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The Immunotoxic Effects of Aldicarb

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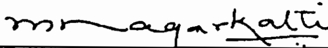

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(ABSTRACT)

In the current studies the effects of administration of 0.1 to 1000 ppb of aldicarb, a carbamate pesticide, on the immune system of C3H mice were investigated. It was observed that aldicarb caused significant immunomodulation of macrophage functions analyzed in a variety of different systems. Initially it was found that aldicarb decreased the stimulatory functions of the macrophages as studied by decreased capacity to stimulate normal autoreactive T cells in the SMLR. This decreased stimulatory activity of the macrophages was found not to be due decrease in the expression of class II MHC-antigens (Ia molecules) nor was it due to the generation of any suppressor macrophages acting to down regulate the immune response. Further investigations revealed that the decreased stimulatory activity of the macrophages correlated with decreased IL-1 production/signal to the T cells by the macrophages. It was also evident that aldicarb did not affect the T cell functions directly. Thus, T cells from aldicarb-treated mice when studied in the SMLR and AlloMLR or when stimulated with ConA or anti-CD3 mAbs, in the presence of normal macrophages, demonstrated normal responses. In contrast, normal T cells exhibited decreased responsiveness in the presence of aldicarb-treated macrophages. The fact that aldicarb did not affect the T cell functions directly was also evident by the fact that aldicarb-treated T cells could respond normally to stimulation with PMA + Ca^{2+} ionophore, a response which is independent of accessory cells. The aldicarb-treated macrophages also exhibited decreased capacity to process and present the antigen, conalbumin, to the T helper cell clone D10.G4. When the mechanism of aldicarb induced defect was investigated, it was observed that aldicarb-treated macrophages produced decreased amounts of IL-1 which was also confirmed by complete reconstitution of the response following addition of exogenous IL-1. With this in mind, macrophage functions in a

number of other systems were examined and demonstrated that aldicarb-treatment also suppressed the macrophage-mediated cytotoxicity of tumor cells, but failed to inhibit the NK cell-mediated cytotoxicity of tumor cells.

Together, these studies suggest that aldicarb selectively affects the macrophage but not NK or T cell functions directly. However, since macrophages play an important role as accessory cells in T cell-mediated responses, it is likely that aldicarb indirectly will also affect the T cell responses.

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Table of Contents

1.0 GENERAL INTRODUCTION AND REVIEW OF LITERATURE 1

1.1 Immune System 1

1.2 Xenobiotics That Modulate the Immune System 6

1.3 Aldicarb 8

1.4 Specific Aims 10

2.0 EFFECT OF ALDICARB ON THE SYNGENEIC MIXED LYMPHOCYTE REACTION 13

2.1 Introduction 13

2.2 Experimental Procedures 15

2.2.1 Mice 15

2.2.2 Aldicarb 15

2.2.3 Macrophage preparation 16

2.2.4 $CD4^+$ T cell Preparation 17

2.2.5 Effects of aldicarb on T cells in mixed lymphocyte reactions 18

2.2.6 Effects of aldicarb on the stimulatory activity of macrophage 19

2.2.7 Fluorescent staining of macrophages for Ia expression 19

2.2.8 Flow cytometry 20

2.2.9 Statistical Analysis 21
2.3 Results 21
2.3.1 Aldicarb treatment decreases the stimulatory activity of macrophages in the SMLR 21
2.3.2 Mechanism of aldicarb-induced suppression of macrophage function 29
2.3.3 Aldicarb treatment does not affect the T cell responses in SMLR and AlloMLR 33
2.4 Discussion 36
2.5 Summary 38

3.0 STUDIES ON EFFECT OF ALDICARB ON MACROPHAGE AND NK CELL ACTIVITY ... 40
3.1 Introduction 40
3.2 Experimental Procedures 42
3.2.1 General Procedures 42
3.2.2 ADCC Assay 42
3.2.3 NK Cell Preparation 43
3.2.4 Tumor Target Cell Preparation 43
3.2.5 Chromium Release Assay 44
3.2.6 Statistical Analysis 44
3.3 Results 45
3.3.1 Aldicarb treatment decreases the activity of macrophages in the macrophage-mediated antibody-dependent cytotoxicity of tumor cells, but does not effect NK cell-mediated cytotoxicity of tumor cells 45
3.4 Discussion 50
3.5 Summary 52

4.0 IMMUNOSUPPRESSION BY ALDICARB OF T CELL RESPONSES TO ANTIGEN-SPECIFIC AND POLYCLONAL STIMULI RESULTS FROM DEFECTIVE IL-1 PRODUCTION BY THE MACROPHAGES 53
4.1 Introduction 53

4.2 Experimental Procedures 54
4.2.1 Mice 54
4.2.2 Aldicarb 55
4.2.3 Monoclonal antibodies (mAb) 55
4.2.4 Purification of T cells and macrophages 56
4.2.5 Responsiveness to Con A 57
4.2.6 Responsiveness to anti-CD3 mAb 57
4.2.7 T cell responsiveness to PMA and calcium ionophore 58
4.2.8 Induction of IL-1 58
4.2.9 Detection of IL-1 59
4.2.10 Antigen-presentation by macrophages to the T helper cells 59
4.2.11 Statistical analysis of the data 60
4.3 Results 60
4.3.1 Responsiveness of spleen cells and purified T cells to Con A in aldicarb-treated mice 60
4.3.2 Responsiveness of spleen cells and purified T cells to anti-CD3 mAb 64
4.3.3 Responsiveness of T cells to PMA and calcium ionophore 64
4.3.4 Antigen-processing and presentation by aldicarb-treated macrophages to T helper cells 66
4.3.5 Measurement of IL-1 produced by aldicarb-treated macrophages 69
4.3.6 Reconstitution of the defective D10.G4 T cell responses with exogenous IL-1 69
4.4 Discussion 71
4.5 Summary 76

LITERATURE CITED **78**

APPENDIX - Abbreviations **86**

VITA: **87**

List of Illustrations

Figure 1. Kinetics of SMLR using aldicarb treated macrophages 23

Figure 2. Decreased stimulatory activity of macrophages from aldicarb treated mice in the SMLR using various effector:stimulator cell densities 24

Figure 3. Stimulatory activity of macrophages in the SMLR from mice treated with varying concentrations of aldicarb 27

Figure 4. Flowcytometric analysis of Ia antigen expression by macrophages in aldicarb treated mice 31

Figure 5. Effect of aldicarb treatment on responder T cells in the SMLR and AlloMLR . 35

Figure 6. Antibody-dependent macrophage-mediated cytotoxicity in mice treated with aldicarb 46

Figure 7. NK cell-mediated cytotoxicity in aldicarb treated mice 47

Figure 8. Antibody-dependent macrophage-mediated cytotoxicity in mice treated with a single dose of aldicarb 48

Figure 9. Responsiveness of whole spleen cells and purified T cells from control and aldicarb-treated mice to Con A 62

Figure 10. Responsiveness of purified T cells to Con A in the presence of accessory macrophages 63

Figure 11. Responsiveness of whole spleen cells and purified T cells to stimulation with anti-CD3 mAbs 65

Figure 12. Responsiveness of purified T cells from aldicarb-treated mice to stimulation with PMA and calcium ionophore 67

Figure 13. Antigen-presentation by macrophages from aldicarb-treated mice 68

Figure 14. Measurement of IL-1 production by macrophages from aldicarb-treated mice 70

Figure 15. Reconstitution of defective macrophage function in aldicarb-treated mice using exogenous rIL-1 72

List of Tables

Table 1. Decreased Capacity of Macrophages From Aldicarb Treated Mice to Stimulate $CD4^+$ T cells Purified Without Using Nylon Wool Treatment 26

Table 2. Stimulatory activity of macrophages in the SMLR from mice treated with varying concentrations of aldicarb 28

Table 3. Decreased Capacity of Aldicarb Treated Macrophages to Stimulate in the SMLR is not Related to Presence of Suppressor Macrophages 30

Table 4. Percent Ia^+ cells and density of Ia antigen expression in macrophage population in mice treated with varying doses of aldicarb 32

Table 5. Reconstitution of defective macrophage function with IL-1 34

1.0 GENERAL INTRODUCTION AND REVIEW OF LITERATURE

1.1 Immune System

The branch of biology involved with the study of defense reactions is called immunology. The word "immunology" derives from the Latin immunis , meaning "free of burden", where the burden may be a tax imposed by Caesar, a law affecting an individual, or a disease (Klein, 1982). Thus people resistant to certain diseases are said to be "immune" to them and this specific resistance to a disease is called immunity.

Over the years the definition of immunology has changed significantly although the basic functions of the immune system still remain the same and that is to guard against disease-causing organisms. With the realization that the cells involved in the immune system

(mainly the lymphocytes and macrophages) have to communicate and cooperate with one another in order to recognize foreign molecules, immunology is defined as a science of self-nonself discrimination (Klein, 1982) and the immune system as a complex network of interacting cells (Jerne, 1984).

Due to the variety of responses available to eliminate pathogens, attempts have been made to categorize the immune responses. Based on the type of response exhibited by the immune system, it has been subdivided into two broad categories of specific and nonspecific responses. The defense systems found in invertebrates are more primitive involving chiefly, phagocytic cells such as macrophages and neutrophils. Such cells also play an important role in defending vertebrates against infections, but they are part of a much more complex immune system involving lymphocytes which exhibit two important features — specificity and memory. Thus, lymphocytes are genetically predetermined to recognize and interact with a specific foreign molecule (antigen) and after the initial encounter, they exhibit a memory response characterized by a strong secondary response to the same antigen enabling the host to eliminate the antigen more efficiently (Klein, 1982).

The specific immune responses can be subdivided into two distinct, but interrelated systems called humoral and cell-mediated immunity. These two systems of specific immunity are based on two critically important subsets of lymphocytes with entirely different functions. The humoral immunity is mediated by antibodies (immunoglobulins) which are proteins produced by a distinct subset of lymphocytes called B cells. The B-cell receptor for antigen (immunoglobulin) can directly bind to soluble antigen in a manner similar to that of many well-characterized receptor-ligand systems. The antibodies produced by the B cells circulate in the blood stream and permeate into the body fluids where they bind to the foreign antigen that induced them. Such a binding neutralizes viruses and inactivates bacterial toxins. The antibody binding also helps the phagocytic cell to ingest the microorganisms easily and to activate a system of blood proteins called complement to kill the microorganisms. Humoral

immunity is most effective in eliminating extracellular pathogens. This ability is based on the activity of a group of bursal derived cells called B lymphocytes that operate as antibody producing cells. This type of immunity is transferable by serum and includes both classical antibody-mediated protective immunity, which combats viruses and bacteria; as well as immediate hypersensitivity reactions, commonly called allergies (Vos, 1977).

In contrast, the cell-mediated immunity is mediated by specialized cells called T lymphocytes. These cells mature and differentiate in the thymus and can recognize an antigen only in association with cell surface molecules encoded by the major histocompatibility complex (MHC). This requirement, known as MHC-restriction, ensures that T cell activation or effector function occurs only in the correct cellular context. Antigen-specific T-cell activation thus results from the formation of a ternary complex involving the T-cell receptor (TcR), nominal antigen, and class-I or class-II encoded MHC molecules (Livingstone and Fathman, 1987; Allison and Lanier, 1987).

As a whole, the mechanisms of cell-mediated immunity act to eliminate pathogens that have taken up intracellular residence as well as the elimination of some types of cancerous cells. (Bick, 1982). Included in cell-mediated immunity are the following processes: Protective immunity effective against fungi, viruses and a number of bacteria; Delayed type hypersensitivity (tuberculin hypersensitivity or contact hypersensitivity); the rejection of tumors and foreign tissues such as transplants (allografts); and graft vs. host diseases (Vos, 1977).

The T cell repertoire can be further subdivided into three functionally distinct populations of T cells based on the phenotype and functions. These subclasses of T cells include cells capable of helper (Th), cytotoxic (Tc) and suppressor (Ts) functions. Each functional class has been demonstrated to employ a distinct set of cellular products in the effector function which can be distinguished from the structure used for antigen recognition (Allison and Lanier, 1987).

The Th cells express a distinct set of cluster of differentiation (CD) antigens called CD4 on the surface of the cells which has been used for their identification and purification (Ahmed & Smith, 1982). These Th cells assist and amplify the immune response by direct or by indirect interactions with all other populations of immune cells. The Th cells recognize and interact with antigen in association with class II MHC molecules on the surface of macrophages or other antigen presenting cells (APC) such as B cells. Following interaction with APC, the Th cells get activated and secrete a variety of protein molecules which help to regulate the functions of other cells (Singer & Hodes, 1983). Included in these functions are the ability to assist B cells to make Ab, inducing cytotoxic T cell (CTL or Tc)-proliferation, and activating the macrophages. Th cells have recently been divided into two broad functional categories, Th1 and Th2 cells. The Th1 cells produce IL-2 and IFN-γ upon stimulation but fail to produce IL-4; in contrast, Th2 cells produce IL-4 but fail to produce IL-2 or IFN-γ (Paul and Ohara, 1987; Mosmann & Coffman, 1989).

In contrast to the Th cells, the cytotoxic T lymphocytes (CTL) primarily protect the host against viral infection and also against cancer. Because viruses proliferate inside the cell, they cannot be attacked by the antibodies. Thus, the CTL recognize the viral antigen processed within an infected cell in association with the class I MHC molecules and kill the infected cells thereby preventing the virus from spreading to other normal cells.

The third subset of T cells called T suppressor cells are not very well characterized, but have been shown to down regulate the immune response. This Ts cell population also carries I-J epitopes, the nature of which is highly controversial. As such, these Ts cells are very important in assuring that the immune system returns to a resting state following up regulation (Murphy, 1987; Dorf & Benaceraff, 1984). Their presence has been clearly shown in certain infections and development of cancer to the disadvantage of the host (Nagarkatti & Kaplan, 1985).

The majority of the T cells have receptors consisting of α and β polypeptide chains. In addition, a small percentage of T cells have been shown to have a distinct type of T cell receptor composed of γ and δ chains. The functions of such cells is not well understood.

In addition to the T and B lymphocytes, a distinct set of cells called natural killer (NK) cells have been described which can recognize and kill tumor cells (Hercend & Schmidt, 1988). Such cells are believed to play an important role in defending the host against cancer. Also, the lymphokines secreted by the T cells can activate some cells to cause lysis of tumor cells. Such cells have been called lymphokine activated killer (LAK) cells. The NK and LAK cells are nonspecific and are MHC-unrestricted (Moretta, 1986).

The antigens recognized by all T cells need to be processed within specialized cells called antigen-presenting cells (APC). The APC that present the antigen to the Th cells express class II MHC encoded antigens called Ia antigens. Such cells include the macrophages, B cells, dendritic cells and Langerhans cells in the skin. The APC phagocytose or endocytoze foreign antigen and partially degrade in the acidic environment of endolysosomes. The cleaved peptides then associate with class-II MHC encoded molecules and are expressed on the cell surface. These events collectively are called antigen-processing (Berzofsky et al., 1988). The APC also secrete several interleukins such as IL-1 and IL-6 which along with the processed antigen and the associated class II MHC molecule can activate the Th cells, a phenomenon called antigen-presentation. The B cells have been shown to be highly efficient in presenting even minute quantities of the antigen since they can internalize the antigen through their Ig-receptor (Pierce et al., 1988). In contrast to the class II restricted antigen-presentation, the class I restricted antigens are derived from proteins, such as viral antigens, that are synthesized within the presenting cell. Many of these antigens are cytosolic proteins which have recently been shown to be processed in the endoplasmic reticulum (ER) and which get

associated with nascent class I molecules before they leave the trans-Golgi complex (Yewdell & Bennink, 1989).

As should be evident from the preceding discussion, the immune system is a very highly interrelated and complex system involving a variety of cells and any alteration in one component of the immune system can lead to changes in all branches of the immune response. Some alterations, such as suppression in Th cell function can lead to effects on virtually every part of the immune system as seen in AIDS patients, leading to increased duration or severity in the frequency of disease caused by a pathogen (Bick, 1982).

1.2 Xenobiotics That Modulate the Immune System

Immunotoxicology examines alterations or modulations of the immune response in a positive or negative direction. In recent years toxicologists have become aware that several environmental pollutants can alter the immune system (Greenlee, 1985; Ward, Murray & Dean, 1985; Vos, Krajnc & Wester, 1985). Although immunotoxicologists do examine upregulations of the immune response following chemical exposure, down regulations of the immune response by xenobiotics are much more prevalent (Spreafico & Vecchi, 1984). Also, most investigators use the term "immunotoxic" to denote detrimental effects on the immune system caused by a compound. Although most xenobiotics investigated cause immunodepression, several non-therapeutic compounds have been identified that augment immune expression following exposure, including vinyl chloride, α-thioglycolate and propyleneglycol (Spreafico et al., 1984). Several compounds have been identified as being immunotoxic. These include herbicides and heavy metals as well as pesticides such as DDT and Lindane. Several chemicals that were previously thought to be innocuous have also been found to act in an

immunosuppressive fashion, these include food additives such as saccharin and vanallin and drugs such as chloroquine (Vos, 1977; Hadden, 1985).

All immunotoxic compounds are thought to mediate their effects through one or more mechanisms (Vos, 1977; Hadden, 1985). These mechanisms include both direct as well as indirect effects. In terms of direct immunomodulatory effects it is known that some immunotoxic compounds are cytotoxic to lymphoid organs such as bone marrow, thymus, lymph nodes and spleen. In addition, these compounds can destroy the cells produced by these organs, and cytotoxicity of several immunotoxic compounds has been demonstrated on stem cells, T cells, B cells, macrophages and other circulating immune cells. Immunomodulators also can have an effect on the antigen recognition, phagocytosis, processing and presentation by antigen presenting cells, as well as causing alterations in the T and B cell receptor functions. Immunomodulators can also affect the production, release or action of mediators such as lymphokines, interleukins and interferrons, by all cells of the immune system. As a result, these and other effects caused by immunomodulators can act to upset immunoregulatory circuits regulated by helper and suppressor T cells (Nagarkatti & Nagarkatti, 1987).

Immunomodulators can also indirectly mediate their effects through the hormones which influence the immune response such as thyroid hormones, corticosterone, gonadotropins and thymic hormones, as well as by causing nutritional deficiencies, e.g. proteins, vitamins, minerals, etc. (Vos, 1977).

1.3 Aldicarb

Aldicarb [(2-methyl-2-(methylthio) propanal O-[methylamino carbaryl] oxime] is a highly effective soil incorporated systemic pesticide that is used against certain mites, nematodes and insects on several commercially important agricultural crops throughout the world (Olson et al., 1986). It is known that aldicarb acts as an acetylcholinesterase inhibitor and has the highest mammalian toxicity of any pesticide registered for use by the Environmental Protection Agency (Worthing & Walker, 1983). This high level of toxicity, coupled with the discovery of aldicarb at levels exceeding 100 parts per billion (ppb) in potable water sources in 11 states, results in a serious threat to mammalian health if not properly controlled (Milex & Delfino, 1985; MMWR 1986; Pant, Tewavir & Gill, 1987; U.S. EPA 1984; Harkin et al., 1984; Jones & Marquardt, 1987; Zake et al., 1982; McWilliams, 1984). Thus, aldicarb has became the first pesticide to be regulated by the Environmental Protection Agency (EPA) to protect the drinking water from aldicarb contamination (Sun, 1988).

In addition, aldicarb is credited with causing the largest North American outbreak of food borne pesticide illness as well as for causing other outbreaks of human illness (Olson et al., 1987; MMWR 1986, Green et al., 1987). A study examining occupational illness associated with pesticide exposure, conducted in California over the period of 1982 to 1985, revealed that aldicarb was responsible for more illnesses per usage than any carbamate pesticide applied over the same period (Brown et al., 1989). These recent examples of the possible detrimental effects of aldicarb have lead to increased governmental and community awareness and concern over the effects of aldicarb on mammalian health in general and human life in specific.

It has been reported that aldicarb alters a number of hematological and biochemical parameters (Pant et al., 1987). In mammals, aldicarb is metabolized rapidly by the liver and

does not appear to be stored in body tissues or fluids. It is excreted primarily by the kidneys (90-95% of total excretion), and to a smaller extent by the intestinal route (Olson et al., 1987). It is highly toxic by the oral, dermal and inhalation routes of exposure and toxic exposure usually leads to uncontrolled movement, paralysis and death (Marshall, 1985). The primary mode of action of aldicarb is via the inhibition of cholinesterase activity and is extremely efficient in this aspect (Casida, 1963; Jackson & Goldman, 1986). This efficiency is due to the fact that it was specifically designed to resemble O-acetylcholine structurally (Worthing & Walker, 1983). Toxicological studies of aldicarb have found it to have an acute oral LD_{50} for rats and mice of 0.6-1.0 mg/kg body weight (U.S. EPA 1984, Worthing & Walker, 1983). It has also been classified as a neurotoxin, with no carcinogenic, reproductive, mutagenic or teratogenic properties reported (U. S. EPA, 1984). In spite of a large amount of toxicological data relating to various impacts of aldicarb on mammalian health, a review of the literature reveals a deficiency of information about the effects of aldicarb on the mammalian immune system.

In terms of data on the specific effects on the mammalian immune response, aldicarb has been shown to significantly suppress the splenic plaque forming response to sheep red blood cells at orally administered concentrations as low as 1 ppb (Olson et al., 1987). In contrast to these findings, Thomas et al. (1987) found that aldicarb had no significant effect on T and B cell responses to mitogens and on T cells responding in the mixed-lymphocyte culture. Later work by Thomas and Ratajczak (1988) also demonstrated that aldicarb does not affect the IgM anti-SRBC B cell response in Swiss-Webster or $B_6C_3F_1$ mice. In this same study they also demonstrated that aldicarb had no immunomodulatory effect on splenic B cell responses to bacterial lipopolysaccharide (LPS) nor did it affect the ability of T cells to respond in AlloMLR assays (Thomas & Ratajczak, 1988).

It has also been shown that aldicarb causes a significant increase in frequency of sister chromatid exchange events (Cid & Matos, 1984) and chromatid and chromosome breaks in

cultured human lymphocytes (Cid & Matos, 1987). An environmentally common derivative of aldicarb, nitroso-aldicarb, has also been shown to increase the incidence of sister chromatid exchange events in a dose dependent manner as well as causing cell-cycle delays in human peripheral blood lymphocytes (Cid, Loria & Matos, 1988). Studies in humans demonstrated that aldicarb may alter responsiveness to candida antigen and alter the T cell subset ratios (Fiore et al., 1986). Beyond this, little is known about the effects and mechanisms of action of aldicarb on various cells of the immune response.

At present there is still a great need for data on the effects of aldicarb on the immune system. These studies are essential due to the fact that aldicarb has been detected at levels exceeding 100 ppb in potable water in several states of the U. S. and any alteration in the immune response caused by environmental contaminants, such as aldicarb, can lead to increased susceptibility to infections, cancer and the development of autoimmune disorders (Reviewed by Nagarkatti & Nagarkatti, 1987).

1.4 Specific Aims

Various immune assay systems measure different levels of cell involvement and complexity in response to a stimulus. As such, it is essential to use assays that measure the overall immune responses as well as those which can delineate the mechanisms of immunomodulation at cellular and molecular levels.

In the present study the effects of aldicarb on the immune system of C3H mice were investigated at concentrations of 0.1 to 1000 ppb. The specific aims were to investigate:

1. The non-specific and specific immune components of the C3H mice injected with single or multiple doses of 0.1 to 1000 ppb of aldicarb intraperitoneally.

2. The specific immune components included the T cells. Of particular interest in this study were the effects of aldicarb on autoreactive T cells which respond to self-class II MHC encoded molecules expressed on APC and on other T cells such as the alloreactive T cells.

3. The nonspecific immune components included macrophage-mediated cytotoxicity of tumor cells and the capacity of NK cells to kill NK-sensitive tumor targets.

4. To activate aldicarb treated T cells using a number of different stimuli, such as, T cell mitogens (Con A), anti-CD3 mAbs or using phorbol myristate acetate (PMA) and Ca^{2+} ionophore and studying their capacity to synthesize DNA.

5. Since T cells cannot respond directly to mitogens or antigens in the absence of accessory cell help, to investigate the effect of aldicarb on the accessory functions of the macrophages.

6. To delineate the accessory functions of macrophages by studying their capacity to produce soluble factors which regulate the T cell functions, such as Interleukin-1; to determine whether aldicarb could induce regulatory suppressor macrophages and to find out whether aldicarb inhibited the expression of class-II MHC-encoded Ia antigens on macrophages.

7. If aldicarb suppressed the accessory functions of the macrophages, to investigate whether such a defect could be reconstituted by using soluble mediators such as interleukins.

In summary the main purpose of this study was to investigate whether aldicarb caused immunomodulation and if so, to identify the molecular and cellular basis of this defect.

2.0 EFFECT OF ALDICARB ON THE SYNGENEIC MIXED LYMPHOCYTE REACTION

2.1 Introduction

The syngeneic mixed lymphocyte reaction (SMLR) is usually defined as a T-cell-proliferative response to *in vitro* stimulation with syngeneic non-T cells bearing Ia antigenic determinants (Weksler, 1981). The responding T cells in the SMLR are $CD4^{+}$ Th cells called autoreactive T cells and the stimulator cells include Ia^{+} B cells, macrophages and dendritic cells.

Autoreactive T cell clones have been isolated from normal, unimmunized mice and used to demonstrate that these cells support cytotoxic T lymphocyte (CTL) precursors differentiating into CTL, enhance antigen-specific T helper cell responses, and induce proliferation and differentiation of resting, unprimed B cells (Nagarkatti, Snow & Kaplan, 1985). Furthermore,

it has also been observed that autoreactive T cells have the capacity to directly stimulate naive $CD4^+$ T cells (Nagarkatti & Kaplan, 1985), thereby suggesting that autoreactive T cells may actively participate in a T-T network proposed by Jerne (1984). There is also ample evidence demonstrating that autoreactive T cells generated in a primary SMLR or autoreactive T cell clones isolated from immunized mice perform a variety of regulatory functions *in vitro*, such as help/amplification (Finnegan et al., 1984; Clayberger et al., 1984; Zauderer et al., 1984; Wolos et al., 1982; Hausman et al., 1979; Chiorazzi et al., 1979; Kotani et al., 1986; Quintans et al., 1986), suppression (Clayberger et al., 1984; Smith et al., 1979; Innes et al., 1979; Kotani et al., 1986; Quintans et al., 1986), or cytotoxicity (Tomonari, 1980). Alternatively, it has been suggested that autoreactive T cells may constitute the precursors of antigen-specific T cells (Dos Reis et al., 1981; Rock et al., 1984). These and the findings that the SMLR has been shown to be defective in patients with various lymphoproliferative and autoimmune diseases, and in autoimmune-susceptible and aged mice (Udhayakumar et al., 1988; Smith et al., 1982), have suggested that autoreactive T cells may perform important immunoregulatory functions and may be essential for initiating an immune response or for maintaining normal immune system homeostasis. (Nagarkatti et al., 1988).

These studies as well as others prompted us to examine the effects of aldicarb on autoreactive T cell responses at both the responder (autoreactive T cell) and the stimulator (macrophage) cell levels to specifically determine the mechanism of immunomodulation.

2.2 Experimental Procedures

2.2.1 Mice

Six to eight week old female C3H/He mice were obtained from NIH (Bethesda, Md.) and housed in groups of five animals in polyethylene cages (Animal Storage Isolators, Nu Aire Inc., Plymouth, MN) containing wood shavings. The cages were shelved in a laminar flow cabinet that provided a sterile environment. The room where the animals were kept were florescently lighted and maintained on a twelve hour light/dark cycle. Room temperatures over the experimental period were maintained at 74± 2 F. The animals were maintained on a animal diet (Purina 5001) and water supplied ad libitum.

2.2.2 Aldicarb

All aldicarb solutions were prepared using high purity aldicarb (Chem Services Ps-734, West Chester, PA) dissolved in phosphate buffered saline (PBS), pH 7.2, and stored at 4° C in the dark. The solutions were freshly prepared every three months. Although the stability of aldicarb solutions at or near neutral pH is well established (Olson et al., 1986) the stability of the solutions were monitored throughout the course of the experiments using HPLC analysis. Briefly, the technique involved analysis using a Beckman 344 system with a 3μ ultra phase column (4.6 x 7 cm). The compound was eluted from the column using 45% methanol: water solvent with a flow rate of 0.5 ml/min. The compound was detected at 210 nm with a retention

time of 5.5 ± 0.09 min. Under these conditions the purity of the aldicarb was established to be 99.4% over the experimental period. Aldicarb solutions were made by serial dilution from a stock concentration of 1000 ppb. And five doses were used in this study: 0.1, 1, 10, 100, and 1000 ppb. All stock solutions and dilutions were prepared with PBS which had been adjusted to a pH of 7.2. The PBS was prepared with Milli-Q purified distilled water and sterilized. Intraperitoneal (ip) injections were given to groups of two to ten animals at a volume of 0.1 ml at one of the five previously mentioned concentrations. Control animals received sterile PBS injections of the same volume and via the same route of administration. Following a period of exposure of either 24 hours or seven days, the test animals were sacrificed by cervical dislocation. General anesthetics were not used in these experiments and all procedures involving animal handling were conducted in a manner so as to minimize stress to the animals.

2.2.3 Macrophage preparation

Macrophages were enriched from the spleens by separating splenic adherent cells (SAC) as described elsewhere (Nagarkatti, Snow & Kaplan, 1985). Whole spleens were aseptically removed from aldicarb or PBS injected animals and immediately transferred to 'complete' RPMI 1640 medium containing 10% FCS (GIBCO Laboratories, Grand Island, NY), 10 mM HEPES buffer (Sigma Chem., St. Louis, Mo.), 1 mM glutamine, 40 μg/ml gentamicin sulfate (GIBCO), and 15 μg/ml 2-mercaptoethanol. Homogenous whole cell suspensions of the splenic tissue were prepared using a lab blender (Stomacher, Tekmar Co., Cincinnati, OH), and subsequently pelleted via centrifugation. The contaminating RBC content of this pellet were removed by lysis in 0.83% ammonium chloride followed by three washing with RPMI. Viable cells were then quantitated by the trypan blue dye exclusion test (Sigma T9520) and plated

onto polystyrene petri plates (Costar 3100, Cambridge, Mass.). Following a 1 hour incubation of these plates in a 37°C, 5% CO_2, humid incubator (Fisher Isotemp 413D) the splenic adherent cells were collected by forceful pipetting, centrifuged, resuspended in 5 ml of media and X-ray irradiated (Minishot II, TFI Corp., Tucker, Ga.) at ~3000 rads and placed on ice. The SAC thus prepared, consisted of > 95% macrophages as determined by non-specific esterase staining and the 5 % contaminating cells consisted mainly of T and B lymphocytes (Nagarkatti, Snow & Kaplan, 1985).

2.2.4 $CD4^+$ T cell Preparation

Single cell suspensions of normal, untreated, C3H splenocytes were obtained, the RBCs lysed and the cells washed three times as detailed previously. These cells were enriched for T-lymphocytes by nylon wool separation as described at length elsewhere (Nagarkatti et al., 1985b). The cell suspension was then centrifuged (1000 rpm for 10 min.) (IEC CRU-5000, IEC, Needham Hts., Mass.) and to the resultant pellet was added appropriate dilutions of monoclonal antibodies 26.7.11s (anti-I-A^k), 3.155 (anti-CD8), J11d (anti-B cell) and 14.4.4 (anti-I-E^k) to effect further purification. The antibodies 26.7.11, J11d and 14.4.4 along with complement were used to eliminate any contaminating Ia^+ B cells or macrophages and 3.155 to eliminate $CD8^+$ T cells. The antibody containing cell suspension was incubated at 4° C for 30 minutes and washed twice. Three mls of complement (Cedarlane CL3051, Hornby, Ontario, Canada), prepared at a final concentration of 1:10 in complete media, was added to the resultant cell pellet and the cells were incubated for an additional 30 minutes at 37° C. The complement at this dilution did not cause any non-specific lysis of cells. Subsequent to this incubation, the cells were pelleted and washed three times, with a final dilution being made to give a volume of 2.0 ml. The $CD4^+$ T cells prepared in this fashion were > 98% pure as

determined by flow cytometric analysis (Nagarkatti, Seth & Nagarkatti, 1988b). In some experiments $CD4^+$ T cells were purified without using nylon wool treatment by first depleting macrophages by plastic adherence followed by treatment with J11d, B220 and anti-I-E^k monoclonal antibodies plus C to deplete the B cells. The $CD8^+$ T cells were depleted as described before using anti-CD8 antibodies and C. The viable cells were purified by centrifugation on ficoll-hypaque gradient.

2.2.5 Effects of aldicarb on T cells in mixed lymphocyte reactions

To study the effects of aldicarb on T cell proliferation, SMLR and allogeneic mixed lymphocyte reaction (AlloMLR) assays were carried out. The mice that were the source of the responder cells were injected with either PBS (control) or with 10 to 1000 ppb of aldicarb, followed by a 24 hour rest period. At the end of the rest period, the spleen cells were isolated and $CD4^+$ T cells prepared as described before. These T cells were next stimulated with irradiated (3000 rad) macrophages from normal untreated C3H mice (SMLR) or with irradiated normal macrophages from C57BL/6 mice (AlloMLR).

Varying numbers of responder and irradiated stimulator cells were cultured in 0.2 ml of complete RPMI medium in 96 well flat bottom plates (Costar 3595) in triplicate at 37° C in a 5% CO_2 incubator. After incubating for various days, the cultures were pulsed with 2 μCi of [^{3}H] thymidine per well during the last 18 hrs. of culture. The cells were collected onto glass fiber filters using a semiautomatic harvester (Skatron 7019, Skatron, Sterling, Va.) and the

amount of radioactivity incorporated into the cells quantitated by scintillation counting (Beta Trac 6895, TM Analytic, Elk Grove Village, Ill.).

2.2.6 Effects of aldicarb on the stimulatory activity of macrophage

To study the effects of aldicarb on macrophage stimulatory activity in SMLR, cultures were established using varying concentrations of responder splenic $CD4^+$ T cells from normal untreated mice and stimulated with irradiated macrophages from PBS-treated (control) or aldicarb-treated C3H mice. Thus, in these assays, the animals serving as a source for macrophages were intraperitoneally injected with varying concentrations of aldicarb or PBS and rested for 24 hours prior to being used in the assay. Thymidine incorporation and quantification of radioactivity were conducted via procedures identical to the methods described above.

2.2.7 Fluorescent staining of macrophages for Ia expression

To examine the effects of aldicarb on cellular Ia expression, flow cytometric analysis of the cell populations were conducted (Nagarkatti, Seth & Nagarkatti, 1988). In these analyses

single cell suspensions from spleens of PBS or aldicarb injected animals were obtained, the red cells were lysed and macrophages were prepared as described before. The cells were washed with PBS containing 0.1 % sodium azide and incubated with monoclonal antibody 14.4.4 (anti-I-E^k) at 4° C for 30 minutes. The cells were washed twice and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG [F(ab')2] for 30 minutes at 4° C, followed by three washings and final dilution to make a suspension of 1 million cells/ ml, at a final volume of 0.5 ml. The negative controls consisted of cells incubated with a non-specific antibody MK-D6 (anti-I-A^d) which was also mouse IgG followed by staining with FITC-labelled anti-mouse IgG.

2.2.8 Flow cytometry

The stained cells were next analyzed using a Epics V, Model 752 (Coulter Electronics, Hileah, Fla.) flow cytometer. The three parameters per cell studied included forward angle light scatter (FALS), 90° light scatter (90LS) and green fluorescence (GFL). Laser excitation was normally 300mW at 488 nm using a 5 watt Innova 90 Argon Laser (Coherent Inc., Palo Alto, Ca.). Data collected, based on 10,000 cells counted per sample, were analyzed with the multiparameter data acquisition and display system (MDADS) and the EASY 88 microcomputer analysis system of Coulter Electronics. FALS were collected linear integral, 90LS, GFL were collected log integral. Histograms showing cell number per channel as a function of fluorescence were collected at a resolution of 256 channels and gated on FALS/90LS and GFL dual parameter histogram 64 x 64 channels resolution defining the cell population of interest.

2.2.9 Statistical Analysis

In all experiments, PEC or spleen cells were pooled from groups of 2 to 10 mice. All assays were performed in triplicate and Δ counts per minute (Δ cpm) and standard errors (S.E.) on these counts were calculated from the data obtained. The Δ cpm represented the proliferative responses of $CD4^+$ T cells to the stimulator cells minus the sum of proliferative responses of the responder $CD4^+$ T cells alone and of the irradiated stimulator cells alone. The various experimental groups were compared with the control group of mice, using Student's t-test. Differences with p values less than 0.05 were considered to be statistically significant. Some data involving multiple experiments using different doses of aldicarb were analyzed using Bonferroni's adjustment in which p values less than 0.01 were considered statistically significant.

2.3 Results

2.3.1 Aldicarb treatment decreases the stimulatory activity of macrophages in the SMLR

Since aldicarb levels found in the drinking water have been reported to be generally less than 100 ppb, studies were conducted to test the effect of aldicarb on T cell and macrophage function at doses significantly lower than this level, but at levels which are still considered to

be environmentally relevant. Groups of mice were injected ip with a single dose of aldicarb at a concentration of 0.1, 1.0, 10, 100 or 1000 ppb. Control mice received PBS and all animals were sacrificed 24 hours after injection to be used in the assay systems. Initial experiments were restricted to studying the effect of aldicarb treatment on the stimulatory abilities of macrophages in the SMLR. First, kinetic assays were conducted to study the peak responses in the SMLR. In this assay, irradiated macrophages from aldicarb treated or untreated (PBS) C3H mice were used as stimulator cells and normal C3H $CD4^+$ T cells as responders. As is evident from Figure 1, when control or aldicarb-treated macrophages were used as stimulators, the responses peaked on day 4. Furthermore, there was a decrease in the stimulatory capacity of aldicarb treated macrophages when compared to the controls on all days tested ($p < 0.05$). Given the fact that the response peaked on day 4, in all subsequent experiments the SMLR was studied on day 4 of incubation.

It was next investigated whether the decreased stimulatory activity of aldicarb treated macrophages was demonstrable at different effector:stimulator cell ratios. In these assays $CD4^+$ responder T cells from normal mice were cultured at one of two concentrations (2×10^5 or 4×10^5/ well) with aldicarb or PBS (control) treated stimulator cells at one of two concentrations (8×10^5 or 4×10^5/ well). The data obtained from these experiments (Figure 2) indicated that aldicarb at 0.1 ppb suppressed stimulatory activity of macrophages in the SMLR at all ratios of stimulator and responder cells examined ($p < 0.05$ at all ratios).

In the above experiments, $CD4^+$ T cells were purified by initially enriching T cells on nylon wool columns. Since this procedure may affect some subsets of T cells which may adhere to the nylon wool, it was investigated whether aldicarb-mediated suppression was demonstrable if T cells were enriched without using nylon wool treatment. In these experiments, spleen cells were depleted of macrophages by plastic adherence and next the B cells were depleted by treatment with anti-B220, J11d and anti-I-E^k monoclonal antibodies plus C. The $CD8^+$ T cells were also depleted by using anti-CD8 antibodies plus C. The $CD4^+$ T cells thus purified were

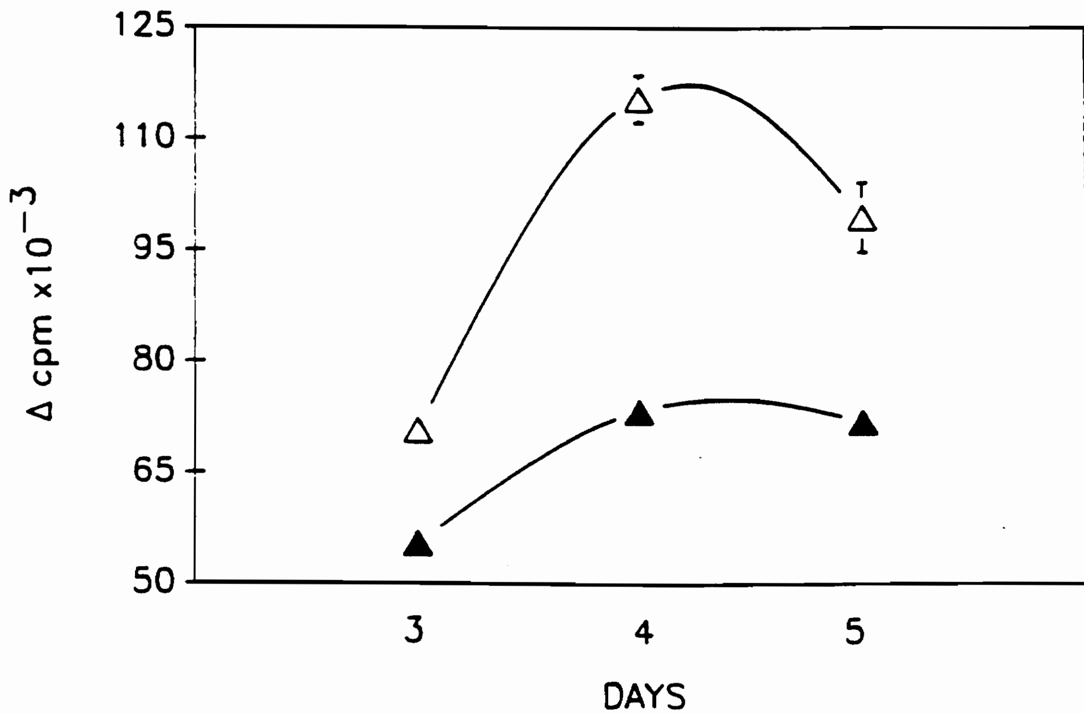

Figure 1. Kinetics of SMLR using aldicarb treated macrophages: Groups of 3 C3H mice were given a single ip injection of either PBS (control) or 10 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and macrophages were separated, irradiated and used as stimulator cells. Responder cells consisted of $CD4^+$ T cells from normal C3H mice. The responder cells (4×10^5/ well) were mixed with either 8×10^5 control (△-△) or 8×10^5 aldicarb treated macrophages (▲-▲). Cell proliferation was measured by the uptake of [^{3}H] thymidine added during the last 18 hours of culture. The assays were terminated on days 3, 4 and 5 and delta cpm values and S.E. of the mean were calculated and depicted.

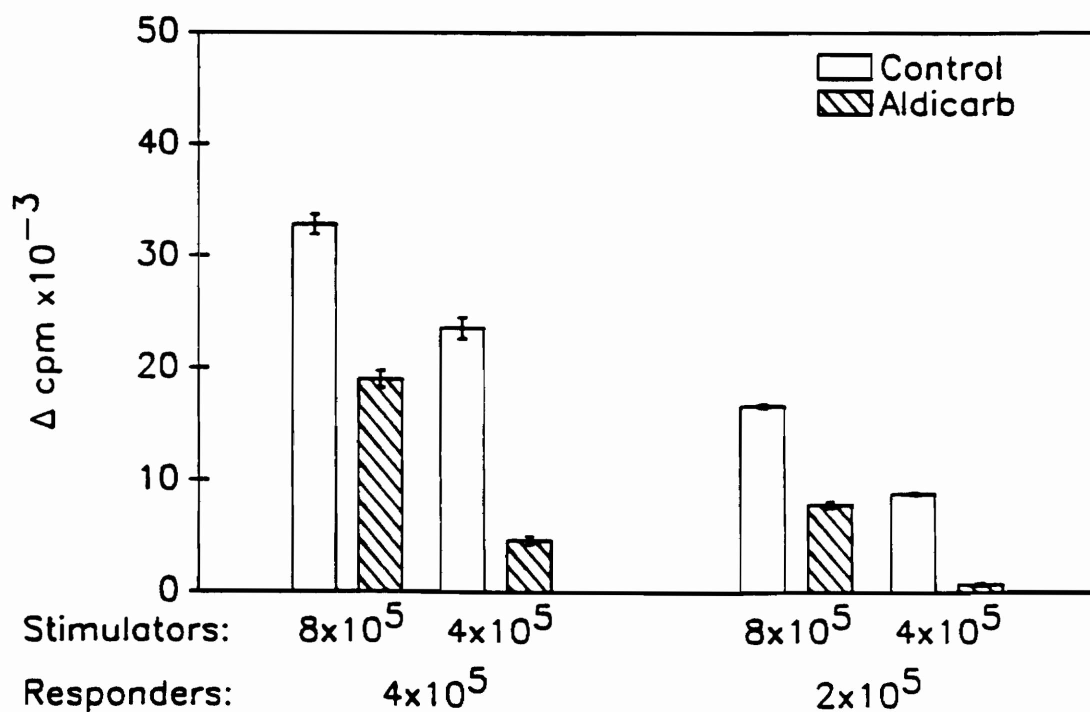

Figure 2. Decreased stimulatory activity of macrophages from aldicarb treated mice in the SMLR using various effector:stimulator cell densities: Groups of C3H mice were given a single ip injection of either PBS (control) or 0.1 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and macrophages separated. Normal $CD4^+$ responder T cells (4×10^5 or 2×10^5/ well) were mixed with either 8×10^5 or 4×10^5 irradiated stimulator cells. Uptake of [^{3}H] thymidine was used to gauge the extent of T cell proliferation as described in Fig. 1. Delta cpm values and S.E. of the mean were calculated. The open vertical bars represent control Δ cpm. and the hatched vertical bars represent experimental (aldicarb treated) Δ cpm values.

tested in SMLR as described before. The data shown in Table 1, suggested that the aldicarb-mediated defect in macrophage function was demonstrable even when the responder T cells were purified without using nylon wool.

To determine if the altered stimulatory activity of the macrophages was dependent on the dose of aldicarb used, several SMLR assays were conducted similar to experiments described in Figure 1, at aldicarb concentrations ranging from 0.1 to 1000 ppb. The percent decrease in the SMLR responses observed using aldicarb treated macrophages when compared to the controls were calculated and plotted in a descending order of suppression. As is evident (Fig. 3 and Table 2), at all concentrations of aldicarb tested and in majority of the assays, aldicarb suppressed the stimulatory activity of macrophages in the SMLR. The percent decrease, however, varied between individual experiments and in some experiments there was indeed an enhancement in the response, the reason for which was not clear. These data were analyzed using Bonferroni's adjustment and the p values obtained for each group were: <0.0005 for 0.1 and 10 ppb of aldicarb treatment and $p<0.0025$ and $P<0.01$ for 100 and 1000 ppb of aldicarb-treatment, respectively. Considering the p values < 0.01 as statistically significant, these data suggested significant suppression in the SMLR assays caused by aldicarb treatment. These data also suggested that, due to varying degrees of suppression observed, one has to perform several experiments before interpreting the immunomodulation caused by aldicarb.

Table 1. Decreased Capacity of Macrophages From Aldicarb Treated Mice to Stimulate CD4$^+$ T cells Purified Without Using Nylon Wool Treatment

Responder Cells (2x10^5)	Stimulator Cells (macrophages) (8x10^5)	SMLR (Cell Proliferation) cpm ± S. E.
CD4$^+$ (Normal)	–	328 ± 50
	PBS treated	23,713 ± 1382
	0.1 ppb Aldicarb	6,647 ± 744
	1 ppb Aldicarb	4,958 ± 1373

T cells Purified Without Using Nylon Wool Treatment CD4$^+$ T cells from C3H mice were purified from normal spleen cells by adhering the cells to plastic plates to remove macrophages followed by treatment with anti-B220, J11d and anti-I-E^k antibodies plus C to deplete B cells and anti-CD8 plus C to deplete CD8$^+$ T cells. The purified CD4$^+$ T cells were incubated with medium alone or with syngeneic irradiated stimulator macrophages from PBS-treated or aldicarb-treated mice. The cultures were incubated for four days. The uptake of ^{3}H-thymidine was used to study the T cell proliferation.

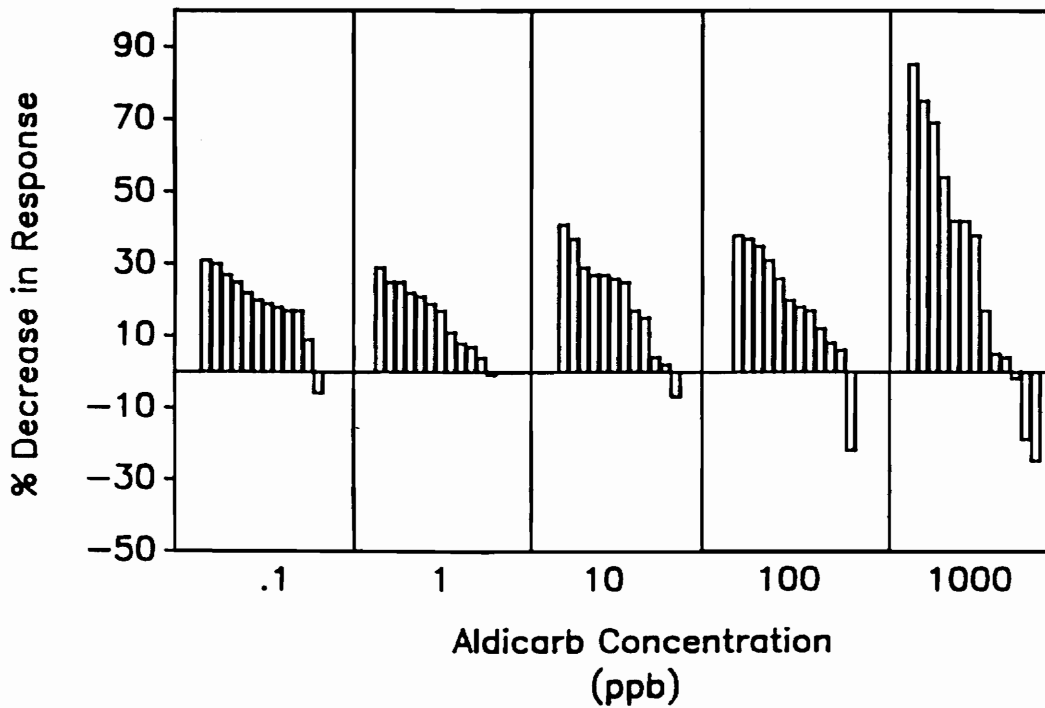

Figure 3. Stimulatory activity of macrophages in the SMLR from mice treated with varying concentrations of aldicarb: Groups of 5 C3H mice were given a single ip injection of either PBS (control) or 0.1, 1.0, 10, 100, or 1000 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and the macrophages isolated were irradiated and used to set up SMLR assays. Normal CD4$^+$ T responder cells (4 x 10^5 or 2 x 10^5) were mixed with either 8 x 10^5 or 4 x 10^5 stimulator cells. Uptake of [^{3}H] thymidine was used to gauge the extent of T cell proliferation. Delta cpm values and S.E. were calculated and considering the response of control macrophages as 100%, the corresponding decrease in responses using aldicarb-treated macrophages were plotted in descending order. A minimum of twelve experiments carried out at each aldicarb concentration were depicted as individual bars.

Table 2. Stimulatory activity of macrophages in the SMLR from mice treated with varying concentrations of aldicarb

Dose of Aldicarb ppb	Number of Experiments	Percentage of the control response	Bonferroni's adjustment (p values)
0.1	16	69.6 ± 6.1	<0.0005
1.0	14	76.4 ± 5.9	<0.0005
10	14	74.9 ± 5.0	<0.0005
100	14	77.5 ± 4.7	<0.0025
1000	14	68.2 ± 9.4	<0.0025

Groups of 5 C3H mice were given a single i.p. injection of either PBS (control) or 0.1, 1.0, 10, 100 or 1000 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and the macrophages isolated were irradiated and used in the SMLR assays shown in Fig 1. In each experiment the SMLR responses obtained in various aldicarb-treated groups were compared with the PBS-treated control group. The data were depicted as percentage of the control response considering the latter as 100 % and the mean percentages ± SEM were calculated based on several experiments performed in each group. These data were analyzed using Bonferroni's adjustment.

2.3.2 Mechanism of aldicarb-induced suppression of macrophage function

Investigations were next undertaken to answer the question as to whether aldicarb induced suppressor macrophages in the culture which caused suppression of the SMLR. Table 3 presents the results of cell-mixing experiments carried out to address this. As before, use of 4×10^5 macrophages from 0.1 or 1.0 ppb of aldicarb-treated mice demonstrated decreased stimulatory activity in the SMLR. However, when the aldicarb-treated macrophages were mixed with 4×10^5 control macrophages, the responses obtained (cpm 29,124 and cpm 20,581) was similar to the control response using 8×10^5 macrophages (cpm 26,464), and was more than the control response using only 4×10^5 macrophages (cpm 11,588) or the additive response of 4×10^5 control + 4×10^5 aldicarb treated macrophages (cpm 16,606 or 15,832). These data demonstrated therefore that aldicarb-treated macrophages were not actively suppressing the SMLR response by producing any suppressor factors, but probably they were defective and provided suboptimal stimulation.

The possibility that aldicarb treatment decreased the expression of Ia antigens on the macrophages was investigated next. Experiments were conducted using macrophages from single injection aldicarb treated animals. Table 4 and Figure 4 demonstrate that the control (PBS) macrophage population contained ~42% Ia^+ cells similar to the percentages of Ia^+ macrophages in various aldicarb treated groups ($p > 0.05$). Thus, there was no significant decrease in the number of Ia^+ macrophages following treatment with aldicarb. Likewise, the Ia density exhibited on the macrophages as measured by mean channel number, was also not diminished following aldicarb exposure when compared to the control cell population.

Table 3. Decreased Capacity of Aldicarb Treated Macrophages to Stimulate in the SMLR is not Related to Presence of Suppressor Macrophages

Stimulator Cells (macrophages)		Cell Proliferation
PBS-treated (Control)	Aldicarb/PBS Treated	(Δ c.p.m $\pm$ S.E.)
4 x 10^5	–	11,588 $\pm$ 636
–	4 x 10^5(0.1 ppb)	5,018 $\pm$ 505
–	4 x 10^5(1.0 ppb)	4,244 $\pm$ 298
4 x 10^5	4 x 10^5(0.1 ppb)	29,124 $\pm$ 129
4 x 10^5	4 x 10^5(1.0 ppb)	20,581 $\pm$ 118
4 x 10^5	4 x 10^5(PBS)	26,464 $\pm$ 662

Groups of C3H mice were given a single ip injection of either PBS (control), 0.1 or 1.0 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and macrophages isolated and used to set up SMLR assays. Normal $CD4^+$ T cell responders (4 x 10^5) from control or aldicarb treated mice were mixed with 4 x 10^5 stimulator cells as described in Fig. 1. Uptake of [^{3}H] thymidine was used to gauge the extent of T cell proliferation. Delta cpm values and S.E. are presented.

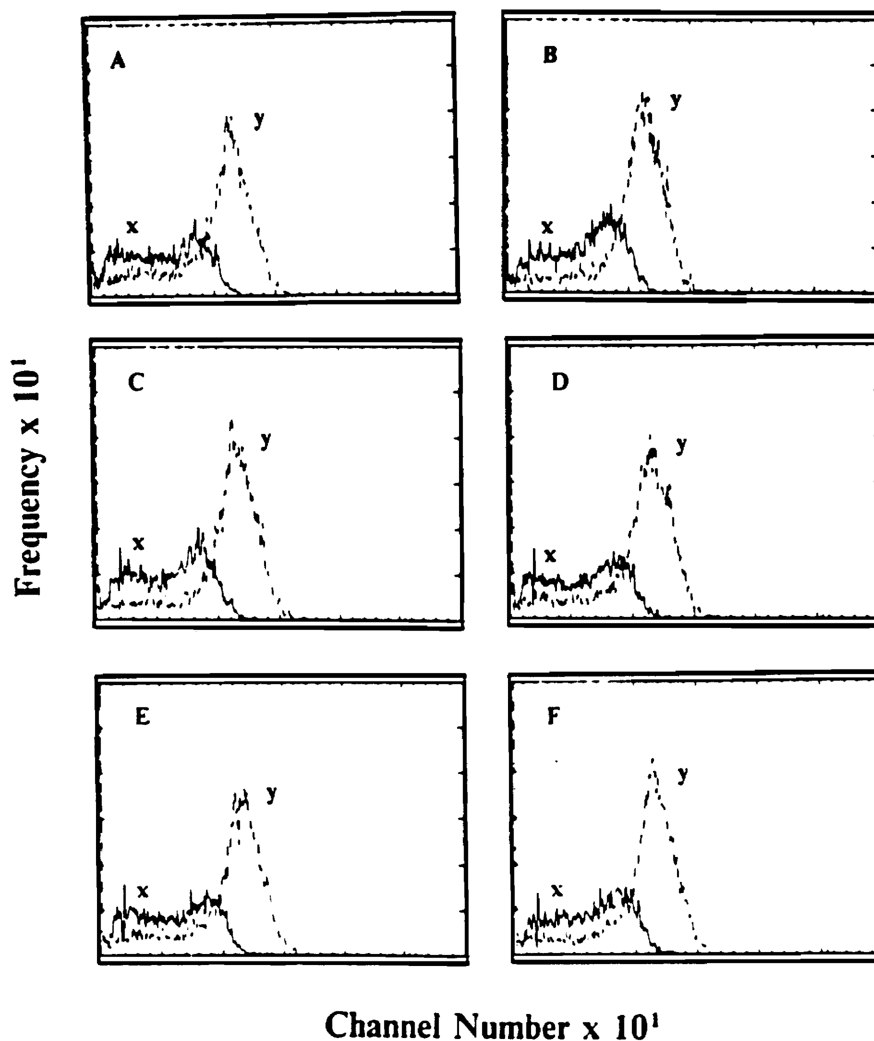

Figure 4. **Flowcytometric analysis of Ia antigen expression by macrophages in aldicarb treated mice:** These figures are a graphical representation of the data presented in Table 2. Macrophages that had been *in vivo* treated with PBS (A), 0.1 ppb (B), 1.0 ppb (C), 10 ppb (D), 100 ppb (E), or 1000 ppb (F) of aldicarb were subsequently stained for Ia. The solid line (x) represents negative controls wherein macrophages were first incubated with a non-specific antibody MKD6 (anti-I-A^d) followed by FITC-conjugated anti-mouse IgG. The broken lines (y) represent cells positively stained with 14.4.4 (anti-I-E^k) followed by FITC conjugated anti-mouse IgG.

Table 4. Percent Ia$^+$ cells and density of Ia antigen expression in macrophage population in mice treated with varying doses of aldicarb

Treatment	% Ia$^+$ Macrophages	Ia Density (Mean Channel Number)
PBS (Control)	42	120
Aldicarb 0.1 ppb	48	118
Aldicarb 1.0 ppb	47	121
Aldicarb 10 ppb	45	121
Aldicarb 100 ppb	41	122
Aldicarb 1000ppb	46	121

Groups of C3H mice were given a single ip injection of either PBS (control), 0.1, 1.0, 10, 100 or 1000 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and macrophages were isolated and labelled as described in the text, and cells were flow cytometrically analyzed.

It was further possible that decreased interleukin 1 (IL-1) secretion by macrophages from aldicarb treated mice was a suggested cause for the observed decrease in T cell proliferation. To delineate this possibility, cultures were established using $CD4^{+}$ responder cells from normal mice and stimulated with PBS or aldicarb-treated macrophages in the presence or absence of exogenous recombinant IL-1 (rIL-1). IL-1 was used at varying doses of 0.5 to 5 U/ml. It had earlier been determined that 5 U/ml of IL-1 was optimal in the thymocyte costimulation assay (Smith, Lachman & Openhiem, 1980). The data shown in Table 5 suggested that suboptimal doses of IL-1 such as 0.5 and 1 U/ml, had no significant effect on the SMLR induced by PBS-treated SAC. However, these doses could augment the SMLR induced by aldicarb treated SAC and restored this response to normal levels. Addition of 2.5 or 5 U/ml of IL-1 caused enhancement in the SMLR stimulated by both PBS-treated and aldicarb-treated SAC and this enhancement was statistically similar. Thus, following the addition of exogenous IL-1, the T cell response in the SMLR using aldicarb-treated macrophages was reconstituted to normal levels. These data therefore suggested that the decreased stimulatory activity of aldicarb-treated macrophages was probably due to defective IL-1 production.

2.3.3 Aldicarb treatment does not affect the T cell responses in SMLR and AlloMLR

Thus far it was observed that aldicarb treatment altered the macrophage functions. Next investigated were the effects of aldicarb on the responder T cells in SMLR and in the AlloMLR.

Table 5. Reconstitution of defective macrophage function with IL-1

Responder cells $2x10^5$	Interleukin-1 (U/ ml)	SMLR (cpm ± S. E.) using stimulator cells (macrophages)	
		PBS treated	Aldicarb-treated
$CD4^+T$ (normal)	–	14,287 ± 1343	2,272 ± 112
	0.5	14,501 ± 1,185	6,451 ± 1,144
	1.0	12,626 ± 1,807	15,435 ± 1,258
	2.5	21,385 ± 2,896	18,025 ± 1,496
	5.0	33,353 ± 3,250	31,916 ± 2,534

$CD4^+$ T cells from normal mice were stimulated with 6 x 10^5 syngeneic irradiated macrophages from PBS-treated or 0.1 ppb aldicarb-treated mice. The cultures were incubated without or with 0.5 to 5.0 U/ml of IL-1. The SMLR was studied on day 4 as described in Figure 1. The $CD4^+$ T cells incubated with medium alone incorporated 270 ± 64 cpm.

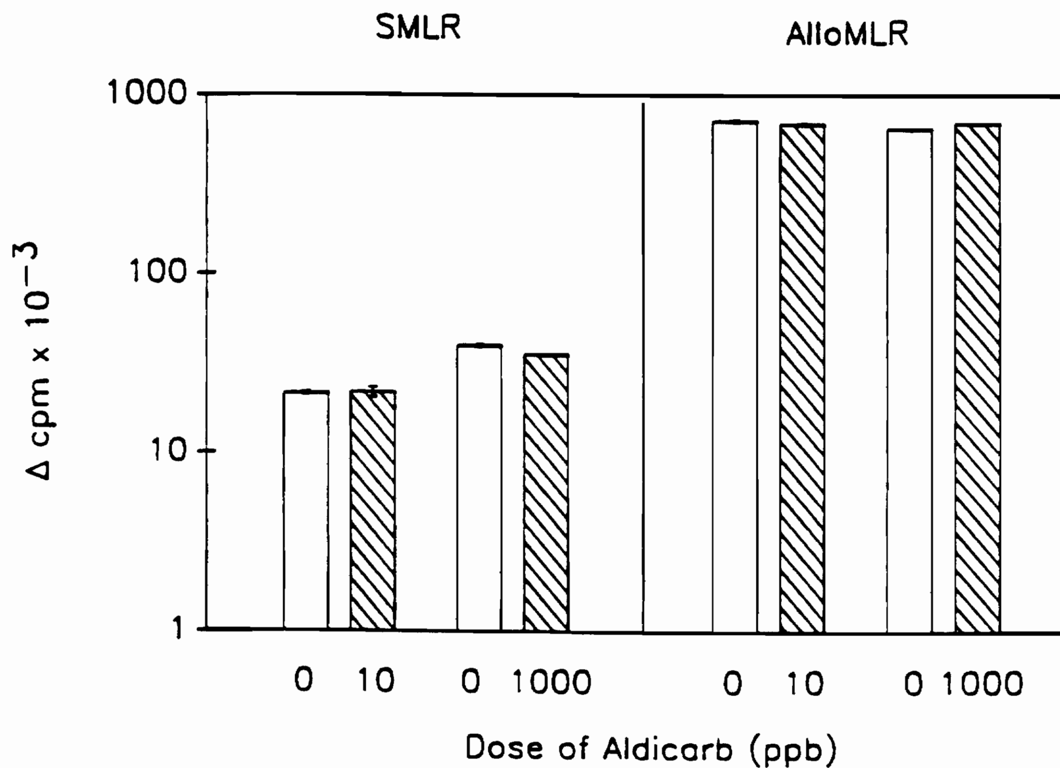

Figure 5. Effect of aldicarb treatment on responder T cells in the SMLR and AlloMLR : Groups of 4 C3H mice were given a single ip injection of either PBS (control) or 10 or 1000 ppb of aldicarb. Twenty-four hours later, spleens were harvested, pooled and $CD4^+$ T cells were isolated and used to set up AlloMLR or SMLR assays. The stimulator cells consisted of normal irradiated macrophages from C57BL/6 (for AlloMLR) or from C3H mice (for SMLR). Responder cells were used at a concentration of 4 x 10^5 cells/ well and stimulator cells at 8 x 10^5 cells/ well. Cell proliferation was measured on day 4 by the uptake of [^{3}H] thymidine, added during the last 18 hours of culture.

For this purpose, $CD4^+$ T cells were purified from aldicarb or PBS (control) treated C3H mice and stimulated with irradiated normal syngeneic (C3H) macrophages (SMLR) or normal irradiated allogeneic (C57BL/6) macrophages (AlloMLR). The data obtained from a representative experiment has been shown in Figure 5. It was found that treatment of mice with 10 or 1000 ppb of aldicarb did not alter the responsiveness of T cells in SMLR or AlloMLR when compared to PBS-treated control mice ($p > 0.05$). Similar data was obtained with other doses of aldicarb (1 and 100 ppb) and using various effector: stimulator cell ratios and has not been depicted.

2.4 Discussion

In the preceding experiments the effects of aldicarb on autoreactive $CD4^+$ T cells and on stimulatory activity of macrophages in the SMLR were investigated. From initial studies at 0.1 to 1000 ppb of aldicarb, it was found that aldicarb suppressed normal macrophage function in its capacity to stimulate T cells in the SMLR. This suppression of normal macrophage function was not attributable to shifts in the kinetics of the SMLR and was demonstrable at all effector:target cell concentrations. In contrast, aldicarb had no direct effect on T cell proliferation in either SMLR or AlloMLR using *in vivo* aldicarb exposed T lymphocytes. These studies therefore demonstrated that aldicarb treatment selectively affected the macrophage functions without affecting the effector T cell functions.

There are three main mechanisms that could be proposed to explain the macrophage defect observed following aldicarb treatment. The first of these mechanisms suggests that if suppressor macrophages are present in the cultures these cells could secrete prostaglandins and other factors that could act to down regulate the SMLR (Stevenson & Battisto, 1986). The possibility of this being a valid mechanism was investigated in the present study. The results

from cell-mixing experiments demonstrated that macrophages from aldicarb treated mice were not actively suppressing the SMLR, since addition of aldicarb-treated macrophages to control cultures, failed to reduce the control SMLR response.

A second logical mechanism to explain the observed suppression of responsiveness was that a decrease in Ia expression on the splenic macrophages could lead to a decreased ability of the $CD4^+$ T cells to interact with the aldicarb treated macrophages. Flow cytometric analysis of macrophages rejected decreased Ia expression as a possible mechanism for the altered response associated with aldicarb exposed macrophages. It was observed that, regardless of the concentration of aldicarb injected, there was no significant change in the percentage of Ia^+ cells in the splenic adherent population. Likewise, there was no alteration of Ia antigen density on the macrophages at any of the aldicarb concentrations administered. Therefore, it can be concluded that the suppression associated with aldicarb treatment was not due to a change in Ia expression by the macrophages.

A third possible mechanism to explain the suppression of responsiveness was that, via alterations in production of interleukin 1 (IL-1) by the macrophages, there was a decrease in the ability of T lymphocytes to proliferate since IL-1 acts in concert with Ia and antigen, leading to T cell proliferation (Mizel, 1982). The present study suggested that the decreased stimulatory activity of macrophages from aldicarb treated mice was eliminated following the aldicarb may affect IL-1 mediated signal to the T cells. Recent studies have demonstrated that IL-1 and IL-6 share several similarities in function (Reviewed by Wong & Clark, 1988) and furthermore, IL-6 is inducible in certain cells by IL-1 (Van Damme et al., 1987). Thus it is possible that in the present study, addition of exogenous IL-1 may induce enhanced production of IL-6 which in turn may activate T cells and thereby reconstitute the decreased SMLR seen in aldicarb-treated cultures.

Alterations in IL-1 activity and the resultant decrease in the functions of macrophages are consistent with, and may be the underlying cause for, the suppression of B cell responses to sheep red blood cells following the oral administration of aldicarb as reported by Olsen et al. (1987). Thomas et al. (1987) reported that aldicarb had no significant effect on T and B cell responses to mitogens and on T cells responding in the MLR. The reason for this discrepancy is not clear. In the current studies it was observed that the percent suppression in the SMLR responses varied between the experiments. Therefore, several experiments were performed in each of the aldicarb treatment groups to arrive at a statistically supported conclusion that aldicarb inhibited the macrophage stimulatory activity.

Since macrophages are important cells involved in the activation of T and B cells, it can be speculated that a defect in the macrophages may affect other T and B cell functions. However, it should also be noted that a defect in macrophage IL-1 synthesis can be reconstituted by the IL-1 produced by the B cells as recently observed in aged mice (Seth et al., 1989).

2.5 Summary

The widely used carbamate pesticide, aldicarb, has been found in drinking water in several areas often exceeding 100 ppb. Recent studies suggesting the possible detrimental effects of aldicarb on mammalian health, prompted an examination of the effects of aldicarb on the immune system. Specifically examined in these studies were the effect of aldicarb on the stimulator and responder cells involved in the SMLR. In the SMLR, $CD4^+$ autoreactive T cells, constituting the responder cell population, interact with syngeneic Ia molecules on stimulator macrophages. It was possible that aldicarb could affect the responses of either or both of these populations. Investigations revealed a decreased capacity of macrophages obtained

from animals treated with 0.1 to 1000 ppb of aldicarb to stimulate normal autoreactive T cells. Further flow cytometric analyses revealed that the decreased stimulatory capacity of the macrophages from aldicarb treated mice was not due to a decrease in the expression of Ia antigens on the surface of macrophages. In addition, cell-mixing experiments failed to demonstrate any suppressor macrophage activity in the macrophages obtained from the mice treated with aldicarb. Addition of exogenous IL-1 could completely restore the defective macrophage stimulatory functions.

In contrast to these effects on macrophages, it was observed that in C3H mice ip treated with single doses of 1 to 1000 ppb of aldicarb, there was no evidence of alterations in the ability of autoreactive T cells to respond to syngeneic Ia molecules expressed by normal macrophages. In addition, responsiveness of T lymphocytes obtained from aldicarb-treated mice to allogeneic Ia antigens, was also unaltered.

Collectively, these data suggested that aldicarb, may selectively suppress the stimulatory activity of macrophages by inhibiting the IL-1 mediated signal to T cells without directly affecting T cell functions.

3.0 STUDIES ON EFFECT OF ALDICARB ON MACROPHAGE AND NK CELL ACTIVITY

3.1 Introduction

Macrophages are a group of non-specific immune accessory cells that serve the primary role of internalizing, degrading and presenting antigenic molecules in association with Ia antigens on their cell surfaces to Th cells, resulting in T cell proliferation. In their role as stimulatory cells, macrophages secrete a number of cytokines that have stimulatory effects on other immune cells. One of the best characterized of these substances, IL-1, stimulates T cell DNA synthesis and thus, T cell proliferation. In addition to the antigen-processing and presenting abilities of macrophages as discussed in the proceeding chapters, activated macrophages can also kill tumor cells thereby suggesting that the macrophages can play an important role in protecting the host against cancer.

The extracellular mechanism by which macrophages cause lysis of tumor cells has been suggested to be due to the production of products such as thymidine (Opitz et al., 1975), complement fragment-C'3a (Ferluga et al., 1978), toxic oxygen metabolites (Nathan & Root, 1977; Nathan & Cohen, 1980; Nathan et al., 1980a, 1980b), arginase (Currie, 1978), protease (Adams et al., 1984), and tumor necrosis factor or cachectin (Beutler et al., 1985). The relative importance of any of these factors appears to be a function of the method of macrophage activation and the cytotoxic system used (Drysdale et al., 1983).

Importantly, the ability of macrophages to secrete and release enzymes and toxic oxygen metabolites that can act cytolytically on the tumor cells can be exploited in the antibody-dependent cell mediated cytotoxicity (ADCC) assay. In this assay Ab coated, Cr^{51} labelled, tumor target cells are mixed with macrophages. The activated macrophages will then kill the tumor targets and this can be detected by the amount of ^{51}Cr-released in the culture (Nagarkatti, Nagarkatti & Kaplan, 1988; Nathan et al., 1980b).

NK cells are a MHC-unrestricted cell population that can spontaneously lyse a variety of tumor cells. Although the target structure recognized by NK cells on the cells destined for destruction has not been characterized, it does have a distinct distribution in several cell populations. The cytolytic abilities of NK cells seems to be based in a cytochalasin-inhibitable, microfilament involved binding step. The NK cell cytotoxicity is increased in the presence of IL-2 or γ-INF. Several lines of evidence suggest that NK cells and CTL may share basically a similar recognition, binding, and lytic mechanism (Henkart, 1985), which may be different from the ones used by activated macrophages. Isolated cytoplasmic granules from NK cells or CTL have similar cytolytic properties and differ from the extracellular mechanisms by which macrophages mediate lysis of tumor cells.

Since macrophages and NK cells constitute important nonspecific effector mechanisms protecting the host against cancer, the effect of aldicarb on the functions of these cells were investigated, since such studies have not been carried out earlier.

3.2 Experimental Procedures

3.2.1 General Procedures

In this group of investigations several of the general procedures employed in the earlier investigations are identical with procedures carried out in these experiments. The housing and maintenance of animals remains unchanged and was described in section 2.2.1. The preparation and analysis of aldicarb solutions was performed as described in section 2.2.2. Procedures unique to the present group of experiments are described in detail in the following sections of this chapter.

3.2.2 ADCC Assay

Peritoneal exudate cells (PEC) were obtained from the peritoneal cavity of control or aldicarb-treated mice by washing the peritoneal cavity repeatedly with cold PBS. To study the ADCC, LSA tumor cells were used as targets. Since LSA tumor cells were Thy-1^{+}, anti-Thy-1

antibodies were used at a final dilution of 1:10 for the ADCC assay (Nagarkatti, Nagarkatti & Kaplan, 1988).

3.2.3 NK Cell Preparation

Single cell suspensions of spleen were prepared using a laboratory blender (Stomacher, Tekmar Co., Cincinnati, OH) in medium RPMI-1640 supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY), 10mM HEPES, 1mM glutamine and 40 μg/ml gentamicin sulfate (Seth et al., 1988). The red cells were lysed using 0.83% ammonium chloride and the cells were resuspended in approximately 5ml of complete medium after two washings.

3.2.4 Tumor Target Cell Preparation

LSA, a spontaneous lymphoma syngeneic to C57BL/6 mice and resistant to NK cell-mediated cytotoxicity and YAC-1, a NK-sensitive target were maintained *in vitro* in complete medium as described elsewhere (Nagarkatti, Nagarkatti & Kaplan, 1988).

3.2.5 Chromium Release Assay

A modification of the ^{51}Cr-release assay (Nagarkatti & Kaplan, 1985; Nagarkatti, Nagarkatti & Kaplan, 1988) was used to study the macrophage and NK cell-mediated cytotoxicity. Varying numbers of effector cells in 0.1 ml were seeded in triplicate, into the wells of 96-well round-bottomed micro-titre plates (Flow Laboratories, Inc., McLean, VA). Tumor targets were labeled with ^{51}Cr by incubating 1 x 10^7 tumor cells in 0.5 ml medium with 20 μCi of sodium chromate (specific activity, 200 to 500 Ci/g; New England Nuclear, Boston, MA) at 37°C for I hr. The tumor cells were next washed 4 times and 0.1ml of the ^{51}Cr-labeled tumor targets were added to each well. The plates were incubated for 4 hr and 37°C. After incubation, the supernatants were harvested with the Titertech collecting system (Flow Laboratories, Rockville, MD) and the radioactivity was measured with a gamma counter (Gamma Trac 1191, TM Analytic, Elk Grove, Illinois). Percentage of specific cytotoxicity was calculated using the formula: % specific cytotoxicity = (experimental release - control release)/(total release - control release) x 100. Control release was measured by incubating ^{51}Cr-labeled targets alone. The control release was usually less than 20% of the maximum release. Maximum release was determined by incubating labeled tumor cells with sodium dodecyl sulfate.

3.2.6 Statistical Analysis

In all experiments, PEC or spleen cells from groups of 5-10 aldicarb treated or control mice were pooled. The cytotoxicity assay was performed in triplicate and the mean percent cytotoxicity and the standard errors (S. E.) were calculated. The various experimental groups were compared with the control group of mice using Student's t-test and any differences with

p values less than 0.05 were considered to be statistically significant. All experiments were repeated at least three times with consistent results.

3.3 Results

3.3.1 Aldicarb treatment decreases the activity of macrophages in the macrophage-mediated antibody-dependent cytotoxicity of tumor cells, but does not effect NK cell-mediated cytotoxicity of tumor cells

The effects of aldicarb on macrophage and NK cell-mediated cytotoxicity were investigated at doses of less than 10 ppb. Initial studies were restricted to short term toxicity of aldicarb in which groups of 5-10 mice were injected ip with 0.01, 0.1, 1 or 10 ppb of aldicarb everyday for 7 days. Control mice received PBS for seven days. Twenty-four hours later mice were sacrificed and the macrophage-mediated antibody-dependent cytotoxicity was studied. The data shown in Figure 6 suggested that at all effector:target (E:T) ratios tested, macrophages from mice treated with 0.01 to 10 ppb, all demonstrated a decreased cytotoxicity against LSA tumor cells when compared to the cytotoxicity observed with control group of mice ($p < 0.05$; all aldicarb treated groups versus control). The percent decrease in cytotoxicity in aldicarb treated groups ranged from 64-100% when compared to the control responses.

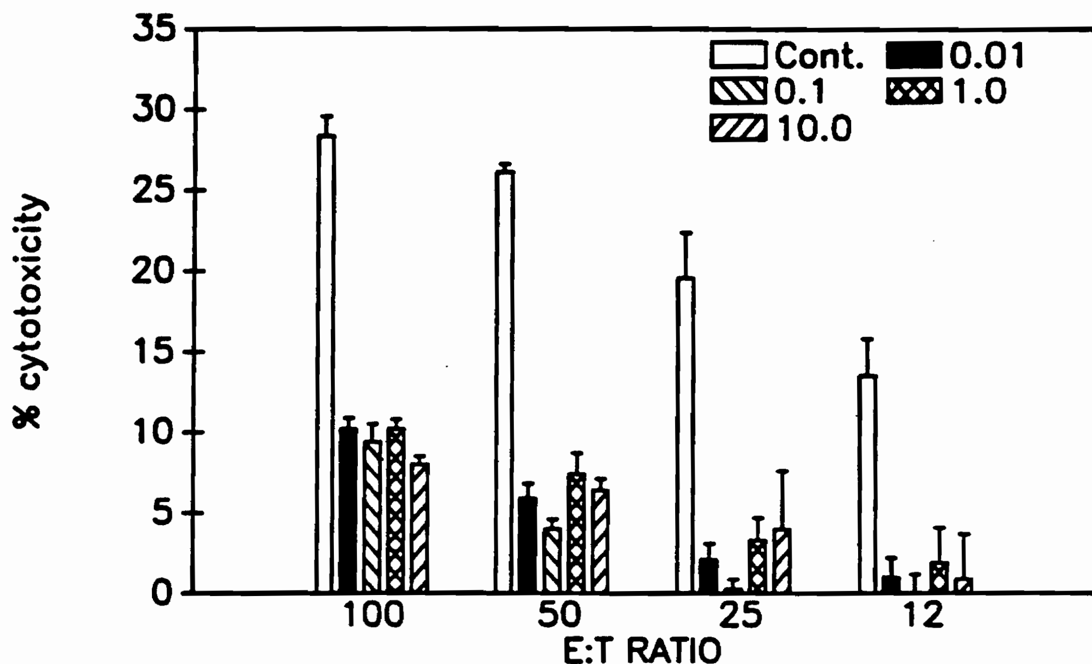

Figure 6. Antibody-dependent macrophage-mediated cytotoxicity in mice treated with aldicarb: Groups of 10 C3H mice were injected either with PBS (control) or with 0.01, 0.1, 1, or 10 ppb of aldicarb ip daily for 7 days. Twenty four hours later, PEC (effector cells) were collected, mixed with antibody-coated ^{51}Cr-labeled LSA tumor targets at different effector cell:target cell (E:T) ratios. The percent cytotoxicity was calculated by measuring the amount of ^{51}Cr-released as described in methods. The vertical bars represent mean percent cytotoxicity ± S.E.

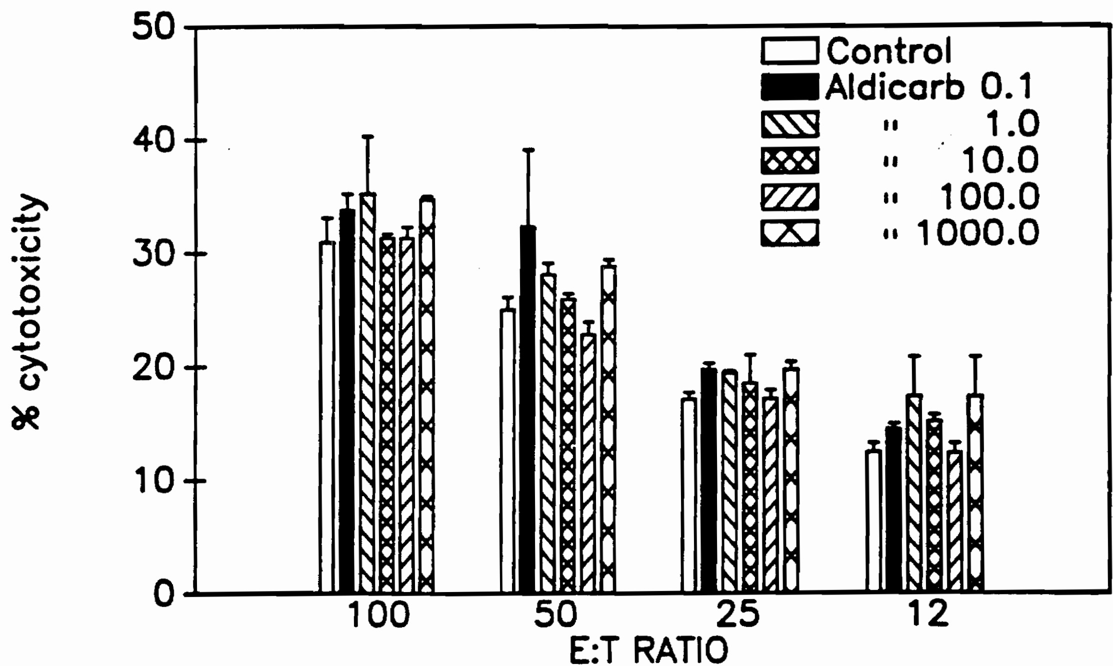

Figure 7. NK cell-mediated cytotoxicity in aldicarb treated mice: Groups of 5 C3H mice were injected either with PBS (control) or with 0.1, 1, 10, 100 or 1000 ppb of aldicarb ip daily for 7 days. Twenty-four hours later, spleens were harvested, pooled and tested for cytotoxicity against ^{51}Cr-labeled YAC-1 tumor cell targets. Varying numbers of spleen cells (effector cells) were mixed with a constant number of tumor cells (target cells) to achieve effector:target (E:T) cell ratios from 100 to 25. The percent cytotoxicity was calculated as described in Fig 6. The vertical bars represent mean percent cytotoxicity ± S.E.

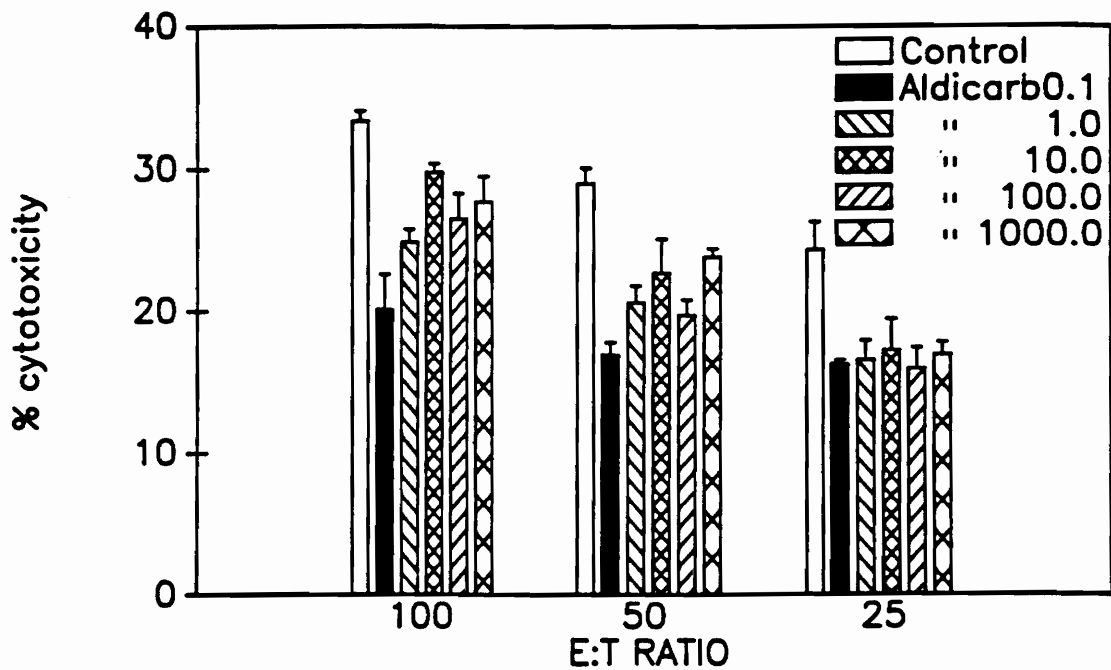

Figure 8. Antibody-dependent macrophage-mediated cytotoxicity in mice treated with a single dose of aldicarb: Groups of 10 C3H mice were injected with either PBS (control) or with a single dose of 0.1, 1, 10, 100 or 1000 ppb of aldicarb. Twenty four later, the cytotoxicity assay was performed as described in fig 6. The vertical bars represent mean percent cytotoxicity ± S.E.

The effect of aldicarb on NK cell-mediated cytotoxicity were next investigated. Mice were injected with different doses of aldicarb or PBS (control) for seven days as described above and 24 hr later, NK cell-mediated cytotoxicity was studied using NK-sensitive YAC-1 targets. The data shown in Figure 7 suggested that aldicarb failed to alter the NK cell-mediated cytotoxicity at all doses tested. In contrast to the macrophage mediated cytotoxicity which was severely suppressed in aldicarb treated mice at 0.01 to 10 ppb (Fig. 6), the NK cell-mediated cytotoxicity was not altered at these doses, and furthermore, increasing the aldicarb doses to 100 or 1000 ppb, still failed to bring about any change in the NK activity.

When acute toxicity testing was performed using a single injection of aldicarb at 0.1 to 1000 ppb and the macrophage-mediated cytotoxicity analyzed 24 hr later, it was observed that all doses of aldicarb from 0.1 to 1000 ppb inhibited the macrophage-mediated killing significantly at all E:T ratios ($p < 0.05$; aldicarb treated groups versus respective controls). However, at lower E:T ratios of 25:1, the percent decrease in cytotoxicity in aldicarb treated mice appeared to be more than the decrease observed at higher E:T ratios of 100:1. Also, at lower E:T ratio, the percent decrease was similar in all aldicarb treated groups (approximately 33%) when compared to the decrease seen at higher E:T ratio of 100:1, where, % suppression ranged from 40% (0.1ppb) to 11% (10ppb). It should be noted that at 100:1 E:T ratio, although the decrease in cytotoxicity was very little in magnitude (for example at 10 ppb of aldicarb, the % decrease was only 11%), it was statistically significant ($p = 0.019$). In summary, following single dose treatment with aldicarb, the % suppression in macrophage-mediated cytotoxicity was to a lesser extent (Fig. 8) than when multiple doses of aldicarb were administered (Fig. 6).

3.4 Discussion

In this section data have been presented on the effects of aldicarb on macrophage and NK-mediated killing of tumor cells. It was found that aldicarb could suppress the macrophage-mediated cytotoxicity but not the NK-mediated cytotoxicity. The effect on macrophages was more prominent during short-term toxicity testing, when, mice received 7 daily injections of 0.01 to 10 ppb of aldicarb (Fig. 6). When tested for acute toxicity, with single injection of aldicarb at 0.1 to 1000 ppb, at lower E:T ratios, the macrophage responses were significantly decreased. However, using higher E:T ratios, although 0.1 ppb of aldicarb could still induce significant inhibition, doses above 1 ppb, caused minimal inhibition of the responses (Fig 8). The differences observed at lower and higher E:T ratios can be explained by the fact that when large number of effector cells were used, there were optimal numbers of effectors in aldicarb treated groups to cause cytotoxicity comparable to that seen in controls. It is interesting to note that at higher E:T ratios, the suppression was more prominent with the lowest dose of aldicarb tested when compared to the higher doses. Recently, a similar inverse dose-dependent suppression in the splenic plaque forming cell responses were reported in aldicarb treated mice, although the reason for this phenomenon observed were not clear (Olson et al., 1987). There have been two reports on the immunomodulating activities of aldicarb. In the first study, it was demonstrated that aldicarb suppressed the B cell responses to sheep red blood cells. In contrast, Thomas et al. (1987) reported that aldicarb at doses of 0.1 to 1000 ppb had no effect on B cell responses to sheep erythrocytes. In addition, these authors found no change in the capacity of B and T lymphocytes to respond to mitogens, and in the gross and histopathologic examination of thymus, spleen, and lymph nodes. The reason for the discrepancy in the above two studies is not clear. One possible reason could be that in the former study outbred white mice were used, while, in the latter study, $B_6C_3F_1$ mice were employed. This may not be uncommon since, mouse-strain

differences have been observed, in the susceptibility to immunotoxic effects of dioxin (Nagarkatti et al., 1984).

In the present study a significant effect was observed on macrophages especially in mice receiving 7 daily doses of 0.1 to 10 ppb of aldicarb. The mechanism by which aldicarb suppresses the macrophage-mediated cytotoxicity is not clear and needs further investigation. The extracellular mechanism by which macrophages cause lysis of tumor targets has been suggested to be due to the production of thymidine, toxic oxygen metabolites, arginase, proteases, complement components, tumor necrosis factor, etc. (Nagarkatti, Nagarkatti & Kaplan, 1988). The relative importance of any of these factors appears to be a function of the method of macrophage activation and the cytotoxic system used. Further studies are essential to delineate whether aldicarb affects the production and release of extracellular factors produced by the macrophages which cause tumor cell-lysis.

In the present study, aldicarb affected selectively the macrophage-mediated cytotoxicity, but not the NK cell-mediated cytotoxicity. Several lines of evidence suggest that NK and cytotoxic T cells may share basically a similar recognition, binding and lytic mechanisms (Henkart, 1987), which may be different from the one used by activated macrophages. Thus isolated cytoplasmic granules from NK or cytotoxic T cells have similar cytolytic properties (Henkart, 1987) and differ from the extracellular mechanism by which macrophages mediate lysis of tumor cells, such as, through reactive oxygen intermediates. It has been observed that treatment of tumor cells with an anti-cancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) affected only the macrophage-mediated cytotoxicity but not that of NK cells. (Nagarkatti, Nagarkatti & Kaplan, 1988).

3.5 Summary

The capacity of macrophages and NK cells to lyse tumor cells was investigated in C3H mice following *in vivo* exposure to aldicarb at low doses, similar to those found in ground water. It was found that the macrophage-mediated cytotoxicity of tumor cells was strongly inhibited following administration of aldicarb interperitoneally at concentrations of 0.1 to 10 ppb over seven consecutive days. This suppression was studied by the *in vitro* antibody-dependent cell-mediated cytotoxicity (ADCC) assay.

A single dose treatment with 0.1, 1, 10, 100, or 1000 ppb of aldicarb inhibited the macrophage mediated cytotoxicity, although considerably to a lesser extent when compared to the suppression brought about by 7 daily injections of aldicarb.

In contrast, 7 daily injections of aldicarb at similar or higher aldicarb concentrations (100 and 1000 ppb) failed to cause significant alterations in the NK cell-mediated lysis of tumor cells. Since activated cytotoxic macrophages play an important role in the defense against intracellular pathogens and against neoplasia, these experiments investigating the effects of aldicarb on macrophages suggests that aldicarb may suppress the natural nonspecific immunity mediated by macrophages.

4.0 IMMUNOSUPPRESSION BY ALDICARB OF T CELL RESPONSES TO ANTIGEN-SPECIFIC AND POLYCLONAL STIMULI RESULTS FROM DEFECTIVE IL-1 PRODUCTION BY THE MACROPHAGES

4.1 Introduction

It is a common practice to use spleen cells stimulated with mitogens such as concanavalin A (Con A) and lipopolysaccharide (LPS) to study the polyclonal activation of T and B cells respectively, during the immunotoxicological evaluations of xenobiotics (Luster et al., 1988). Such assays although useful in the overall assessment of the immune system, fail to pinpoint the defect on T or B cells, since,

such responses involve multiple cell-types, particularly the accessory cells. For example, the effect on T cells can be investigated by studying the spleen cell responsiveness to mitogens such as Con A. However, any suppression observed cannot be interpreted to suggest a defect in the T cell responsiveness because this response, in addition, also depends on the accessory cell functions of the macrophages particularly their capacity to secrete IL-1 and IL-6. In the present study we therefore investigated the effect of aldicarb on mixed and purified cell populations and observed that when mixed populations of cells were used, aldicarb suppressed the T cell activation. Interestingly however, this suppression was not found to be due to any intrinsic defect in the responsiveness of T cells but appeared to be due to a defect in the accessory functions of the macrophages.

4.2 Experimental Procedures

4.2.1 Mice

Eight week old female C3H/HeJ and C57BL/6 mice were obtained from National Institute of Health (Bethesda, Md). The mice were housed in cages which were kept in a laminar flow cabinet that provided a sterile environment as described in detail in section 2.2.1.

4.2.2 *Aldicarb*

Also in keeping with previous experimental techniques, all aldicarb solutions were prepared by dissolving high purity aldicarb (Chem Services Ps-734 West Chester, PA) in PBS and stored at 4°C in the dark. The stability of the solutions were monitored using HPLC analysis. Groups of 3-5 mice were injected intraperitoneally (ip) with 0.1 ml volume of aldicarb and after 24 hrs. the mice were sacrificed by cervical dislocation and used in various experiments. Control animals received 0.1 ml of PBS by the i.p. route.

4.2.3 *Monoclonal antibodies (mAb)*

The mAb J11d, B220, 14.4.4 (anti-IE^k), and 145.2 C11 (anti-CD3) were grown *in vitro* and used as antibody concentrated from the culture supernatants (Seth et al., 1988; Nagarkatti et al., 1988).

4.2.4 Purification of T cells and macrophages

Spleens were collected and single cell suspensions were made using a tissue blender (Stomacher, Tekmar Co., Cincinnati, OH) in 'complete' RPMI 1640 medium containing 10% heat-inactivated fetal-calf serum (GIBCO Laboratories, Grand Island, NY), 10 mM glutamine, 40 μg/ml gentamycin sulfate (GIBCO) and 50 mM 2-mercaptoethanol. The red blood cells were lysed using 0.85% ammonium chloride solution and the spleen cells were washed thrice and resuspended in complete medium. T cells were purified as described before (Nagarkatti et al., 1988). Briefly, the spleen cells were passed over nylon-wool columns. The nonadherent cells were collected and subjected to plastic adherence on petri plates (Costar 3100, Cambridge, MA) at 37°C for 1 hour to remove further, any contaminating macrophages. The nonadherent cells were treated with J11d, B220 and anti-IE^k mAbs plus complement to deplete any contaminating Ia$^+$ B cells. The cells prepared in this fashion were >98% T cells as determined by staining the cells with anti-CD3 mAbs and analyzing the cells using a flow cytometer. The macrophages were prepared by adhering the spleen cells over plastic petri plates for 2 hours at 37°C. The adherent cells were removed by forceful pipetting. Such cells were found to be >90% macrophages as determined by nonspecific esterase staining (Dean et al., 1989).

4.2.5 Responsiveness to Con A

Responsiveness of whole spleen cells or purified T lymphocytes to Con A was carried out as described before (Nagarkatti et al., 1989). When using purified T cells for Con A activation, irradiated (2000 rads) syngeneic macrophages were added to the cultures (2-4x10^5 cells/well). The cultures were carried out in triplicate with 2-4x10^5 responder cells in 96 well flat bottom tissue culture plates, in 0.2 ml of complete medium containing 2 μg/ml of Con A. The cultures were pulsed with 2μCi of ^{3}H-thymidine/well, 30 hours after the initiation of cultures and harvested 18 hours later using a cell harvester (Skatron Inc., Sterling, VA) and the radioactivity incorporated was determined in a liquid scintillation counter (TM Analytic, Elk Grove Village, IL). The results were expressed as mean counts per minute (cpm) of triplicates ± standard error (SE) of the mean.

4.2.6 Responsiveness to anti-CD3 mAb

The responsiveness to anti-CD3 mAb was studied in an identical manner to the assay used for studying Con A responses, except that in the place of Con A, anti-CD3 mAb (1:100 final dilution) was added to the cultures and the cell proliferation was measured by ^{3}H-thymidine-incorporation assay (Leo et al., 1987).

4.2.7 T cell responsiveness to PMA and calcium ionophore

T cells were purified from the spleens as described earlier. Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore, A23187 were purchased from Sigma Chemical Co., St. Louis, Mo., dissolved and stored in dimethyl sulfoxide at -20°C. T cells (2-4x10^5 cells/well) were next incubated in 0.2 ml complete medium in 96 well tissue culture plates in the presence of 10ng/ml of PMA and 0.5μm of A23187. The cultures were incubated at 37°C for 48 hours, and were pulsed with 2.0μCi of ^{3}H-thymidine 18 hours before the termination. The incorporation of ^{3}H-thymidine was detected as described earlier.

4.2.8 Induction of IL-1

Aldicarb was injected as before into C57BL/6 mice. The spleen cells were harvested 24 hours later and splenic macrophages were purified by adhering the cells on plastic plates for 2 hours. The adherent cells were next cultured in 96 well plates at a density of 10^6 cells/well in 0.2 ml medium containing 1μg/ml of LPS. After overnight culture, the supernatants from the wells were harvested, pooled and stored at -70°C.

4.2.9 Detection of IL-1

IL-1 was detected using an IL-1 sensitive T cell clone D10.G4.1. This T helper cell line was maintained by weekly passage in culture containing 100μg/ml of conalbumin and irradiated H-2^k spleen cells, and 5% human T cell growth factor (TCGF), (Kaye et al., 1983). To assay for IL-1, 2×10^4 D10 cells/well in 0.1 ml medium were cultured with 2μg/ml of Con A and 0.1 ml of the culture supernatant to be tested for IL-1 activity. After 2-3 days of culture, cell proliferation was measured by ^{3}H-thymidine-incorporation assay as described above.

4.2.10 Antigen-presentation by macrophages to the T helper cells

Splenic macrophages were isolated as before, irradiated (2000 rads) and 4-8×10^5 cells were mixed with 5×10^4 D10 T cells in the presence of 100μg/ml of conalbumin in 0.2 ml complete medium in 96 well plates. The cultures were incubated at 37°C for 2-3 days and cell proliferation was measured by ^{3}H-thymidine incorporation assay.

4.2.11 Statistical analysis of the data

All assays were performed in triplicate and the mean ± S.E. of the counts per minute (cpm) were calculated. The various groups were compared for statistical significance by Student's t-test and p values less than 0.05 were considered to be statistically significant. In some experiments the data was presented as Δcpm which represented the total cpm minus the sum of cpm detected in cultures containing responder cells alone and stimulator cells alone.

4.3 Results

4.3.1 Responsiveness of spleen cells and purified T cells to Con A in aldicarb-treated mice

Initially, whole spleen cells from PBS-treated (control) or aldicarb-treated mice were studied for their responsiveness to Con A. The data depicted in Fig. 9 (upper panel) showed that spleen cells demonstrated decreased responsiveness in all aldicarb-treated groups when compared to the controls ($p<0.05$). This decrease was not dose-dependent and was found to be similar in all aldicarb-treated groups.

To address whether the decreased responsiveness seen in aldicarb-treated mice was due to a defect in the responder T cells or the accessory cells such as macrophages which are critical for the Con A responsiveness, further studies were conducted using purified cell populations. Thus, T cells were purified from the spleens of control and aldicarb-treated mice and stimulated with Con A in the presence of irradiated splenic macrophages isolated form normal syngeneic mice. Interestingly, in such assays, the purified T cell responsiveness to Con A in aldicarb-treated groups was found to be normal and comparable to that of the control T cell responsiveness (Fig. 9, lower panel) These data suggested that the decreased responsiveness to Con A seen using whole spleen cells was possibly caused by a defect in the stimulator macrophages. It should be noted that purified T cells stimulated with Con A alone in the absence of accessory cells failed to proliferate (data not shown).

The T cells were next purified from aldicarb-treated or untreated (control) mice and mixed with irradiated macrophages from aldicarb-treated or control mice in the presence of Con A. The data depicted in Fig. 10 clearly demonstrated that aldicarb-treated T cells responded normally to Con A in the presence of control macrophages, whereas they showed decreased responsiveness in the presence of aldicarb-treated macrophages ($p < 0.05$). Similarly, T cells from control groups of mice showed significantly decreased reactivity to Con A in the presence of aldicarb-treated macrophages. These data together confirmed the observation that aldicarb altered the macrophage but not the T cell function directly.

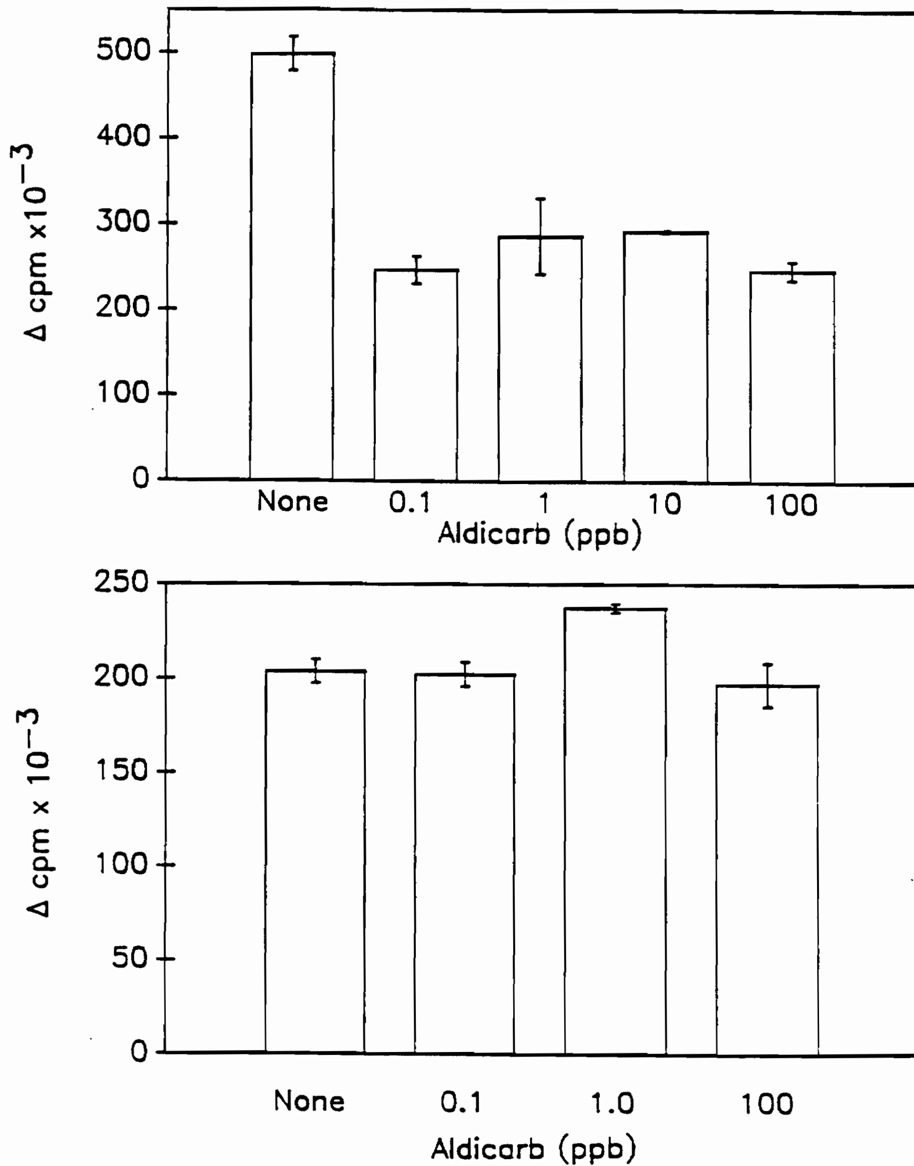

Figure 9. Responsiveness of whole spleen cells and purified T cells from control and aldicarb-treated mice to Con A: In the upper panel, whole spleen cells (4×10^5) from aldicarb treated or control mice were stimulated with Con A for 48 hours and cell proliferation was measured by ^{3}H-thymidine incorporation assay. In the lower panel, T cell (2×10^5) were purified and stimulated with Con A in the presence of normal irradiated macrophages (4×10^5). Responder or stimulator cells cultured alone incorporated generally less than 5000 cpm. These values were subtracted to obtain the Δcpm. The cultures were harvested after pulsing the plates with 2.0 μCi of ^{3}H-thymidine/well during the last 18 hours of culture.

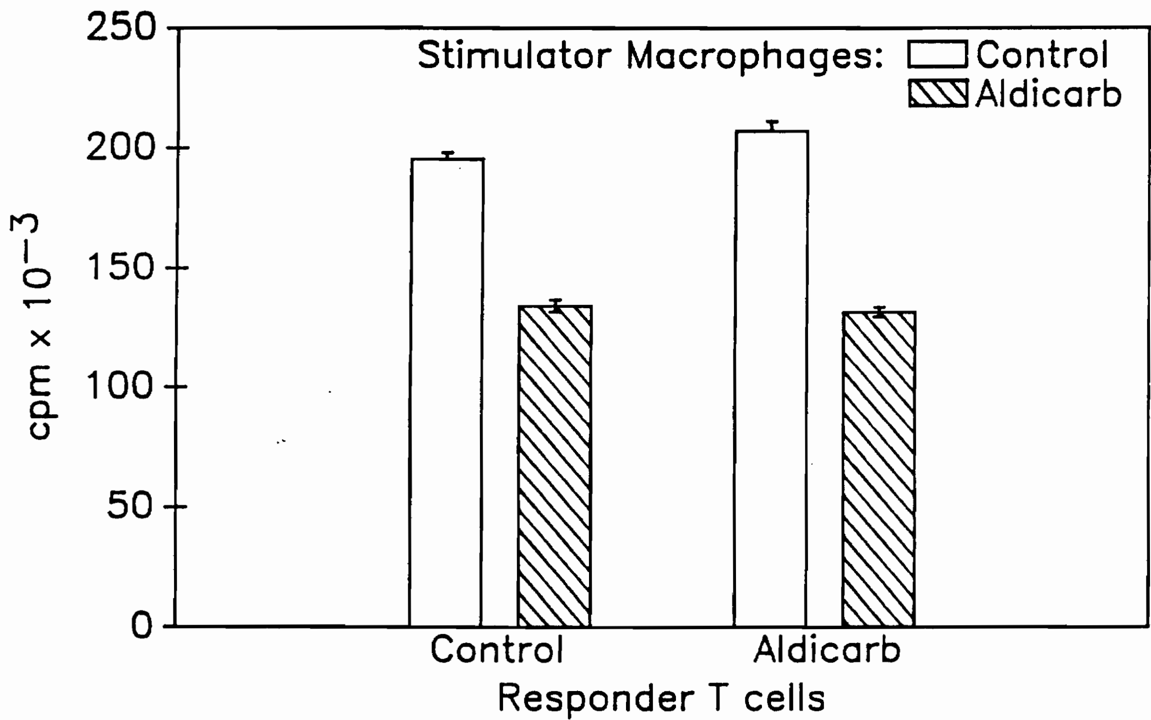

Figure 10. Responsiveness of purified T cells to Con A in the presence of accessory macrophages: Responder T cells ($2x10^5$) were purified from control or aldicarb-treated mice and stimulated with Con A in the presence of irradiated splenic macrophages ($4x10^5$) from control or aldicarb-treated mice. The assay was performed and studied as in Fig. 9.

4.3.2 Responsiveness of spleen cells and purified T cells to anti-CD3 mAb

Antibodies directed against CD3 antigens on T cells have been shown to activate the T cells (Leo et al., 1987). Although soluble anti-CD3 mAbs do not by themselves stimulate the T cells, cross-linking the CD3 molecules as achieved by use of Fc receptor-bearing macrophages can activate the T cells. To study the anti-CD3 response, whole spleen cells from aldicarb-treated or control mice were stimulated with anti-CD3 mAbs and T cell proliferation was measured. It was observed as shown in Fig. 11 (upper panel) that aldicarb at all concentrations tested (0.1, 1, 10 and 100ppb) significantly suppressed the anti-CD3 responses ($p<0.05$). However, when T cells were purified from aldicarb-treated mice and stimulated with anti-CD3 mAbs in the presence of normal control macrophages, the suppression observed earlier was no longer demonstrable (Fig. 11, lower panel).

4.3.3 Responsiveness of T cells to PMA and calcium ionophore

Purified T cells have been shown to proliferate directly in response to stimulation with PMA and calcium ionophore in the absence of additional signals from the accessory cells. The T cells were therefore purified from aldicarb-treated mice and stimulated with PMA and calcium ionophore and the T cell proliferation measured. The data shown in Fig. 12 suggested that

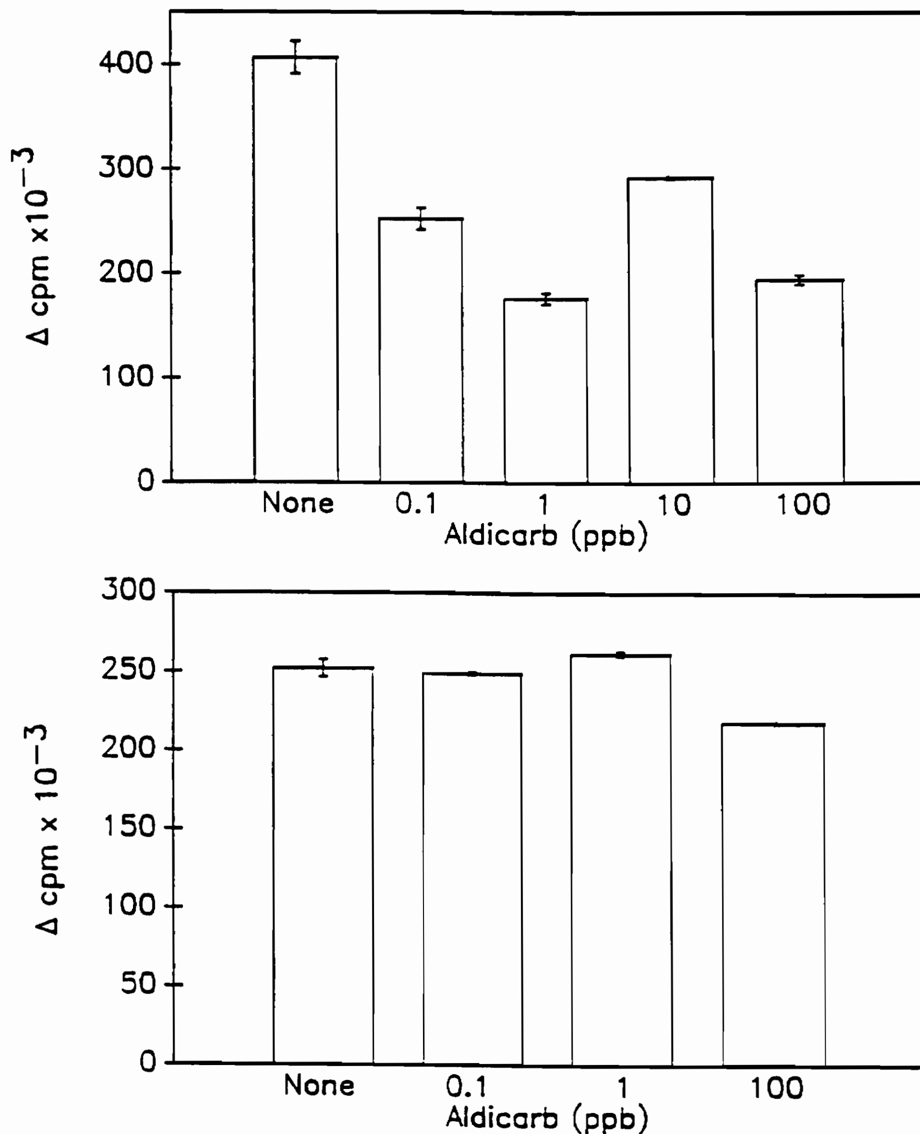

Figure 11. Responsiveness of whole spleen cells and purified T cells to stimulation with anti-CD3 mAbs: In the upper panel, whole spleen cells ($4x10^5$) from control or aldicarb-treated mice were cultured with 1:100 final dilution of anti-CD3 mAbs. In the lower panel purified T cells from aldicarb-treated mice were cultured in the presence of anti-CD3 mAbs and irradiated normal macrophages ($4x10^5$cells/well). The cultures were incubated for 48 hours at 37°C, and cell proliferation was measured as described in the legend to Fig. 9. Responder cells cultured with medium alone incorporated less than 5000 counts and these values were subtracted from the experimental cpm to obtain the Δcpm. Also, purified T cells stimulated with anti-CD3 mAbs alone incorporated less than 5000 cpm.

aldicarb-treated T cells did not exhibit any defective responsiveness when compared to the control T cells ($p > 0.05$). These data confirmed that aldicarb does not induce any direct immunosuppressive effect on T cells.

4.3.4 Antigen-processing and presentation by aldicarb-treated macrophages to T helper cells

To study the antigen-processing and presenting abilities of the macrophages, a T helper cell clone D10.G4 was used which responds to the conalbumin in association with I-A^k molecules (Kaye et al., 1983). The D10.G4 cells were cultured in the presence of irradiated control or aldicarb-treated macrophages and 100 μg/ml of conalbumin for 2-3 days and the T cell proliferation was measured. The data shown in Fig. 13 suggested that aldicarb-treated macrophages demonstrated decreased antigen-processing and presentation abilities when compared to the control macrophages ($p < 0.05$).

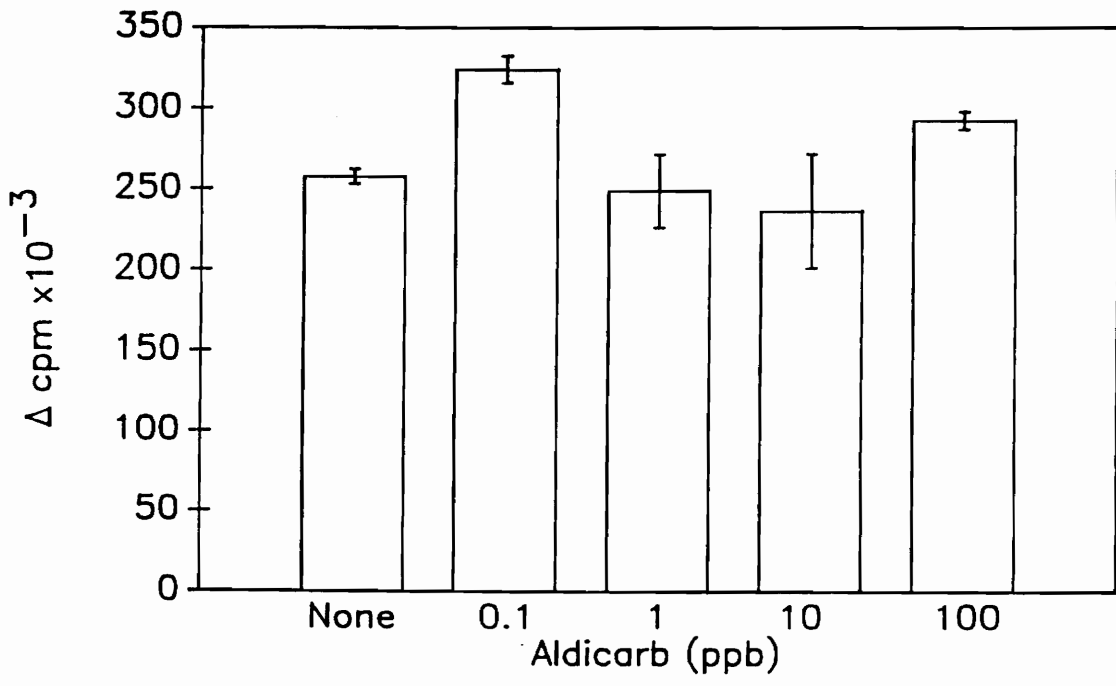

Figure 12. Responsiveness of purified T cells from aldicarb-treated mice to stimulation with PMA and calcium ionophore: T cells were purified from the spleens of aldicarb-treated mice and $2x10^5$ cells were cultured with PMA (10ng/ml) and calcium ionophore (0.5μM) for 48 hours at 37°C and cell proliferation was measured as described in the legend to Fig. 9.

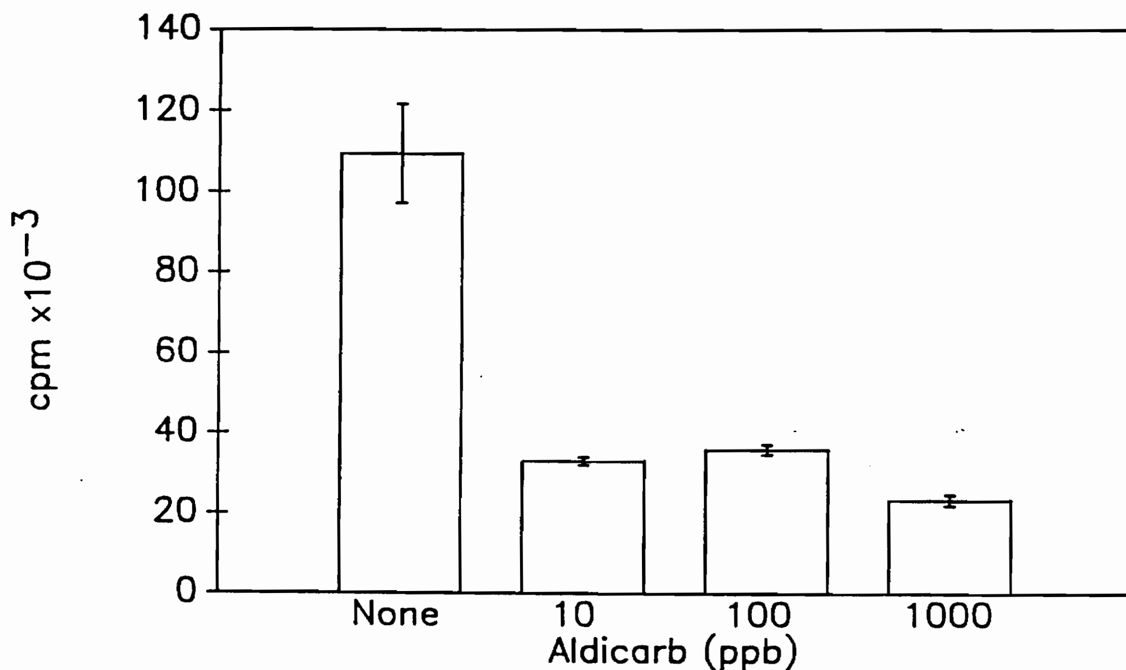

Figure 13. Antigen-presentation by macrophages from aldicarb-treated mice: Macrophages ($5x10^5$/well) from aldicarb-treated or control mice were irradiated and added to D10.G4 T cells (10^5/well) in the presence of 100μg/ml of conalbumin. The cultures were incubated at 37°C for 2 days and cell proliferation was studied as described in the legend to Fig. 9.

4.3.5 Measurement of IL-1 produced by aldicarb-treated macrophages

It was next investigated whether aldicarb-treatment caused a decrease in the IL-1 production by the macrophages. To address this possibility, splenic macrophages from C57BL/6 mice were stimulated with LPS and the culture-supernatants collected 24 hours later were tested for the IL-1 activity. The IL-1 was detected using IL-1 sensitive D10.G4 T cells. These T cells were cultured in the presence of Con A (2μg/ml) and recombinant IL-1 (positive control) or various culture supernatants and the T cell proliferation was measured. The data shown in Fig. 14 suggested that the supernatants from aldicarb-treated macrophages contained decreased amounts of IL-1 when compared to the supernatants from control macrophages ($p < 0.05$). These data suggested that aldicarb may suppress the IL-1 production by the macrophages.

4.3.6 Reconstitution of the defective D10.G4 T cell responses with exogenous IL-1

Exogenous recombinant IL-1 (rIL-1) was added to D10.G4 T cells stimulated with irradiated macrophages from aldicarb-treated or control mice to determine whether such an addition would restore the defective responses seen in aldicarb-treated macrophages. It was observed

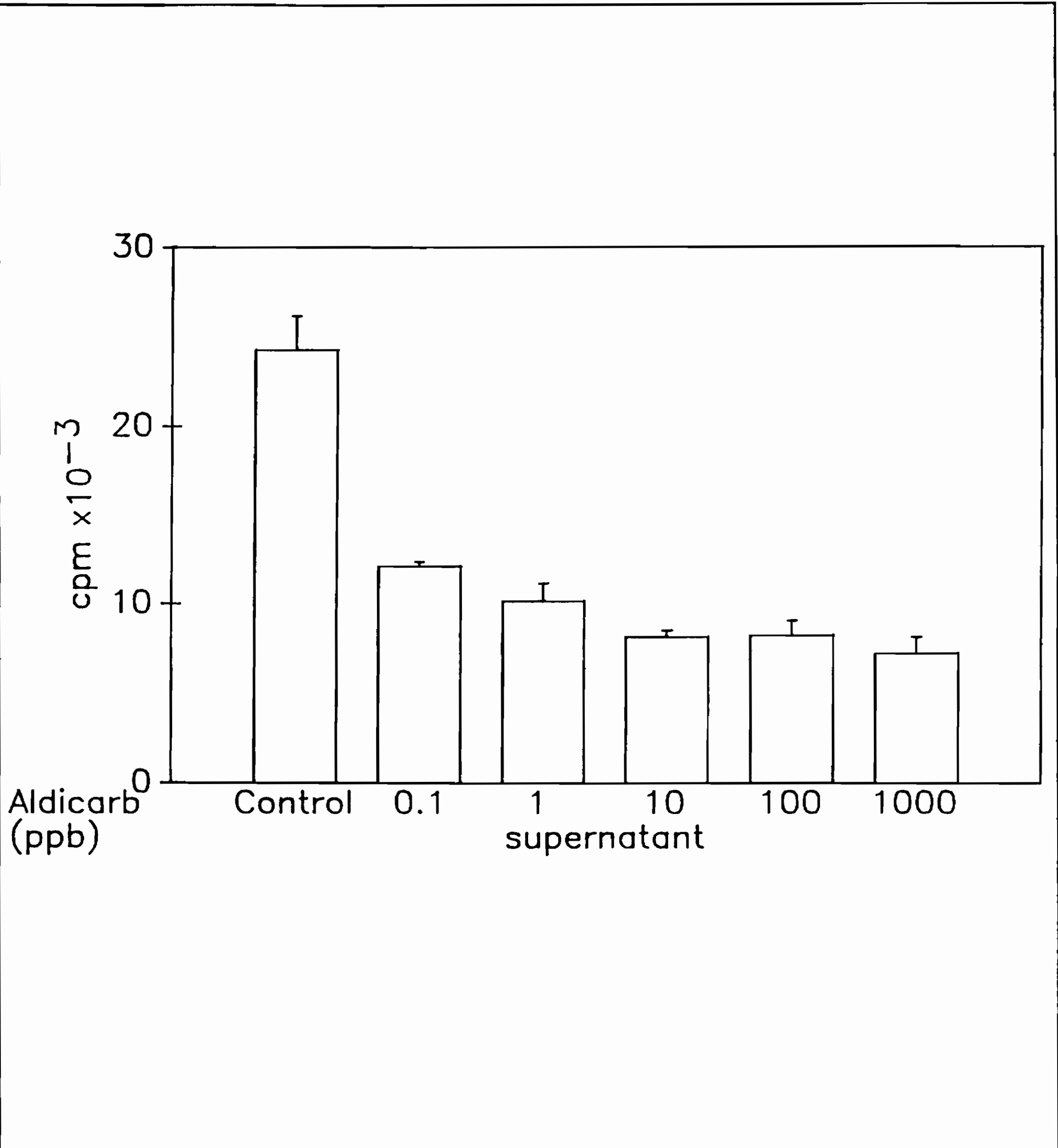

Figure 14. Measurement of IL-1 production by macrophages from aldicarb-treated mice: Splenic macrophages from control or aldicarb-treated mice were stimulated with LPS as described in methods and the culture supernatants were collected and assayed for IL-1 activity. To measure IL-1, D10.G4 T cells ($5x10^4$) were cultured with Con A (2μg/ml) in the presence of various culture supernatants for 2 days and the cell proliferation was measured as described in the legend to Fig. 9. The D10.G4 cells stimulated with Con A alone or IL-1 alone failed to demonstrate significant proliferation (less than 2000 cpm). As a positive control D10.G4 T cells stimulated with Con A and IL-1 (200 units/ml) demonstrated 72,136 $\pm$ 5,191 CPM.

that addition of increasing quantities of rIL-1 reconstituted the defective response and caused a significant increase in the D10.G4 T cell responses of aldicarb-treated spleen cells comparable to those obtained using control spleen cells (Fig. 15). These data confirmed that aldicarb-mediated immunomodulation of macrophage functions may include a defect in IL-1 production by the macrophages.

4.4 Discussion

In this section are described studies involving the immunotoxicity of aldicarb on T cell activation using a number of different approaches to activate the T cells. When whole spleen cells from aldicarb-treated mice were used as responders, a defect in the responsiveness was demonstrable in both Con A and anti-CD3 mAb-mediated activation but not when the cells were stimulated with PMA plus calcium ionophore. Since the T cell activation requires accessory cell functions, the T cells were further purified from control or aldicarb-treated mice and stimulated with Con A or anti-CD3 mAbs in the presence of macrophages from control or aldicarb-treated mice. Such studies clearly demonstrated that aldicarb did not affect the T cell functions directly but altered the macrophage accessory cell functions. This defect in the macrophage functions was attributed to decreased IL-1 production. Recent studies have suggested that several environmental contaminants can alter the immune system of the host (Recently reviewed by Luster et al., 1988). It is often a common practice to use whole spleen cells as responders while studying T cell activation with mitogens such as Con A and a decrease in responsiveness to Con A is then interpreted as a defect in T cell responsiveness. However, since the T cell responsiveness to Con A, also depends upon the accessory cells

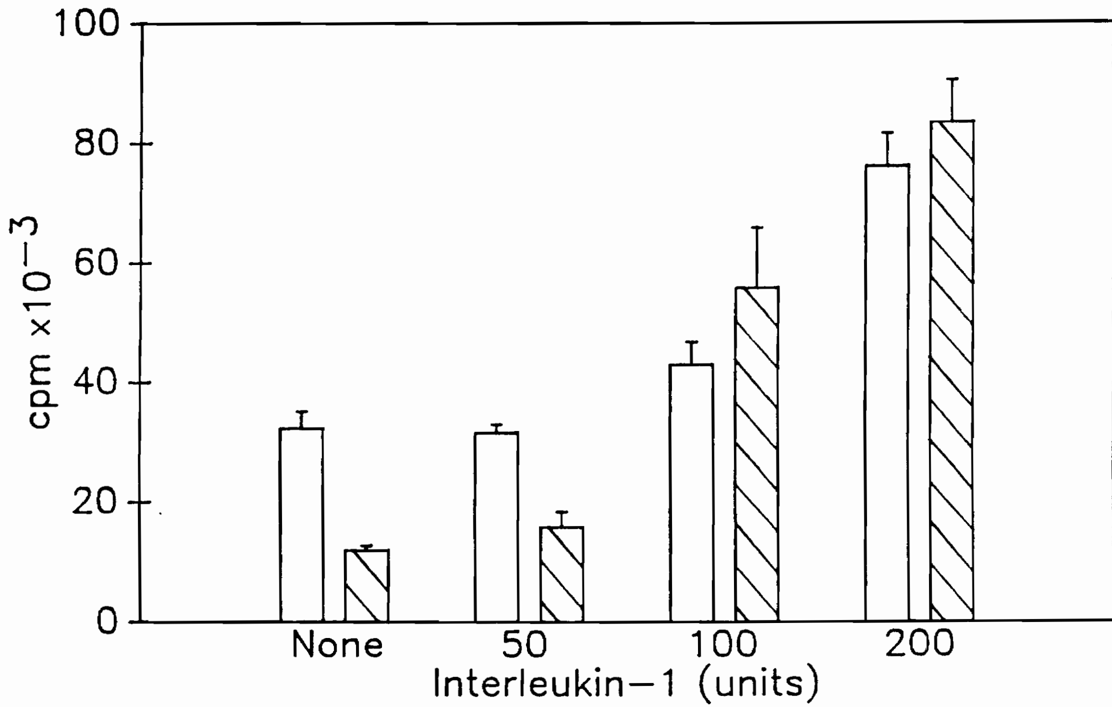

Figure 15. Reconstitution of defective macrophage function in aldicarb-treated mice using exogenous rIL-1: D10.G4 T cells ($5x10^4$/well) were stimulated with irradiated macrophages ($5x10^5$/well) from control (open bars) or (slashed bars) aldicarb-treated mice in the presence of 100μg/ml of conalbumin as described in Fig. 13. These cultures were further incubated in the absence or presence of 50, 100 or 200 units/ml of rIL-1. The cell proliferation was measured as described in legend to Fig. 9.

such as macrophages (Rosenstreich et al., 1976), such a conclusion may be inaccurate. This fact was well demonstrated in the current study.

The T cell receptor (TcR) is associated on the surface of the cell to CD3, a multichain complex which is thought to transduce signals from the TcR to the cytoplasm of the cell. Recent studies have demonstrated that mAb directed against a subunit on the CD3 complex can activate the T cells to proliferate and that such a stimulation mimics that of the antigen plus major histocompatibility (MHC) product (Leo et al., 1987). Soluble anti-CD3 mAb fails to activate the T cells. However, multi-valent cross-linking of anti-CD3 mAb with the use Fc receptor-bearing macrophages, can activate the T cells (Hirsch et al., 1989). In the present study we observed that when whole spleen cells from aldicarb-treated mice were stimulated with anti-CD3 mAbs, they demonstrated a decreased response. However, this decrease was not due to a direct effect of aldicarb on the T cells, inasmuch as, when purified T cells from aldicarb-treated mice were stimulated with anti-CD3 mAb in the presence of normal macrophages, the defective response observed earlier was no longer demonstrable. These data suggested that the decreased anti-CD3 responses may have resulted from a defect in the accessory functions of the macrophages.

Several recent studies have demonstrated that calcium ionophores along with phorbol esters such as PMA can directly activate T lymphocytes to proliferate. In this system, PMA is known to activate protein kinase C (PKC) directly and the calcium ionophore causes an increase in intracellular Ca^{2+}. In combination, these events can bypass the early signal transduction of lymphocyte activation and provide a strong signal for the activation of normal mature T cells (Truneh et al., 1985). This activation of T cells is independent of the accessory cells since purified T cells can respond to PMA plus calcium ionophore, whereas, the same cells fail to respond to Con A or anti-CD3 mAb in the absence of accessory cells. The fact that aldicarb-treatment does not directly affect the T cell functions was confirmed by stimulating purified T cells with PMA plus calcium ionophore, at which time, no significant suppression in

the T cell responses were seen. Our data also suggested that stimulation of T cells with PMA plus calcium ionophore may represent a better assay than the use of Con A for studying direct T cell activation.

Recently, a few studies have reported the immunomodulating properties of aldicarb. Olson et al. (1987), reported that mice given aldicarb in drinking water at 1-1000 ppb concentrations for 14-34 days, demonstrated significantly suppressed B cell responses to sheep red blood cells. Studies in humans have demonstrated that aldicarb may alter responsiveness to Candida antigen and alter the ratios of T cell subsets (Fiore et al., 1986). In contrast to these findings, Thomas et al. (1987) reported that aldicarb had no significant effect on T and B cell responses to mitogens and on T cells responding in the mixed-lymphocyte culture. The reason for this discrepancy is not clear. Since the immunotoxicity of xenobiotics depends on the strains of mice used and the Ah phenotype (Nagarkatti et al., 1983), use of different mouse strains may help to resolve this question. In the previous studies we found that aldicarb does have an effect on the immune system. It was observed that aldicarb suppressed the self-Ia reactive (autoreactive) T cell responses. This was however caused not by the direct action on T cells but by altering the macrophage functions (Dean et al., 1989). Aldicarb also altered the macrophage but not NK-cell mediated cytotoxicity of tumor cells (Selvan et al., 1989). In addition to providing accessory cell functions, the macrophages also play a critical role in processing a foreign antigen and presenting the antigen in association with self-Ia molecules (Harding et al., 1988). In the present study, therefore, the capacity of the macrophages to process conalbumin and present it to the D10.G4 T cell clone was analyzed. Although aldicarb-treated macrophages were defective in stimulating the D10.G4 T cells, this defect was probably not related to any decrease in the antigen-processing and presenting abilities of the macrophages but was probably related to defective IL-1 production inasmuch as addition of exogenous IL-1 could completely reconstitute the D10.G4 T cell responsiveness.

In earlier studies three main mechanisms were looked at to explain decreased capacity of aldicarb-treated macrophages to stimulate autoreactive T cells (Dean et al., 1989). The first possibility was that aldicarb-treatment induced suppressor macrophages which would secrete prostaglandins or other factors which would down regulate the T cell responses. The second possibility was that the aldicarb-treated macrophages expressed decreased levels of Ia antigens necessary to trigger the autoreactive T cell responses. Lastly, the aldicarb-treated macrophages could produce decreased amounts of IL-1 critical for T cell activation. These studies excluded the first two possibilities and it was found that addition of exogenous IL-1 could completely reconstitute the defective macrophage function (Dean et al., 1989). In the present study, it has been further demonstrated that aldicarb-treated macrophages do indeed produce decreased amounts of IL-1. The decrease in IL-1 production may indirectly have several effects on T cell proliferation. Recent studies have demonstrated that IL-1 and IL-6 share several similarities in function (Wong and Clark, 1988) and furthermore, IL-6 is inducible in certain cells by IL-1 (Van Damme et al., 1987). Thus it is possible that addition of exogenous IL-1 may induce enhanced production of IL-6 which in turn may activate the T cells and reconstitute the defective response (Dean et al., 1989). Decreased IL-1 production may also result in suboptimal activation of T cells which in turn may produce less autocrine growth factors such as IL-2 or IL-4 leading to decreased T cell proliferation.

It should be noted that in the present study as well as in previous studies, a dose dependent suppression of the immune response was not observed. However, it was observed that when multiple doses of aldicarb were injected, they caused increased suppression of macrophage-mediated cytotoxicity when compared to single-dose treatments (Selvan et al., 1989). Olson et al. (1987) observed an inverse dose response after multiple exposure with aldicarb. Thus they observed that lower doses of aldicarb caused greater immunosuppression than higher doses of aldicarb, the reasons for which were not clear.

In summary, the present study demonstrates that aldicarb affects mainly the macrophages without affecting the T cells. However, since most T cell responses are dependent on the accessory cells, aldicarb may indirectly affect the T cell functions also. The present study also demonstrates the importance of the use of purified lymphocyte and macrophage population while analyzing the immunotoxicity of xenobiotics. Such studies help to pinpoint the defect at individual cell populations and enable to shed new light on the mechanisms of immunomodulation.

4.5 Summary

In this section, studies on the immunomodulatory effects of aldicarb on T cells activated by a number of different ways have been described. When C3H mice were injected intraperitoneally with a single dose of 0.1 to 1000 ppb of aldicarb and their spleen cells were stimulated with T cell mitogens such as concanavalin A (Con A), a decreased responsiveness was detected when compared to the control mice. However, when purified T cells from aldicarb-treated mice were stimulated with Con A in the presence of irradiated control macrophages, the defective T cell response was no longer demonstrable. Also, purified control T cells stimulated with Con A in the presence of irradiated macrophages from aldicarb-treated mice showed decreased responsiveness. Similar observations were made using anti-CD3 monoclonal antibodies (mAb) to activate the T cells inasmuch as whole spleen cells from aldicarb-treated mice showed decreased responsiveness to anti-CD3 stimulation, whereas, purified T cells when stimulated with anti-CD3 antibodies in the presence of irradiated control macrophages, showed normal reactivity. Together these data suggested that aldicarb altered the accessory cell functions of the macrophages without directly affecting

the T cells. This was further confirmed by stimulating purified T cells from aldicarb-treated mice with phorbol myristate acetate (PMA) and calcium ionophore, a response which is independent of the accessory cells and which was found to be normal in aldicarb-treated mice. When macrophage functions were further analyzed in aldicarb-treated mice, it was observed that the macrophages demonstrated decreased capacity to process and present conalbumin to the T helper cell clone, D10.G4. When splenic macrophages from aldicarb-treated mice were activated with lipopolysaccharide (LPS), they produced decreased amounts of IL-1 when compared to similarly activated control macrophages. Also, the decreased antigen-presentation by aldicarb-treated macrophages was reconstituted when exogenous recombinant IL-1 was added to the cultures. These data together suggested that aldicarb affects the macrophage functions by interfering with IL-1 production and that it does not affect the T cell functions directly.

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APPENDIX - Abbreviations

Press ADCC, antibody-dependent cell-mediated cytotoxicity assay; AIDS, Acquired Immunodeficiency Syndrome; AlloMLR, allogeneic mixed lymphocyte reaction; APC, antigen presenting cell; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CD, cluster of differentiation; Con A, concanavalin A; CPM, counts per minute; CTL, cytotoxic lymphocyte; EPA, Environmental Protection Agency; ER, endoplasmic reticulum; FALS, forward angle light scatter; IL, interleukin; IFN-γ, gamma interferon; ip, intraperitoneal; LAK, lymphokine activated killer cell; LPS, lipopolysaccharide; mAb, monoclonal antibody; MDADS, multiparameter data acquisition and display system; MHC, major histocompatibility complex; NK, natural killer cell; PEC; peritoneal exudate cell; PKC, protein kinase C; PMA, phorbol myristate acetate; ppb, parts per billion; rIL, recombinant interleukin; SAC, splenic adherent cell; SE, standard error; SMLR, syngeneic mixed lymphocyte reaction; Tc, cytotoxic T cell; TcR, T cell receptor; Th, T helper cell; Ts, T suppressor cell.

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