role for normal human circulating eosinophils in the antibody-dependent, complement-independent destruction of the schistosomula of Schistosoma mansoni. The effector

cell in this system was purified by density gradient centrifugation and was identified as a granulocyte. Selective treatment of unpurified effector cells (i.e. a mixed population of human peripheral blood leukocytes) with anti-eosinophil antiserum and complement pointed to the eosinophil as being the principal, if not the sole, effector of the ADCC destruction of the antibody-sensitized schistosomula.

Direct evidence of the ADCC cytotoxic effects of eosinophil-enriched cell preparations was presented by Butterworth et al in 1977. In this investigation, cell suspensions containing up to 98.5 percent human eosinophils and devoid of neutrophils, were effective in mediating ADCC cytotoxicity towards antibody-sensitized schistosomula. On the other hand, comparable preparations enriched in either mononuclear cells or in neutrophils, and devoid of eosinophils, were inactive. It has also been demonstrated, in parallel studies, that the ADCC mechanism operative in man is also operative in the baboon, the natural host for S. mansoni in Africa (Butterworth et al, 1976).

It would also appear that a similar mechanism operates in the mouse. This is based on evidence presented by Mahmoud et al (1975) that in vivo ablation of circulating eosinophils by treatment with anti-eosinophil antiserum abolishes immunity in mice towards

the schistosomular target organisms, whereas anti-lymphocyte, anti-macrophage and anti-neutrophil antisera have no effect. Additional evidence of eosinophil involvement is presented by von Lichtenberg et al (1976) who report that a greater number of eosinophils are present at the sites of cercarial challenge in the ears of mice immune to S. mansoni than at comparable sites in normal mice.

Kassis et al (1979) documented that adherence to and killing of schistosomula by murine peritoneal exudate cells (PEC) was mediated through complement-independent opsonizing IgG. Both PEC-derived eosinophils and macrophages were seen to adhere firmly to the surface of the organisms, however only the macrophages were observed to invade them. Selective depletion of eosinophils by treating the PEC suspensions with mono-specific anti-eosinophil antiserum resulted in blocking the early adherence phase of the cellular reaction to the parasite but a substantial part of the PEC ability to kill the schistosomula was retained. The above evidence in the mouse might suggest that although eosinophils to play a role in the ADCC destruction of the schistosomula, cooperation between these and other leukocytes, notably macrophages, cannot be ruled out.

The evidence implicating the rat eosinophil in the ADCC destruction of S. mansoni antibody-sensitized schistosomula is less clear. Mackenzie et al (1977) reported that rat eosinophils could adhere to S. mansoni schistosomula sensitized with infected rat serum, however no cytotoxic activity against the target was clearly evidenced (Capron et al, 1975; 1978a; 1978b). Sher (1976), on the other hand, found that rat eosinophils were inactive against the schistosomula of S. mansoni and postulated that rat neutrophils were the major effector cell. Capron et al (1975) and Perez (1974; Butterworth et al, 1977) have also reported that macrophages are active in the antibody-dependent cell-mediated destruction of antibody-sensitized schistosomula. It is interesting to note that in Capron et al's (1975) experiments, the antibody involved was IgE, while in Perez' (1974; Butterworth et al, 1977), it was IgG.

Capron et al (1978b) presented evidence supporting the existence, in the rat, of an ADCC mechanism in which eosinophils were the effector cells in the presence of IgG_{2a}, the major subclass of rat IgG and the second class of anaphylactic antibody in the rat (the first is IgE). This suggested that the class of the antibody and not only that of the target cell determined the effector cell involved in the ADCC reaction.

These workers proposed that rat eosinophils interacted with mast cells to kill S. mansoni schistosomula. In fact, ultrastructural studies revealed the presence of mast cells and of eosinophils in contact with the schistosomula (Capron et al, 1978a; 1978b). Preliminary experiments (Capron et al, 1978a) indicated that mast cell depletion of eosinophil-rich cell populations decreased eosinophil-dependent ADCC cytotoxicity.

These studies, along with others (Capron et al, 1978a; 1978b) demonstrated that a minimum ratio of mast cells to eosinophils was required in order for eosinophils to express ADCC cytotoxic activity. Furthermore, the ADCC cytotoxic activity exhibited by eosinophil-enriched cell populations could be inhibited in part by the pretreatment of mast cells with heat aggregated IgG_{2a} immunoglobulins or with Fc fragments, but not by Fab' fragments, prior to their addition to a mast cell depleted eosinophilic cell suspension. This latter may suggest that adherence of the mast cell to the antibody-sensitized schistosomula may take place through an Fc cell surface receptor for the Fc piece of IgG_{2a}.

These data are reminiscent of that obtained in earlier investigations in other experimental systems where it was found that ADCC cytotoxic activity was sensitive to abrogation by pretreatment of effector

cells with aggregated IgG or by the addition of these soluble aggregates of IgG directly to the ADCC cell cultures (Basten et al, 1972a, 1972b; Dickler and Kunkel, 1972; MacLennan, 1972a; Perlmann et al, 1972). However, it must be pointed out that the data obtained in the former case serve to propose a role for mast cells as cooperators with eosinophils rather than as the principal effectors of antibody-dependent cellular cytotoxicity towards antibody-sensitized S. mansoni schistosomula in the experimental rat.

Butterworth et al (1977), working in the human experimental system, did not find evidence of cooperation between eosinophils and neutrophils, monocytes, lymphocytes, basophils or mast cells in the destruction of S. mansoni schistosomula, in contrast with the findings in mice (Kassis et al, 1979) and in rats (Capron et al, 1978a; 1978b). Enhancement of eosinophil-mediated cytotoxic activity was not observed following the addition of eosinophil-enriched cell preparations to eosinophil-depleted cell preparations of lymphocytes, neutrophils and monocytes, from whence the above conclusion was drawn. Likewise the pretreatment of a cell suspension(s) known to contain basophils, with a monospecific anti-basophil antiserum and complement, did not alter the cytotoxicity subsequently observed. This was in marked contrast to the documentation of

a 90 percent reduction in ADCC cytotoxic activity when anti-eosinophil antiserum was used under similar circumstances.

Although the early work implicating eosinophils as effectors in ADCC in man and other experimental animals was directed at outlining the immunological features of parasitic infection and/or immunity to reinfection by Schistosoma mansoni, later work has shown that the same mechanism(s) may be operative in infections with other parasitic organisms among these Trypanosoma cruzi, the causative agent of Chagas' disease in man (Olabuenaga et al, 1979).

A role has been described for neutrophils and eosinophils in ADCC with a possible supportive-cooperator role for mast cells. All of these cell types are unique in that they possess Fc cell surface receptors (Henson, 1969; Messner and Jelinek, 1970). The same is also true for platelets (Henson, 1969) and speculation as to the role of these latter in ADCC is not surprising. Lovchik and Hong (1977), working with human platelets prepared from spleen by differential centrifugation, demonstrated that platelets were capable of displaying ADCC cytotoxicity against a panel of appropriately antibody-sensitized target cells, notably human and chicken erythrocytes and P815 murine mastocytoma cells. ADCC cytotoxic activity appeared to be a function of

platelet concentration. In addition, platelet activity was seen to vary with the target cell utilized in the assay. Most marked activity was directed against the human erythrocyte target cells and comparatively less towards the chicken erythrocytes and the P815 target cells with the least activity being shown against these latter.

The combined evidence presented in this section would outline a definitive role in ADCC for the leukocyte, whether neutrophil (PMN) or eosinophil, alone or in concert with K lymphocytes, monocytes-macrophages or mast cells in man and/or other animal species. A role for the human platelet in ADCC is also proposed. The plethora of experimental systems utilized to delineate leukocyte function in ADCC is reminiscent of the previously stated theme that the nature of the target cell specifies the identity of the effector cell. Appropriate caution against the interpretation and extrapolation of data between man and animals must therefore be reiterated.

The complexity of the information on ADCC may allow speculation as to its role in the immune response in vivo. This very same complexity may in fact underline the importance of this mechanism to host survival. That so many cell types involved in other immunologic functions are participants in ADCC cytotoxicity may allow for refinement and modulation in the immune

response through multiple interactions between these different immunologic mechanisms. This would accord flexibility and adaptability to the immune system which would in turn profit the host and ensure its survival. The case may also be presented that such interactions may occasionally be detrimental to host survival and be intimately involved in the pathogenesis(es) of disease.

2.6 The Role of Antibody in ADCC Cytotoxicity

The early investigative work into the nature of ADCC demonstrated that the ADCC cytotoxic reaction was mediated primarily by antibodies of the IgG class of immunoglobulins (MacLennan, 1972a; Perlmann et al, 1972; Cerottini and Brunner, 1974). More recently, however, antibodies of other classes, notably IgM and IgE, have been reported to initiate ADCC under special circumstances (Dennert and Lennox, 1973; Capron et al, 1975; Lamon et al, 1975a, 1975b, 1975c; Blair et al, 1976; Wahlin et al, 1976; Fuson and Lamon, 1977; Pearson, 1978). Regardless of the antibody class implicated in ADCC in any given experimental system, it is apparent that the integrity of the antibody molecule, particularly with reference to the Fc portion, is essential in order that ADCC be demonstrated.

Removal, by pepsin digestion, of the Fc fragment of rabbit immunoglobulin G to yield $F(ab)'_2$ (cleavage at the level of $C\gamma 2$) was found to significantly decrease ADCC cytotoxicity (Gelfand et al, 1972; Moller and Svehag, 1972). It was concluded, therefore, that the recognition site on the antibody molecule for the effector cell surface receptors did not reside in the $F(ab)'_2$ antibody moiety. Similarly, Fab fragments were observed not to have ADCC inductive capability (Moller and Svehag, 1972; Larsson and Perlmann, 1972; Perlmann et al, 1972). Treatment of target cell sensitizing IgG antibody with 2-mercaptoethanol to cleave disulfide bonds in the Fc portion of the immunoglobulin molecule was also observed to reduce ADCC cytotoxicity (MacLennan, 1972a; Moller and Svehag, 1972; Perlmann, 1972; Van Boxel et al, 1972; Denk et al, 1974; Lovchik and Hong, 1977; Pearson, 1978). Likewise, treatment of rabbit target cell sensitizing IgG antibody with the enzyme plasmin to cleave the chains of the molecule in order to remove 107 amino acid residues on the C-terminal of the antibody molecule was followed by a decrease in ADCC cytotoxic activity. This suggested that the last 107-C-terminal amino acid residues, i.e. the $C\gamma 3$ domain, were essential for the initiation of ADCC cytotoxicity (MacLennan, 1972a).

This contrasted with the observations of Wisloff et al (1974a; 1974b) who concluded that the $C\gamma 2$ region

of the antibody molecule was more critical to the ADCC reaction than the Cγ3 domain. These workers reported that the "Fc fragment of the antibody molecule containing a part of the N-terminal part of the Fc portion inhibited an ADCC reaction directed at ChRBC target cells better than did the C-terminal half (Cγ3 domain) of the Fc fragment." The discrepancy between these studies is unexplained. Lovchik and Hong (1977) present data which suggest that the nature of the ADCC cytotoxic effector cell-anti-target cell antibody interaction is different for ADCC systems mediated by phagocytic and/or non-phagocytic cells. It has also been proposed that the ADCC destruction of antibody-sensitized target cells of tissue culture origin, e.g., Chang liver cells (MacLennan, 1973) is mediated by non-phagocytic cells whereas that of erythrocytes, notably ChRBC, is mediated by phagocytic as well as non-phagocytic cells. The difference in the models chosen by MacLennan (1972a) and Wisloff et al (1974a; 1974b) and the conflicting experimental results may well be a reflection of the different effector cell mechanisms operative in these experimental systems.

Taken collectively, these findings indicate that the structural integrity of the Fc region of the IgG immunoglobulin, rather than that of the antigen-binding region, is critical to the initiation of the ADCC

reaction. Furthermore, it is suggested that within the Fc region, the necessary structural-chemical features of the Fc piece do not reside exclusively within the Cγ3 domain. The integrity of the Cγ2 domain may also be required to stabilize Cγ3 or to enhance the affinity of Cγ3 for the corresponding effector cell membrane receptor (Lovchik and Hong, 1977) in order to trigger the ADCC reaction.

The interaction between the Fc piece of the antibody molecule and the corresponding effector Fc cell surface receptor has been shown to be critical for the demonstration of ADCC cytotoxic effector cell activity by several of the workers cited above. Additionally, it has been shown that any manipulation of the immunoglobulin Fc piece which was successful in disrupting its chemical and structural integrity was also successful in decreasing the ADCC cytotoxic activity exhibited by the effector cells. It would therefore be expected that any manipulation of the Fc cell surface receptor which likewise succeeded in disrupting its chemical and structural integrity would also be successful in interfering with its ADCC cytotoxic capability. This is indeed the case.

Blocking of the ADCC cytotoxic effector cell surface receptor was observed to be followed by a decrease in ADCC cytotoxic activity. Blocking could be

achieved either by the pretreatment of ADCC cytotoxic effector cells and/or by the addition of immune complexes (related and/or unrelated antigen-antibody complexes) and/or heat or otherwise aggregated immunoglobulin and anti-immunoglobulin (Basten et al, 1972a, 1972b; Dickler and Kunkel, 1972; MacLennan, 1972a; Perlmann et al, 1972; Van Boxel et al, 1972; Dickmeiss, 1974; Trinchieri et al, 1975a, 1975b) to the culture vessels but could not be inhibited by the comparable use of native immunoglobulin.

Soluble aggregated immune complexes and/or immunoglobulin are proposed to act through competition with target cell-bound immunoglobulin for Ig-Fc cell surface receptors on the membranes of the ADCC cytotoxic effector cells. The failure of native Ig to act to inhibit ADCC is unresolved. It is postulated that the preferred action of immune complexes or aggregated immunoglobulin is a reflection of the higher avidity of ADCC cytotoxic effector cell surface Fc receptors for the complementary sites of antibody molecules held together either by antigen or through physico-chemical aggregation (Cerottini and Brunner, 1974). This data further suggests that the antibody responsible for ADCC cytotoxic activity is, for the most part, not cytophilic for the ADCC cytotoxic effector cells (Perlmann and Perlmann, 1970; Cerottini and Brunner, 1974).

Notwithstanding this, Perlmann et al (1972) and Perlmann (1976) have shown that lymphocyte effector cells may sometimes pick up antibody complexed with antigen from the surface of target cells and furthermore use these antibodies in an ADCC cytotoxic reaction with fresh target cells of relevant antigenicity. These workers reported that lymphocytes incubated with anti-ChRBC IgG alone had little antibody remaining on their surface as compared to lymphocytes incubated with ChRBC target cells plus anti-target cell IgG antibody. Furthermore, when fresh ChRBC target cells were added to these latter preparations, it was found that only those lymphocytes pretreated with anti-ChRBC antiserum in the presence of target cells were noted to demonstrate significant levels of ADCC cytotoxicity. It was also observed at that time that the cytotoxic activity of the lymphocyte effector cells was specific for the chicken erythrocyte target cell. Lymphocytes incubated either with anti-ChRBC alone, with anti-ChRBC and duck erythrocytes (a non-cross-reacting target cell) or with normal rabbit serum IgG and ChRBC did not acquire this type of cytotoxicity. It was concluded on the basis of these experiments that lymphocytes could utilize antibody acquired from antigen-antibody complexes to effect the ADCC cytotoxic destruction of fresh antigen added to the culture system. It was considered, therefore,

that human lymphocytes could be "armed" to effect ADCC cytotoxicity. It must be stated, however, that the level of ADCC cytotoxic activity observed in this type of experimental system was, nevertheless, much reduced when compared to that observed in a more conventional experimental system (Perlmann et al, 1972).

Perlmann et al (1972) also reported that pretreatment of human lymphocytes with antigen-antibody complexes (ChRBC-anti-ChRBC antiserum) was followed by a reduced level of ADCC cytotoxicity when these ADCC cytotoxic effector cells were added to culture vessels containing anti-target cell antiserum (anti-ChRBC IgG) and target cells (ChRBC). Pretreatment of these same lymphocytes with anti-target cell antiserum alone did not alter the ADCC cytotoxic activity subsequently observed. It was proposed that the inhibition of ADCC cytotoxic activity was attributable to blocking of the lymphocytic Fc cell surface receptors and the ensuing inability of the anti-target cell antibody to form the necessary bridge between effector cell and target cell so essential to the initiation of the ADCC cytotoxic event.

Blocking of Fc cell surface receptors can be achieved not only with antigen-antibody complexes and aggregated immunoglobulin but also with naturally-occurring Fc reactive materials such as rheumatoid factor and staphylococcal protein A (Austin and Daniels,

1976; Isturiz et al, 1976; Pearson, 1978). It is interesting to note that in the case of these latter, the inhibitory effects on ADCC cytotoxicity were observed when antibody-sensitized target cells were pre-treated with these Fc reactive substances. It was proposed that these Fc reactive materials depressed ADCC cytotoxic activity through alteration or covering of the Fc piece(s) on the target cell-bound antibody to prevent the linking of the target cell to the ADCC cytotoxic effector cell by way of the anti-target cell antibody (Pearson, 1978). This is unlike the proposed mechanisms of action for antigen-antibody complexes and aggregated immunoglobulin where inhibition of ADCC cytotoxic activity was attributed to blocking at the effector (by way of effector Fc cell surface receptors) rather than target cell level.

Inhibition of ADCC cytotoxic activity by anti-immunoglobulin antiserum as well as by immune complexes, aggregated immunoglobulin and Fc reactive substances is also a feature of ADCC (Chapuis and Brunner, 1972; Van Boxel et al, 1972; Dickmeiss, 1974a; Trinchieri et al, 1975a, 1975b; Lovchik and Hong, 1977). The inhibitory effects of anti-immunoglobulin antiserum on ADCC may well reflect the formation of Ig-anti-Ig immune complexes in culture medium and/or the shedding of such complexes from

the target cell surfaces into the culture medium. This may be followed in turn by the occupation of Fc cell surface receptors on the ADCC cytotoxic effector cells and prevent these from interacting with the antibody-sensitized target cells to effect target cell destruction. Mikulski and Billing (1977) have additionally observed ADCC inhibition following the blocking of effector Fc cell surface receptors by means of an anti-B lymphocyte allo-antiserum (Pearson, 1978). Whatever the mechanism by which effector Fc cell surface receptor blockade is achieved, this obstruction is sufficient to prevent an ADCC reaction from ensuing or to reduce the level of ADCC activity exhibited, provided that a critical number of such receptors are occupied.

This early investigative work into the identity of the anti-target cell antibodies capable of triggering an ADCC reaction revealed that these antibodies belonged principally to the IgG class. These conclusions were drawn from studies in which anti-target cell antiserum was fractionated on the basis of molecular size and charge by passage through Sephadex columns (MacLennan et al, 1969; MacLennan, 1972a). The eluates capable of initiating an ADCC reaction principally contained IgG antibody. The macroglobulin containing eluate was essentially

inactive (MacLennan et al, 1969; MacLennan, 1972a; Moller and Svehag, 1972; Perlmann et al, 1972; Cerottini and Brunner, 1974; Lovchik and Hong, 1977; Pearson, 1978).

Additional studies with myeloma proteins of antibody classes M, A, D and E revealed that aggregates of these antibodies did not have inhibitory effects on ADCC as would be expected if they were participants in the reaction (Perlmann et al, 1972; MacLennan et al, 1973). This presumably is a reflection of the inability of portions of these antibody molecules to bind to effector Fc cell surface receptors (Lovchik and Hong, 1977). This latter is an essential prerequisite for ADCC cytotoxic potential. However, macroglobulins have been occasionally observed to have a suppressive effect on ADCC when present in quantity, along with IgG, in anti-target cell antiserum (Perlmann, 1976). In retrospect this may have been, in fact, a reflection of the ability of IgM antibody to participate in ADCC under special circumstances.

Further experimental work attempted to elucidate the identity(ies) of the IgG subclasses participating in ADCC. It is well noted that the various IgG subclasses (IgG1, 2, 3 and 4) possess differing abilities to participate in certain immunological reactions, among these:

(i) fixation of complement (IgG1 and 3);

(ii) binding to monocytes (IgG1 and 3); and

(iii) facilitation of intracellular bacterial killing by polymorphonuclear leukocytes (IgG1, 2 and 3) (MacLennan, 1972a).

Aggregates of human myeloma proteins belonging to IgG subclasses 1 through 4 were all found to be capable of inhibiting ADCC cytotoxicity (MacLennan, 1972a; Perlmann et al, 1972; Larsson et al, 1973; Pearson, 1978). This inhibitory effect was likewise considered to be a direct reflection of the ability of these aggregates to bind to effector Fc cell surface receptors and thereby block the effector-antibody-target cell interaction so essential for the demonstration of ADCC cytotoxicity. It was additionally demonstrated that IgG2 and IgG4 had a lower affinity for effector cells than IgG1 and 3, suggesting that more than one site on the Fc piece could react with Fc cell surface receptors (Spiegelberg et al, 1976; Pearson, 1978). It was interesting to note that the sole immunological function of IgG4 was in ADCC cytotoxicity (MacLennan, 1972a).

When the role of IgG subclasses in ADCC was examined from the perspective of effector cell ability to bind to these antibodies, it was observed that only myeloma aggregates of the subclasses IgG1 and IgG3 had the

ability to inhibit ADCC by human macrophage-monocyte cells, whereas only those of the IgG subclasses 2 and 4 could inhibit the ADCC activity of non-phagocytic, lymphoid effector cells (MacLennan et al, 1973; Larsson et al, 1975a, 1975b; Lovchik and Hong, 1977). This information adds to the already large compendium of data suggesting that lymphocyte and macrophage-monocytic cells are triggered to act in ADCC by different mechanisms.

It must also be noted that although ADCC cytotoxic effector cells and anti-target cell antisera of different animal species origin react with seemingly equal efficiency, some differences in the level of ADCC cytotoxic activity exhibited can be attributed to the species heterogeneity of the components in the assay system. For instance, it is well documented that murine lymphoid ADCC effector cells are more discriminating in their antibody requirements for triggering ADCC destruction of non-erythrocyte target cells than are effectors of rat, guinea pig, monkey or human origin (Trincheri et al, 1973; Halloran and Festenstein, 1974; Lovchik and Hong, 1977). A requirement for homologous/isologous antiserum is noted (Perlmann et al, 1972; Forman and Moller, 1973; Harada et al, 1973; Kiessling and Klein, 1973; Lamon et al, 1974).

Trinchieri et al (1973) examined the ability of human cells and those derived from several other animal species (chicken, sheep, mouse rat, guinea pig and rabbit) to mediate ADCC cytotoxic activity against alloantibody-sensitized human lymphoblastoid target cells. They reported that human lymphocytes were in general more efficient ADCC cytotoxic effectors than were ADCC cytotoxic effector cells of the other animals investigated. They concluded on the basis of this study that interspecies variability in ADCC capability did exist.

Zighelboim and Gale (1974) subsequently reported that the requirement for syngeneic/allogeneic anti-target cell antiserum in ADCC was not unique to the mouse. In a study comparing the ADCC cytotoxic activities of effector cells from rat, rabbit and man against a battery of target cells, notably EL-4 (a murine leukemia tissue culture cell line syngeneic to C57/BL6 mice), RPMI-4265 (an established lymphoblast cell line derived from a patient with chronic myelogenous leukemia) and SRBC, they observed that each species of ADCC cytotoxic effector cell evaluated was most active in mediating ADCC when the target cells were sensitized with antiserum allogeneic to the effector cell donor (Zighelboim and Gale,

1974). They further went on to report that although human cells appeared to be generally more efficient as ADCC cytotoxic effectors than cells derived from either the rat or rabbit, no truly distinctive pattern of activity was discernible when xenogeneic serum was utilized. These findings were in partial agreement to those of Trinchieri et al (1973).

In a brief article discussing ADCC in various orders of vertebrates, Imir et al (1977) commented that little species specificity was shown within mammalian classes with regard to association between the Fc piece of the anti-target cell antibody and the Fc cell surface receptor on the ADCC cytotoxic effector cell. They stated that effector cells of the Orders Primates, Lagomorpha and Rodentia were all capable within reason of interacting with lagomorph or rodent antibodies (Imir et al, 1977). Mammalian ADCC cytotoxic effectors were not capable, on the other hand, of interacting with avian antibodies. The converse was also observed to be true by these workers but not by others (Calder et al, 1974a). It was suggested on the basis of some of the above information that there was indeed evidence for inter-species variation in the ADCC reactive determinant of the anti-target cell ADCC-inducing antibody (MacLennan, 1972a; Imir et al, 1977).

Although it had been determined with relative certainty that human antibodies of the IgG subclasses 1, 2, 3 and 4 are all capable of initiating, with greater or lesser efficiency, ADCC cytotoxicity, less is known about the IgG subclasses in animal species, particularly with respect to binding of the Fc piece to the effector Fc cell surface receptor and the subsequent induction of ADCC (Pearson, 1978). Nevertheless, Chapuis and Brunner reported in 1971 that guinea pig antibodies of the IgG subclass 2 were capable of sensitizing ChRBC target cells to the ADCC action of normal guinea pig lymphoid cells. They observed, however, that IgG1 was incapable of doing so although both subclasses showed equal haemagglutinating abilities. However, Larsson and Ohlander in 1975 reported that guinea pig IgG antibodies of subclasses 1 and 2 were successful in inducing ADCC. This partial contradiction is unresolved.

The early investigative work into the nature of the antibody capable of initiating an ADCC reaction revealed that this antibody belonged to the IgG(7S) class of immunoglobulins (MacLennan et al, 1969, 1973; MacLennan, 1972a; Moller and Svehag, 1972; Perlmann et al, 1972). IgM, A, D and E were found to be inactive in ADCC at that time. Subsequent to this, antibodies of the classes M and E have been designated as being

capable of participating in ADCC. The first convincing evidence of IgM participation in ADCC was presented by Dennert and Lennox in 1973. These workers reported that murine-derived IgM (as well as IgG) anti-ChRBC immunoglobulin was capable of initiating the ADCC cytotoxic destruction of ChRBC targets by murine effector cells. However, the bulk of the evidence implicating IgM participation in ADCC was outlined in 1975 by Lamon and co-workers utilizing a murine Moloney-sarcoma virus system.

These investigators measured the ability of sera obtained from mice with regressed Moloney-sarcoma virus-induced tumours and from mice with progressively growing tumours, to initiate ADCC by normal murine lymphoid cells against Ha2 target cells (an established cell line from an MSV-induced tumour of a CBA mouse, which produces infectious virus and possesses the MSV-determined cell surface antigen). The 19S as well as the 7S Sephadex G-200 fractions of the above antisera were found to trigger ADCC cytotoxicity by normal murine splenic lymphocytes, as measured through the microcytotoxicity assay. These Sephadex G-200 fractions corresponded to IgM and IgG immunoglobulins, respectively (Lamon et al, 1975a). This was determined on the basis of the G-200 fractions' molecular size, immunologic specificity and through the use of Sepharose-coupled anti-mouse

IgM and anti-mouse IgG columns. Passage of the 19S or 7S fraction over the corresponding columns resulted in a depletion of the ADCC triggering ability of these Sephadex fractions respectively, thereby implicating ADCC initiating activity to murine IgM as well as to murine IgG (Lamon et al, 1975a; 1975b; 1975c). Further studies revealed that IgM was optimally active at relatively high concentrations in culture whereas the opposite was true for IgG. Furthermore, IgM and IgG in combination appeared to interfere with each other in the ADCC assay (Lamon et al, 1975a; 1975b; 1975c). This latter was reminiscent of the observation made by Perlmann (1976) that IgM antibodies were observed on occasion to have a suppressive effect on ADCC when present in quantity, along with IgG, in anti-target cell antiserum. Lamon et al (1975a; 1975b; 1975c) additionally went on to identify a number of lymphocytic categories, among which thymic lymphocytes, as being capable of exhibiting ADCC cytotoxic activity against IgM-sensitized Ha2 target cells.

The work of Lamon et al (1975a; 1975b; 1975c) was confirmed by Blair et al (1976) using yet another murine tumour cell system. In this instance sera from female mice bearing virus-induced (MTV) mammary tumours were capable of triggering normal murine lymphocytes to effect the ADCC destruction of tumour cells in culture. Gel filtration of these

sera and subsequent examination of these fractions indicated that 19S (IgM) as well as 7S (IgG) antibodies were capable of participating in this ADCC reaction.

The participation of IgM in ADCC is not unique to the murine experimental model. Wahlin et al (1976) demonstrated that purified human peripheral blood lymphocytes were capable of effecting the ADCC destruction of bovine erythrocytes sensitized with either rabbit IgG or IgM anti-target cell antiserum in a plaque assay described by Perlmann et al (1975) and Biberfield et al (1975). It was also reported that, in this instance, the formation of plaques in the presence of IgG anti-target cell antiserum took place within, and was complete by, 20 hours whereas that in the presence of IgM antibodies was complete only by 40 hours. Not only did this discovery corroborate the earlier work in the murine experimental model, it also seemed to suggest that certain differences did exist with respect to the cytotoxic reaction proper between the IgG and the IgM ADCC systems. Again, the discovery that human ADCC cytotoxic effector lymphocytes could act with IgM antibody to effect ADCC led to speculation as to the identity of the ADCC cytotoxic effector cell. It was determined subsequently to be a subset of the human T lymphocyte uniquely possessing Fc receptors specific for IgM and not IgG antibody (Moretta et al, 1975; 1976)

corroborating Lamon et al's (1975a; 1975b; 1975c) findings in the mouse. Fuson and Lamon (1977) have additionally reported that human peripheral blood lymphocytes were capable of effecting the destruction of human heterophile IgM-coated sheep erythrocyte target cells. Rabbit IgM antibodies were also shown to be active in the system (Fuson et al, 1978). This mechanism of ADCC in which IgM is the antibody class implicated in the reaction is not unique to mice and man. It is also operative in the sub-human primate (Prevost et al, 1975, 1976; Pearson, 1978).

To date there is evidence that IgG as well as IgM antibodies directed against target cells and/or target cell-associated antigens are capable of initiating the ADCC phenomenon ultimately effected by lymphocytes and/or other cell types from animals of diverse origin and measured through a variety of assays (microcytotoxicity assay, plaque assay, ^{51}Cr-release assay). These two antibody classes are nevertheless not unique in this capability. IgE antibody has also been implicated in the ADCC destruction of parasitic target organisms and/or associated antigens (Schistosoma mansoni schistosomula, among others, Capron et al, 1975). A role for IgD in ADCC has yet to be described, however a role for secretory IgA has recently been demonstrated by Shen and Fanger (1981).

Although pure secretory IgA in this model was inactive by itself, it was capable of acting synergistically with IgG to enhance ADCC cytotoxicity by IgG 100 percent. This synergism was seen at IgA to IgG ratios approximating those at mucosal surfaces and in mucosal secretions. It was also observed with lymphocytic, monocytic and PMN effector cells possessing Fc receptors for IgA as well as IgG.

Regardless of the source of the antibody, it is apparent that the integrity of the molecule, particularly with reference to the Fc portion, is essential in order that ADCC be demonstrated. Furthermore, it has been shown that the interaction of the Fc piece of the antibody molecule with the corresponding ADCC cytotoxic effector Fc cell surface receptor is also critical for ADCC. Blocking of the Fc cell surface receptor with immune complexes or aggregated immunoglobulin interferes with the expression of ADCC cytotoxic activity. Similarly manipulation of the antibody Fc piece by Fc reactive substances or antiglobulin also interferes with ADCC cytotoxic activity.

It is not, however, essential that antibody be presented directly to the effector cell in order to initiate the ADCC cytotoxic reaction. Human lymphocytes can be "armed" through the acquisition of antibody from immune complexes and subsequently interact with

target cells to effect ADCC. It is presumed that this mechanism is also operative in experimental animals.

Suffice to say that antibodies in general, and more particularly antibodies of the classes IgG, IgM and IgE are essential components of the ADCC cytotoxic reaction in a variety of experimental animals and in man. In the context of ongoing research in ADCC, a role for IgA and/or IgD has yet to be defined.

2.7 The Mechanism of Cytolysis of Antibody-Coated Target Cells in ADCC Cytotoxicity

The precise mechanism of cytolysis in ADCC cytotoxicity has, as yet, not been elucidated. However, it is well established that contact between effector cell and antibody-coated target cell is essential for the initiation of the cytotoxic event. This was first demonstrated by Perlmann and Holm in 1969. Chicken erythrocytes coated with either PPD (purified protein derivative of tuberculin) or thyroglobulin, were co-cultured with antiserum specific for one or the other of the target cell-bound antigens and were then exposed to human lymphocytes. Only those target cells which had been complexed with the antigen specific for the antiserum in culture were subsequently lysed. This illustration of the necessity for effector-target cell contact achieved by way of anti-target cell antibody was repeated by Holm and Perlmann

(1969) and MacLennan and Harding (1970) using a similar experimental model with Chang liver target cells and later by Scornik (1974) who, like Perlmann and Holm (1969), utilized hapten-complexed chicken erythrocytes as target cells and the appropriate anti-hapten antibody. Although these early experiments illustrated the necessity for effector-target cell contact in ADCC, they served additionally to underline the role played by the anti-target cell antibody in the elaboration of this contact. The anti-target cell antibody would appear to act as a bridge linking effector and target cell. This bridging activity would also appear to be:

(i) a function of the serum specificity for target cell antigenic determinants;

(ii) a function of the ability of antiserum and target cell to interact to form antigen-antibody complexes, and

(iii) a function of the ability of effector cells to recognize and to interact with these antigen-antibody complexes and so be triggered to effect the ADCC destruction of the target cells (Holm and Perlmann, 1969; MacLennan and Harding, 1969; Perlman and Holm, 1969; Scornik, 1974).

However, the exact mechanism of target cell destruction was not elucidated by these experiments.

Complement has often been suggested to act in the antibody-dependent cellular destruction of target cells. In the experiments in which ADCC was first described (Moller, 1965; MacLennan and Loewi, 1968; Perlmann and Holm, 1968), the antiserum dilutions utilized were in fact capable of supporting complement-mediated target cell destruction. However, it was later pointed out by Perlmann and Holm (1969), after much refinement of experimental procedures designed to measure ADCC, that the concentration of antiserum capable of supporting, for example, ChRBC target cell destruction by purified human lymphocytes in an acceptable conventional ADCC assay system, was insufficient to permit complement-mediated erythrolysis. Complement was therefore judged by these workers not to participate in ADCC. It must be additionally noted that all sera utilized in conventional ADCC assays were reported to be heat-inactivated at 56°C. for 1 hour. This effectively destroyed the first two complement proteins, C'1 and C'2, thereby preventing ADCC target cell destruction through activation of the conventional complement pathway by the proteins C'1 and C'2.

The dissociation of complement and ADCC was further supported by the results of experiments conducted with pharmacologic inhibitors of the complement cascade; notably carrageenan. Carrageenan effectively blocks the cascade by binding to the first component of complement, C'1 (Lovchik and Hong, 1977). The incorporation of carrageenan into ADCC cell cultures was not found to alter in any way the ADCC cytotoxic activity subsequently observed (Perlmann et al, 1972; Pollack and Nelson, 1973). However, the participation of complement by way of the alternate pathway was not ruled out by these experimental results.

Van Boxel et al (1974) have thoroughly investigated the role of complement in ADCC by means of an experimental system utilizing mice genetically deficient in certain complement proteins. It is important to note that the in vitro culture system was designed to be devoid of complement either through introduction via tissue culture reagents or through endogenous synthesis by normal murine cells in culture. Effector cells and serum from complement-deficient mice were used exclusively to support the ADCC cell cultures and enabled the complement-free status of the experimental system to be maintained. Van Boxel et al (1974) demonstrated that lymphoid cells and immune sera obtained from

mice deficient in the complement components C'4 to C'6 were capable of mediating ADCC against ChRBC target cells. A further depletion of the complement components C'3 through C'9 by cobra venom factor had no effect on the ADCC exhibited by these murine effector lymphocytes and immune sera. The additional elimination of the C'1 and C'2 components through heat-inactivation at 56°C for 1 hour effectively created a complement-free ADCC culture system in which the ADCC activity of murine lymphocytes was unaltered. Complement was, therefore, ruled out as an active cytolytic factor in this experimental model.

Although the complement cascade has been shown to be inactive in the ADCC cytotoxic mechanism on the basis of the above reports, the participation of other cytotoxic factors in target cell destruction is still a consideration. This point was investigated early on in the ADCC literature by Perlmann and Holm (1969). These workers designed an experimental system in which chicken as well as duck erythrocytes were designated as target cells in an ADCC reaction by normal human lymphocytes. They observed that chicken erythrocytes were the sole target cells lysed when equal numbers of ^{51}Cr-radiolabelled chicken and unlabelled duck erythrocytes were pretreated with rabbit anti-ChRBC antiserum (there was no cross-reactivity between these

antibodies and duck erythrocytes) and co-cultured with normal human lymphocytes in an ADCC system. When equal numbers of unlabelled chicken erythrocytes and, conversely, radiolabelled duck erythrocytes were similarly pretreated with rabbit anti-ChRBC antiserum and likewise used as target cells in an ADCC reaction with normal human lymphocytes, no evidence of duck erythrocyte disruption was measurable by the release of the radionuclide ^{51}Cr into the culture medium. The converse, also held true when rabbit anti-duck erythrocyte antiserum was substituted for the anti-ChRBC antiserum. The participation of soluble cytotoxic factors in the culture medium was, therefore, ruled out by Perlmann and Holm (1969) on the basis that "innocent" bystander target cells in a target cell mixture (either unsensitized radiolabelled duck or chicken erythrocytes) were not destroyed during the course of an ADCC reaction with the antibody-sensitized target cells. However, it is important to note that action of such factors only at the local or cellular level cannot be excluded on the basis of the preceding experimental observations. Moller and Svehag (1972) arrived at similar conclusions from data generated in an experimental system which used a sheep and human fibroblast target cell mixture in an ADCC reaction by normal human lymphocytes in the presence of the appropriate corresponding anti-target cell

antisera.

Despite the failure to demonstrate the participation of complement and/or other soluble cytotoxic factors in target cell killing by ADCC, investigators have, nevertheless, been able to document the necessity for effector cell viability as well as on-going metabolic activity in order for ADCC to occur (Perlmann and Holm, 1969; Calder et al, 1974b). Calder et al (1974b) reported that human lymphoid cells twice frozen and thawed, and confirmed to be non-viable as a result of this treatment, were unable to destroy rabbit antibody-coated ChRBC target cells. Other workers have documented further that lysis of antibody-coated target cells was an energy dependent phenomenon as pharmacologic inhibitors of cellular respiration also successfully inhibited ADCC cytotoxic activity. This was illustrated by Perlmann and Holm (1969) and Strom et al (1975a) with antimycin A, by Strom et al (1975a) with oligomycin and by Henney (1974) with sodium azide.

Strom et al (1975a) found that antimycin A, an antibiotic that inhibits the respiratory chain, caused a drug dose-dependent inhibition of ADCC cytotoxic activity when it was added to effector-target cell mixtures. Perlmann and Holm (1969) had earlier reported that pretreatment of ADCC cytotoxic effector cells with antimycin A was followed by an inhibition of

ADCC cytotoxicity thereby leading to the suggestion that the antibody-induced reaction is dependent on energy-requiring effector processes.

Additional evidence that this indeed was the case was again provided by Strom et al (1975a) using yet another antibiotic, namely oligomycin. This latter is known to inhibit cellular respiration by acting upon the mitochondrial enzyme ATPase. Oligomycin, like antimycin A, was found to produce a drug dose-dependent inhibition of ADCC. That the action was at the effector and not at the target cell level was illustrated by the data obtained following pretreatment of either of these cell groups with the antibiotic. The inhibitory effect of oligomycin was seen only when the effector cells were treated (Strom et al, 1975a). Henney (1974) likewise corroborated the necessity and dependence of the ADCC cytotoxic mechanism on energy requiring effector cell processes by demonstrating that sodium azide was inhibitory to the ADCC erythrolysis of sheep erythrocytes. Strom et al (1975a) additionally reported that a pharmacologic inhibitor of glycolysis, notably 2-deoxyglucose (2-DG) was also observed to inhibit ADCC, reiterating the energy-dependent nature of the effector cellular processes required for the demonstration of ADCC cytotoxic activity.

ADCC cytotoxic activity has also been found to be abrogated by inhibitors of microtubule function. Strom et al (1973a) reported that lysis of Lewis rat antibody-sensitized BN-rat thymocyte target cells by Lewis rat spleen cells was partially suppressed by non-toxic concentrations of colchicine and vinblastine. No effect was recorded when the drugs were used to pretreat the target cells prior to culture for ADCC suggesting that the action of the drugs was at the effector cell level only.

Trinchieri et al (1975a; 1975b) also reported that colcemid, another drug toxic to microtubule function, partially inhibited the ADCC destruction of lymphoblastoid target cells. In contrast to the findings of the above investigators with colchicine, vinblastine and colcemid, Gelfand et al (1975) found that the ADCC destruction of ChRBC was not inhibited by these drugs: The possibility exists that these conflicting results are a reflection of the different experimental systems utilized in the above studies. The selection of erythrocyte target cells by Gelfand et al (1975) is in contrast to the use of lymphoid target cells by Strom et al (1973a) and Trinchieri et al (1975a; 1975b) with the consequence that different effector cells may be acting in these experimental models. This may very well be ultimately reflected in subtle differences in effector

cell susceptibility to microtubular disruption by these drugs. It is nevertheless particularly significant that ADCC cytotoxic activity is sensitive to microtubular disruption by colchicine, vinblastine and colcemid as microtubules are cellular organelles known to be intimately involved in cellular secretory processes, among others (Bloom and Fawcett, 1975).

The use of the fungal metabolites cytochalasin A (CA) and cytochalasin B (CB) in the investigation of the ADCC cytotoxic mechanism has revealed that intact microfilament function is also essential for the cytotoxic event. Cytochalasin A disrupts microfilament function by cleaving disulfide bonds whereas cytochalasin B prevents the cellular uptake of hexose, necessary for microfilament activity (Wessells et al, 1971; Kletzien et al, 1972; Mizel, 1973). Strom et al (1975a) have reported that both of these chemicals inhibit the ADCC destruction of Lewis rat antibody-sensitized BN rat thymocyte target cells by Lewis rat splenic lymphocytes. This is in agreement with work by several investigators with cytochalasin A (Gelfand et al, 1975) and with cytochalasin B (Perlmann et al, 1972; Cerottini and Brunner, 1972, 1974; Dickmeiss, 1974a, 1974b; Gelfand et al, 1975; Greenberg et al, 1975a, 1975b; Trinchieri et al, 1975a, 1975b) in a variety of experimental models.

Perlmann et al (1972) early on, demonstrated that

CB had a profound inhibitory effect on the ADCC destruction of rabbit antibody-sensitized ChRBC by normal human peripheral blood lymphocytes. Cerottini and Brunner (1972) in the same year corroborated these results using an identical experimental model and noted, additionally, that CB could also inhibit the ADCC destruction of murine-allo-antibody-coated tumour cells by normal murine splenic effector cells. Dickmeiss (1974a; 1974b) later illustrated that CB consistently and almost totally inhibited the erythrolysis of human allo-antibody-sensitized human erythrocyte target cells by human peripheral blood leukocytes. Similarly, Gelfand et al (1975) reported that CB inhibited the lysis of rabbit-antibody-coated ChRBC by rabbit splenic and mesenteric lymph node effector cells.

It would appear then that treatment of ADCC cytotoxic effector cell preparations with CB, presumably leading to disruption of microfilament function, is associated with loss of ADCC effector cell capability in a variety of experimental systems. This would imply therefore that ADCC cytotoxic activity requires intact microfilament activity. However, the exact role of the microfilament(s) in the ADCC cytotoxic mechanism is still open to discussion. It is reasonable to suggest that the ADCC cytotoxic mechanism is intimately associated with cellular motility and surface movement as microfilaments

are closely involved with these latter (Bloom and Fawcett, 1975) and both ADCC and microfilament activity are susceptible to the inhibitory action of CB.

Gelfand et al (1975) and Trinchieri et al (1975a; 1975b) have in fact documented that this may indeed be the case on the basis of parallel studies on the pharmacologic effects of CB on ADCC and on the antibody-dependent cellular digestion of immune complexes (bound to latex beads in this case). This latter is a known secretory phenomenon, activated like ADCC, through the interaction of cellular Fc cell surface receptors with immunoglobulin Fc pieces (Trinchieri et al, 1975a; 1975b). Furthermore, it has been previously demonstrated that this latter reaction was enhanced in the presence of CB as noted by an increased release of lysosomal enzymes by effector cells (PMN in this case) in the presence of antigen-antibody complexes (Hawkins, 1973; Henson and Oades, 1973).

Trinchieri et al (1975a; 1975b) reported that CB inhibited both ADCC as well as the antibody-dependent cellular digestion of antigen (by human monocytes, granulocytes and lymphocytes), this latter contrasting with the findings of Hawkins (1973) and Henson and Oades (1973). Trinchieri et al (1975a; 1975b) therefore concluded that the antibody-dependent cellular digestion of antigen was not effected through cellular secretory

processes. They postulated rather that this inhibitory phenomenon was effected by interference with immune complex formation through the action of CB on microfilament function and hence effector cell motility. Furthermore, as ADCC was known to be triggered in a manner similar to antibody-dependent cell-mediated antigen digestion, Trinchieri et al (1975a; 1975b) postulated that the action of CB on ADCC was likewise achieved through interference with microfilament function and consequently effector cellular motility resulting in the inability of ADCC cytotoxic effector cell surface Fc receptors to bind to immunoglubulin Fc pieces. A secretory mechanism in ADCC cytotoxicity was therefore ruled out by these authors (Trinchieri et al, 1975a; 1975b).

Gelfand et al (1975), however, further qualified the mode of action of CB on ADCC, suggesting that CB may act at an early membrane-associated step in the triggering of the ADCC reaction because CB acts rapidly to effect an inhibition of ADCC which is reversible upon removal of the drug from culture. They also commented, on the basis of time course studies, that CB acted presumably after effector-target cell interaction, but before the initiation of the cytotoxic mechanism. They additionally considered that the membrane movement and uropod formation initiated through

effector-target cell binding through Fc interaction may be a prerequisite for triggering the effector cell to become cytotoxic.

The fact remains that CB does inhibit ADCC presumably through interference with cellular motility by acting at the level of the microfilament. That inhibition of secretory activity by action of the drugs colchicine, vinblastine and colcemid at the level of the microtubule was also followed by an abrogation of ADCC cytotoxic activity together with the observation that cytotoxic factors have not been found in culture fluids is also significant. One can postulate, therefore, that ADCC cytolysis may be achieved by means of a secretory mechanism acting at the local cellular level only, subsequent to effector-target cell binding through Fc interaction.

In contrast to the effects of colchicine, vinblastine and colcemid on the one hand, and cytochalasin A and B on the other hand, mitomycin C, an antibiotic that selectively inhibits DNA synthesis by cross-linking DNA strands (Strom et al, 1975a), has no effect on ADCC (Moller and Svehag, 1972; Calder et al, 1974b; Dickmeiss, 1974a, 1974b; Strom et al, 1975). However, actinomycin D, an antibiotic that at low concentrations selectively inhibits DNA-dependent RNA synthesis (Strom et al, 1975a) was found to cause a modest inhibition of ADCC

by some workers (Strom et al, 1975a) but not by others (Dickmeiss, 1974a; 1974b). Likewise ouabain, a cardiac glycoside that specifically inhibits plasma membrane Na-K-dependent ATPase leading to restriction of Na-K transport, had no effect on ADCC (Strom et al, 1975a) cytotoxicity. It may be concluded therefore that DNA synthesis or ATPase-dependent Na-K transport are not critical to the cytolytic mechanism in ADCC. The data on DNA-dependent RNA synthesis is equivocal in this respect.

Protein synthetic mechanisms were also investigated with a view to determining their role in the cytotoxic mechanisms involved in ADCC. Cycloheximide and puromycin, antibiotics capable of inhibiting protein synthesis in intact cells (Strom et al, 1973; 1975a) were found by Strom et al (1975a) to cause a dose-dependent inhibition of ADCC when added to ADCC effector-target cell mixtures. MacLennan and Harding (1970) however had reported earlier that puromycin did not interfere with the ADCC destruction of Chang liver target cells while Dickmeiss (1974a; 1974b) concluded that inhibitors of protein synthesis yielded inconsistent results in this respect. The evidence gathered to implicate DNA, RNA, protein synthetic mechanisms as well as cellular ionic transport mechanisms as pathways critical to the cytolytic destruction of target cells in ADCC

is not overwhelming.

It has, however, been noted by several workers (Strom et al, 1973a, 1973b, 1975a; Garovoy et al, 1975; Perlmann, 1976; Pearson, 1978) that ADCC is modulated through alteration in the intracellular levels of cAMP and cGMP. Intracellular accumulation of cAMP has been observed to abrogate ADCC through an effect produced on the ADCC cytotoxic effector cell (Strom et al, 1973a, 1973b, 1975a; Garovoy et al, 1975; Perlmann, 1976; Pearson, 1978) whereas exogenous cGMP was found to enhance ADCC cytotoxicity (Strom et al, 1973b, 1975a; Garovoy et al, 1975; Perlmann, 1976; Pearson, 1978).

Likewise, it would appear that cations, notably Ca^{++} and Mg^{++} are critical for the manifestation of lymphocyte-mediated ADCC cytotoxicity. Dickmeiss (1974a; 1974b) reported the consistent inhibition of ADCC cytotoxicity by 5 mM of EDTA (ethylenediaminetetra-acetate, a chemical compound that chelates cations) and the reversal of this by the addition of equimolar amounts of Ca^{++} to the ADCC cultures in question. The addition of 2.5 mM of Ca^{++} and 2.5 mM of Mg^{++} was found to restore the EDTA-inhibited ADCC cytotoxic reaction to only one-half of the original level of cytotoxicity whereas Mg^{++} alone (5 mM) had no effect. These data suggested that Ca^{++} alone was critical for the manifestation of ADCC in this experimental system utilizing lymphocyte

target cells. Pross et al (1974) likewise reported that EDTA at concentrations of greater than 2 mM was found to decrease the ADCC cytotoxicity towards antibody-sensitized ChRBC target cells to very low levels.

Goldstein and Fewtrell (1975) and Goldstein and Gomperts (1975) reported that the ADCC-mediated destruction of erythrocyte and non-erythrocyte target cells was variably dependent on cations. The erythrolysis of SRBC by human ADCC cytotoxic effector cells was noted to be Mg^{++}-independent as well as Mg^{++}-dependent. However, the destruction of Chang liver target cells was shown to be Ca^{++}-independent. This segregation of cation dependence in ADCC was further investigated by Lovchik and Hong (1977).

These workers reported that the lysis of P815 target cells by human ADCC cytotoxic effector lymphoid cells could be abrogated by EDTA but that this inhibition was reversible by the addition of Mg^{++} ions to the cultures rather than by the additon of Ca^{++} ions as reported by Dickmeiss (1974a; 1974b). Lovchik and Hong (1977) went on to report that erythrocyte target cells (ChRBC and HuRBC) were partially resistant to the effects of EDTA. When ADCC cytotoxic effector cell preparations were depleted of adherent cells and then tested for ADCC in the presence of EDTA, these

remaining cells were incapable of mounting an ADCC cytotoxic reaction. It was proposed then that non-adherent ADCC cytotoxic effector cells required divalent cations in order to effect ADCC. It was proposed that adherent cells presumably did not, on the basis of circumstantial evidence gathered through similar analyses of ADCC with erythrocyte target cells. These findings of Lovchik and Hong (1977) supported the conclusions of earlier work by other investigators. Scornik and Cosenza (1974) had previously reported that murine PEC-derived macrophages were not inhibited by EDTA whereas the opposite was true for non-adherent splenic ADCC cytotoxic effector cells. The exact cation(s) involved in this system were not identified.

On the basis of the combined evidence cited above, one could confidently conclude that ADCC cytotoxicity has variable cation dependence. Lovchik and Hong (1977), similarly concluded that this was indeed the case and went on to propose that this variable cation dependence was a manifestation of three different ADCC cytotoxic mechanisms, in particular:

(i) a divalent cation-independent mechanism effected by adherent cells capable of lysing erythrocyte target cells;

(ii) a Mg^{++} requiring system involved in the destruction of erythrocyte and non-erythrocyte

(e.g. P815 cells) target cells; and

(iii) a Ca^{++} requiring mechanism active against other non-erythroid target cells.

Importantly, these data illustrate that adherent and non-adherent cells have different cation requirements in order to effect the ADCC destruction of target cells. Furthermore, as more than one cell type is capable of effecting ADCC, it is probable that more than one target cell damage mechanism is operative.

This is particularly the case for the adherent cell, where phagocytosis, an intrinsic property of this category of cells, must not be overlooked when contemplating cytotoxic mechanisms. However, Temple et al (1973) demonstrated that the ADCC-mediated lysis of fowl erythrocytes by guinea pig macrophages was independent of phagocytosis as the addition of cytochalasin B (which prevents phagocytosis but not immunocyto-adherence) to cell cultures did not alter the degree of ADCC cytolysis observed. Holm and Hammarstrom (1973) found, on the other hand, that sodium iodoacetate, an agent which blocks phagocytosis, was capable of abolishing the ADCC destruction of HRBC target cells. This data is questionable, however, as sodium iodoacetate has been previously reported to suppress immune cell lysis by non-phagocytic effector cells (Perlmann and Holm, 1969; Lovchik and Hong, 1977). Scornik et al (1974) found

that phagocytosis by murine PEC was depressed by EDTA while ADCC cytotoxicity was unaffected. Allison et al (1972) had earlier suggested that phagocytosis of erythrocyte target cells by adherent murine PEC were not the preferred mechanism of target cell destruction in ADCC. This contrasts with reports which indicate that phagocytosis follows the attachment of macrophages to opsonized erythrocytes. Taken collectively, these data suggest that phagocytosis is not a preferred mechanism of ADCC target cell damage by adherent cells, monocytes-macrophages. Gale and Zighelboim (1975) also reported that this was not the case for PMN-mediated ADCC, based on serial light microscopic studies.

Gale and Zighelboim (1974; 1975) did, nevertheless, speculate on alternate mechanisms of ADCC destruction of target cells by PMN. They concluded that the reaction was divalent cation dependent (Ca^{++}) and enhanced by elevations of intracellular or exogenous cGMP and inhibited by elevations of intracellular or exogenous cAMP. Colchicine in concentrations inhibitory to microtubule formation and/or lysosome release by PMN was inhibitory to PMN ADCC cytotoxic activity. Serial light microscopic examination of tissue cultures revealed that PMN incubated with EL-4 target cells and normal rabbit serum (NRS) were randomly distributed in cultures. It was also noted that contact between effector and target

cell occurred in a random fashion. Examination of cultures containing immune rabbit serum (IRS) instead of NRS revealed that PMN were closely associated with EL-4 target cells. Rosette formation (a central EL-4 target cell surrounded by 3-6 PMN) was also observed. Some PMN in this close association with target cells were noted to be degranulating. Phagocytosis was not observed even after prolonged periods of incubation.

In the continuing investigation of PMN-mediated ADCC cytotoxic activity, Gale and Zighelboim (1975) additionally reported that target cell destruction by PMN was complement independent, based on studies with carrageenan and with bovine serum albumin (BSA) as a substitute for FBS to rule out the involvement of the alternate complement pathway. Likewise the reaction was observed to be independent of DNA replication and "de novo" protein synthesis based on studies with X-irradiation and mitomycin C on the one hand and puromycin, cycloheximide and chloramphenicol on the other. It was also reported that only viable cells were capable of effecting this reaction.

It would appear then that PMN-mediated ADCC, analogous to lymphocyte-mediated ADCC, is an immunologically specific reaction requiring viable cells. It is also cation dependent and subject to modulation by cyclic nucleotides (cGMP and cAMP) as well as sensitive to

inhibitors of microtubule function and lysosome release. It is also complement independent as well as independent of DNA replication and protein synthesis. Based on these data, it was concluded that the PMN, in its own right, possessed the necessary "armamentarium" to effect ADCC cytotoxic activity (Gale and Zighelboim, 1974; 1975).

It would appear that this latter may also be the case for the eosinophil-mediated ADCC destruction of IgE-sensitized parasitic target organisms. Butterworth et al (1979) indicated that damage to S. mansoni schistosomular targets by the eosinophil was complement independent and owing to the glycolysis-dependent release of a preformed effector molecule of presumed lysosomal origin. Electron microscopic studies confirmed the above supposition by revealing that parasitic damage was intimately associated with the close adherence of the eosinophil to the parasite's surface. This was seen to be followed by degranulation of eosinophil onto the worm's surface. These data suggested strongly that a compound of granular origin in the eosinophil was responsible for the cytopathologic effects on the schistosomula. Butterworth et al (1979) went on to identify "major basic protein" (MBP: an arginine-rich material localized in the crystalloid area of the eosinophilic granule, accounting for greater

than 50 percent of the total granular protein content; Butterworth et al, 1979) of human or guinea pig eosinophilic origin as being capable of damaging schistosomula in concentrations as low as 2×10^{-5} M. The cytotoxic capability of MBP was in part attributed to the basic properties of the molecule. MBP was also determined to be non-specific in action as mammalian target cells were also subject to damage by this protein. MBP-mediated target damage was also determined to be associated with the uptake of MBP onto the parasite's surface with both uptake and damage subject to inhibition by the action of heparin. It was also determined that MBP was released during a reaction between eosinophils and schistosomula and was detected both on the surface of the organism as well as in the supernatant tissue culture fluid (SNF).

Target damage mediated directly by eosinophils was found, however, not to be sensitive to the inhibitory effects of heparin. This would suggest that the ADCC cytotoxic effect of the eosinophil is due to the local release of high concentrations of MBP into a sequestered site adjacent to and/or on the schistosomula rather than due to a generalized release of MBP into the tissue culture medium. These findings further suggest that the release of MBP may well represent a major mechanism of eosinophil-mediated damage to parasitic organisms in general and S. mansoni

schistosomula in particular (Butterworth et al, 1979).

The precise mechanism of ADCC-mediated target cell damage has not yet been determined. Suffice it to say that as many mechanisms have been proposed as there are ADCC effector cell types. Nevertheless, there is quite some body of evidence to suggest strongly that (phagocytosis of opsonized target cells excluded) the cytotoxic mechanism is a secretory process occurring at the local or cellular level. The strongest case for this is presented by the eosinophil with MBP being identified as the cytotoxic agent in question. The general consensus for the rest remains, however, that the ADCC reaction in general is immunospecific, dependent on viable cells, energy and cations, independent of DNA or "de novo" protein synthesis, subject to the reciprocal regulation by cGMP and cAMP and sensitive to the disruption of cell secretory systems and organelles as well as the disruption of cellular motility. Elucidation of the mechanism(s) of ADCC cytotoxicity is projected to rest with the development of biochemical methods to detect minute quantities of biochemically active substances in the microenvironment of the cell.

2.8 The Lymphoid Tissue Distribution of ADCC Cytotoxic Effector Cells

ADCC cytotoxic activity has been examined in a number of animal species as well as in birds and in man. Additionally, ADCC cytotoxic activity has been studied in a variety of lymphoid tissues taken from this diversity of experimental models and ADCC capability has been attributed to a number of cell types within individual animals and experimental models. Data is available for man, subhuman primates, rodents (rats, mice, guinea pigs), lagomorphs and fowl (chickens). Among the tissues investigated are peripheral blood, thymus, spleen, lymph node, thoracic duct lymph, bone marrow, peritoneal exudate and fetal tissues. The variety of tissues examined is second only to the variety of assay systems used to investigate ADCC. Consequently, unanimity with respect to effector cell type and its respective distribution among the lymphoid tissues within a given species is difficult to realize. Even more difficult is the interspecies comparison of effector cell distribution. This part of the review will nevertheless attempt to give a summary of the variety of data obtained and the conclusions thereby derived.

In man, ADCC cytotoxic activity has been ascribed to circulating leukocytes (lymphocytes and granulocytes) against appropriately sensitized erythrocytes

as well as other target cells (Chang liver cells: MacLennan et al, 1969; fibroblasts: Moller and Svehag, 1972; carcinoma cells: Hakala et al, 1974; herpes-virus infected cells: Shore et al, 1974; and endothelial cells: Hirschberg et al, 1975; Lovchik and Hong, 1977; among others). The data available on ADCC for other human lymphoid tissues is more limited, however. Lovchik and Hong (1977) did run a series of comparisons between the ADCC capabilities of a variety of lymphoid tissues tested against a battery of target cells (ChRBC, HuRBC and P815 cells). Cells from some human thymuses were noted to demonstrate low levels of ADCC cytotoxic activity towards all three antibody-sensitized target cells whereas cells from others were observed to be inactive. They also reported that human splenic cells were more strongly active against these three target cells than were PBL, although these latter were indeed active. Lymph node cells were comparably inactive. Additional data gathered by other workers (Holm et al, 1974) would seem to indicate that human thoracic duct lymphocytes were also inactive in ADCC.

Lovchik and Hong (1977) also reported that human bone marrow was poorly active against antibody-sensitized ChRBC, HuRBC and P815 target cells. However, when these same bone marrow cells were processed through a ficol-

hypaque density gradient to remove granulocytes and erythrocytes, the remaining cells were found to be effective in ADCC against these same three target cells. It is possible that the earlier observation was owing to a dilution effect of granulocytes, erythrocytes and other immature cell types on the ADCC effector cell population in the unfractionated bone marrow rather than to the direct inhibition by any one or combination of these cell groups on the ADCC cytotoxic effector cell proper.

Lovchik and Hong (1977) also examined lymphoid tissue of thymic, splenic and hepatic origin from 21 to 22 week old human fetuses and found that these tissues were poorly active or inactive in ADCC against ChRBC, HuRBC and P815 target cells. These data corroborated those reported by other workers (Trinchieri et al, 1975a) who observed that cells prepared from human fetal (9 to 14 weeks of gestation) liver, spleen, bone marrow and thymus were also inactive in ADCC against lymphoblastoid target cells. The variation between the fetal and mature condition particularly with respect to those tissues known to be active in man, especially spleen and bone marrow, may very well suggest that a maturation process is involved in the acquisition of ADCC capability.

ADCC cytotoxic activity in man has been attributed to PBL (lymphocytes and granulocytes) as well as to cells derived from the spleen and bone marrow. Cells of thymic origin have been shown to be variably active in ADCC while lymph node cells and cells prepared from thoracic duct lymph were found to be inactive. The data of Lovchik and Hong (1977) do not only ascribe ADCC cytotoxic effector cell status to various lymphoid tissue cells, but also serve to illustrate the concept that individual lymphoid tissues do not react with equal efficiency in different target cell systems. For example, in the above study, human granulocytes were observed to demonstrate ADCC cytotoxic activity only towards the antibody-sensitized erythrocyte target cells (ChRBC, HuRBC). This theme reappears throughout this review and is substantiated by the observations of many of the authors cited in this work.

Peripheral blood leukocytes from subhuman primates were also found (by Lovchik and Hong, 1977) to act in ADCC against ChRBC, HuRBC and P815 target cells. In some instances, it was noted that the level of activity shown by the monkey PBL exceeded that shown by human PBL.

In the investigation of rodent experimental systems, de Landazuri et al (1974) and Lovchik and Hong (1977)

reported that rat peripheral blood leukocytes were more active in ADCC towards lymphoblastoid target cells than were cells taken from any other rat lymphoid tissue. This was in contradistinction to the data of Zighelboim and Gale (1974) who showed that rat PBL were poorly active in ADCC against EL-4 target cells. MacLennan (1973), on the other hand, found rat PBL to be intermediate in activity, along with bone marrow and lymph node cells, between peritoneal exudate cells on the one hand, and thoracic duct lymph and/or thymus cells on the other in an ADCC assay with Chang liver target cells. These divergent observations reiterate the theme presented throughout this literature review that target cell identity in turn specifies in part the nature of the cell capable of effecting its destruction in the ADCC reaction.

Several workers, despite differences in their experimental systems, have presented data to the effect that rat thymus cells were inactive in ADCC (Lovchik and Hong, 1974, 1977; de Landazuri et al, 1974; MacLennan and Harding, 1970). Rat splenic cells on the other hand were reported to be capable of effecting ADCC against a variety of target cells (ChRBC, HuRBC, P815 cells: Lovchik and Hong, 1977; SRBC: Ormerod and Jolley, 1976). Although rat splenic cells were capable of effecting ADCC, Lovchik and Hong (1977)

reported that these cells were, nevertheless, less active than their human counterparts against the same battery of target cells.

Rat lymph node cells have been demonstrated to be variably active against lymphoma, erythrocyte (ChRBC, HuRBC) and P815 target cells (Lovchik and Hong, 1977). They were found in fact to be mildly effective against lymphoma target cells (de Landazuri et al, 1974; Lovchik and Hong, 1977), poorly active against erythrocyte target cells and inactive towards P815 cells (Lovchik and Hong, 1977). Thoracic duct lymph was also inactive in the rat (Perlmann and Holm, 1969; MacLennan and Harding, 1970; Lovchik and Hong, 1977) as it was in man. Rat PEC have been found to be active in ADCC by some (MacLennan, 1973) but not by others (Ormerod and Jolley, 1976).

However, the most comprehensive study of the lymphoid tissue distribution of ADCC cytotoxic effector cells in the rat was conducted by MacLennan, 1973. The data was generated through an assay in which Chang liver cells were designated as target cells. The results were expressed as the ratio of the number of cytotoxic cells contained in the test population relative to that number contained in the spleen cell population. MacLennan indicated that the bulk of ADCC activity in the rat resided in the spleen

and peritoneal exudate whereas thymus and thoracic duct lymph contained few or no cytotoxic cells. It is interesting to note that the spleens of rats tested after 3 days of thoracic duct drainage showed remarkably high levels of ADCC cytotoxic activity, far in excess of that observed as the normal baseline for the rat spleen. The elimination of a group of ADCC-inactive cells by thoracic duct drainage may have very well led to the concentration of ADCC reactive cells within rat lymphoid tissues and particularly the spleen. Blood, bone marrow and lymph node cells were also examined and it was found that these were intermediate in activity between spleen and peritoneal exudate cells on the one hand and thymus cells and cells from thoracic duct lymph on the other. These data were in partial agreement with data on the rat referred to earlier, with differences possibly being attributable to variations in the experimental systems used by different groups of workers to detect ADCC.

These findings in the rat were reminiscent of those reported for man where PBL, spleen and bone marrow were found to contain ADCC cytotoxic effector cells while thoracic duct lymph was not. There is, nevertheless, a distinction to be made between this

animal species and man with regard to the ADCC cytotoxic activity of lymph node and thymic cells. In the case of the former, rat lymph node cells possess ADCC capability whereas those of man are poorly active in ADCC. In the case of the latter, rat thymic cells are poorly ADCC reactive whereas those of man show variable levels of ADCC activity.

Thymic cells taken from newborn mice or adult mice were reported by Ralph et al (1973) to be relatively inactive in ADCC. Adult liver was also examined in this study and was likewise found to be inactive. However, murine thymic cells have been also reported to be the designated ADCC cytotoxic effector cell in a special assay using Moloney-virus induced tumour target cells and IgM anti-target cell antibody (Lamon et al, 1975a; 1975b; 1975c). This was a unique experimental system by virtue of the IgM identity of the inducing antibody. The point remains, nevertheless, that murine thymic cells could effect ADCC cytotoxicity given the appropriate experimental conditions. Splenic cells have likewise been reported to be active in this experimental system as well as in more conventional systems in which a variety of target cells (P815 targets in the presence

of isologous antiserum: Kiessling and Klein, 1973; P815 mastocytoma cells: Greenberg et al, 1975a; chicken erythrocytes: Britton et al, 1973; sheep erythrocytes: Ormerod and Jolley, 1976) were utilized.

However, murine splenic cells have also been reported to be poor ADCC cytotoxic effectors against murine lymphoma target cells. These conflicting results reiterate the distinction between erythrocyte and mammalian target cell destruction in ADCC (Ormerod and Jolley, 1976) and re-emphasize the necessity of rigid scrutinization of data before drawing definitive conclusions as to the presence or absence of ADCC cytotoxic effector cells in any given lymphoid tissue.

Murine peritoneal exudate cells have also been found by several workers to be an excellent source of ADCC cytotoxic effector cells against a variety of target cells (Allison, 1972; Cerottini and Brunner, 1974; Ormerod and Jolley, 1976).

ADCC cytotoxic activity in the mouse has been described for spleen and peritoneal exudate cells. Additionally, ADCC capability has been ascribed to fetal and neonatal liver. On the other hand, variable results have been obtained with thymic cells. Nevertheless, these cells in some instances have been demonstrated to be active in ADCC. The identification of ADCC cytotoxic effectors in the murine spleen is

consistent with findings in man and in the rat. Likewise, the identification of ADCC cytotoxic effector cells in murine peritoneal exudate is reminiscent of observations made for the rat. The variability in the ADCC cytotoxic activity demonstrated by murine thymic cells is not surprising as similar observations have been noted for man and for the rat. With respect to the study of fetal tissues, only in the mouse has ADCC capability been ascribed to fetal liver cells (Britton et al, 1973; Ralph et al, 1973).

Although the mouse and rat are important to experimental work in biology and medicine, the guinea pig as well as the rabbit play equally significant roles in the investigation of medical dilemmae and immunological mechanisms. These animals have also been used to further the understanding of ADCC as have the mouse and rat, and ADCC in the guinea pig has been reported to be present in cells of splenic origin. This was confirmed through testing of splenic cell preparations against a variety of target cells (PPD-coated ChRBC: Perlmann and Holm, 1968; ChRBC, HuRBC and P815 target cells: Lovchik and Hong, 1977). Guinea pig PBL have also been reported to be active in ADCC against PPD-coated chicken erythrocyte target cells while thymic cells have not. In the guinea pig as in other rodents and man, ADCC cytotoxic capability has been described

for cells prepared from the spleen and peripheral blood. Again, thymic cells have proven to be unpredictable in their behaviour and found, by Perlmann and Holm (1969) to be inactive in ADCC.

ADCC cytotoxicity has also been investigated in a variety of strains of rabbit, notably Bouscat rabbits (Bona et al, 1975; 1976), Silver-greys (Gelfand et al, 1972; Resch et al, 1974) and in species not specified (Zighelboim and Gale, 1976). A number of lymphoid tissues have been investigated in these species utilizing a variety of target cells. Among the lymphoid tissues investigated were spleen, thymus and bone marrow (Bona et al, 1975, 1976;) PBL (Zighelboim and Gale, 1976) and spleen, thymus and MLN (Gelfand et al, 1972; Resch et al, 1974). ADCC cytotoxic activity has been reported to be present in all of the above tissues. It is interesting to note that lymph node cells and particularly thymic cells were definitively active in ADCC in the rabbit, in contradistinction to the previous experiences of workers with cells of similar origin in man as well as in the rat and guinea pig. This information also gives weight to those reports attributing ADCC capability to murine thymic cells.

The study of the ADCC phenomenon has not been restricted to mammalian subjects only. Fowl have also been examined in this light (Calder et al, 1974a) and

ADCC capability has been ascribed to chicken peripheral blood leukocytes (lymphocytes). ADCC was additionally investigated in chickens bursectomized in ovo at 17 days incubation. Little difference was noted in the ADCC expressed by PBL of either bursectomized or normal chickens despite the fact that those bursectomized in ovo were severely hypogammaglobulinemic or agammaglobulinemic suggesting that they were severely depleted of B cells. Despite the relative absence of B cells, these chickens were as active in ADCC as their normal counterparts thus ruling out a role for the bursa-processed lymphocyte in ADCC in the chicken.

Although this review of the interspecies lymphoid tissue distribution of ADCC cytotoxic effector cells is rather simplistic in that it does not take into account the nature of the assay used to measure ADCC nor the identification of the cell type effecting ADCC in a given animal, it nevertheless does reveal certain common features between species while at the same time underlines differences. In general, ADCC cytotoxic capability can be ascribed to PBL and to those tissues of principal haematopoietic activity in man, animals and fowl. Data is less conclusive for thymus, thoracic duct lymph and lymph node. However, this survey has emphasized that ADCC cytotoxic activity is not unique to any one "Order" and further that consistencies

are evident between "Orders" and "Species". It remains to determine the biological relevance of ADCC cytotoxicity.

2.9 Summary

The ADCC cytotoxic reaction has been reviewed in the context of a discussion of its individual components (notably ADCC cytotoxic effector cells, target cells and anti-target cell antibodies), the lymphoid tissue localization of ADCC cytotoxic effector cells and the ADCC cytotoxic mechanism proper. The immunospecificity of the ADCC reaction has been considered in the context of several discussions of the components essential for the demonstration of ADCC cytotoxic reactivity, i.e.,

(i) ADCC cytotoxic effector cells with immunoglobulin Fc cell surface receptors;

(ii) target cells and/or target cell associated antigens, and

(iii) anti-target cell antibodies of the appropriate immunoglobulin class capable of interacting with Fc receptors on ADCC cytotoxic effector cells (Zighelboim and Gale, 1976).

Immunospecificity is concluded to reside with the immunoglobulin molecule, because it alone is directed in an immunologically specific way toward target cell and/or target cell-associated antigens. Furthermore, it alone acts in a manner to permit the linking of target cell to ADCC cytotoxic effector cell, an interaction essential for the initiation of the cytotoxic event. The immunospecificity of the ADCC reaction together with the overwhelming documentation of ADCC cytotoxic capability in all of the mammals and fowl thus far analyzed lends considerable weight to the biological relevance of ADCC and its eventual emergence and academic consideration as a "true" immunologic entity and vital component of the immune system. A brief consideration of the possible role of ADCC in immune responsiveness and/or immunocompetence is therefore in order.

It is important to note that the ADCC reaction is mediated by seemingly immunocompetent cells normally present in the circulation and/or other lymphoid tissues. It is also important to note that these ADCC cytotoxic effector cells have not been previously exposed to, or sensitized with target cells. This suggests that the ADCC reaction can only be triggered following sensitization of the target cell by the appropriate antibody. The ADCC reaction may be considered

therefore as either a forerunner of the humoral immune response, designed as a protective mechanism against invasive pathogenic agents or conversely as an adaptation of humoral immunity.

Investigations concerned with the phylogeny of the immune response have disclosed that antibodies of the IgG type appear to have evolved prior to the appearance of complement. These antibodies in the absence of complement, were incapable of causing the lysis and/or destruction of target organisms. However, these antibodies acting with Fc receptor bearing cells were capable of destroying antibody-sensitized target organisms. Once the complement system made its appearance, it probably displaced the Fc receptor bearing cell as the major naturally-occurring, non-immunologically induced protagonist in antibody-mediated lysis for reasons of accessibility to discrete target cell sites and energy efficiency. Thus the ADCC effector cell may represent a primitive improvisation by nature to serve as a stop-gap, in an evolutionary sense, between the appearance of circulating antibodies and the later appearance of the complement system (Richter, 1980).

Alternatively, the ADCC cytotoxic effector cell may be considered to represent a more highly evolved immune mechanism. It is interesting to note that the target cell need be sensitized with very few antibody molecules

in order to be susceptible to lysis by Fc receptor bearing lymphocytes. In fact, the number of antibody molecules required for ADCC cytotxicity is 100 to 1,000 times less than is required for complement-mediated target cell lysis. Thus ADCC is very effective in the presence of a limited antibody response and may therefore have great survival value. It may therefore be that Nature has retained both antibody-dependent cellular cytotoxic and antibody-dependent complement mediated mechanisms to ensure the survival of the host in the event that one of these mechanisms fails.

The precise niche occupied by ADCC in the immune response is as yet undecided. In light of the above considerations it is possible that ADCC may also serve to link humoral immunity on the one hand, to cell-mediated immunity on the other and therefore occupy a position intermediate to these two "classical" components of the immune response. This would compel the recognition of ADCC as a third component of the immune response whose function may be postulated to lie in the enhancement (particularly in the face of an inadequate conventional immune response) and/or modulation of the mammalian or avian response to challenge with any given antigen(s). In so doing ADCC would promote the ultimate goal of the immune response, survival.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Saline Solution--0.9 percent in pyrogen-free sterile water was obtained from Abbott Laboratories Limited, Montreal, Quebec, and stored at room temperature. The approximate pH of the solution was 5.7.

Dulbecco's Phosphate - Buffered Saline (PBS)--was obtained as a ten times concentrate from Grand Island Biological Company, Grand Island, New York and stored at room temperature.

Sterile Water (pyrogen-free)--was obtained from Abbott Laboratories Limited, Montreal, Quebec and stored at room temperature.

Hank's Balanced Salt Solution (HBSS)--was obtained in 500 ml quantities from Microbiological Associates Inc., Bethesda, Maryland and stored at 4°C.

Tissue Culture Medium 199--modified with 1.4 g of sodium bicarbonate and containing HBSS and L-glutamine, was obtained in 500 ml quantities from Microbiological Associates Inc., Bethesda, Maryland and stored at 4°C.

Tissue Culture Medium RPMI 1640--with L-glutamine was obtained in 500 ml quantities from Microbiological Associates Inc., Bethesda, Maryland and stored at 4°C.

Tissue Culture Medium CMRL 1066--with L-glutamine was obtained in 500 ml quantities from Microbiological Associates Inc., Bethesda, Maryland and stored at 4°C.

Penicillin - Streptomycin Mixture (PS)--containing 5,000 IU of potassium penicillin G per ml and 5,000 µg per ml of streptomycin sulfate was obtained in 100 ml quantities from Microbiological Associates Inc., Bethesda, Maryland and stored at -20°C.

Fetal Bovine Serum [Rehatuin] (FBS)--was obtained in 500 ml quantities as a filtered sterile solution from Connaught Laboratories, Willowdale, Ontario and stored at -20°C.

Rabbit Serum--was freshly prepared from adult New Zealand White rabbits prior to immunization with chicken erythrocytes, filtered and stored in 5 ml aliquots at -20°C.

Rabbit Anti-Chicken Erythrocyte Antiserum (RACE)--was freshly prepared by inoculating adult New Zealand White rabbits with a 10 percent suspension of chicken erythrocytes in saline at various time intervals. These rabbits were subsequently bled at 7, 10 and 15 days after the last immunizing injection and the antiserum was filtered and stored in 5 ml aliquots at -20°C.

Rabbit Gamma-Globulin Fraction II--was obtained from Pentex Biochemicals, Kankakee, Illinois and stored at 4°C.

Chicken Erythrocytes (ChRBC)--were obtained fresh weekly in 5 ml quantities with 100 IU of heparin sodium as an anticoagulant per ml of blood collected. These were stored at 4°C.

Sheep Erythrocytes (SRBC)--were obtained fresh weekly from Qualicum, Ottawa, Ontario in 10 ml quantities (100 IU of heparin sodium as an anticoagulant per ml of blood). The cells were stored at 4°C.

Horse Erythrocytes (HRBC)--were obtained fresh weekly from Qualicum, Ottawa, Ontario in 10 ml quantities (100 IU of heparin sodium as an anti-coagulant per ml of blood). The cells were stored at 4°C.

Bacto Adjuvant, Complete Freund's (CFA)--was obtained from Difco Laboratories, Detroit, Michigan and stored at 4°C.

Radioactive Sodium Chromate (^{51}Cr) Solution--was obtained in 3 ml quantities bi-monthly from NEN Canada, Lachine, Quebec, at a concentration of 37kBq/ml of sodium chromate in normal saline (pH 8). It was stored at 4°C.

Ficoll 400 (MW 400,000)--was obtained in 500 g quantities from Pharmacia Fine Chemicals AB, Uppsala, Sweden and stored at room temperature.

Hypaque Sodium (diatrizoate, 50% W/V, pH 6.5 to 7.7)--was obtained from Winthrop Laboratories, Aurora, Ontario and stored in the dark at room temperature.

Methyl Cellulose--was obtained in 500 g quantities from BDH Chemicals Ltd., Poole, England and stored at room temperature.

Dextran T250 (MW 250,000)--was obtained in 500 g quantities from Pharmacia Fine Chemicals AB, Uppsala, Sweden and stored at room temperature.

GAF Carbonyl Iron Powder (grade SF)--was obtained from Chemical Developments of Canada Limited, Montreal, Quebec and stored at room temperature.

Heparin Sodium--was obtained in a concentration of 1,000 U.S.P. units per ml from A Damon Company, Downsview, Ontario and in a concentration of 10,000 U.S.P. units per ml from Organon Canada Ltd./Ltee., Toronto, Ontario. The heparin sodium was stored at 4°C.

Hematall --azide-free isotonic diluent for Coulter counting was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Zap-o-globin--hi-speed stromatolysing and haemaglobin reagent was obtained from Coulter Electronics of Canada Ltd., Mississauga, Ontario and stored at room temperature.

Trypan Blue Stain--was obtained as a 0.4 percent solution in normal saline from Grand Island Biological Company, Grand Island, New York and stored at room temperature.

Wright's Stain--in crystalline form, was obtained from Harleco, Gibbstown, New Jersey and stored at room temperature.

Methyl Green Stain--in crystalline form, was obtained from Harleco, Gibbstown, New Jersey and stored at room temperature.

Pararosanilin--in anhydrous form, was obtained from the Sigma Chemical Company, St. Louis, Missouri and stored at room temperature.

Buffer Mixture--pH 6.8 for staining reaction control, was obtained from BDH Chemicals (Canada) Ltd., Montreal, Quebec and stored at room temperature.

Tris-Buffer--or tris (hydroxymethyl) aminomethane, was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Potassium Phosphate, Monobasic--was obtained from the J. T. Baker Chemical Co., Phillipsburg, New Jersey and stored at room temperature.

Sodium Phosphate, Dibasic--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Sodium Nitrite--in crystalline form, was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Sodium Hydroxide--electrolytic pellets were obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Sodium Azide--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at

room temperature.

Sodium Acetate--in fused-anhydrous form, was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Alpha-Naphthyl Acetate--in anhydrous form, was obtained from the Sigma Chemical Company, St-Louis, Missouri and stored at room temperature.

Acetic Acid, Glacial--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Hydrochloric Acid (specific gravity at 15.5°C minimum 1:1854, maximum 1:1923)--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored in a cool location.

Ethylene Glycol Monomethyl Ether--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Ethanol--reagent alcohol containing methanol, was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Methanol--acetone-free histological grade methyl alcohol, was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored in a cool location.

Glycerol--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and stored at room temperature.

Dry Ice--was obtained from Union Carbide Canada Limited, Ottawa, Ontario.

Nembutal--sodium pentobarbital injection U.S.P., was obtained from Abbott Laboratories Limited, Montreal, Quebec and stored at room temperature.

Surital--sodium thiamylal, was obtained in powdered form from Parke, Davis and Company Ltd., Brockville, Ontario. It was stored at room temperature and dissolved in distilled water prior to use.

New Zealand White Rabbits--were obtained from the Laboratory Animal Farm Reg'd., Wendover, Ontario.

Specific Pathogen-Free (SPF) White Leghorn Roosters--were obtained from the Animal Diseases Research Institute, Agriculture Canada, Ottawa, Ontario

3.1.2 Apparatus

Sterile Universal Containers (20 ml)--were obtained from Sterilin Ltd., Richmond, Surrey, England.

Conical Centrifuge Tubes (50 ml)--were obtained

from Falcon Plastics, Oxnard, California and sterilized by autoclaving prior to use.

Sterile Test Tubes (17 x 100 mm and 12 x 75 mm)--were obtained from Falcon Plastics, Oxnard, California.

All Glassware--was obtained from the Fisher Scientific Company, Fair Lawn, New Jersey and sterilized by autoclaving before use.

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

Hypodermic Needles--sterile disposable Luer-Lok hypodermic needles of all sizes were obtained from Becton, Dickinson Co., Canada Ltd., Mississauga, Ontario.

Pipettes--1, 2, 5 and 10 ml Pyrex disposable serological pipettes were obtained from Corning Glass Works, Corning, New York.

Automatic MLA Pipettes--50, 100, 200 and 400 µl pipettes were obtained from Medical Laboratory Automation Inc., Mount Vernon, New York.

Automatic MLA Pipette Tips--were obtained from the Fisher Scientific Company, Fair Lawn, New Jersey.

Nalgene Filters (0.22 m, 0.45 m)--were obtained from Sybron Corporation, Rochester, New York.

Whatman Filters--of all sizes were obtained from the Fisher Scientific Company, Fair Lawn, New Jersey.

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

Light Microscopes--were obtained from Wild Leitz Canada Ltd./Ltee. through the Fisher Scientific Company, Fair Lawn, New Jersey.

Haemacytometer Counting Chambers--were obtained from the Fisher Scientific Company, Fair Lawn, New Jersey.

Incubator--cultures were maintained in a National incubator at 37°C in 5 percent CO_2 in air. The interior of the incubator was humidified by placing a large tray of distilled water on the bottom shelf. Compressed air was passed through a Nogren filter (Littleton, Colorado) to remove suspended oil and water.

Gamma Counter--a Beckman "Biogamma" was obtained from Beckman, Fullerton, California.

"Biogamma" Vials--were obtained from Beckman, Fullerton, California.

3.2 Methods

3.2.1 The Preparation of Tissue Culture Medium

The tissue culture media used in this study, namely medium 199, RPMI 1640 and CMRL 1066 were fortified with 100 IU of potassium penicillin G per ml, 100 µg per ml of streptomycin sulfate and varying amounts of heat-inactivated (56°C, 30 minutes) fetal bovine serum to final concentrations of 0, 2, 4, 6, 10 and 20 percent of the culture medium.

3.2.2 The Preparation of Serum

Fetal Bovine Serum--one hundred ml aliquots of fetal bovine serum (FBS) were heat-inactivated in a water bath at 56°C for 30 minutes. The FBS was distributed in 12 ml aliquots once cooled to room temperature and stored at -20°C.

Rabbit Anti-Chicken Erythrocyte Antiserum (RACE)--rabbit anti-chicken erythrocyte antiserum was prepared by inoculating normal adult New Zealand White rabbits (2 to 4 kg) with 1 ml of a suspension of chicken erythrocytes in saline (10^9 cells per ml) at 2 or 3 day intervals. A total of 3 injections were administered. The rabbits were bled 7, 10 and 15 days after the last immunizing injection. These bleedings were not pooled. The

antisera were heat-inactivated at 56°C for 30 minutes and stored in 5 ml aliquots at -20°C. Appropriate dilutions were made as required for use in the cytotoxicity assay in medium 199 fortified with 100 IU of potassium penicillin G per ml and 100 μg per ml of streptomycin sulfate (M-199-PS).

Normal Rabbit Serum--Normal rabbit serum was obtained by bleeding the rabbits a number of times prior to immunization. The serum samples from any individual rabbit were pooled and heated at 56°C for 30 minutes, absorbed twice with chicken erythrocytes at 37°C (0.1 ml of packed ChRBC per ml of serum) and stored in 5 ml aliquots at -20°C. Appropriate dilutions were made in M199-PS or other fortified culture media as required for use in the cytotoxicity assay.

Haemagglutination--Heat-inactivated (56°C) serum obtained from individual rabbits immunized with saline or with SRBC (109 SRBC) was serially diluted (doubling dilutions) in PBS. A 0.1 ml aliquot of a 2 percent SRBC preparation was added to each of these serial dilutions and the preparations were mixed gently. These were then allowed to stand at room temperature overnight. Anti-SRBC antibodies were detected by the presence of a diffuse rather than button-like

pellet in the test tubes. Controls consisted of PBS to which SRBC were added.

3.2.3. The Preparation of Cell Separation Reagents

Dextran Solution--Dextran T250 was made up as a 6 percent solution in saline. After autoclaving, this solution was supplemented with heparin sodium (10,000 U.S.P. units per ml) to a final concentration of 150 U.S.P. units of heparin sodium per ml of dextran solution. This preparation was stored at 4°C.

Ficoll-Hypaque Solution--Ficoll 400 was prepared as an 8 percent solution in water. It was supplemented with 0.1 percent methylcellulose and adjusted to a specific gravity of 1.1 at 18°C with hypaque sodium. It was then filtered through a 0.45 μm Nalgene filter and stored in the dark at 4°C.

3.2.4 The preparation of Soluble Aggregates of Rabbit Gamma-Globulin (RAGG)

Phosphate Buffered Saline--one liter of Dulbecco's Phosphate Buffered Saline (PBS) was prepared by diluting the PBS tenfold in distilled water and then adding 1 ml of 5 N sodium hydroxide. The pH of the final solution was 7.2.

Soluble Aggregates of Rabbit Gamma-Globulin (RAGG)--five hundred milligrams of rabbit gamma-globulin (fraction II) were dissolved in 10 ml of PBS, pH 7.2. Five ml aliquots were heat-aggregated in a water bath for 25 minutes at 63°C and centrifuged at 850 g for 10 minutes to remove any clumps. The supernatant fluid was pipetted off and diluted in M199-PS to a final concentration of 5 mg/ml and stored at -20°C until use. Further dilutions were made as required in M199-PS for use in the inhibition of ADCC cytotoxicity assay.

3.2.5 The Preparation of ^{51}Chromium-labelled Chicken Erythrocyte Target Cells

Erythrocytes were obtained from young SPF White Leghorn roosters by bleeding from the wing vein into a syringe containing heparin sodium (100 USP units of heparin sodium per ml of blood collected). The cells were allowed to settle at 4°C for a minimum of 24 hours prior to use. The erythrocytes were washed 3 times in saline with centrifugation at 540 g for 10 minutes between the washes. The supernatant fluid was discarded. The pelleted erythrocytes were then diluted 10 fold in the appropriate culture medium and an aliquot was counted in a Coulter counter. A

0.1 ml volume of this 10 percent suspension of chicken erythrocytes was incubated with an equal volume of sodium chromate (3700 Bq of ^{51}chromium) specific activity 14,800 Bq/mg and rotated in a tissue culture rotator for 1 hour at 37°C and 5 percent CO_2 in air. The cells were suspended in a 10 ml volume of HBSS fortified with 100 IU of potassium penicillin G per ml and 100 μg of streptomycin sulfate (HBSS-PS) and centrifuged at 480 g for 10 minutes. The supernatant fluid was discarded and this washing procedure was repeated twice more. The ^{51}Cr-labelled chicken erythrocytes were then suspended in 10 ml of the appropriate culture medium and diluted to 2 x 10^5 cells per ml just prior to use in the ADCC cytotoxicity assay.

3.2.6 The Preparation of Lymphoid Tissue Cell Suspensions

The Preparation of Cell Suspensions of Thymus, Spleen, Appendix, Sacculus Rotundus, Peyer's Patches, Mesenteric and Popliteal Lymph Nodes and SRBC and/or HRBC Challenge Sites--outbred adult New Zealand White rabbits weighing approximately 2 kilograms were used throughout this study. The rabbits were sacrificed by the intravenous injection of Nembutal or Surital (50 mg/kg body weight). Surital was the preferred

anaesthetic agent as Nembutal caused a moderate amount of haemolysis. The cells were prepared as outlined in figure 4. The thymus, spleen gut-associated lymphoid tissues (GALT: appendix, sacculus rotundus, Peyer's patches), mesenteric lymph nodes (MLN), popliteal lymph nodes (PLN) and SRBC and/or HRBC skin challenge sites were rapidly extirpated, rinsed with sterile saline fortified with 100 IU of potassium penicillin G per ml and 100 µg per ml of streptomycin sulfate and placed into sterile disposable plastic test tubes containing HBSS-PS. Each organ was then individually transferred to a sterile vessel containing 2 ml of HBSS-PS. Cell suspensions were prepared by chopping each organ into small fragments. This was followed by shaking the contents of the tube vigorously to liberate cells from the organ fragments. The fragments were allowed to settle and the supernatant fluid containing the lymphoid cells was pipetted off and passed through sterile gauze into 50 ml sterile centrifuge tubes. The volume was made up to 50 ml with HBSS-PS, and the cells were centrifuged at 480 g for 10 minutes. The supernatant fluid was discarded. This washing procedure was repeated twice. The pelleted cells of the final wash were then suspended in the appropriate culture medium and an

aliquot was counted in a Coulter counter. The cells were then diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay. All cell suspensions were kept in an ice bath at 4°C until introduction into tissue culture in the ADCC cytotoxicity assay. Cellular viability was determined by the trypan blue dye exclusion technique just prior to culture.

The Preparation of White Blood Cell Suspensions--circulating white blood cells (WBC) were obtained by bleeding the rabbits directly from the heart (33 ml) into a sterile 50 ml syringe containing heparin sodium in a 6 percent dextran solution. The contents of two 50 ml syringes containing the blood-dextran mixture were transferred to a sterile graduated cylinder and kept at 37°C in air for 30 to 45 minutes or until the red blood cells (RBC) had settled. The leukocyte-lymphocyte rich upper layer was then transferred to 50 ml sterile centrifuge tubes containing 30 ml of HBSS-PS. The cells were centrifuged at 850 g for 20 minutes. The supernatant fluid was decanted and the pelleted WBC were suspended in 50 ml of HBSS-PS and centrifuged at 480 g for 10 minutes. This washing procedure was repeated twice. After the last wash, the pelleted WBC were suspended in the appropriate

culture medium and an aliquot was counted in a Coulter counter. The cells were then diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay. All cell suspensions were kept in an ice bath at 4°C until introduction into tissue culture in the ADCC cytotoxicity assay. Cellular viability was determined by the trypan blue dye exclusion technique just prior to culture.

The Preparation of Bone Marrow Cell Suspensions--bone marrow was obtained by splitting the tibia and femur of the rabbit with a bone cutter. The marrow was suspended in HBSS-PS in a sterile Universal container and this was shaken vigorously for 1 to 2 minutes. The supernatant fluid containing the liberated bone marrow cells was passed through sterile gauze into 50 ml sterile centrifuge tubes. The volume was made up to 50 ml with HBSS-PS and the bone marrow cells were centrifuged at 480 g for 10 minutes and the supernatant fluid was discarded. The washing procedure was repeated twice. The pelleted cells of the final wash were suspended in the appropriate culture medium and an aliquot was counted in a Coulter counter. The cells were diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay.

All cell suspensions were kept in an ice bath at 4°C until introduction into tissue culture in the ADCC cytotoxicity assay. Cellular viability was determined by the trypan blue dye exclusion technique just prior to culture.

Ficoll-Hypaque Density Gradient Centrifugation of Rabbit WBC, Spleen and Bone Marrow Cell Suspensions--20 ml volumes of cell preparations of WBC, spleen and bone marrow at a concentration of 5 x 10^6 cells per ml in HBSS-PS were layered onto 10 ml of ficoll-hypaque and centrifuged at 1,000 g at 18°C for 30 minutes. The cells at the blood ficoll-hypaque gradient interphase (FHI) were pipetted off, diluted in 50 ml of HBSS-PS and centrifuged at 850 g for 20 minutes and the supernatant fluid was discarded. The pelleted cells of this first wash were suspended in 50 ml of HBSS-PS and centrifuged at 480 g for 10 minutes. These cells were similarly washed once more, suspended in the appropriate culture medium and an aliquot was counted in a Coulter counter. The cells were diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay.

The cells in the pellet of the ficoll-hypaque discontinuous density gradient (FHP) were similarly

washed, suspended in the appropriate culture medium and counted in a Coulter counter. They were then diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay.

All cell suspensions were kept at an ice bath at 4°C until introduction into tissue culture in the ADCC cytotoxicity assay. Cellular viability was determined by the trypan blue dye exclusion technique just prior to culture.

3.2.7 The Treatment of Lymphoid Tissue Cell Suspensions with Tris-Buffered Ammonium Chloride Solution to Lyse Contaminating Erythrocytes

Tris-Buffered Ammonium Chloride Solution--tris buffer (2.059 g) was dissolved in 100 ml of distilled water and the pH of this preparation was adjusted to 7.65 with hydrochloric acid. Ammonium chloride (8.3 g) was dissolved in one liter of distilled water and nine parts of this preparation (900 ml) were mixed with one part (100 ml) of tris buffer. The pH was adjusted to 7.2 with hydrochloric acid. The solution was filtered through a Nalgene filter (0.22 µm) and stored at 4°C.

The Treatment of Lymphoid Tissue Cell Suspensions with Tris-Buffered Ammonium Chloride Solution--rabbit cell suspensions were centrifuged at 480 g for 10 minutes and the supernatant fluids were discarded. Ten ml volumes of heated (37°C) tris-buffered ammonium chloride solution (0.15M) were added to the pelleted cells to lyse the contaminating erythrocytes in the cell suspensions. These preparations were incubated in a water bath at 37°C for 10 minutes and then made up to a volume of 50 ml with HBSS-PS. They were centrifuged at 480 g for 10 minutes and the supernatant fluid was discarded. This washing procedure was repeated twice more and the pelleted cells of the final wash were suspended in the appropriate culture medium. An aliquot was counted in a Coulter counter and the cells were diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay. All cell suspensions were kept in an ice bath at 4°C until introduction into tissue culture in the ADCC cytotoxicity assay. Cellular viability was determined by the trypan blue dye exclusion technique just prior to culture.

3.2.8 The Depletion of Phagocytic Cells from Lymphoid Tissue Cell Suspensions by Carbonyl Iron Uptake

WBC, spleen and bone marrow cells were suspended in a 50 percent heat-inactivated fetal bovine serum--saline solution, containing 2 to 8 mg of carbonyl iron per ml. The final cell concentration of the preparation was 2×10^6 cells per ml. Five ml aliquots of these cell suspensions were incubated in a tissue culture rotator for 30 minutes at 37°C in 5 percent CO_2 in air. At the termination of this incubation period, the cells were resuspended. The iron particles and the cells containing iron particles were separated from the balance of the cell suspension by pressing the test tube against a magnet and pipetting off the supernatant fluid and non-iron-containing cells into a conical sterile centrifuge tube(s). This cell suspension was made up to a volume of 50 ml with HBSS-PS and centrifuged at 480 g for 10 minutes. The supernatant fluid was discarded. The pelleted cells were similarly washed twice more, suspended in the appropriate culture medium and an aliquot was counted in a Coulter counter. The cells were diluted in the appropriate culture medium for use in the ADCC cytotoxicity assay. All suspensions were kept in an ice bath at 4°C until introduction into tissue culture in the ADCC cytotoxicity assay.

Cellular viability was determined by the trypan blue dye exclusion technique just prior to culture.

3.2.9 The Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay

One-fifth ml volumes of rabbit lymphoid tissue cell suspensions (10^6 cells or as otherwise specified) or WBC were mixed with 0.2 ml of ^{51}Cr-ChRBC (4×10^4 cells) target cells and 0.2 ml of heat-inactivated rabbit anti-ChRBC antiserum (RACE) or normal control serum. The optimal dilution of RACE utilized (1:1,000) was determined in preliminary experiments.

Controls consisted of unlabelled ChRBC (10^6 cells in 0.2 ml volumes or as otherwise specified) or medium (0.2 ml: control no.1) as substitutes for rabbit lymphoid cells or WBC. A final control (control no.2) consisted of culture medium (0.4 ml) and ^{51}Cr-ChRBC. All cultures were set up in duplicate or triplicate as cell numbers would allow.

The results obtained from the control cultures, together with the determination of the spontaneous release (S.R.) of ^{51}Cr from the ^{51}Cr-ChRBC target cells, indicate the degree of target cell stability during the culture period. A high degree of stability must be ensured in order to be able to consistently measure the ADCC activity of various cell preparations with

any degree of accuracy.

Unless otherwise stated, the cultures were incubated for 24 hours at 37°C in 5 percent CO_2 in air. The tubes were then centrifuged at 480 g for 10 minutes. Aliquots of the culture supernatants (400 μl) were transferred to appropriate vials and counted for 5 minutes in a gamma counter. The data was recorded as counts per culture per minute and the results were expressed as the cytotoxic index (C.I.) calculated as follows:

$$\text{C.I.} = \frac{\text{Test Release} - \text{Spontaneous Release}}{\text{Total Release} - \text{Spontaneous Release}} \times 100$$

Test Release--refers to the ^{51}Cr detected in the supernatant fluid of the cultures containing RACE and rabbit lymphoid cells, WBC or their substitutes (notably ChRBC or medium).

Spontaneous Release (S.R.)--refers to the ^{51}Cr detected in the supernatant fluid of the cultures containing normal control serum, rabbit lymphoid cells, WBC or their substitutes (notably ChRBC or medium).

Total Release--refers to the ^{51}Cr contained in the supernatant fluid following freeze-thawing of the ^{51}Cr-ChRBC suspension 3 to 4 times in an alcohol-dry ice mixture (-70°C).

3.2.10 The Inhibition of ADCC Assay by Soluble Aggregates of Rabbit Gamma-Globulin (RAGG)

One tenth ml volumes of rabbit WBC, spleen, bone marrow, thymus, appendix and mesenteric lymph node cells (10^7 cells per ml) were incubated with RAGG for 2 hours at 37°C and 5 percent CO_2 in air with an equal volume of culture medium M199-PS to achieve a final concentration of 1,000, 500, 100, 50 or 5 μg per ml of RAGG. At the end of the incubation period, appropriate concentrations of ^{51}Cr-ChRBC target cells (4×10^4 cells in 0.2 ml) and RACE (1:1,000, 0.2 ml) or normal control serum (1:1,000, 0.2 ml) were added to these cells as per the ADCC cytotoxicity assay. The cells were further incubated for 24 hours at 37°C and 5 percent CO_2 in air and processed for radioactive counting as previously described. The percent inhibition (% Inh) was calculated according to the following formula:

$$\text{Inhibition} = \frac{\text{Control C.I.} - \text{Test C.I.}}{\text{Control C.I.}} \times 100$$

Control C.I.--refers to the cytotoxic index obtained in the cultures containing rabbit lymphoid cells or WBC, RACE and ^{51}Cr-ChRBC target cells.

Test C.I.--refers to the cytotoxic index obtained in the cultures containing RAGG, rabbit lymphoid cells or WBC, RACE and ^{51}Cr-ChRBC target cells.

3.2.11 Histological Techniques

The Trypan Blue Dye Exclusion Technique for Measuring Cellular Viability--two parts trypan blue histological stain (0.2 percent trypan blue in physiological saline) were added to 1 part of the rabbit lymphoid tissue cell suspension (10^6 cells) and allowed to stand 5 to 15 minutes and then counted in a haemacytometer counting chamber under light microscopy. The cells excluding the dye were considered to be viable and those incorporating the stain were considered to be non-viable. The viability was expressed as a percentage of the total number of cells counted and examined.

The Methods for Wright's Stain--the smears to be stained were placed on a rack over a sink. Twenty to 40 drops (0.5 to 1 ml) of Wright's stain were carefully added to the slides in order to cover the smears. The stain was allowed to stand for 2 minutes. Twenty drops (0.5 ml) of buffer solution (pH 6.8) were then pipetted onto the slides and mixed with Wright's stain by gently blowing on several portions of the slide. This mixture was allowed to stand for 5 minutes. After

5 minutes, the excess staining solution was removed from the slides by flooding these freely with distilled water. They were then removed from the rack and allowed to air dry.

Wright's Staining Reagent--Wright's stain was prepared by blending 0.5 g of Wright's stain powder with 3 to 5 ml of glycerol and mixing these with mortar and pestle. One hundred ml of acetone-free methanol was added to this mixture and this was allowed to stand for 2 to 3 days before filtering. It was then passed through a Whatman number 1 cellulose filter and stored at room temperature.

Buffer for Wright's Stain--buffer mixture for staining reaction control (pH 6.8) was made up as directed. One vial of this preparation was dissolved in distilled water and made up to a volume of 500 ml. It was prepared freshly as required for use with Wright's, Leishman's and Giemsa's staining methods.

Non-specific Esterase Staining for Monocytes--a 1.5 ml volume of a 4 percent sodium nitrite solution (0.2 g of sodium nitrite in 5 ml of distilled water) was added to an equal volume of pararosanilin solution and allowed to stand for 1 minute. After 1 minute, 2.4 ml of this preparation was added to a beaker containing a mixture of 40 mg of alpha-naphthyl acetate

and 2 ml of ethylene glycol monomethyl ether. This was followed by the addition of 35.5 ml of phosphate buffer (0.1M, pH 7.6). This caused a buff coloured precipitate to form. The pH was adjusted to 6.1 (5.8 to 6.5) with concentrated hydrochloric acid or 1N sodium hydroxide and the solution was filtered through a Whatman number 1 cellulose filter. The precipitate was discarded.

Air dried unfixed smears of rabbit lymphoid tissue cell preparations (rapid drying with cold air is essential) were covered with the above staining reagent and incubated at room temperature for 45 minutes, washed with distilled water, blow dried with hot air and fixed in methanol for 2 minutes and again blow dried. They were then counterstained for 2 minutes with the 1 percent methyl green staining solution, washed with distilled water, blow dried and examined under a light microscope.

Several leukocyte types in the rabbit stain for esterase and hence this staining technique cannot be accurately used to enumerate rabbit monocytic cells exclusively. It is, however, an excellent stain for revealing cytological differences between the various leukocyte types in the rabbit and was used with this in mind.

Pararosanilin Staining Reagent--pararosanilin staining reagent was prepared by weighing out 1 g of

pararosanilin followed by the addition of 20 ml of distilled water and 5 ml of concentrated hydrochloric acid.

Methyl Green Staining Reagent--a 1 percent solution of methyl green staining reagent was prepared by weighing out 1.4 g of sodium acetate and 1.0 g of methyl green stain followed by the addition of distilled water to a final volume of 100 ml. The pH was adjusted to 4.2 with concentrated acetic acid.

Phosphate Buffer--phosphate buffer (0.1M, pH 7.6) was prepared by mixing 1,000 ml of a 0.1M solution of Na_2HPO_4 (14.1 g in 1,000 ml of distilled water) and 190 ml of a 0.1M solution of KH_2PO_4 (2.72 g in 200 ml of distilled water).

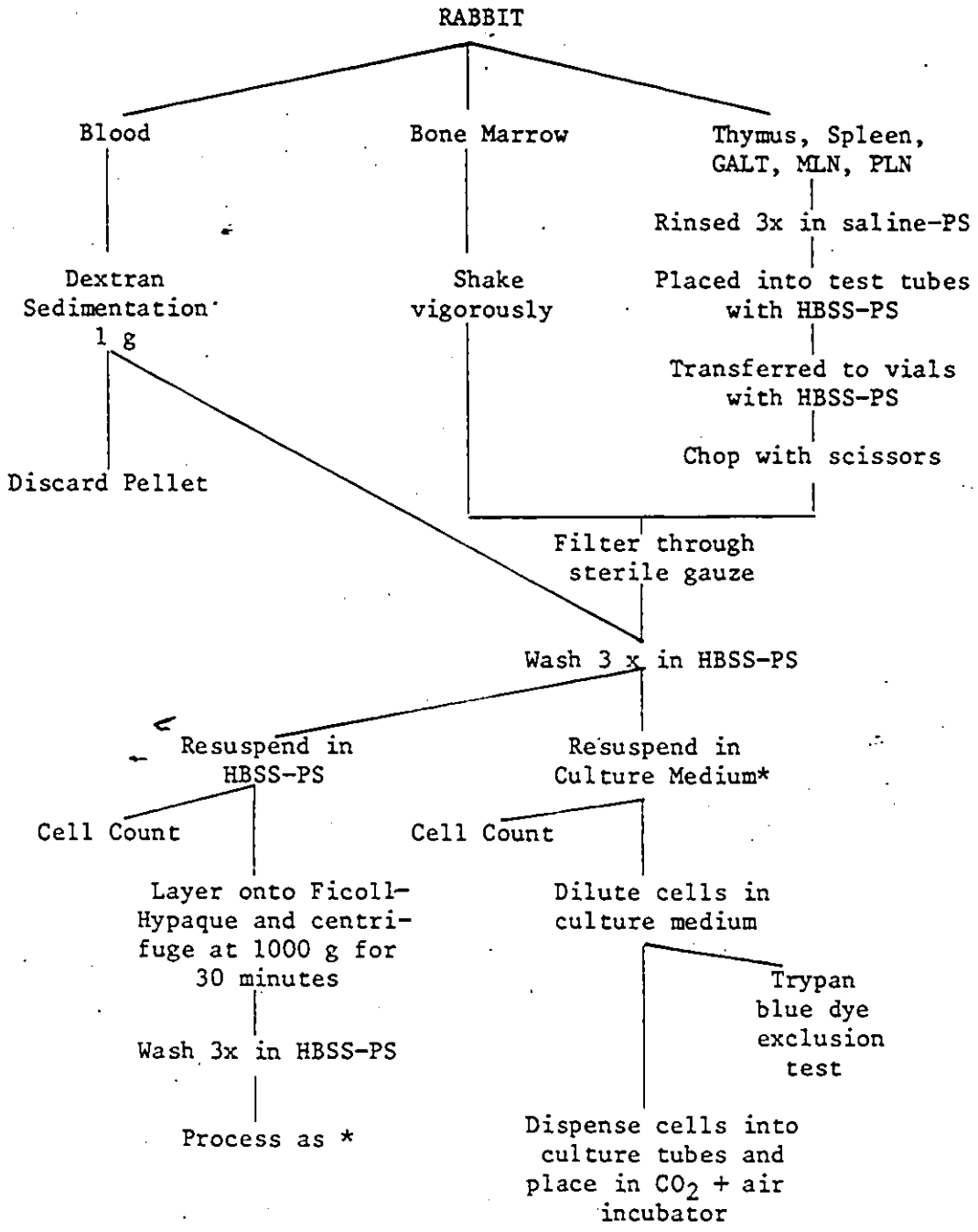

Figure 4:-- Preparation of Rabbit Lymphoid Cells for Tissue Culture.

CHAPTER IV

THE OPTIMAL CONDITIONS FOR ADCC CYTOTOXICITY IN THE RABBIT

4.1 Introduction and Objectives

In order to ascertain the nature of ADCC in the Lagomorph with a view to the acceptability of members of this Order as models for ADCC in man, it was deemed essential to define the conditions which permit maximum ADCC cytotoxic activity by rabbit cells. The objectives of the experiments carried out in this part of the investigation were:

(i) to evaluate the effectiveness of the rabbit anti-ChRBC antiserum (RACE), culture media and medium supplements in the ADCC cytotoxicity assay;

(ii) to determine the activity profile (optimal effector to target cell ratio and time course) of the cells of the various rabbit lymphoid tissues in the ADCC cytotoxicity assay, and

(iii) to determine the lymphoid tissue distribution of ADCC cytotoxic effector cells by simultaneous examination of the lymphoid tissues of a given rabbit(s).

4.2 Experimental Protocols and Results

The data presented in the following tables are representative of results obtained in individual experiments. Each experimental protocol was repeated a minimum of 5 times with the majority repeated 10 times. Each value represents the mean of duplicate or triplicate determinations. Since the viability of all cell preparations at the time of culture was 85 to 95 percent as determined by the trypan blue dye exclusion technique, the number of cells introduced into culture were not corrected for viability.

4.2.1 The Evaluation of the Rabbit Anti-ChRBC Antiserum (RACE)

Heat-inactivated RACE antiserum and normal rabbit control serum were diluted in M199-PS and titrated in the ADCC assay with a fixed concentration of spleen cells (5×10^6 cells per ml in the culture medium M199-PS supplemented with heat-inactivated FBS to a final concentration of 6 percent) (table I). The ADCC assay was conducted as described in chapter III: Materials and Methods, section 3.2.9.

Spleen cells (E) co-cultured with ^{51}Cr-ChRBC target cells (T) at the effector to target cell ratio (E:T) of 25:1 showed marked ADCC cytotoxic activity

TABLE I

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE CONCENTRATION OF ANTI-TARGET CELL (ChRBC) ANTISERUM

Exp. No.+	Cells tested	E:T*	E/ml**	T/ml***	The cytotoxic index of the following anti-ChRBC antiserum concentrations						
					10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	Spleen	25:1	$5x10^6$	$2x10^5$	85	78	66	13	0	1	0
2	Spleen	25:1	$5x10^6$	$2x10^5$	74	67	52	14	1	0	0
3	Spleen	25:1	$5x10^6$	$2x10^5$	94	84	70	23	4	0	0

+ The medium used was M-199, fortified with penicillin (100 units per ml) and streptomycin (100 μg per ml) and fetal bovine serum to a final concentration of 6 percent.

* E:T = Effector to target cell ratio.

** E/ml = Effector cell concentration per ml of stock cell suspension.

*** T/ml = Target cell concentration per ml of stock cell suspension.

with RACE dilutions as low as 10^{-4}, but no ADCC cytotoxicity at RACE dilutions of 10^{-5} and lower. The ADCC cytotoxic activity was four-fold greater at a RACE dilution of 10^{-3} as compared to the activity observed with RACE at a dilution of 10^{-4}. Cell cultures with RACE at dilutions of 10^{-2} and 10^{-1}, respectively, resulted in obvious but not remarkable increases in the ADCC cytotoxic activity of the spleen cells. Therefore, RACE antiserum was used in all subsequent experiments at a dilution of 10^{-3}.

4.2.2 The Evaluation of Different Culture Media and Medium Supplements

The different media (M199, RPMI 1640 and CMRL 1066) were tested to evaluate the culture conditions for optimal expression of ADCC cytotoxic activity. They were all supplemented with 100 IU of potassium penicillin G and 100μg per ml of streptomycin sulfate. These media were also fortified with heat-inactivated FBS to final concentrations of 0, 2.5, 6, 10 and 20 percent.

The ADCC assay was performed as previously outlined (chapter III: Materials and Methods, section 3.2.9) using various cell preparations (thymus, WBC, spleen, appendix, MLN and bone marrow) and ^{51}Cr-ChRBC target

cells. RACE antiserum and normal rabbit control serum were diluted in the appropriate serum-free medium.

Tables II, III, IV illustrate the effects of different culture media and variations in FBS concentration on ADCC cytotoxic activity. At 0 and 2.5 percent FBS in M199-PS, a relatively high S.R. was observed in controls no. 1 and no. 2 (81, 94 and 25 percent, respectively) as well as a cytotoxic effect of the RACE antiserum on the target cells (C.I.'s in control no. 1 are 72 and 45 percent, respectively) (table II).

However, at a concentration of FBS in M199-PS of 6 percent or greater, the S.R. for all cell preparations, for the ChRBC controls and controls no. 1 and no. 2 were very low (mean S.R. of 4 percent) and no cytotoxic activity was observed in control no. 1. In addition, the C.I.'s of the cell suspensions changed only slightly with increasing FBS concentrations in M199-PS (table II). It can be concluded, therefore, that 6 percent FBS in M199-PS provides the necessary stabilizing conditions for the target cells over a 24 hour incubation period.

When the concentration of FBS was varied in culture medium RPMI 1640-PS (table III), the target cells were stable only at the FBS concentrations of 20 percent or greater as reflected by the figures for the S.R. in the cell cultures, and especially control cultures

TABLE II

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE CONCENTRATION OF FETAL BOVINE SERUM USING CULTURE MEDIUM 199

Cells tested	E:T*	Fetal bovine serum used in the following concentrations (%) 0 C.I.**	0 S.R.***	2.5% C.I.	2.5% S.R.	6% C.I.	6% S.R.	10% C.I.	10% S.R.	20% C.I.	20% S.R.
Thymus	25:1	6	5	9	3	9	3	4	4	3	3
WBC	25:1	61	6	62	6	55	5	55	5	60	4
Spleen	25:1	27	5	38	4	32	4	31	4	15	3
Appendix	25:1	16	16	24	14	19	13	14	12	12	8
MLN	25:1	1	11	11	4	6	3	8	3	6	4
Bone marrow	25:1	86	5	91	4	76	4	62	3	61	4
ChRBC	25:1	9	7	5	3	0	4	0	3	1	3
Control No. 1	0:1	72	81	45	25	1	4	0	3	0	3
Control No. 2	0:1	N/A****	94	N/A	25	N/A	4	N/A	3	N/A	4

*E:T = Effector to target cell ratio.
Effector cells were constant at 10^6 cells per culture.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199.

**C.I. = Cytotoxic Index (see Materials and Methods).

***S.R. = Spontaneous release = $\frac{\text{cpm in control}}{\text{cpm in total}} \times 100$.

****N/A = Not applicable.

TABLE III

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE CONCENTRATION OF FETAL BOVINE SERUM USING CULTURE MEDIUM RPMI 1640

Cells tested	E:T*	Fetal bovine serum used in the following concentrations (%)									
		0		2.5%		6%		10%		20%	
		C.I.**	S.R.***	C.I.	S.R.	C.I.	S.R.	C.I.	S.R.	C.I.	S.R.
Thymus	25:1	8	29	0	29	0	29	4	20	6	14
WBC	25:1	45	15	48	12	36	13	21	11	38	13
Spleen	25:1	19	21	29	14	24	13	28	13	21	13
Appendix	25:1	0	100	10	47	0	31	0	24	5	23
MLN	25:1	8	23	11	14	4	11	2	13	5	10
Bone marrow	25:1	91	21	100	12	99	12	97	11	84	11
ChRBC	25:1	0	80	0	48	1	25	0	16	0	11
Control											
No. 1	0:1	0	83	0	94	0	100	0	89	1	13
No. 2	0:1	N/A****	79	N/A	100	N/A	73	N/A	26	N/A	16

*E:T = Effector to target cell ratio.
Effector cells were constant at 10^6 cells per culture.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was RPMI 1640.

**C.I. = Cytotoxic Index (see Materials and Methods).

***S.R. = Spontaneous release = $\frac{\text{cpm in control}}{\text{cpm in total}} \times 100$.

****N/A = Not applicable.

TABLE IV

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE CONCENTRATION OF FETAL BOVINE SERUM USING CULTURE MEDIUM CMRL 1066

Cells tested	E:T*	Fetal bovine serum used in the following concentrations (%)									
		0		2.5%		6%		10%		20%	
		C.I.**	S.R.***	C.I.	S.R.	C.I.	S.R.	C.I.	S.R.	C.I.	S.R.
Thymus	25:1	13	16	21	16	27	12	18	11	11	13
WBC	25:1	35	15	37	9	37	8	19	8	17	9
Spleen	25:1	63	13	63	9	66	7	59	7	60	8
Appendix	25:1	32	47	44	37	44	26	23	25	32	31
MLN	25:1	35	23	44	12	34	10	26	9	29	8
Bone marrow	25:1	78	11	91	8	97	8	69	8	73	8
ChRBC	25:1	64	58	69	41	14	22	9	22	12	19
Control											
No. 1	0:1	0	100	0	100	0	100	0	100	49	71
No. 2	0:1	N/A****	100	N/A	100	N/A	100	N/A	100	N/A	100

*E:T = Effector to target cell ratio.
Effector cells were constant at 10^6 cells per culture.
Target cells (ChRBC) were constant at $4x10^4$ cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was CMRL 1066.

**C.I. = Cytotoxic Index (see Materials and Methods).

***S.R. = Spontaneous release = $\frac{\text{cpm in control}}{\text{cpm in total}} \times 100$.

****N/A = Not applicable.

no. 1 and no. 2. The mean value for the S.R. in medium RPMI 1640-PS containing 20 percent FBS was 14 percent. The cytotoxic index of the cytotoxic effector cells remained virtually unchanged as the concentration of FBS was increased to 20 percent (table III). Therefore, medium RPMI 1640-PS supplemented with 20 percent FBS also constitutes optimal culture conditions for the analysis of ADCC activity over a 24 hour period. However, the values for S.R. in this medium are higher than those for the former (table II).

Similarly, when the concentration of FBS was varied in culture medium CMRL 1066-PS, the stability of the ^{51}Cr-ChRBC target cells, as reflected by the figures for the S.R., was observed only at the FBS concentration of 20 percent (table IV). However, the S.R. was very high in control cultures no. 1 and no. 2 (71 and 77 percent, respectively). Furthermore, RACE was observed to exert cytotoxic effects on the ^{51}Cr-ChRBC target in control no. 1. The C.I. of the various rabbit cell preparations also appeared to decrease somewhat as the concentration of FBS was increased in culture medium (table IV). It would appear, therefore, that culture medium CMRL 1066-PS fortified with FBS to a final concentration of 20 percent of the culture medium is not very effective in promoting ADCC. While RPMI 1640-PS fortified with FBS to a final concentration of

20 percent of the culture medium is significantly superior to the former, M199-PS supplemented with FBS to a final concentration of 6 percent of the culture medium is by far the most effective culture medium for the ADCC assay and is heretofore referred to as culture medium.

4.2.3 The Determination of the Optimal Cell Concentration and Range of ADCC Cytotoxic Activity for the Cells of Each of the Rabbit Lymphoid Tissues

Lymphoid tissues (thymus, WBC, spleen, appendix, sacculus rotundus, Peyer's patches, MLN, PLN and bone marrow) were extirpated from several rabbits and cell suspensions were prepared from these tissues (chapter III: Materials and Methods, section 3.2.6). The cells were assayed for ADCC cytotoxic activity (as outlined in chapter III: Materials and Methods, section 3.2.9) at varying cell concentrations in order to determine the dose response profile for the cells of each tissue. Spleen cells which consistently demonstrate marked ADCC cytotoxic activity were used as controls in each assay.

As can be seen in table V, thymus cells show minimal ADCC activity at effector to target cell ratios (E:T) of 25:1 and higher. WBC exhibit marked ADCC activity at E:T cell ratios in the range from 100:1 to 1:1. Some residual activity can still be observed at an E:T cell ratio of 1:5.

It may therefore be concluded that whereas thymus cells are only minimally cytotoxic at very high E:T cell ratios, optimal ADCC cytotoxic activity for WBC and spleen cells is observed at E:T cell ratios of 25:1. Since WBC and spleen effector cells at an E:T ratio of 25:1 consistently exhibit ADCC cytotoxic activity similar to that observed at higher E:T cell ratios, and since the number of rabbit cells needed to attain this ratio is easily obtainable, subsequent experiments were carried out with WBC and spleen cell suspensions at the E:T cell ratio of 25:1.

Table VI illustrates the dose response profiles of the cells of the appendix, sacculus rotundus and Peyer's patches (gut-associated lymphoid tissues or GALT organs) of several rabbits assayed for ADCC cytotoxic activity, with spleen cells serving as a reference cytotoxic effector cell preparation. Cells of the appendix, sacculus rotundus and Peyer's patches display maximum ADCC cytotoxic activity over the range of E:T cell ratios of 100:1 to 25:1. Some activity was

TABLE V

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE THYMUS, WHITE BLOOD CELLS AND SPLEEN EFFECTOR CELL CONCENTRATIONS

Rabbit no.	Cells tested	The cytotoxic index at the following E:T ratios*									
		100:1	50:1	25:1	10:1	5:1	1:1	1:5	1:25	1:100	0:1
1	Thymus	14**	8	5	2	1	0	0	0	0	0
2		11	7	4	2	1	1	0	0	1	0
3		15	9	5	2	1	1	1	0	0	0
1	WBC	100	100	100	100	92	32	8	2	0	0
2		85	83	86	77	66	21	6	1	0	0
3		67	64	62	46	39	13	2	2	1	0
1	Spleen	75	75	72	66	51	13	3	1	0	0
2		71	68	64	41	25	8	2	1	0	0
3		58	62	86	46	29	8	5	0	1	0

*E:T = Effector to target cell ratio.
Target cells (ChRBC) constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The concentration of fetal bovine serum was constant at 6 percent of the culture medium.
The medium used was M-199.

** Each value given represents the Cytotoxic Index (C.I.)--see Materials and Methods.

TABLE VI

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE APPENDIX, SACCULUS ROTUNDUS, PEYER'S PATCHES AND SPLEEN EFFECTOR CELL CONCENTRATIONS

Rabbit no.	Cells tested	The cytotoxic index at the following E:T ratios*									
		100:1	50:1	25:1	10:1	5:1	1:1	1:5	1:25	1:100	0:1
4	Appendix	22**	20	16	8	5	1	1	0	0	0
5		20	12	16	7	5	2	0	0	1	0
6		22	15	11	6	5	3	0	1	4	1
4	Sacculus	7	14	10	5	3	1	0	0	1	0
5	rotundus	35	36	27	21	14	3	0	0	0	0
6		3	0	3	1	0	1	0	2	3	1
4	Peyer's	5	5	5	2	0	0	0	0	0	0
5	patches	8	8	7	4	0	0	0	1	0	0
6		25	20	15	11	5	7	4	3	1	1
4	Spleen	42	39	28	13	11	3	1	0	0	0
5		59	57	50	43	56	16	3	2	0	0
6		51	37	22	12	6	1	2	0	0	1

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The Anti-ChRBC antiserum was constant at 10^{-3}.
The concentration of fetal bovine serum was constant at 6 percent of the culture medium.
The medium used was M-199.

** Each value given represents the Cytotoxic Index (C.I.)--see Materials and Methods.

observed below the E:T cell ratio of 25:1, but it would appear to vary greatly between similar cell preparations from different rabbits.

Only 6 of 24 preparations of MLN cells were active (C.I.'s greater than 10 percent) in the ADCC assay at an E:T cell ratio of 25:1, the results of 4 of which are documented in table VII. PLN cells were difficult to assess at high E:T cell ratios due to the limitations of cell numbers. Nevertheless, PLN cells appear to be relatively inactive in the ADCC assay. Bone marrow cells are considerably cytotoxic down to the E:T cell ratio of 1:1, with very little ADCC cytotoxic activity recorded below this ratio (table VII).

A number of cell preparations (thymus, WBC, spleen, GALT, MLN, PLN and bone marrow) (table VIII) of different rabbits were assayed at the E:T cell ratio of 25:1 in order to determine the mean cytotoxic index and range of ADCC activity for each tissue under investigation. The data in table VIII indicate that rabbit lymphoid tissues can be classified into three categories on the basis of the levels of ADCC cytotoxic activity exhibited. The first category (a) consists of bone marrow, WBC and spleen cells which show uniformly high levels of ADCC cytotoxic activity. The second category (b) consists of Peyer's patches, PLN and thymus cells which are inactive or show negligible

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The concentration of fetal bovine serum was constant at 6 percent of the culture medium.
The medium used was M-199.

** Each value given represents the Cytotoxic Index (C.I.)--see Materials and Methods.

*** ND = Not done, due to insufficient numbers of cells.

TABLE VII

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF VARYING THE MESENTERIC LYMPH NODE, POPLITEAL LYMPH NODE, BONE MARROW AND SPLEEN EFFECTOR CELL CONCENTRATIONS

Rabbit no.	Cells tested	The cytotoxic index at the following E:T ratios*									
		100:1	50:1	25:1	10:1	5:1	1:1	1:5	1:25	1:100	0:1
1	MLN	40**	37	32	21	14	4	1	0	1	0
7		10	5	4	5	2	0	1	3	1	2
8		6	5	2	1	1	0	0	0	0	0
9		4	2	0	10	0	0	0	0	0	0
7	PLN	ND***	ND	2	1	2	1	2	3	2	2
8		ND	ND	ND	2	1	0	1	0	1	0
9		ND	ND	ND	ND	3	0	1	1	0	0
7	Bone marrow	89	86	85	68	48	22	5	5	2	2
8		78	77	73	47	25	ND	ND	ND	1	0
9		47	44	38	27	16	3	1	1	1	0
10		74	78	83	71	38	13	4	1	0	0
1	Spleen	75	75	72	66	51	13	3	1	0	0
7		52	50	50	34	23	9	4	2	1	2
8		32	29	17	11	8	4	1	2	0	0
9		49	45	38	22	12	4	1	1	0	0
10		51	44	35	10	9	3	0	0	1	0

TABLE VIII

THE ADCC CYTOTOXIC INDICES (C.I.) OF LYMPHOID TISSUE CELL PREPARATIONS FROM NORMAL RABBITS

Cells tested*	Number of samples tested	Mean value of C.I.	Standard error of the mean (S.E.M.)	Standard deviation
Bone marrow	54	69	2	13
WBC	35	59	4	23
Spleen	40	46	3	20
Appendix	28	15	2	10
Sacculus rotundus	22	12	2	11
Peyer's patches	21	6	1	5
MLN**	24	12	3	15
PLN	18	3	1	3
Thymus	28	3	0	2

* The effector to target cell ratio is 25:1.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.
The duration of culture was 24 hours.

** Only 6 of 24 preparations of MLN gave a C.I. of greater than 10 percent.

levels of ADCC cytotoxicity and the third category (c) consists of appendix, sacculus rotundus and MLN which show levels of ADCC cytotoxic activity intermediate to that seen in the first two categories. Note, however, that only 6 of the 24 preparations of MLN assayed had a cytotoxic index of greater than 10 percent.

4.2.4 The Tissue Distribution of the ADCC Cytotoxic Effector Cells

The lymphoid tissue distribution of ADCC cytotoxic effector cells was determined by analyzing simultaneously, thymus, WBC, spleen, GALT, MLN, PLN and bone marrow cells of a number of rabbits (table IX). The cell suspensions were assayed at variable E:T cell ratios (25:1, 5:1, 1:1 and 1:5) and the ADCC assay was performed as outlined in chapter III: Materials and Methods, section 3.2.9. Bone marrow, WBC and spleen cells consistently demonstrated high levels of ADCC activity while cells of the Peyer's patches, thymus, MLN and PLN demonstrated very little, if any, ADCC cytotoxic activity. These results are similar to those presented in table VIII.

TABLE IX

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE DISTRIBUTION OF EFFECTOR CELLS AMONG THE DIFFERENT LYMPHOID TISSUES OF THE SAME RABBIT

Cells tested	The cytotoxic index at the following E:T ratios*				
	25:1	5:1	1:1	1:5	0:1
Bone marrow	59**	42	18	5	0
WBC	55	41	15	5	0
Spleen	37	17	5	3	0
Appendix	14	10	3	1	0
Sacculus rotundus	26	18	14	3	0
Peyer's patches	3	2	1	0	0
Thymus	2	0	0	0	0
MLN	3	1	0	0	0
PLN	5	1	0	0	0

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

** Each value given represents the Cytotoxic Index (C.I.)--see Materials and Methods.

4.2.5 The Relationship of ADCC Cytotoxic Activity to Duration of Culture

The ADCC cytotoxic activity exhibited by cells of the various lymphoid tissues (thymus, WBC, spleen, GALT, MLN, PLN and bone marrow) was investigated to determine the relationship of ADCC cytotoxic activity to time in culture (table X). The ADCC assay was performed as outlined in chapter III: Materials and Methods, section 3.2.9. The cultures were maintained for ½, 2, 6 to 8, 16 to 18, 24, 48 and 72 hours respectively, prior to processing for radioactive counting.

Bone marrow, WBC and spleen cells showed marked ADCC cytotoxic activity after 24 hours in culture and demonstrated an increase in ADCC cytotoxic activity when cultured for up to 72 hours. Thymus, GALT, MLN and PLN cells consistently showed appreciable cytotoxicity only after 48 and 72 hours of culture.

Thymus, WBC, spleen, GALT, MLN, PLN and bone marrow cells were also cultured at concentrations of 5×10^6 cells per ml for 2, 24, 48 and 72 hours respectively, prior to the addition of the ^{51}Cr-ChRBC target cells and RACE antiserum and/or normal rabbit serum. The cell suspensions were then incubated for a further 24 hours prior to processing for radioactive counting (table XI).

TABLE X

THE RELATIONSHIP OF ADCC CYTOTOXIC ACTIVITY TO THE DURATION OF CULTURE. CELLS OF THE DIFFERENT RABBIT LYMPHOID TISSUES CULTURED WITH ANTIBODY-SENSITIZED TARGET CELLS FOR UP TO 72 HOURS

		C.I. at time (hours) of culture*						
Cells tested	E:T**	1/2	2	6-8	16-18	24	48	72
Bone marrow	25:1	0	2	25	52	58	70	77
WBC	25:1	0	4	25	48	53	67	71
Spleen	25:1	0	1	17	34	38	53	63
Appendix	25:1	0	0	2	6	9	33	46
Sacculus rotundus	25:1	0	0	3	4	8	32	45
Peyer's patches	25:1	0	0	3	8	10	23	47
Thymus	25:1	0	0	4	1	2	26	41
MLN	25:1	0	0	2	5	8	20	39
PLN	25:1	ND***	1	2	2	3	9	24

* The rabbit cells were co-cultured with antibody-coated ChRBC during the period of culture.

** E:T - Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

*** ND = Not done, due to insufficient number of cells.

TABLE XI

THE RELATIONSHIP OF ADCC CYTOTOXIC ACTIVITY TO THE DURATION OF CULTURE. CELLS OF THE DIFFERENT RABBIT LYMPHOID TISSUES INCUBATED WITHOUT ANTIBODY-SENSITIZED TARGET CELLS PRIOR TO CULTURE WITH ANTIBODY-SENSITIZED ^{51}Cr-ChRBC TARGET CELLS

Cells tested	E:T	Cells preincubated for the following time periods prior to the addition of antibody-sensitized ^{51}Cr-ChRBC			
		0	24	48	72
Bone marrow	25:1	64**	80	75	73
WBC	25:1	63	70	56	17
Spleen	25:1	37	30	32	34
Appendix	25:1	11	10	9	43
Sacculus rotundus	25:1	3	5	5	24
Peyer's patches	25:1	1	1	0	5
MLN	25:1	1	2	4	4
PLN	25:1	0	1	2	1
Thymus	25:1	2	3	3	4
Thymus***	25:1	0	2	26	46

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199, supplemented with fetal bovine serum to a final concentration of 6 percent.

** Each value given represents the Cytotoxic Index (C.I.)--see Materials and Methods.

*** In this experiment, the thymus cells were also incubated with antibody-sensitized ^{51}Cr-ChRBC during the entire period of culture.

WBC, spleen and bone marrow cells displayed a marked level of ADCC cytotoxic activity after an initial 24 hours in culture with RACE antiserum and ^{51}Cr-ChRBC target cells. Appendix and sacculus rotundus cells exhibited ADCC cytotoxic activity only if RACE antiserum and ^{51}Cr-ChRBC target cells were not added until 72 hours of culture. Peyer's patches, MLN, PLN and thymus cells failed to exhibit ADCC cytotoxic activity under these conditions. However thymus cells showed appreciable ADCC cytotoxic activity when co-cultured for 48 hours with ^{51}Cr-ChRBC target cells and RACE antiserum.

4.3 Discussion and Conclusions

The analysis of the ADCC cytotoxic activity of cells of the lymphoid tissues and WBC over a wide range of E:T cell ratios in the standard 24 hour ADCC assay indicated that the cells could be classified into three categories:

(i) "highly" cytotoxic cells (bone marrow, WBC and spleen cells);

(ii) "mildly to moderately" cytotoxic cells (appendix, sacculus rotundus and MLN cells), and

(iii) "non-cytotoxic" cells (Peyer's patches, PLN

and thymus cells).

However, when these cells were co-cultured with RACE antiserum and ^{51}Cr-ChRBC target cells for varying intervals of time, it was noted that the "highly" cytotoxic cells showed ADCC cytotoxic activity early in the culture period (6 to 8 hours) which increased over 72 hours. The "mildly to moderately" cytotoxic cells, as well as the "non-cytotoxic" cells, exhibited ADCC cytotoxic activity only after co-culture with RACE antiserum and ^{51}Cr-ChRBC target cells for 48 and 72 hours.

Preculture of the cells for varying periods of time in the absence of RACE antiserum and ^{51}Cr-ChRBC target cells (24, 48 and 72 hours) followed by the addition of RACE antiserum and ^{51}Cr-ChRBC target cells in the standard 24 hour ADCC assay revealed that rabbit bone marrow, WBC and spleen cells displayed ADCC cytotoxic activity irrespective of the duration of the preculture period. Appendix and sacculus rotundus cells were markedly active only if RACE antiserum and ^{51}Cr-ChRBC target cells were added at 72 hours of culture. The other lymphoid cells, including the thymus cells, showed no ADCC cytotoxic activity irrespective of the duration of preculture (table XI).

Interestingly enough, since the same preparation of thymus cells was markedly active when

co-cultured for 48 and 72 hours with RACE antiserum and ^{51}Cr-ChRBC target cells, it may be concluded that the thymic cytotoxic effector cells die in culture in the absence of RACE antiserum and target cells.

On the basis of the results of the time course experiments, one must be cautious before classifying certain lymphoid tissues as being "non-cytotoxic" (Peyer's patches, PLN and thymus). A more precise classification would categorize the cells of the lymphoid tissues and WBC as being either rapidly cytotoxic or as exhibiting a lag phase in the expression of ADCC cytotoxic activity.

Gelfand et al (1972) and Bona et al (1975, 1976) have previously attributed ADCC cytotoxicity to rabbit thymus, spleen, bone marrow, peritoneal exudate and MLN cells on the basis of their ability to lyse ^{51}Cr-ChRBC target cells during a 15 to 20 hour incubation period. In the above-mentioned studies, GALT or PLN cells were not assayed for ADCC cytotoxic activity. The experimental findings in this study have confirmed that WBC, bone marrow and spleen cells were consistently cytotoxic. Appendix and sacculus rotundus cells, not previously investigated, displayed only marginal ADCC cytotoxic activity, while thymus, MLN, Peyer's patches and PLN cells (these latter two not having been previously investigated for ADCC cytotoxic activity) displayed

very low or no ADCC cytotoxic activity in the standard 24 hour ADCC assay.

The ADCC cytotoxic activity displayed by the MLN cells was very inconsistent as activity was detected in only 6 of 24 MLN cell preparations. The results with the thymus and MLN cells would therefore appear to be in conflict with those of Gelfand et al (1972) and Bona et al (1975, 1976) who consistently detected ADCC cytotoxic activity in rabbit thymus and MLN cells (Gelfand et al, 1972) and in thymus cells (Bona et al, 1975; 1976) when cultured for 24 hours with ^{51}Cr-ChRBC target cells and RACE antiserum.

Gelfand et al (1972) have suggested, on the basis of studies investigating the relationship of ADCC cytotoxic activity to duration of culture, that rabbit phagocytic cells are rapidly acting in ADCC while lymphocytes show a delayed onset of ADCC cytotoxic activity.

To date, information derived from the present study reveals that cells prepared from several of the rabbit lymphoid tissues do not react with equal speed in the ADCC cytotoxicity assay. Cells derived from rabbit lymphoid tissues can be considered to be either rapidly cytotoxic or to exhibit a lag phase in the expression of ADCC cytotoxic activity. The earlier observations of Gelfand et al (1972) may be applicable in this case. With this in mind, it was considered

necessary to delve into some of the physico-chemical properties of the cells effecting ADCC in the rabbit in order to ultimately identify the ADCC cytotoxic effector cell in the Lagomorph as well as its tissue distribution.

CHAPTER V

THE FUNCTIONAL AND MORPHOLOGICAL HETEROGENEITY OF THE ADCC CYTOTOXIC EFFECTOR CELL IN THE RABBIT

5.1 Introduction and Objectives

In the previous chapter the optimal conditions for the ADCC cytotoxic reaction in the rabbit were defined and the ADCC cytotoxic effector cells in the rabbit were classified as rapidly acting in ADCC or as exhibiting a lag phase in the onset of ADCC cytotoxic activity. In this chapter the functional and morphologic properties of ADCC cytotoxic effector cells of the different lymphoid tissues are investigated.

The objectives were:

(i) to isolate and identify relatively homogeneous populations of cells through discontinuous density gradient centrifugation (based on the property of cellular specific gravity) for further analysis in the ADCC assay;

(ii) to determine the role of the phagocyte in ADCC in the rabbit;

(iii) to determine the response of ADCC cytotoxic effector cells to treatment with soluble aggregates of rabbit gamma-globulin (RAGG); and,

(iv) to attempt correlation of selected functional and morphologic characteristics of rabbit ADCC cytotoxic effector cells.

5.2 Experimental Protocols and Results

The data presented in the following tables are representative of results obtained in individual experiments. Each experiment was repeated a minimum of 5 times with the majority repeated 10 times. Each value represents the mean of duplicate or triplicate determinations. Since the viability of all cell preparations at the time of culture was 85 to 95 percent as determined by the trypan blue dye exclusion technique, the numbers of cells introduced into the cultures were not corrected for viability.

5.2.1 The ADCC Cytotoxic Activity of Mononuclear and Heterophil Cells Following Separation by Centrifugation in a Ficoll-Hypaque Discontinuous Density Gradient

Bone marrow, WBC and spleen cell suspensions were fractionated by centrifugation in a ficoll-hypaque discontinuous density gradient (chapter III: Materials and Methods, section 3.2.6) and the mononuclear (interphase) and heterophil (pellet) cells were assessed for ADCC cytotoxic activity at varying E:T cell ratios (100:1, 50:1, 25:1, 10:1, 5:1, 1:1 and 1:5). The ADCC assay was performed as outlined in chapter III: Materials and Methods, section 3.2.9.

Smears were made of all cell suspensions and stained for non-specific esterase or with Wright's stain and analyzed microscopically to characterize, on a morphological basis, the cells of the different fractions.

Unfractionated bone marrow, WBC and spleen cells consistently showed high levels of ADCC cytotoxic activity in the conventional 24 hour ADCC assay (table XII). As can also be seen in table XII, the pelleted bone marrow cells (FHP), the majority of which appeared to be heterophils (data not presented), were almost twice as active as the mononuclear cell preparations when used in equal cell numbers (E:T 100:1 to 25:1; $p \geq 0.01$). The interphase cells which consisted of

* Target cells (ChRBC) are constant at 4×10^4 cells per culture.
Effector lymphoid cells are constant at 10^6 cells per culture.
Anti-target cell (ChRBC) antiserum is constant at 10^{-3}.
The medium used is M-199 supplemented with fetal bovine serum to a final concentration of 6 percent of the culture medium.

** Cytotoxic index ± standard error of the mean.

*** ND = Not done.

TABLE XII

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE ADCC CYTOTOXIC INDICES OF UNFRACTIONATED AND FICOLL-HYPAQUE DENSITY GRADIENT FRACTIONATED RABBIT BONE MARROW AND SPLEEN EFFECTOR CELLS AT DIFFERENT E:T RATIOS

	E:T Ratios*						
Cells tested	100:1	50:1	25:1	10:1	5:1	1:1	1:5
Unfractionated bone marrow cells	72±14**	73±14	74±13	67±17	54±23	13± 5	3±1
Interphase cells	33	48± 8	43± 5	46± 8	40± 7	19± 8	8±6
Pellet cells	80±10	80± 9	74± 8	91	48±14	15± 7	5±4
Unfractionated spleen cells	27± 5	38±12	42±21	30±15	27±16	27±16	5±3
Interphase cells	ND***	37±13	33±16	10± 4	31±24	14±10	7±2
Pellet cells	ND	43±17	24±16	14± 9	13± 8	8± 4	3±0

a high percentage of mononuclear cells (data not presented), were somewhat less active than the unfractionated bone marrow cells when compared on an equal cell basis (E:T 100:1 to 25:1; $p \geq 0.01$).

The splenic interphase cells, which similarly consisted of a high percentage of mononuclear cells, expressed similar ADCC cytotoxic activity as the unfractionated and the pelleted spleen cells (E:T 100:1 to 1:5; no significant difference between interphase and unfractionated spleen cells). The majority of the pelleted spleen cells appeared to be heterophils (data not presented).

Table XIII presents the relative distribution and ADCC cytotoxic activity of the mononuclear and heterophil cells in the unfractionated and fractionated WBC. In general, all of the WBC preparations (table XIII) demonstrated marked levels of ADCC cytotoxic activity (E:T 25:1 to 0.31:1; the differences between the cell preparations were not statistically significant).

It should be noted that the WBC were treated with tris-buffered ammonium chloride to lyse contaminating erythrocytes (see chapter III: Materials and Methods, section 3.2.7). In preliminary experiments (data not presented), it was consistently observed that the ADCC cytotoxic activity of the WBC was not affected by this procedure. Furthermore, it was also observed that

TABLE XIII

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE ADCC CYTOTOXIC INDICES OF UNFRACTIONATED AND FICOLL-HYPAQUE DENSITY GRADIENT FRACTIONATED RABBIT WHITE BLOOD CELLS AT DIFFERENT E:T RATIOS

Cells tested	E:T Ratios*							Differential WBC Count	
	25:1	10:1	5:1	2.5:1	1.25:1	.62:1	.31:1	Mononuclear cells (%)	Heterophils (%)
Unfractionated WBC	45±5**	36±6	30±5	19±5	14±3	9±4	9±6	54±8	46±8
Mononuclear cells	34± 9	24±6	15±6	10±4	5±3	8±7	8±6	86±3	14±3
Heterophils	56±17	50±7	36±11	19±3	13±5	11±5	12±7	23±6	77±6

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent of the culture medium.

** Cytotoxic index ± standard error of the mean.

the intentional addition of graded numbers of erythrocytes to any of the syngenic cell cultures did not alter the ADCC cytotoxic activity of these cell preparations, thus demonstrating that erythrocytes have no effect on the activity of the ADCC cytotoxic effector cells in the rabbit.

5.2.2 The Effect of Phagocyte Depletion on the ADCC Cytotoxic Activity of Rabbit Lymphoid Cells

Cell suspensions of bone marrow, WBC, spleen, appendix, MLN and thymus were depleted of phagocytic cells by incubation with carbonyl iron particles (as outlined in chapter III: Materials and Methods, section 3.2.8). These cells were then cultured in a standard 24 hour ADCC assay as well as for longer periods of time at varying E:T cell ratios. The ADCC assay was performed as previously described (chapter III: Materials and Methods, section 3.2.9). All of the cell preparations were stained and examined microscopically both before and after phagocyte depletion (as described in chapter III: Materials and Methods, section 3:2.11).

The percentages of circulating monocytes and heterophils were markedly reduced in phagocyte-depleted WBC suspensions (0.5 and 4 percent, respectively) as compared to the untreated WBC (4 and 16 percent,

respectively). Similar results were obtained with spleen cells. With respect to the bone marrow cells, phagocyte depletion resulted in a drop in the percentage of monocytes (6 percent to 1 percent) unaccompanied by a concomitant decrease in the percentage of heterophils.

Table XIV illustrates the ADCC cytotoxic activity of untreated and phagocyte-depleted bone marrow, WBC and spleen cells in the standard 24 hour assay. Phagocyte-depleted WBC and spleen cells exhibited negligible ADCC cytotoxic activity as compared to the untreated WBC cells and spleen cells ($a > b$, $p \geq 0.1$; $c > d$, $p \geq .005$). Furthermore, phagocyte-depleted WBC and spleen cells failed to show significant ADCC cytotoxic activity when the assay was extended for up to 72 hours (table XV). On the other hand, phagocyte-depleted bone marrow cells displayed ADCC cytotoxic activity similar to that of the untreated cells at 6, 24, 48 and 72 hours of culture (tables XIV and XV), (the differences between the cell preparations were not statistically significant).

Phagocyte-depleted thumus cells, like the untreated thymus cells, were inactive in the standard ADCC assay but active in the 48 hour assay ($p \geq .05$), whereas phagocyte-depleted MLN cells failed to show the characteristic lag phase (table XV) in the onset

TABLE XIV

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE ADCC CYTOTOXIC INDICES OF UNTREATED AND PHAGOCYTE DEPLETED RABBIT SPLEEN, BONE MARROW AND WHITE BLOOD CELLS AT DIFFERENT E:T RATIOS

Cells tested	E:T Ratios*					
	50:1	25:1	10:1	5:1	1:1	0:1
WBC--untreated (a)	57**	48	19	5	2	0
WBC--phagocyte depleted (b)	5	5	5	3	3	0
Spleen--untreated (c)	34	28	11	8	11	0
Spleen--phagocyte depleted (d)	5	5	2	1	1	0
Bone marrow--untreated (e)	59	62	54	32	10	0
Bone marrow--phagocyte depleted (f)	71	62	45	25	6	0

* Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.
The duration of culture was 24 hours.

** Identical results were obtained irrespective of whether 2 or 8 mg of iron particles were incubated with each ml of cells.

* Target cells (ChRBC) were constant at 4×10^4 cells per culture.
Effector cells were constant at 10^6 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

** ND = Not done.

*** Identical results were obtained irrespective of whether 2 or 8 mg of iron particles were added to each ml of cells.

TABLE XV

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE ADCC CYTOTOXIC INDICES OF PHAGOCYTE-DEPLETED WHITE BLOOD CELLS, SPLEEN, BONE MARROW, THYMUS, APPENDIX AND MESENTERIC LYMPH NODE CELLS AS A FUNCTION OF THE DURATION OF CULTURE

	Duration of culture (hrs)*			
Cells tested	6	24	48	72
WBC--untreated	ND**	24***	28	38
WBC--phagocyte depleted	ND	8	12	12
Spleen--untreated	ND	26	34	45
Spleen--phagocyte depleted	ND	5	10	12
MLN--untreated	ND	3	13	ND
MLN--phagocyte depleted	ND	0	2	ND
Appendix--untreated	ND	25	46	ND
Appendix--phagocyte depleted	ND	10	22	ND
Bone marrow--untreated	19	38	58	66
Bone marrow--phagocyte depleted	23	31	61	70
Thymus--untreated	ND	1	15	ND
Thymus--phagocyte depleted	ND	1	12	ND

of ADCC cytotoxic activity. Phagocyte-depleted appendix cells consistently displayed 50 percent of the ADCC cytotoxic activity exhibited by the untreated appendix cells.

5.2.3 The Effect of Soluble Aggregates of Rabbit Gamma-Globulin (RAGG) on the ADCC Cytotoxic Activity of Rabbit Lymphoid Cells

The ADCC cytotoxic activity of bone marrow, WBC, spleen, appendix, MLN and thymus cells was evaluated following incubation with varying amounts of RAGG (as described in chapter III: Materials and Methods, section 3.2.10). The ADCC assay with appendix, MLN and thymus cells was extended to 48 hours since it was observed that these cells demonstrate a lag in the onset of ADCC cytotoxic activity (see chapter IV: The Optimal Conditions for ADCC in the Rabbit).

As can be seen in table XVI, the ADCC cytotoxic activity of WBC and spleen cells could be inhibited to an extent of 87 percent and 81 percent respectively, at the highest concentration of RAGG utilized (1,000 μg per ml) whereas the ADCC cytotoxic activity of bone marrow and MLN cells was much less affected (35 percent inhibition and 52 percent inhibition, respectively). Similar concentrations of RAGG did not appear to affect the ADCC cytotoxic activity of the

* Effector to target cell ratio was constant at 25:1.
  Effector cells were constant at 10^6 cells per culture.
  Target cells (ChRBC) were constant at $4x10^4$ cells per culture.
  The anti-ChRBC antiserum was constant at 10^{-3}.
  The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent of the culture medium.

** C.I. = Cytotoxic index (see Materials and Methods) ± standard error of the mean.

*** %Inh = Percent inhibition (see Materials and Methods).

+ N/A = Not applicable.
++ ND = Not done.

TABLE XVI

THE ADCC REACTION WITH RABBIT LYMPHOID CELLS. THE EFFECT OF SOLUBLE AGGREGATES OF RABBIT GAMMA-GLOBULIN ON THE ADCC CYTOTOXIC ACTIVITY OF RABBIT WHITE BLOOD CELLS, SPLEEN, BONE MARROW, THYMUS, APPENDIX AND MESENTERIC LYMPH NODE CELLS

	Inhibition of the ADCC cytotoxic activity of the mononuclear effector cells following incubation with aggregated rabbit gamma-globulin in the following concentrations*											
	0 μg/ml		5 μg/ml		50 μg/ml		100 μg/ml		500 μg/ml		1000 μg/ml	
Cells tested	C.I.**	%Inh***	C.I.	%Inh	C.I.	%Inh	C.I.	%Inh	C.I.	%Inh	C.I.	%Inh
WBC	62±44	N/A+	47±33	23±16	32±23	49±35	30±21	53±37	28±20	59±41	10± 7	87±61
Spleen	42±19	N/A	35±25	28±20	23±16	52±37	15± 7	63±28	12± 5	69±31	14± 6	81±36
Bone Marrow	80±56	N/A	71±50	10± 7	63±45	21±15	60±42	25±17	53±37	53±24	51±36	35±25
MLN	20±14	N/A	ND++	ND	ND	ND	21±15	0± 1	20±14	2± 1	21±15	2± 1
Appendix	20±14	N/A	ND	ND	ND	ND	13± 9	28±20	14±10	31±22	9± 7	50±37
Thymus	46±32	N/A	ND	ND	ND	ND	39±38	27±26	38±27	20±14	39±28	17±12

thymus cells (2 percent inhibition). Appendix cells were inconsistently inhibited in the ADCC assay.

5.3 Discussion and Conclusions

The findings presented indicate that the ADCC cytotoxic effector cells in the rabbit are heterogeneous and constitute functional and morphologically distinct subpopulations of cells which can be classified into a number of distinct categories on the basis of their morphology and properties in the ADCC reaction (table XXI).

The cells of the bone marrow, WBC and spleen show a rapid onset of ADCC cytotoxic activity by 6 to 8 hours of culture which begins to level off by 24 hours and increases only slightly over a further 48 hours in culture. The ADCC cytotoxic effector cells in the WBC and spleen appear to be largely phagocytic as the phagocyte-depleted WBC and spleen cells were essentially inactive, whereas those in the bone marrow appear to be non-phagocytic heterophils and/or lymphocytes. However, the additional existence of an ADCC cytotoxic effector macrophage in the bone marrow cannot be ruled out, as purified monocytes were not analyzed.

A further distinction between the bone marrow ADCC cytotoxic effector cells on the one hand, and

the WBC and spleen effector cells on the other, is that the ADCC cytotoxicity exhibited by bone marrow cells was inhibited by soluble aggregates of rabbit gamma-globulin to only a minor degree, whereas the activity of WBC and of spleen cells was almost completely inhibited by RAGG.

The effector cells in the Peyer's patches, thymus, MLN and PLN require a period of 24 to 48 hours of culture before showing ADCC cytotoxic activity which increases markedly after 72 hours of culture. These cells attain significant ADCC cytotoxic activity after 48 to 72 hours in culture only if they are co-cultured with RACE antiserum and target cells for the entire culture period. The treatment of thymus cells to deplete phagocytes did not alter in any way the ADCC cytotoxic activity exhibited. Thymic ADCC activity was not inhibited by RAGG. On the other hand, MLN cells lost all of their ADCC cytotoxic activity following the removal of phagocytic cells and ADCC cytotoxic activity could be markedly inhibited by RAGG. It is therefore concluded that the ADCC cytotoxic effector cells in the thymus and MLN are lymphocytes and phagocytic cells, respectively (table XXI).

The ADCC cytotoxic effector cells in the appendix and sacculus rotundus also demonstrate delayed ADCC cytotoxic activity, but they do not require prior

co-culture with RACE antiserum and target cells to-facilitate the expression of ADCC cytotoxic activity (table XXI). Since the depletion of phagocytic cells from the appendix cell suspensions consistently resulted in a 50 percent reduction of ADCC cytotoxic activity, and since appendix ADCC cytotoxic effector cells were inconsistently inhibited by RAGG, it would appear that the ADCC cytotoxic effector cells in the appendix are both lymphocytes and phagocytes.

The ADCC cytotoxic effector cells in the rabbit, therefore, appear to be the heterophils and monocytes in the circulation, phagocytic cells in the spleen and MLN, lymphocytes and/or non-phagocytic heterophils in the bone marrow, lymphocytes in the thymus and lymphocytes and phagocytic cells in the appendix.

Gelfand et al (1972) observed an early onset of ADCC cytotoxic activity by PEC cells which was attributed to a phagocytic cell component since phagocyte-depleted PEC cells exhibited a delayed onset of ADCC cytotoxic activity. The lymphocyte was identified as the cell demonstrating delayed (24 hour) ADCC cytotoxic activity (Gelfand et al, 1972). The results presented above do not support the interpretation of Gelfand et al (1972) since phagocytic cells were shown to exhibit both immediate (WBC and spleen cells) and delayed (MLN cells) ADCC cytotoxic activity and

non-phagocytic bone marrow cells were found to exhibit immediate ADCC cytotoxic activity only. Like Gelfand et al (1972), non-phagocytic mononuclear cells were shown to demonstrate delayed (thymus and appendix cells) ADCC cytotoxic activity.

CHAPTER VI

THE ROLE OF THE ADCC CYTOTOXIC EFFECTOR CELL IN HUMORAL IMMUNITY AND IN CELL-MEDIATED IMMUNITY (DELAYED HYPERSENSITIVITY) IN THE RABBIT

6.1 Introduction and Objectives

I have already established that a variety of cell types (lymphocytes, heterophils and phagocytes) from a variety of lymphoid tissues (thymus, WBC, spleen, bone marrow, MLN, appendix) in the rabbit are capable of effecting ADCC. It remains to be determined whether these cells participate in the immune response (humoral and/or cell-mediated) in the rabbit. Rabbits were immunized to induce either a humoral or a cell-mediated immune response following which cells of the various lymphoid tissues were assessed for ADCC cytotoxic activity. It was considered that any marked change from the normal in the organ distribution of ADCC cytotoxic effector cells would be indicative of their recruitment and/or participation in the immune response.

6.2 Experimental Protocols and Results

The data presented in the following tables are representative of results obtained in individual experiments. Each experimental protocol was repeated a minimum of 3 times. Each value represents the mean of duplicate determinations. Since the viability of all cell preparations at the time of culture was 85 to 95 percent as determined by the trypan blue dye exclusion technique, the numbers of cells introduced into culture were not corrected for viability.

6.2.1 The Lymphoid Tissue Distribution of ADCC Cytotoxic Effector Cells Prior to and During a Humoral Immune Response

New Zealand White rabbits were immunized intravenously (marginal ear vein) with 1 ml of a 10 percent suspension of sheep erythrocytes (SRBC) in saline (10^9 cells). Control rabbits were inoculated with 1 ml of sterile saline instead of the SRBC. Serum samples were taken prior to immunization and at the time of sacrifice. The rabbits were sacrificed at days, 1, 3, 5, 7 or 12 post-inoculation. The lymphoid tissues were extirpated and cell suspensions of bone marrow, WBC, spleen, GALT, MLN, PLN

and thymus (prepared as outlined in chapter III: Materials and Methods, section 3.2.6) were assayed for ADCC cytotoxic activity (as described in chapter III: Materials and Methods, section 3.2.9).

As can be seen in table XVII, haemagglutinating antibodies were detected by day 7 post-immunization. The lymphoid tissue distribution of ADCC cytotoxic effector cells in these rabbits was comparable to that of control rabbits (tables XVII and VIII). Two of the MLN cell suspensions prepared from the control rabbits exhibited unusually high levels of ADCC cytotoxic activity (C.I.'s of 47 and 54 percent, respectively) (tables XVII and VIII).

6.2.2 The Failure to Detect ADCC Cytotoxic Effector Cells in the PLN at Various Times Following Ipsilateral Immunization (footpad) With SRBC in Saline to Induce a Humoral Immune Response

As PLN cells are consistently inactive in the standard 24 hour ADCC assay (chapter IV: The Optimal Conditions for ADCC in the Rabbit), experiments were conducted to determine whether or not ADCC cytotoxic effector cells were recruited into PLN actively engaged in antibody synthesis. The experimental protocol

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at $4x10^4$ cells per culture.
The anti-target cell antiserum was constant at 10^{-3} of the stock solution.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

** ND = Not done.

TABLE XVII

THE EFFECT OF IMMUNIZATION ON THE LYMPHOID TISSUE DISTRIBUTION OF ADCC CYTOTOXIC EFFECTOR BY THE INTRAVENOUS INOCULATION OF SHEEP ERYTHROCYTES IN SALINE

Rabbit no.	Antigen dose (1 ml)	Haemagglutination titre		Day of sacrifice post-immunization	The cytotoxic index of the lymphoid tissues at an E:T ratio of 25:1*								
		Before immunization	At time of sacrifice		Bone marrow	WBC	Spleen	Appendix	Sacculus rotundus	Peyer's patches	MLN	PLN	Thymus
1	Saline	0	0	1	84	75	71	8	4	3	7	2	6
2	10^9 SRBC	0	0	1	85	72	73	2	ND**	1	5	2	4
3	10^9 SRBC	0	0	1	76	82	65	5	2	1	3	1	1
4	Saline	0	0	3	75	89	64	9	8	16	22	2	4
5	10^9 SRBC	0	0	3	77	80	60	4	4	0	10	2	12
6	10^9 SRBC	0	0	3	79	69	48	1	2	1	5	1	4
7	Saline	0	0	5	70	ND	44	7	12	4	24	0	3
8	10^9 SRBC	0	0	5	58	71	38	16	14	13	17	1	2
9	10^9 SRBC	0	0	5	48	47	27	10	10	11	17	0	1
10	Saline	0	0	7	66	66	78	29	24	6	47	4	6
11	10^9 SRBC	0	160	7	59	59	47	28	22	18	22	2	2
12	10^9 SRBC	0	160	7	65	55	47	20	13	1	14	3	1
13	Saline	0	0	12	74	40	49	20	12	4	54	2	2
14	10^9 SRBC	0	80	12	77	61	54	20	24	6	25	1	2
15	10^9 SRBC	0	320	12	77	81	56	17	15	4	19	2	2

involved the unilateral subcutaneous immunization of rabbits with SRBC in saline to induce a humoral immune response in the lymph node draining the site of antigenic challenge (ipsilateral PLN), followed by analysis of the ADCC cytotoxic activity of the cells of the PLN. The contralateral PLN served as a control.

Antigen (5×10^8 SRBC in saline) was inoculated subcutaneously into the left hind metatarsal footpad of test rabbits. Control rabbits were similarly injected with an equal volume of sterile saline. The ipsilateral and contralateral PLN were excized, weighed and processed for testing in the ADCC assay (as outlined in Chapter III: Materials and Methods, sections 3.2.6 and 3.2.9) at day 7 or 12 post-inoculation. WBC and spleen cells were similarly assayed for ADCC cytotoxic activity. Serum samples taken prior to immunization with SRBC and at the time of sacrifice were also assayed to determine the presence of circulating antibodies to SRBC.

As can be seen in table XVIII, the test rabbits possessed circulating anti-SRBC antibodies at the time of sacrifice. The popliteal lymph node ipsilateral to the inoculation site was very much larger in every case. Despite this, the ADCC cytotoxic activity of the cells of either the ipsilateral or contralateral

TABLE XVIII

THE DISTRIBUTION OF ADCC CYTOTOXIC EFFECTOR CELLS IN THE IPSILATERAL AND CONTRALATERAL POPLITEAL LYMPH NODES, CIRCULATING LEUKOCYTE POOL AND SPLEEN FOLLOWING THE SUB-CUTANEOUS INJECTION OF SHEEP ERYTHROCYTES INTO THE METATARSAL FOOTPAD

Rabbit no.	Antigen dose (0.5 ml)	Haemagglutination titre		Day of sacrifice post-immunization	Wet weight of lymph node		The cytotoxic index at an E:T ratio of 25:1*			
		Before immunization	At time of sacrifice		Ipsilateral lymph node (g)	Contralateral lymph node (g)	Ipsilateral lymph node	Contralateral lymph node	WBC	Spleen
1	Saline	0	0	7	0.15	0.16	0	0	34	21
2	10^9 SRBC	0	320	7	0.33	0.17	9	0	4	18
3	10^9 SRBC	0	160	7	0.32	0.17	3	0	55	41
4	Saline	0	0	12	0.25	0.24	16	14	15	41
5	10^9 SRBC	0	640	12	0.34	0.20	6	5	42	35
6	10^9 SRBC	0	320	12	0.31	0.18	5	2	14	22

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

PLN was well within the normal low values for this tissue (tables XVIII and VIII).

The spleen cells of the control and test rabbits displayed normal ADCC cytotoxic activity while the WBC of 2 of 4 of the test rabbits and 1 of 2 of the control rabbits demonstrated unusually low ADCC cytotoxic activity (tables XVIII and VIII).

6.2.3 The Failure to Detect ADCC Cytotoxic Effector Cells in the PLN at Various Times Following Ipsilateral Immunization (footpad) With SRBC in CFA to Induce a Cell-Mediated Immune Response

The experiments conducted in this portion of the investigation were essentially identical to those outlined in section 6.2.2, except that the rabbits were immunized with SRBC in CFA to induce a cell-mediated immune response to the SRBC.

Antigen (5×10^8 SRBC in CFA) was inoculated subcutaneously into the left hind metatarsal footpad of test rabbits. Control rabbits were similarly injected with an equal volume of CFA. The ipsilateral and contralateral PLN were excized, weighed and processed for testing in the ADCC assay (as outlined in Chapter III: Materials and Methods, sections 3.2.6 and 3.2.9) at day 7 or 12 post-inoculation. WBC and spleen cells were similarly assayed for ADCC cytotoxic activity.

Serum samples taken prior to immunization with SRBC and at the time of sacrifice (days 7 or 12 post-immunization) were assayed to determine the presence of circulating antibodies to the SRBC. Skin tests were also performed on the control and test rabbits by the intradermal injection of 0.1 ml of a suspension of 10^9 SRBC per ml in saline on the dorsum of the rabbits. The skin tests were read 24 to 36 hours after challenge and the rabbits were sacrificed when these were positive. A positive skin reaction was characterized by erythema, induration and heat.

As can be seen in table XIX, all of the test rabbits were positive for circulating anti-SRBC antibodies at the time of sacrifice. They all reacted positively upon intradermal challenge with the immunizing SRBC antigen. The control rabbits which received CFA subcutaneously instead of the SRBC in CFA possessed no circulating antibodies to SRBC and were unresponsive following intradermal skin challenge with SRBC.

The PLN ipsilateral to the site of inoculation in the control and test rabbits was at least three-fold larger than its contralateral counterpart in every case. However, there was little difference in the ADCC cytotoxic activity between the cells of the ipsilateral and contralateral PLN of the control or test rabbits when assayed at day 7 or 12

TABLE XIX

THE DISTRIBUTION OF ADCC CYTOTOXIC EFFECTOR CELLS IN THE IPSILATERAL AND CONTRALATERAL POPLITEAL LYMPH NODES, CIRCULATING LEUKOCYTE POOL AND SPLEEN FOLLOWING THE SUBCUTANEOUS INJECTION OF SHEEP ERYTHROCYTES IN COMPLETE FREUND'S ADJUVANT INTO THE METATARSAL FOOTPAD

Rabbit no.	Antigen dose (0.5 ml)	Haemagglutination titre: Before immunization	Haemagglutination titre: At time of sacrifice	Skin test (24 hr.)	Day of sacrifice post-immunization	Wet weight of lymph node: Ipsilateral lymph node (g)	Wet weight of lymph node: Contralateral lymph node (g)	The cytotoxic index at an E:T ratio of 25:1*: Ipsilateral lymph node	The cytotoxic index at an E:T ratio of 25:1*: Contralateral lymph node	The cytotoxic index at an E:T ratio of 25:1*: WBC	The cytotoxic index at an E:T ratio of 25:1*: Spleen
1	CFA**	0	0	-	7	0.5	0.18	6	7	77	64
2	10^9SRBC-CFA	0	320	+	7	0.28	0.10	7	0	20	47
3	10^9SRBC-CFA	0	2,560	+	7	0.63	0.16	9	4	78	56
4	CFA	0	0	-	12	0.78	0.23	7	6	65	67
5	10^9SRBC-CFA	0	10,240	+	12	0.46	0.17	11	9	66	68
6	10^9SRBC-CFA	40	640	+	12	0.87	0.17	9	6	69	61

* E:T = Effector to target cell ratio.
Target cells (ChRBC) were constant at $4x10^4$ cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

** CFA = Complete Freund's Adjuvant.

post-inoculation. The ADCC cytotoxicity exhibited by these cells was negligible in every case. WBC and spleen cells showed normal levels of ADCC cytotoxic activity (tables XIX and VIII).

6.2.4 The ADCC Cytotoxic Activity of the Cells Infiltrating Sites Exhibiting a Delayed Hypersensitivity Reaction

New Zealand White rabbits were inoculated with SRBC in CFA (10^9 SRBC in CFA into each biceps femoris muscle group). At day 7 or 12 post-immunization they were challenged intradermally with 0.1 ml of a suspension of SRBC and HRBC in saline (10^8 cells). The rabbits were sacrificed on day 8 or 13 post-immunization at the time when the skin reaction was optimally positive. A positive skin reaction was characterized by erythema, induration and heat. Cell suspensions of bone marrow, WBC, spleen and the SRBC and/or HRBC challenge sites were prepared according to the methods described in chapter III: Materials and Methods, section 3.2.6, and were assayed for ADCC cytotoxic activity (as outlined in chapter III: Materials and Methods, section 3.2.9).

Control rabbits were inoculated with CFA (1 ml into each biceps femoris muscle group) and in every case were unresponsive upon challenge with the above

antigens (SRBC and HRBC). They, therefore, were not processed for the assessment of ADCC cytotoxic activity.

As can be seen in table XX, all of the rabbits immunized with CFA showed positive skin reactions 24 hours following the intradermal challenge with the immunizing SRBC antigen. The bone marrow, WBC and spleen cells of all of the rabbits assayed were normally cytotoxic (tables XX and VIII). Seven of 8 preparations of SRBC challenge sites were very active in the ADCC assay. The cells obtained from the HRBC or saline challenge sites were unreactive.

6.3 Discussion and Conclusions

There was no alteration observed in the distribution of ADCC cytotoxic effector cells among the various rabbit lymphoid tissues during the course of a humoral immune response following antigenic stimulation with SRBC. Similarly, ADCC cytotoxic effector cells were not found to be recruited into normally ADCC negative PLN (in the standard ADCC assay) during the course of either humoral or cell-mediated immune responses following the unilateral (footpad) immunization with SRBC in saline or in CFA, respectively.

Significantly, however, ADCC cytotoxic effector cells were found to be present in sites undergoing

TABLE XX

THE EFFECT OF IMMUNIZATION ON THE LYMPHOID TISSUE DISTRIBUTION OF ADCC CYTOTOXIC EFFECTOR CELLS. THE APPEARANCE OF EFFECTOR CELLS IN SKIN CHALLENGE SITES DURING THE COURSE OF A DELAYED HYPERSENSITIVITY SKIN REACTION TO SHEEP ERYTHROCYTES IN SALINE

	Skin challenge with SRBC			The cytotoxic index at an E:T ratio of 25:1*			
Rabbit no.	Day post-immunization	Skin reaction (24 hours)	Day of sacrifice post-immunization	Bone marrow cells	% WBC	Spleen cells	SRBC challenge sites
1	7	+	8	61	60	47	37
2	7	+	8	58	44	51	22
3	7	+	8	61	57	36	16
4	7	+	8	74	46	26	33
5	12	+	13	78	14	46	34
6	12	+	13	67	72	44	42
7	12	+	13	61	57	36	16
8	12	+	13	62	37	25	1

* E:T = Effector to target cell ratio.
The target cells (ChRBC) were constant at 4×10^4 cells per culture.
The anti-ChRBC antiserum was constant at 10^{-3}.
The medium used was M-199 supplemented with fetal bovine serum to a final concentration of 6 percent.

TABLE XXI

THE ADCC REAGTION WITH RABBIT LYMPHOID CELLS. A SUMMARY OF THE MORPHOLOGIC AND FUNCTIONAL PROPERTIES OF ADCC CYTOTOXIC EFFECTOR CELLS IN THE RABBIT

	Property				
		Duration of culture (hrs) for		Requirement for co-culture with target cells to elicit ADCC activity	Inhibition of ADCC by RAGG (50%)
Tissue	Morphology	Significant activity	Maximum activity		
Blood					
(i) unfractionated cells	N/A	6-8	48	NO	YES
(ii) FHI cells***	monocyte	24	ND*	NO	YES
(iii) FHP cells ****	heterophil	24	ND	NO	YES
Spleen	macrophage	6-8	48	NO	YES
Bone marrow	mononuclear cell (non-phagocytic)	6-8	48	NO	NO
Thymus	lymphocyte	48	72	YES	NO
Appendix					
(i) unfractionated cells	N/A	24	72	NO	NO
(ii) macrophage depleted cells	lymphocytes	24	ND	NO	NO
Mesenteric lymph node	macrophage	48	72	YES	YES
Peyer's patches	?	48	72	YES	ND**
Sacculus rotundus	?	48	72	YES	ND
Popliteal lymph node	?	48	72	YES	ND**

* ND = Not done.

** ND = Not done due to insufficient number of cells.

*** FHI = Cells at interface following centrifugation in the ficol-hypaque density gradient.

**** FHP = Cells in pellet following centrifugation in the ficol-hypaque density gradient.

a delayed hypersensitivity reaction to intradermal challenge with the immunizing antigen (SRBC) but not with an unrelated antigen (HRBC). Skin biopsies taken from these areas (figure 5) showed a "classical" delayed hypersensitivity lesion characterized by an infiltration of mononuclear leukocytes (lymphocytes and macrophages) into the dermis of the skin and perivascular cuffing around the venules and arterioles.

Pudifin et al (1971) observed even though rats subjected to 850 rads whole body irradiation were unable to mount a humoral immune response, ADCC cytotoxic effector cells were detected in their spleens. A similar observation in the rodent has been reported by Harding and MacLennan (1972). Pudifin et al (1971) have also shown that the rat PLN draining a site of immunization is a poor source of ADCC cytotoxic effector cells. It was, therefore, concluded by these workers that the presence of ADCC cytotoxic effector cells was not singularly crucial in the induction of humoral immune response, nor was the presence of humoral immune response followed by the recruitment of ADCC cytotoxic effector cells into the reactive tissue(s). Similarly in my investigation, ADCC cytotoxic effector cells were not detected in cell suspensions prepared from PLN undergoing either a humoral or additionally a cell-mediated immune response following

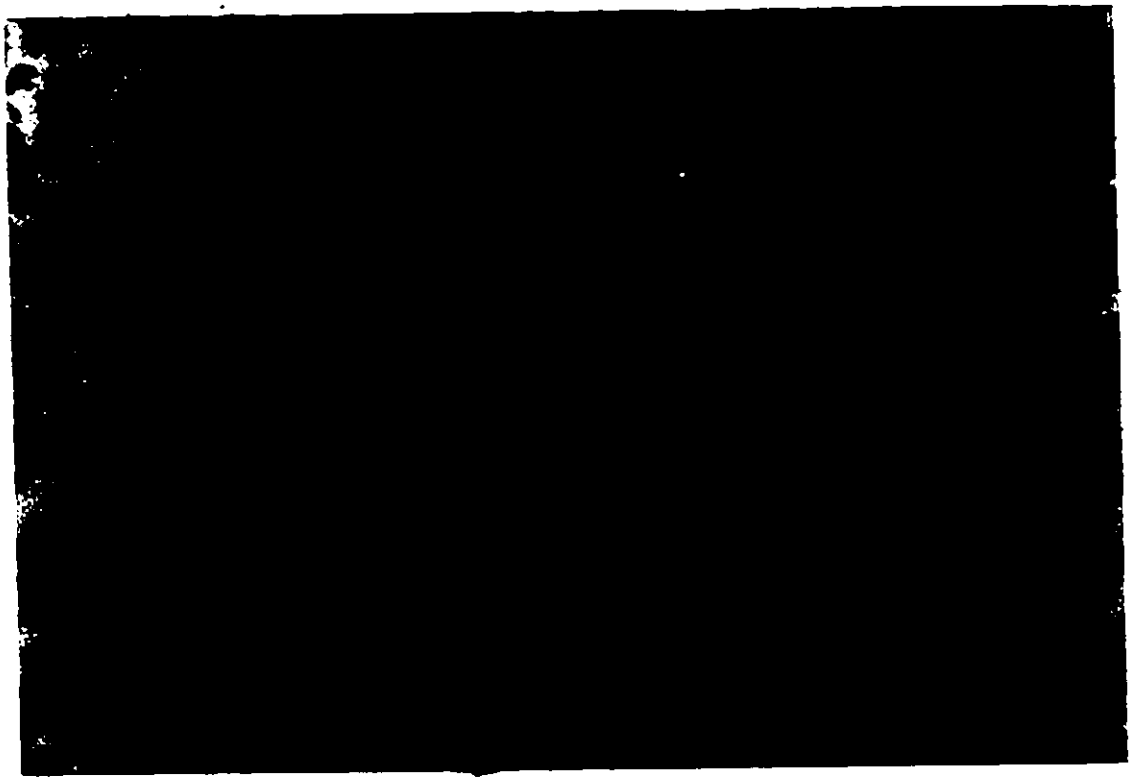

Figure 5A:-- Skin Biopsy of a Rabbit, Previously Sensitized with SRBC in CFA, 24 hours after Intradermal Challenge with Sheep Erythrocytes in Saline. Magnification: 10 x 10.

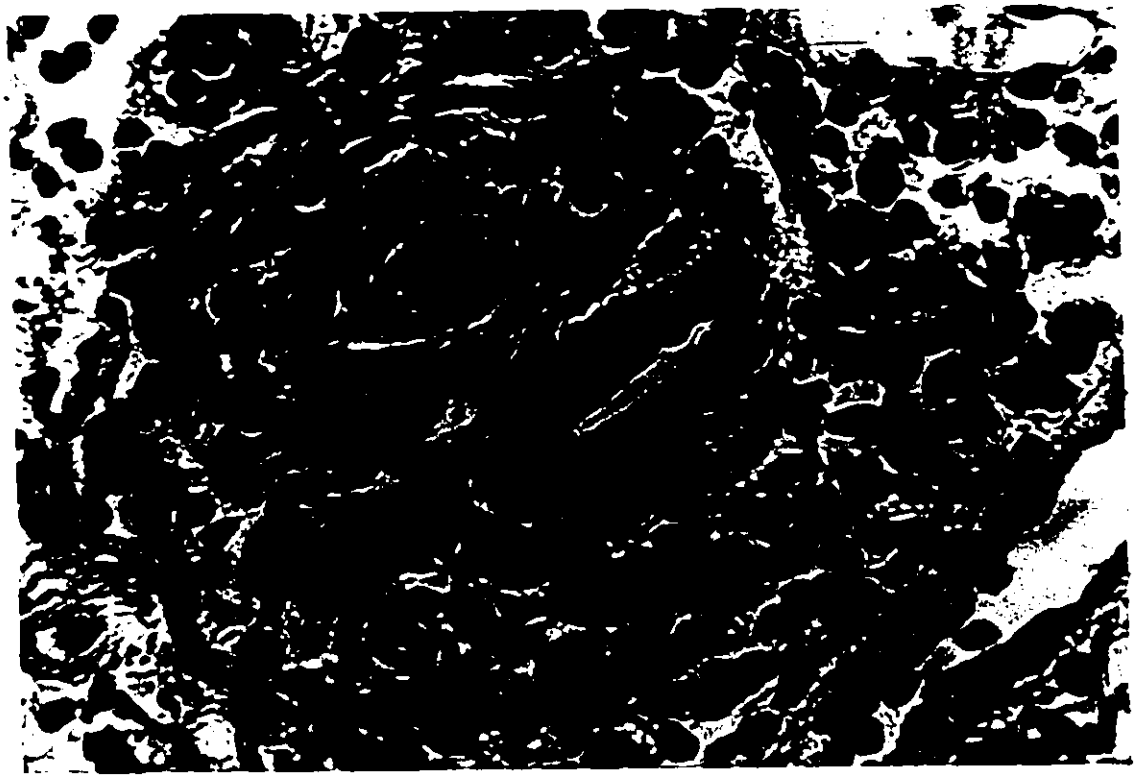

Figure 5B:-- Skin Biopsy of a Rabbit, Previously Sensitized with SRBC in CFA, 24 hours after Intradermal Challenge with Sheep Erythrocytes in Saline. Magnification: 10 x 40.

unilateral (footpad) immunization with SRBC or SRBC in CFA, respectively (tables XVIII and XIX).

The experiments conducted in this portion of the investigation have, therefore, failed to demonstrate any correlation between ADCC cytotoxic effector cells and humoral or cell-mediated immunity in the rabbit, since ADCC cytotoxic effector cells were not detected in lymph nodes engaged in antibody synthesis or undergoing a cell-mediated (delayed hypersensitivity) immune response. Furthermore, no marked alteration in the distribution of ADCC cytotoxic effector cells was noted among the other lymphoid tissues investigated.

However, of more than academic interest is the detection, in the rabbit, of ADCC cytotoxic effector cells infiltrating skin sites showing erythema and induration following challenge with the sensitizing antigen (delayed hypersensitivity reaction). Stiller et al (1975) have also recovered ADCC cytotoxic effector cells from human renal allografts undergoing rejection. These data suggest the participation of ADCC cytotoxic effector cells in cell-mediated immune responses. However, their contribution to the inflammatory reaction and the pathology associated with the cell-mediated or delayed hypersensitivity reaction still remains to be clearly elucidated.

It would appear then that the ADCC mechanism of target cell destruction may, in fact, provide a link between the two "classical" components of the immune response, namely humoral immunity on the one hand and cell-mediated immunity on the other. This would compel the recognition of a third category of immune response presumably serving to refine, modulate and/or enhance the response of an animal to challenge with any given antigen or battery of antigens.

CHAPTER VII

GENERAL DISCUSSION

The ADCC reaction was first described by Möller in 1965. He demonstrated that cells exist in the normal animal capable of effecting lysis of antibody-sensitized target cells. This finding was quickly corroborated by other investigators and it laid to rest the dogma that the presence of cells capable of mediating lysis of a specific target cell must necessarily be generated as a consequence of prior immunization of the host. Today, it is accepted that sub-populations of circulating and non-circulating human and animal cells can lyse antibody-sensitized nucleated and non-nucleated cells, thus implying a prominent role for these cytotoxic cells in the phylogeny and ontogeny of the immune response. Furthermore, at least one other cytotoxic reaction is known to be carried out by normally-occurring circulating cells. This is the naturally-occurring cell-mediated cytotoxic (NOCC) reaction.

In the NOCC reaction circulating cells acquire, within a few hours in culture, the capacity to lyse target cells to which they had not been previously exposed (Behelak and Richter, 1974; Behelak et al, 1976; Pross et al, 1977; Pross and Baines, 1977). These two reactions, the ADCC and NOCC reactions, may be grossly categorized as "para-immunologic" reactions as the effector cells exist in the circulation independent of prior sensitization of the host, in contradistinction to the conventional immunologic reactions which are totally dependent upon induced antibodies or sensitized cells. The "non-specific" immunity originally described by Eli Metchnikoff more than 80 years ago and subsequently elaborated on by Sir Almroth Wright, takes on a new meaning in the context of present day concepts revolving around the naturally-occurring cytotoxic cells.

In view of the cytotoxic nature of the ADCC reaction, the effector cell has been referred to as a "killer" or "K" cell (Perlmann et al, 1975; Perlmann, 1976). However, since the NOCC reaction also involves the participation of normally-occurring cytotoxic cells, the term "K" cell should rationally be used to identify all normally-occurring cells mediating cytotoxic reactions and not be reserved only for the cell(s) mediating the ADCC reaction. The term

ADCC cytotoxic effector cell is therefore utilized here.

One undisputed fact which has survived intensive investigation is that the ADCC cytotoxic effector cell(s) can only lyse the target cell(s) following the interaction of the target cell(s) with anti-target cell antibodies. It is generally agreed that the initial event triggering off the ADCC cytolytic reaction is the interaction between the Fc region on the target cell-fixed antibody molecule and the surface receptor on the ADCC cytotoxic effector cell which bears a configurationally complimentary structure, the Fc receptor. Although the results of the majority of investigations support the IgG identity of the antibodies (MacLennan and Loewi, 1968; Perlmann and Perlmann, 1970; Gelfand et al, 1972; Hallberg, 1974; Wisloff et al, 1974a, 1974b; Larsson et al, 1975a; MacDermott et al, 1975; Shore et al, 1976), a not insignificant number of investigations have also implicated IgM antibodies (Dennert and Lennox, 1973; Lamon et al, 1975a, 1975b, 1975c; Blair et al, 1976; Wahlin et al, 1976; Fuson and Lamon, 1977; Fuson et al, 1978).

For the purposes of this study, the immunologically mature outbred rabbit constituted an excellent animal in which to investigate immunologic phenomena and characterize and evaluate the mechanisms (cellular

and non-cellular) involved in antibody-dependent cell-mediated cytotoxicity. The rabbit is a relatively large animal compared with other laboratory rodents (mice, rats, guinea pigs) and it is therefore possible to obtain large numbers of cells for each of the easily accessible lymphoid tissues. This permits the simultaneous investigation of different lymphoid tissues and several experimental parameters. Furthermore, mice, rats and guinea pigs utilized in research are often inbred, in contradistinction to the rabbit. Results obtained with animals of one strain are not often observed in animals of a different strain and therefore, more often than not, the investigator is tempted to select the strain of animal which has been previously shown to act in the desired manner.

Additionally, man, like the rabbit, is an outbred animal and the physiological anatomy of the rabbit is more closely related to that of the human than is that of other experimental animals used, especially mice, rats and guinea pigs. Furthermore in all of these latter animal species, the spleen and lymph nodes are haematopoietic organs, quite unlike the situation in the human and rabbit where only the bone marrow in the adult is haematopoietic. The above similarities between rabbit and man are important when viewed in the light of the considerations, cited below, which

constitute the rationale for this investigation.

In the assessment of ADCC cytotoxic activity in man it is invariably only the circulation that is evaluated. It is generally assumed, but not yet proven, that circulating ADCC cytotoxic effector cells in man are representative of similar cells present or generated in other lymphoid tissues. The question therefore asked is whether in fact human ADCC cytotoxic effector cells exist in parenchymal lymphoid tissues which are similar in activity to circulating ADCC cytotoxic effector cells? If so, in which tissues are these cells located?

The answers to these questions can be best formulated in the experimental animal in which circulating cells and cells of the different lymphoid tissues can be simultaneously examined for ADCC cytotoxic activity. The rabbit is the experimental animal of choice for the reasons previously cited.

The objectives of this investigation were:

(i) to determine the optimal culture conditions for the demonstration of ADCC cytotoxic activity with rabbit cells;

(ii) to define precisely the tissue distribution of ADCC cytotoxic effector cells in the rabbit;

(iii) to determine the functional and morphological homogeneity or heterogeneity of the ADCC cytotoxic effector cell in the rabbit; and

(iv) to define the role played by ADCC cytotoxic effector cells in humoral immunity, cell-mediated immunity and local immune responses.

The results of this study indicate that:

(i) ADCC cytotoxic effector cells are present in rabbit WBC, bone marrow, spleen, appendix, saccu-lus rotundus, Peyer's patches, MLN, PLN and thymus;

(ii) that the ADCC cytotoxic effector cells of the WBC, bone marrow and spleen are considered to be rapidly acting in the ADCC assay whereas those of the other lymphoid tissues demonstrate a lag in the onset of ADCC cytotoxic activity; and

(iii) that the ADCC cytotoxic effector cells in the rabbit are heterogeneous and constitute functional and morphologically distinct sub-populations of cells.

Furthermore, in vivo data would suggest:

(iv) that ADCC cytotoxic effector cells may be attracted to the site of a cell-mediated immune reaction (delayed hypersensitivity reaction) in the rabbit.

ADCC cytotoxic activity has been previously attributed to rabbit thymus, spleen, bone marrow, PEC and MLN cells (Gelfand et al, 1972; Resch et al, 1974; Bona et al, 1975, 1976), and PBL (Zighelboim and Gale, 1974), following the cytolysis of radionuclide-labelled

target cells (^{51}Cr-ChRBC) subsequent to their incubation with the various lymphoid cells and rabbit anti-target cell antiserum for periods of 15 to 20 hours under standard tissue culture conditions. So far, neither the rabbit GALT (appendix, sacculus rotundus and Peyer's patches) nor the PLN were, or have been so far, investigated for ADCC cytotoxic activity.

This investigation has confirmed the observations of Gelfand et al (1972), Resch et al (1974), Bona et al (1965; 1976) and Zighelboim and Gale (1976), and has likewise demonstrated that ADCC cytotoxic effector cells can be consistently detected in the WBC, bone marrow and spleen under culture conditions similar to those described by Gelfand et al (1972). Additionally, this work has shown that appendix and sacculus rotundus cells are marginally active in ADCC while the cells of the thymus, MLN, PLN and Peyer's patches are poorly active or inactive in ADCC. The ADCC cytotoxic activity of MLN cells was observed to be very inconsistent as activity was detected in only 6 of 24 MLN cell preparations. Comparison of data would indicate therefore that the present results with the thymus and MLN cells in ADCC would appear to be in conflict with those of Gelfand et al (1972), Resch et al (1974) and Bona et al (1975; 1976) respectively.

Furthermore, the analysis in this study of ADCC cytotoxic activity of cells of the lymphoid tissues and WBC over a wide range of E:T cell ratios in a standard 24 hour ADCC assay suggested the classification of these cells into three categories:

(i) "highly" cytotoxic cells (bone marrow, WBC and spleen cells);

(ii) "mildly to moderately" cytotoxic cells (appendix, sacculus rotundus and MLN cells), and

(iii) "non-cytotoxic" cells (Peyer's patches, PLN and thymus cells).

It was noted, however, that when these cells were co-cultured with target cells and anti-target cell antibodies for varying intervals of time, the "highly" cytotoxic cells showed ADCC cytotoxic activity early in the culture period (6 to 8 hours) which increased incrementally over 72 hours. The "mildly to moderately" cytotoxic cells as well as the "non-cytotoxic" cells, exhibited ADCC cytotoxic activity only after co-culture with the target cells and anti-target cell antibodies for 48 and 72 hours.

Preculture of the cells for varying periods of time in the absence of target cells and anti-target cell antibodies (24, 48 and 72 hours) followed by the addition of these reactants in the standard 24

hour ADCC assay showed that rabbit bone marrow, WBC and spleen cells displayed ADCC cytotoxic activity irrespective of the duration of the preculture period. Appendix and sacculus rotundus cells were markedly active only if the target cells and anti-target cell antiserum were added at 72 hours of culture. The other lymphoid cells, including the thymus cells, showed no ADCC cytotoxic activity irrespective of the duration of preculture. Interestingly enough, since the same preparation of thymus cells was significantly active when co-cultured for 48 and 72 hours with target cells and anti-target cell antibodies, it can be concluded that the thymic cytotoxic effector cells die in culture in the absence of target cells and anti-target cell antibodies.

On the basis of the results of the time course studies, the cells of the various lymphoid tissues can be additionally classified as being:

(i) rapidly cytotoxic in the ADCC assay, or as

(ii) demonstrating a lag phase in the onset of ADCC cytotoxic activity.

Although the initial observations in this investigation lead to the conclusion that thymus and MLN cells were "mildly" or "non-cytotoxic" respectively, in contrast with the findings of Gelfand et al (1972), Resch et al (1974) and Bona et al (1975, 1976), the time

course studies of ADCC cytotoxic activity in the New Zealand White rabbit would indicate that these two tissues are in fact slow-acting in the ADCC assay but do nevertheless possess ADCC cytotoxic effector cells.

Gelfand et al (1972) investigated the kinetics of the ADCC cytotoxic reaction. They analyzed the ADCC cytotoxic activity exhibited by PEC and phagocyte-depleted spleen, thymus and MLN cells over a period of 72 hours in culture with antibody-coated target cells. They observed that PEC (containing 70 to 80 percent macrophages, 10 to 20 percent granulocytes and 10 to 20 percent lymphocytes) on the one hand demonstrated an early onset (by 4 to 6 hours of culture) of ADCC cytotoxic activity which dropped off markedly by 20 hours of culture. Column-purified thymus and MLN cells, on the other hand (containing less than 1 percent macrophages, respectively), were found to effect appreciable target cell lysis only after 15 and 20 hours of culture. Spleen cells (containing 1 percent phagocytic cells) were shown to exhibit a rapid onset (by 4 to 6 hours of culture) of ADCC cytotoxic activity which was noted to level off by 15 to 20 hours of culture although a slight increase in activity was observed up to 72 hours. They also reported that the addition of PEC to

phagocyte-depleted thymus and MLN cells (0.5 percent of these cell preparations) resulted in a rapid onset (by 4 to 6 hours of culture) of ADCC cytotoxic activity which they attributed to the phagocytic cells since the phagocyte-depleted cell preparations exhibited a delayed onset of ADCC cytotoxic activity. The lymphocyte was identified as the cell demonstrating delayed (20 hours) ADCC cytotoxic activity. Additionally, Gelfand et al (1972) concluded that ADCC cytotoxic activity could be attributed to both a phagocytic and a non-phagocytic cell (lymphocyte) in the spleen and to a lymphocyte in the thymus and MLN. The presence of a single ADCC cytotoxic effector cell population in the thymus and MLN, and of 2 ADCC cytotoxic effector cell populations in the spleen, suggested that the ADCC cytotoxic effector cell(s) in the rabbit is heterogeneous with respect to its capacity to effect ADCC cytotoxic destruction of antibody-coated target cells.

The findings in this investigation similarly indicate that the ADCC cytotoxic effector cells in the rabbit are in fact heterogeneous and constitute functionally and morphologically distinct subpopulations. These subpopulations can be classified into a number of categories on the basis of their cellular morphology and properties in the ADCC reaction, as outlined in

table XXI and reiterated below.

Cells of the bone marrow, WBC and spleen were found to demonstrate a rapid onset of ADCC cytotoxic activity by 6 to 8 hours of culture which was seen to almost level off by 24 hours and increase slightly over a further 48 hours in culture. The ADCC cytotoxic effector cells in the WBC and spleen appeared to be largely phagocytic as phagocyte-depleted WBC and spleen cells were essentially inactive. The ADCC cytotoxic effector cells in the bone marrow appeared to be non-phagocytic heterophils and/or lymphocytes, but the additional existence of an effector macrophage in the bone marrow was not ruled out as purified monocytes were not analyzed.

A further distinction was noted between the bone marrow ADCC cytotoxic effector cells on the one hand, and the WBC and spleen effector cells on the other hand. The ADCC cytotoxic reaction with bone marrow cells was inhibited by soluble aggregates of rabbit gamma-globulin to only a minor degree, whereas that with WBC and spleen cells was almost completely inhibited by RAGG.

The ADCC cytotoxic effector cells in the Peyer's patches, thymus, MLN and PLN were found to require a period of 24 to 48 hours in culture before showing ADCC cytotoxic activity. This was noted to increase markedly by 72 hours of culture. These cells attained

significant ADCC cytotoxic activity at 48 to 72 hours only if they were co-cultured with target cells and anti-target cell antibodies for the entire culture period. The phagocyte-depleted thymus cells did not show any ADCC cytotoxic activity. Thymic ADCC activity was not inhibited by RAGG. On the other hand, MLN cells lost all of their ADCC cytotoxic activity following the removal of phagocytic cells and ADCC cytotoxic activity could be totally inhibited by RAGG. It can be therefore concluded that the ADCC cytotoxic effector cells in the thymus and MLN are lymphocytes and phagocytic cells, respectively. The ADCC cytotoxic effector cells in the appendix and in sacculus rotundus were also shown to demonstrate delayed ADCC cytotoxic activity but did not appear to require prior co-culture with target cells and with anti-target cell antibodies in order to facilitate the expression of ADCC cytotoxic activity. Since the depletion of phagocytic cells from the appendix cell suspensions consistently resulted in a 50 percent reduction of ADCC cytotoxic activity, and since appendix ADCC cytotoxic effector cells were inconsistently inhibited by RAGG, it can be concluded that the ADCC cytotoxic effector cells in the appendix are both lymphocytes and phagocytes.

It is therefore concluded that the ADCC cytotoxic effector cells in the rabbit are heterophils and

monocytes in the circulation, phagocytic cells in the spleen and MLN, lymphocytes and/or non-phagocytic heterophils in the bone marrow, lymphocytes in the thymus and lymphocytes and phagocytic cells in the appendix.

The conclusion arrived at by Gelfand et al (1972), that phagocytic cells act rapidly (by 4 to 6 hours of culture) in the ADCC assay and that lymphocytes demonstrate a delayed (20 hours) onset of ADCC cytotoxic activity was only partially supported by my investigation. Although in my investigation phagocytic cells in the WBC and spleen were found to express ADCC cytotoxic activity in the absence of a significant latent period, the non-phagocytic bone marrow cells (lymphocytes and/or non-phagocytic heterophils) also expressed ADCC cytotoxic activity in the absence of an extended latent period. On the other hand, thymic lymphocytes did not demonstrate ADCC cytotoxic activity until 48 hours of culture, whether or not macrophages were initially removed. Gelfand et al (1972) also reported that pure thymic lymphocyte preparations were slow to act in ADCC. They additionally claimed that ADCC cytotoxic activity could be attributed to both a phagocytic and a non-phagocytic cell in the spleen whereas the results of my investigation strongly indicate that the ADCC cytotoxic activity of spleen cells can be solely attributed to a splenic phagocytic cell. Furthermore, MLN cells in my

investigation were inconsistently shown to display only marginal ADCC cytotoxic activity (cells from only 6 of 24 animals were active), whereas the results of Gelfand et al (1972) imply that rabbit MLN cells are consistently active. Thus, the findings of my investigation do not support the conclusions arrived at by Gelfand et al (1972) since in my experiments the phagocytic cells have been shown to exhibit both immediate (WBC and spleen cells) and delayed (MLN cells) ADCC cytotoxic activity and non-phagocytic cells in the bone marrow have been shown to exhibit immediate ADCC cytotoxic activity. Like Gelfand et al (1972), however, I have found that non-phagocytic mononuclear cells in several organs (thymus and appendix cells) have been shown to exhibit delayed ADCC cytotoxic activity.

My investigation has also presented evidence to support an ADCC function for the rabbit heterophil (tables XII and XIII). This conclusion is based on the repeated observation that the heterophil (derived from either the WBC, bone marrow or spleen) consistently failed to lyse target cells in the absence of anti-target cell antibodies. However, in the presence of antibodies directed against the target cells, marked ADCC cytotoxic activity was observed by the heterophil of either the peripheral blood, bone marrow or spleen in the standard 24 hour ADCC assay. Furthermore, the

ADCC cytotoxic activity exhibited by heterophil cells from peripheral blood-derived heterophil can be inhibited by soluble aggregates of rabbit gamma-globulin (data not presented). It would therefore appear that heterophils in general, or a class of heterophils in particular, possess a definite immunologic function, quite aside from a non-specific phagocytic function. Similar results have been obtained with human neutrophils implying that polymorphonuclear leukocytes possess a definite immunologic role(s) (Gale and Zighelboim, 1974, 1975; MacDonald et al, 1975; Hunninghake and Fauci, 1971; Zighelboim et al, 1976; Clark and Klebanoff, 1977).

The confusion regarding the precise nature of the cell which mediates ADCC cytotoxic activity may be a reflection of current findings which indicate that although the cytotoxic reaction cannot occur in the absence of anti-target cell (IgG) antibodies, or in some cases IgM antibodies--(Dennert and Lennox, 1973; Lamon et al, 1975a, 1975b, 1975c; Blair et al, 1976; Wahlin et al, 1976; Fuson and Lamon, 1977; Fuson et al, 1978), nevertheless the identity of the ADCC cytotoxic effector cell appears to vary with the type of target cell used in the assay system (Perlmann and Holm, 1969; MacLennan, 1973; Perlmann, 1976; Lovchik and Hong, 1977; Pearson, 1978). For example, MacLennan and Harding (1970) and MacLennan (1972a) excluded the

participation of the rat macrophage in the ADCC destruction of Chang liver target cells. Greenberg et al (1975a; 1975b) reported that antibody-sensitized chicken erythrocytes were lysed by phagocytic and non-phagocytic murine spleen cells, whereas antibody-coated SL2 lymphoma cells were lysed primarily by non-phagocytic murine spleen cells. Holm and Hammarstrom (1973) demonstrated that in man, the monocyte was implicated in the cytolysis of human erythrocytes sensitized with alloantiserum.

Recent evidence suggests that the identity of the human ADCC cytotoxic effector cell is dependent upon the concentration of anti-target cell antibodies utilized in the ADCC assay (see Appendix). In this study, the lymphocyte and/or neutrophil appeared to be the effector cell(s) if target cells were sensitized with antiserum at an optimal sensitizing dilution (10^{-2} or 10^{-3}), whereas the monocyte was found to be the sole effector cell if the target cells were sensitized with antiserum in a suboptimal sensitizing dilution (2×10^{-4} or 10^{-4}) (table XXII).

These data indicate that the ADCC cytotoxic activity of the monocyte is effectively masked by that of the lymphocyte in the presence of optimal numbers of target cell-bound antibodies. However, as the number of antibody molecules per target cell was decreased,

it was observed that the monocyte asserted itself owing to:

(i) its greater affinity for the Fc region of the antibody molecule as compared to that of the lymphocyte or neutrophil and/or

(ii) its activation following interaction with a single target cell-fixed antibody molecule whereas

(iii) the ADCC cytotoxic effector lymphocyte or neutrophil may require the interaction of 2 or more adjacent target cell-fixed antibody molecules to become activated.

This interpretation may offer an explanation for some of the controversy surrounding the identity of the ADCC cytotoxic effector cell and carries implications which cannot be ignored. It suggests that it is imperative that, in any study, the target cells be incubated with the corresponding antiserum in varying dilutions before concluding that a given cell is the only effector cell in antibody-dependent cell-mediated cytotoxicity.

The apparent discrepancies in my findings and the observations of Gelfand et al (1972) may, in part, be explained by the differences in the concentrations of the anti-chicken erythrocyte antibody.

Although chicken erythrocyte target cells and rabbit anti-chicken erythrocyte antiserum were used in both studies, Gelfand et al (1972) pretreated the chicken erythrocytes with the unpurified rabbit anti-chicken erythrocyte antiserum or the column-fractionated antiserum (IgG) prior to their introduction into tissue culture, whereas unfractionated rabbit anti-chicken erythrocyte antiserum was directly added to the lymphoid and chicken erythrocyte cell cultures and allowed to remain in culture in my investigation. This would suggest that a net difference in IgG antibody concentration between the two ADCC assays was likely. One cannot rule out, however, the possibility of "species differences" among the supposedly outbred rabbits. Gelfand et al (1972) used Silver-Grey rabbits in their study, whereas New Zealand White rabbits were utilized in this investigation.

The first part of my investigation was designed to establish the organ source of ADCC cytotoxic effector cells in the mature outbred rabbit and to determine whether or not such cells constituted unique populations, thus permitting them to be distinguished one from the other. This information was essential in order to be able to evaluate the role(s) played by the ADCC cytotoxic effector cell(s) in the humoral and cell-mediated immune responses in the rabbit. Do ADCC

cytotoxic effector cells migrate to and subsequently concentrate in organs involved in the immune response and/or infiltrate sites undergoing delayed hypersensitivity reactions? An attempt to resolve this question constitutes the second part of my investigation.

The experiments carried out to evaluate the role of the ADCC cytotoxic effector cell in humoral and cell-mediated immunity (delayed hypersensitivity) in the mature outbred rabbit, showed that:

(i) there was no alteration in the distribution of ADCC cytotoxic effector cells among the various lymphoid tissues during the course of a humoral immune response following antigenic stimulation with SRBC;

(ii) injection of the antigen, in saline or in CFA, did not result in any changes in reactivity of cells in the draining PLN.

Significantly, however, it was demonstrated that:

(iii) ADCC cytotoxic effector cells were present in sites undergoing a delayed hypersensitivity reaction to intradermal challenge with immunizing antigen but not with an unrelated antigen.

Pudifin et al (1971) observed that rats subjected to 850 rads whole body irradiation were unable to mount a humoral immune response although ADCC cytotoxic

effector cells were detected in their spleens. They have also shown that the rat PLN draining a site of immunization is a poor source of ADCC cytotoxic effector cells. This suggests that the presence of ADCC cytotoxic effector cells is not singularly crucial to the induction of the humoral immune response, nor is the presence of a humoral immune response singularly crucial to the recruitment of ADCC cytotoxic effector cells into the reactive site.

My investigation has likewise shown that ADCC cytotoxic effector cells are not present in cell suspensions prepared from PLN undergoing either a humoral or a cell-mediated immune response following unilateral (footpad) immunization with SRBC or SRBC in CFA. These data also failed to demonstrate any relationship between ADCC cytotoxic effector cells and humoral or cell-mediated immunity in the rabbit. Furthermore, no marked alteration in the distribution of ADCC cytotoxic effector cells was noted among the other lymphoid tissues concurrently investigated.

Of more than academic interest was the detection of ADCC cytotoxic effector cells infiltrating skin sites exhibiting erythema and induration following challenge with sensitizing antigen (delayed hypersensitivity reaction). Stiller et al (1975) also reported the recovery of ADCC cytotoxic effector cells from

human renal allografts undergoing rejection. Similarly, Strom et al (1975b) noted that as many as 50 percent of cells recovered from rejected human renal allografts bore Fc receptors, suggesting a role for ADCC cytotoxicity in the rejection process.

However, this evidence is largely circumstantial in nature. Recently, von Willebrand and Hayry (1978) analyzed cellular infiltrates of allografted human kidneys during primary acute rejection episodes. These cellular infiltrates, which were mixtures of macrophages, monocytes, lymphocytes and neutrophils, were only weakly, if at all, cytotoxic to transplant parenchymal cells. Although the authors speculated that mechanisms other than those dependent upon direct cell-mediated cytotoxicity were perhaps responsible for the deterioration of an allograft during the rejection phase, they neglected to test their cells for ADCC cytotoxic activity against endothelial cells of the transplant which, in fact, may have been the target cells. Such a finding would be consistent with the observed pathology of a renal allograft undergoing rejection in which the first signs of rejection-edema, sludging of blood, thrombosis and eventual cessation of blood flow, suggest vascular and not parenchymal disturbances. The evidence presented by von Willebrand and Hayry (1978) points, at least circumstantially, to the potential involvement

of Fc receptor-bearing cells in the pathology associated with renal allograft rejection in man. My investigation has demonstrated the presence of ADCC cytotoxic effector cells in sites exhibiting delayed hypersensitivity reactions in the rabbit, thereby suggesting that an ADCC cytotoxic effector mechanism, similar to that documented in man, may be involved in allograft rejection in the rabbit.

In conclusion, it has been demonstrated that the ADCC cytotoxic effector cells of the rabbit lymphoid tissues under test in this investigation display:

(i) different patterns of reactivity on a temporal and quantitative basis;

(ii) varying degrees of sensitivity to inhibition by aggregated gamma-globulin; and

(iii) varying morphology.

Preliminary results also suggest:

(iv) a role for the ADCC cytotoxic effector cell in the cell-mediated immune response but not in the humoral immune response.

It would also appear that only the splenic ADCC cytotoxic effector cell resembles its circulating counterpart and may indeed be represented by the circulating ADCC cytotoxic effector cell. One might therefore propose that the circulating ADCC cytotoxic effector cell is generated in the spleen. The ADCC cytotoxic

effector cell in the other lymphoid tissues would appear to be confined to these tissues.

A fundamental problematic must now be addressed. Is the information regarding ADCC cytotoxicity in the rabbit also valid for man? On the basis of the findings in my investigation with ADCC cytotoxic effector cells of the peripheral blood pool in the rabbit and concurrent investigation of the ADCC cytotoxic effector cells in the human circulating WBC population (see Appendix), the answer would have to be a qualified no. Although both the rabbit heterophil and human neutrophil are active in the ADCC assay, the human circulating mononuclear ADCC cytotoxic effector cells are both lymphocytes and phagocytic monocytes, whereas the rabbit circulating mononuclear cytotoxic effector cell is strictly a phagocytic cell. Since, in both studies, the same target cell and anti-target cell antiserum were used to define the circulating ADCC cytotoxic effector cell and since investigations of both rabbit and human WBC were carried out simultaneously, these results would indicate a species difference in this regard.

Therefore, although work aimed at understanding the nature and interactions of immunocompetent cells in the experimental animal needs further defining, one must be on guard not to extrapolate from animal to man without due consideration of their inherant

differences and in the absence of unequivocal supporting empirical evidence. Albeit the differences in ADCC cytotoxic activity between the rabbit and man are significant, the study of ADCC cytotoxicity in the rabbit and other experimental animals may nevertheless provide insights and shed some light on the role of ADCC and related immunological mechanisms (MICC and NOCC) in the normal Immune Response.

CHAPTER VIII

CONTRIBUTION TO KNOWLEDGE

1. The candidate has determined the culture conditions required for the demonstration of reproducible ADCC cytotoxic activity by the various lymphoid tissues under investigation in the New Zealand White rabbit.

2. The candidate has determined that the optimal effector cell concentration in the ADCC assay, as well as the range of ADCC cytotoxic activity, is characteristic of each of the different lymphoid tissues in the New Zealand White rabbit.

3. The candidate has determined that the optimal time of culture required for the expression of ADCC cytotoxic activity by the WBC, bone marrow and spleen cells is 24 hours whereas it is 48 hours for the appendix, sacculus rotundus, Peyer's patches, MLN and thymus cells and 72 hours for the PLN cells.

4. The candidate has determined that ADCC cytotoxic effector cells are present in appendix, sacculus rotundus, Peyer's patches and PLN, tissues which

have not been previously assessed for ADCC cytotoxic activity in the New Zealand White rabbit.

5. The candidate has determined that ADCC cytotoxic effector cells in the New Zealand White rabbit are heterogeneous and constitute functionally and morphologically distinct subpopulations of cells which can be classified into a number of distinct categories on the basis of their morphology and properties in the ADCC reaction.

6. The candidate has determined that ADCC cytotoxic effector cells in the New Zealand White rabbit appear to be heterophils and monocytes in the circulation, phagocytic cells in the spleen and MLN, lymphocytes and/or non-phagocytic heterophils in the bone marrow, lymphocytes in the thymus and lymphocytes and phagocytic cells in the appendix.

7. The candidate has determined that an immunological role for the rabbit heterophil does exist.

8. The candidate has shown that there is no alteration in the distribution of ADCC cytotoxic effector cells among the various rabbit lymphoid tissues during the course of a humoral immune response following antigenic stimulation with SRBC.

9. The candidate has shown that ADCC cytotoxic effector cells are not recruited into normally ADCC negative PLN during the course of either a humoral or a cell-mediated immune response following the unilateral (footpad) immunization with SRBC in saline or in CFA, respectively.

10. The candidate has shown that ADCC cytotoxic effector cells are present in sites undergoing a delayed hypersensitivity reaction to intradermal challenge with immunizing antigen (SRBC) but not with an unrelated antigen (HRBC).

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APPENDIX

The antibody-dependent cell-mediated cytotoxic reaction

II. THE EFFECT OF THE CONCENTRATION OF ANTI-TARGET CELL ANTIBODIES ON THE IDENTITY OF THE HUMAN EFFECTOR CELLS

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Summary. The circulating leukocytes of normal adults (lymphocytes, monocyte-lymphocyte mixtures and neutrophils) were investigated for their capacity to induce antibody-dependent cell-mediated cytotoxicity (ADCC). The target cell was the rabbit antibody-sensitized chicken erythrocyte. Under conditions of optimal target cell sensitization by anti-target cell antibodies, ADCC cytotoxic activity was exhibited by the neutrophils and lymphocytes to the apparent exclusion of monocytes.

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The lymphocytes exhibit activity more rapidly than do the neutrophils and they are more active on an equal cell basis. However, when the effector cells were investigated using target cells sensitized with the anti-target cell antiserum in a threshold concentration, the monocyte and not the lymphocyte or neutrophil displayed ADCC cytotoxic activity. It was concluded that the effector cells in the circulation are heterogeneous and include subclasses of lymphocytes, monocytes and neutrophils and that the identity of the cytotoxic effector cells appears to vary with the concentration of anti-target cell antibodies.

INTRODUCTION

Although 14 years have elapsed since the antibody-dependent cell-mediated cytotoxic reaction (ADCC) was first described (Möller, 1965; Perlmann and Holm, 1969; MacLennan, 1972; Perlmann et al, 1972; Cerottini and Brunner, 1974; Perlmann, 1976; Lovchik and Hong, 1977), the precise identity of the cell(s) mediating this reaction and its role in the aetiology and pathogenesis of disease is still very controversial. The majority of studies have disclosed that the ADCC effector cell is neither a B nor a T cell (defined by convention as possessing surface immunoglobulins

and theta antigens, respectively) nor an adherent (phagocytic?) cell (Perlmann and Holm, 1969; MacLennan, 1972; Perlmann et al, 1972; Greenberg et al, 1973; Cerottini and Brunner, 1974; Wisloff et al, 1974; Zighelboim et al, 1974; MacDermott et al, 1975; Lovchik and Hong, 1977). The cell has therefore been referred to as a 'null' cell (Greenberg et al, 1973).

Work in this laboratory is concerned with relating the presence and functional state of the Fc receptor-bearing ADCC effector cells in the circulation with the immune status of the patient. In order to assess the role of these cells in the provision of immunity, it is essential that they be unequivocally identified. In this investigation, rabbit antibody-sensitized ChRBC were used to identify the human circulating ADCC effector cells. However, even with a single antibody-sensitized target cell, it was observed that the identity of the effector cell varies depending upon the number of antibody molecules on the sensitized target cell.

MATERIALS AND METHODS

Preparation of circulating effector cells

Mononuclear cells (at the interface) and neutrophils (in the pellet) were obtained by centrifuging whole blood and buffy coat cells, respectively, in a ficoll-hypaque (FH) discontinuous density gradient (9 percent ficoll in distilled water adjusted to a specific gravity of 1.077 at 18°C with sodium hypaque) (Boyum, 1968). Red cells were eliminated by incubating the unfractionated cells, the mononuclear cells and neutrophils in 10 ml of tris-buffered ammonium chloride solution (0.15 M) at 37°C for 10 min. The cells were then diluted to 50 ml with HBSS and centrifuged at 480 g for 10 min. This wash procedure was repeated twice. The cells were then suspended in the culture medium and an aliquot was counted in a Coulter counter. The cells were diluted in the culture medium to the required concentration for use in the cytotoxicity assay.

Depletion of phagocytic cells by carbonyl iron uptake

The WBC were suspended in autologous human serum (diluted two-fold in saline) at a cell concentration of 2×10^6 cells per ml. Eight milligrams of carbonyl

iron particles were added to each millilitre of the cell suspension and the cells were incubated in a tissue culture rotator for 30 min at 37°C in 5 percent CO_2 in air. The cells were then resuspended and the iron-containing cells were separated from the non-phagocytic cells by pressing the tube against a magnet and pipetting off the supernatant containing the non-phagocytic cells. These latter cells were suspended in HBSS and centrifuged at 480 g for 10 min. The cells were washed once more, suspended in culture medium and an aliquot was counted in a Coulter counter. The cells were diluted in the culture medium to the required concentration for use in the cytotoxicity assay.

Smears were stained for non-specific esterase using the technique of Yam et al (1971) for the detection of monocytes. Approximately 8-20 percent of the mononuclear cells gave the characteristic staining pattern attributed to monocytes (strong granular cytoplasmic staining) prior to elimination of monocytes, whereas less than 1 percent of the cells stained in this fashion following elimination of the monocytes by the carbonyl iron ingestion method.

Rabbit anti-chicken erythrocyte antiserum (RACE)

The anti-target cell antiserum was prepared as described previously (see chapter III: Materials

and Methods, section 3.2.2).

Cytotoxicity (ADCC) assay

The assay has been described in detail (see chapter III: Materials and Methods, section 3.2.9). Briefly, 0.2 ml of a suspension of circulating effector (E) cells (10^6 cells) was mixed with 0.2 ml of ^{51}Cr-ChRBC target (T) cells (4×10^4 cells) and 0.2 ml of heat-inactivated rabbit anti-ChRBC antiserum (RACE) or normal control serum in sterile plastic tubes (No. 2003, Falcon Plastics). At the end of the incubation period, the tubes were centrifuged at 480 g for 10 min. Aliquots of the culture supernatants (400 µl) were transferred to appropriate vials and counted for 5 min in a Beckman Biogamma counter. The data were recorded as counts per culture per minute and the results are expressed as the cytotoxic index (CI) calculated as follows:

$$C.I. = \frac{\text{Test release-Spontaneous release}}{\text{Total release-Spontaneous release}} \times 100$$

"Test release" refers to the ^{51}Cr detected in the supernatant of the culture containing RACE, effector cells and ^{51}Cr-ChRBC. "Spontaneous release" refers to the ^{51}Cr detected in the supernatant of the culture containing normal control serum, effector cells and

^{51}Cr-ChRBC. The "total release" of ^{51}Cr refers to the ^{51}Cr in the supernatant following freeze-thawing of the ^{51}Cr-ChRBC suspension three to four times in an alcohol-dry ice mix (-70°C).

RESULTS

Preliminary experiments were carried out using different media (M199, RPMI-1640, NCTC-109 and CMRL-1066) supplemented with penicillin and streptomycin and fetal bovine serum in varying concentration. The medium which consistently provided optimal conditions for ADCC activity was M199 fortified with fetal bovine serum (FBS) to a final concentration of 6 percent (results not presented in tables). It is referred to as culture medium.

The RACE antiserum was found not to be inhibitory irrespective of the dilution. The cytotoxic activity of the effector cells decreased incrementally as the RACE antiserum was diluted from 10^{-1} (CI:60-80) to 10^{-7} (CI:20-40) (table XXII). Maximum or optimal cytotoxicity by unfractionated mononuclear cells and neutrophils was attained with the antiserum used in a concentration of 10^{-1} to 10^{-3}.

As can be seen in table XXIII, the cytotoxic activity of the unfractionated mononuclear cells was minimal

TABLE XXII

THE ADCC REACTION WITH HUMAN CIRCULATING MONONUCLEAR CELLS. THE EFFECT OF VARYING THE CONCENTRATION OF ANTI-TARGET CELL ANTISERUM ON THE ADCC CYTOTOXIC ACTIVITY OF HUMAN CIRCULATING MONONUCLEAR CELLS

Expt. No.	The cytotoxic index of human mononuclear cells at the following concentrations of rabbit anti-target cell (chicken RBC) antiserum*						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
1	60	63	74	59	51	47	20
2	70	62	61	54	45	38	29
3	77	60	63	55	48	42	36

* The medium used was culture medium. E:T = 10:1. Duration of culture was 24 hrs.

TABLE XXIII

THE ADCC REACTION WITH HUMAN CIRCULATING WHITE BLOOD CELLS. THE ADCC CYTOTOXIC ACTIVITY OF FICOL-HYPAQUE DENSITY GRADIENT SEPARATED WHITE BLOOD CELLS AS A FUNCTION OF TIME IN CULTURE WITH THE ANTIBODY-SENSITIZED TARGET CELLS

Expt. No.	Cells tested (E:T = 10:1)	The cytotoxic index at the following times of culture (hrs)*								
		0	½	1	2	6-8	16-18	24	48	72
1	Mononuclear cells	1	7	16	26	76	76	74	72	80
	Neutrophils	0	1	1	1	3	35	54	59	64
2	Mononuclear cells	0	2	6	12	46	54	51	68	69
	Neutrophils	0	1	0	1	18	57	66	68	67
3	Mononuclear cells	1	19	28	41	64	71	69	70	83
	Neutrophils	0	0	1	0	3	19	23	31	39

*The medium used was culture medium.

at 1-2 hrs in culture, increased dramatically by 6-8 hrs in culture, began to plateau at 24 hrs and increased only incrementally over the next 48 hrs. On the other hand the neutrophils displayed very little cytotoxic activity by 6-8 hrs in culture. The cytotoxic activity increased markedly at 16-18 hrs and increased marginally over the next 48 hrs. Therefore, effector and target cells were co-cultured for 24 hrs in the experiments reported upon in this communication.

Phagocyte-depleted mononuclear cells appear to be as effective as unfractionated mononuclear cells in their ability to lyse optimally sensitized ^{51}Cr-ChRBC target cells (table XXIV). However, monocyte-depleted mononuclear cells showed decreased ADCC activity as compared to the unfractionated mononuclear cells at low effector:target cell ratios (2.5:1) even when the anti-target cell antiserum was used at an optimal concentration (table XXV).

The phagocyte-depleted mononuclear cells and the neutrophils failed to induce antibody-dependent target cell cytolysis as compared to the unfractionated (monocyte-containing) mononuclear cells when the target cells were sensitized with the anti-target cell antiserum at a threshold (sub-optimal) concentration (10^{-4}) (table XXV). These results, which were consistently obtained in five consecutive experiments,

TABLE XXIV

THE ADCC REACTION WITH HUMAN CIRCULATING MONONUCLEAR CELLS FOLLOWING THE DEPLETION OF PHAGOCYTIC CELLS

Cells of individual tested	Treatment of cells tested	E:T ratio	The cytotoxic index of the effector cells at the following times (hrs) in culture with target cells*					
			2	6-8	18	24	48	72
S.B.	Untreated cells	10:1	20	51	100	69	68	58
		5:1	14	44	60	53	65	51
		2.5:1	6	28	38	70	63	54
	Phagocyte-depleted cells	10:1	12	33	67	81	91	61
		5:1	6	22	55	79	84	57
		2.5:1	3	12	31	57	88	75
H.H.	Untreated cells	10:1	22	51	53	100	72	62
		5:1	18	53	55	56	77	57
		2.5:1	9	39	52	54	77	39
	Phagocyte-depleted cells	10:1	ND**	88	83	86	100	ND
		5:1	9	55	61	66	100	77
		2.5:1	4	26	35	41	90	74
N.L.	Untreated cells	10:1	ND	42	59	58	60	67
		5:1	11	39	59	64	62	63
		2.5:1	6	25	51	56	57	66
	Phagocyte-depleted cells	10:1	6	31	64	76	76	77
		5:1	4	27	56	68	76	76
		2.5:1	2	7	36	54	76	77

* The anti-target cell antiserum was used in an optimal concentration (10^{-3}).

** ND = Not done.

TABLE XXV

THE ADCC REACTION WITH HUMAN CIRCULATING WHITE BLOOD CELLS. THE IDENTITY OF THE ADCC CYTOTOXIC EFFECTOR CELL AS A FUNCTION OF THE CONCENTRATION OF THE ANTI-TARGET CELL ANTISERUM

Expt. no.	Dilution of anti-target cell antiserum	Cells analyzed	The ADCC cytotoxic index at the following effector to target cell ratios (time of incubation is 24 hrs)					
			10:1	5:1	2.5:1	1.25:1	0.62:1	0.31:1
1	10^{-3}	Unfractionated M.C.*	44	51	50	50	33	17
		Monocyte-depleted M.C.**	66	61	42	25	14	9
		Neutrophils	33	31	19	10	3	1
	10^{-4}	Unfractionated M.C.*	32	24	24	16	10	3
		Monocyte-depleted M.C.**	11	4	6	3	2	0
		Neutrophils	3	2	6	2	4	0
2	10^{-3}	Unfractionated M.C.*	44	38	28	17	10	4
		Monocyte-depleted M.C.**	35	32	23	9	2	1
		Neutrophils	28	31	25	15	5	2
	10^{-4}	Unfractionated M.C.*	26	22	10	7	4	1
		Monocyte-depleted M.C.**	3	4	2	1	1	2
		Neutrophils	4	9	4	9	5	0

* Unfractionated mononuclear cells.

** Monocyte-depleted mononuclear cells.

indicate that the lymphocytes, neutrophils and monocytes are the effector cells when the anti-target cell antiserum is utilized at the optimal concentration and that the monocytes are the only effector cells when the anti-target cell antibodies are present in sub-optimal or threshold concentration.

DISCUSSION

The ADCC reaction was first described by Möller (1965) who demonstrated that cells exist in the normal animal capable of effecting lysis of antibody-sensitized target cells. This finding was quickly corroborated by other investigators and it laid to rest the dogma that the presence of cells capable of mediating lysis of a specific target cell must necessarily be generated as a consequence of prior immunization of the host.

Using a variety of antibody-sensitized target cells, investigators have shown that the effector cells may be lymphocytes (Perlmann and Holm, 1969; MacLennan, 1972; Perlmann et al, 1972; Greenberg et al, 1973; Cerottini and Brunner, 1974; Wisloff et al, 1974; Zighelboim et al, 1974; MacDermott et al, 1975; Perlmann, 1976; Lovchik and Hong, 1977) and/or monocytes (Perlmann and Holm, 1969; Dennert and Lennox, 1973; Holm and Hammarstrom, 1973; Greenberg et al, 1975; Kovithavongs

et al, 1975; Larsson and Ohlander, 1975; MacDonald et al, 1975) and/or neutrophils (Gale and Zighelboim, 1974, 1975; MacDonald et al, 1975; Hunninghake and Fauci, 1976; Nelson et al, 1976; Zighelboim et al, 1976; Clark and Klebanoff, 1977). In the present communication, it has been demonstrated that, even using a single antibody-sensitized target cell, the apparent identity of the effector cell may vary by altering the concentration of the antibodies on the target cells, a subtle change in the assay procedure.

The results indicate that, with the anti-target cell antiserum used at an optimal sensitizing concentration (10^{-3} dilution), removal of monocytes from the human mononuclear cell preparation did not diminish the lytic activity of the remaining cells except at low effector to target cell ratios, thus appearing to deny a role in ADCC cytolysis to the circulating human monocyte. Both lymphocytes and neutrophils were consistently able to induce marked lysis of optimally sensitized ChRBC target cells.

A consistent but disconcerting finding is that the monocytes appear to be the sole effector cells when the target cells are sensitized with the antiserum in a sub-optimal sensitizing concentration (10^{-4}

dilution). These data indicate that the ADCC activity of the monocytes may be masked by that of the lymphocytes when the target cells are optimally sensitized with antibodies. However, as the number of antibody molecules per target cell is decreased, the monocyte asserts itself as the pre-eminent cytotoxic cell possibly due to:

(i) its greater affinity for the Fc region of the antibody molecule as compared to that of the lymphocyte or neutrophil, and/or

(ii) its activation following interaction with a single target-cell fixed antibody while the effector lymphocyte or neutrophil may require interaction with two or more adjacent target-cell fixed antibody molecules to become activated.

It is therefore possible that the utilization of a weak anti-target cell antiserum, even when used in the undiluted state in the ADCC assay, may implicate only the monocytes as the cytotoxic cells. On the other hand, a second antiserum may be a strong antiserum and its utilization in the ADCC assay may implicate the lymphocytes and neutrophils to the apparent exclusion of the monocytes. Thus, the concentration of anti-target cell antibodies in the antiserum is a factor in the identification of the effector cells.

This finding may account for some of the controversy surrounding the identity of the effector cells. It is imperative that the target cells be incubated with the anti-target cell antiserum in varying dilutions before advocating a role only for the lymphocyte and/or the monocyte and/or the neutrophil in the ADCC cytolytic reaction.

The demonstration by other investigators (Gale and Zighelboim, 1974, 1975; MacDonald et al, 1975; Hunninghake and Fauci, 1976; Nelson et al, 1976; Zighelboim et al, 1976; Clark and Klebanoff, 1977) and confirmed in this communication that neutrophils mediate the ADCC reaction carries implications which cannot be ignored. The participation of the neutrophil in the ADCC cytolytic reaction is a specific one since the target cells are not lysed in the absence of anti-target cell antibodies. Furthermore, the neutrophil-mediated ADCC reaction is inhibited by soluble aggregates of human gamma-globulin much in the same manner as is the mononuclear cell-mediated ADCC reaction (unpublished results). It must therefore be inferred that the neutrophil effector cell, like the mononuclear effector cell, expresses its activity following interaction with the Fc region of the target cell-localized antibody molecule. The neutrophil should therefore be considered to have a specific immunological role

in addition to a non-specific phagocytic one.

It remains to be determined whether subclasses of neutrophils and monocytes can be distinguished on the basis of surface-bearing Fc receptors and whether the presence and functional state of the Fc-receptor-bearing lymphocytes, monocytes and neutrophils correlate with the clinical condition under investigation and with the immunocompetent (or immunoincompetent) state.

Abbreviations: NOCC, naturally occurring cell-mediated cytotoxicity; ChRBC, chicken erythrocytes; RACE, rabbit anti-ChRBC antiserum; HBSS, Hanks's balanced salt solution; culture medium, M199 fortified with penicillin (100 units/ml), streptomycin (100 μg/ml) and FBS to a concentration of 6 percent; FBS, fetal bovine serum.

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ABSTRACT

Rabbit peripheral blood leukocytes and cells of various lymphoid tissues (thymus, bone marrow, spleen, appendix, sacculus rotundus, Peyer's patches, mesenteric and popliteal lymph nodes) were investigated for their ability to effect antibody-dependent cell-mediated cytotoxicity (ADCC) of rabbit antibody-coated ^{51}chromium-radiolabelled (^{51}Cr) chicken erythrocyte (ChRBC) target cells. Rabbit white blood cells (WBC), spleen and bone marrow cells demonstrate ADCC cytotoxic activity in the absence of a prolonged latent period. Appendix and sacculus rotundus cells attain cytotoxic activity only after 48 hours in culture, whether or not they are co-cultured with the rabbit antibody-coated ChRBC target cells whereas Peyer's patches, thymus and lymph node cells demonstrate a delayed onset of cytotoxic activity only if they are co-cultured with the target cells for the entire culture period. The bone marrow and thymus ADCC cytotoxic effector cells appear to be non-phagocytic whereas those of the spleen, mesenteric lymph nodes and WBC appear to be totally phagocytic. The cells of the appendix appear to be both non-phagocytic as well as phagocytic. The ADCC activity of the bone marrow

and thymus cells is inhibited only marginally by soluble aggregates of rabbit gamma-globulin (RAGG) whereas that exhibited by WBC, spleen and mesenteric lymph node cells is almost totally inhibited. The ADCC cytotoxic activity of appendix cells was inconsistently inhibited by RAGG.

Alteration of the lymphoid tissue distribution of ADCC cytotoxic effector cells was not observed following the induction of a humoral immune response by intravenous inoculation with sheep erythrocytes (SRBC) in saline or by unilateral subcutaneous (footpad) inoculation with SRBC in saline. The same was also true for rabbits undergoing a cell-mediated immune response following unilateral subcutaneous immunization (footpad) with SRBC in complete Freund's adjuvant (CFA). ADCC cytotoxic effector cells were detected in skin challenge sites undergoing a delayed hypersensitivity reaction to SRBC in rabbits previously immunized by the intramuscular inoculation of SRBC in CFA.