THE EFFECTOR CELLS IN *IN VITRO* SEMI-SYNGENEIC CYTOTOXICITY AND THEIR FUNCTIONS IN THE *IN VIVO* PHENOMENON OF THE GRAFT-VERSUS-HOST REACTION

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A Thesis Submitted to The Faculty of Graduate Studies
In Partial Fulfilment of The Requirement For The
Degree of Master of Science

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TABLE OF CONTENTS

ABBREVIATIONS

GVH	=	Graft-versus-host
PEC	=	Peritoneal exudate cell
PLN	=	Peripheral lymph node
PMN	=	Polymorphonuclear
^{51}Cr	=	Chromium 51
MHC	=	Major Histocompatibility Complex
Mls	=	M - Locus
MLR	=	Mixed leukocyte reaction
H-2	=	Histocompatibility - 2 locus
Hh	=	Hybrid Histocompatibility (Hemopoietic Histocompatibility)
Ir	=	Immune response gene
CMC	=	Cell-mediated-cytotoxicity
PFC	=	Plaque-forming-cell
SRBC	=	Sheep red blood cell
LATS	=	Long Acting Thyroid Stimulator
LT	=	Lymphotoxin
LAD	=	Lymphocyte Activating Determinant
LAF	=	Lymphocyte Activating Factor
ITL	=	Initiator T lymphocyte
RTL	=	Recruited T lymphocyte
MIF	=	Migration Inhibitory Factor
AEF	=	Allogeneic Effect Factor
MF	=	Mitogenic Factor
CPM	=	Counts Per Minute

RS	=	Recognition Structure
RS_A	=	Recognition structure for the A antigen
RS_B	=	Recognition structure for the B antigen
Anti-RS	=	Anti-recognition Structure
RPMI	=	Rosewell Park Memorial Institute
HBSS	=	Hank's Balanced Salt Solution
BBS	=	Borate Buffered Saline
DS	=	Dulbecco's Solution
PPD	=	Purified Protein Derivative
EC	=	Effector Cell
TC	=	Target Cell

Semi-syngeneic = Semi-allogeneic
e.g., parental A/A cells are semi-syngeneic or semi-allogeneic to the (A x B) F_1 cells

LIST OF TABLES

LIST OF FIGURES

Figures

ABSTRACT

Graft-versus-host reaction was induced in adult F_1 hybrid mice with the transplantation of one of the two strains of parental spleen cells. The immunocompetent cells from these GVH-induced F_1 hosts showed a semi-syngeneic cytotoxicity or F_1 anti-parent immune reaction which was quantitated by the in vitro assays of the lysis of ^{51}Cr-labelled target cells. The route of induction of the GVH reaction was important in determining the degree of semi-syngeneic cytotoxicity, and the peritoneal exudate cells were observed to be most effective in eliciting the in vitro F_1 anti-parent immune response.

Investigations on the mechanical aspect of the semi-syngeneic cytotoxicity reaction revealed that the GVH reaction appeared to activate the F_1 macrophages to become cytotoxic effector cells since they were shown to possess surface adherence and were exclusively sensitive to silica particles. Non-adherent F_1 immunocompetent cells were found incapable of initiating the semi-syngeneic cytotoxicity reaction. Moreover, irradiation of the F_1 effector cells abrogated the semi-syngeneic cytotoxicity reaction.

The semi-syngeneic cytotoxicity reaction, mediated by the GVH-induced F_1 macrophages, could be enhanced by both in vivo and in vitro addition of normal syngeneic F_1 amcrophages. The adoptive transfer of GVH-induced F_1 PECs into other syngeneic F_1 recipients undergoing GVH reactions resulted in an increased semi-syngeneic cytotoxicity response as measured by the CMC assays, and also a decrease of the in vivo GVH reaction as evidenced by ; (1) decreased spleen indices relative to those

GVH-induced F_1 animals without receiving additional GVH-activated F_1 PECs, and (2) increased survival rates of lethally irradiated and GVH-induced F_1 recipients relative to those without receiving additional GVH-activated PECs. Such capacity to suppress an in vivo GVH reaction by the GVH-activated F_1 cells could be abrogated by irradiation. Moreover, certain degree of specificity seems to exist in the in vivo GVH reaction suppression. This type of speciificity is reflected by the in vitro observation in the preferential target cell lysis experiments. The GVH activated F_1 immunocompetent cells were shown to mediate the host-versus-graft reaction in producing the spontaneous resolution of the in vivo GVH reaction.

The underlying mechanism of the semi-syngeneic cytotoxicity reaction was explored. Investigations on the immunological aspect of the mechanism revealed that, during a GVH reaction, the F_1 host immunocompetent cells exhibited a preferential cytotoxic effect on the parental H-2 genotype target cells; i.e., when the H-2 genotype of the target cells used in CMC assays and the H-2 genotype of the parental cells used in GVH induction were identical, the lysis of target cells was significantly higher than the situation in which the H-2 genotype of the target cells and the parental cells were different. In addition, the F_1 immunocompetent cells were found capable of reacting against the histocompatibility antigens of the parental cells, demonstrating the mediation of the semi-syngeneic cytotoxicity or F_1 anti-parent reaction via the antigenic determinants of the H-2 complex.

INTRODUCTION

A graft-versus-host reaction results from the recognition of host tissue-antigens which do not exist in the transplanted immunocompetent donor cells. Circumstances initiating GVH reactions include situations in which the host will accept a graft without the capacity of rejection. The principle of GVH reaction is classically illustrated by the condition that F_1 hybrid animals will accept immunocompetent cells from either parental strains, but the grafted cells from one parental strain are confronted in the tissues of the F_1 host with antigens inherited from the other parental strain. The grafted cells proceed to attack the F_1 host tissues bearing such foreign antigenic determinants resulting in the experimental form of graft-versus-host reaction (Oliner *et al* 1961). The principle of such a unidirectional reaction is illustrated in Figure 1.

There are basically two categories of GVH reaction; systemic and localized. The immunologists have done considerable work on the systemic GVH reactions, while the pathologists are more interested in studying the localized GVH reactions. Experimental evidence of systemic GVH reaction was initially provided by the production of "runting diseases" in newborn mice which were injected with adult lymphoid cells (Simonsen,1957). Apart from this type of classical hybrid wasting disease, other types of systemic GVH reactions have been described. For example, adult mice previously made tolerant to another strain by neonatal inoculation with immunoincompetent cells from the other strain, were noted to develop "runting diseases" when they were grafted with immunocompetent cells from

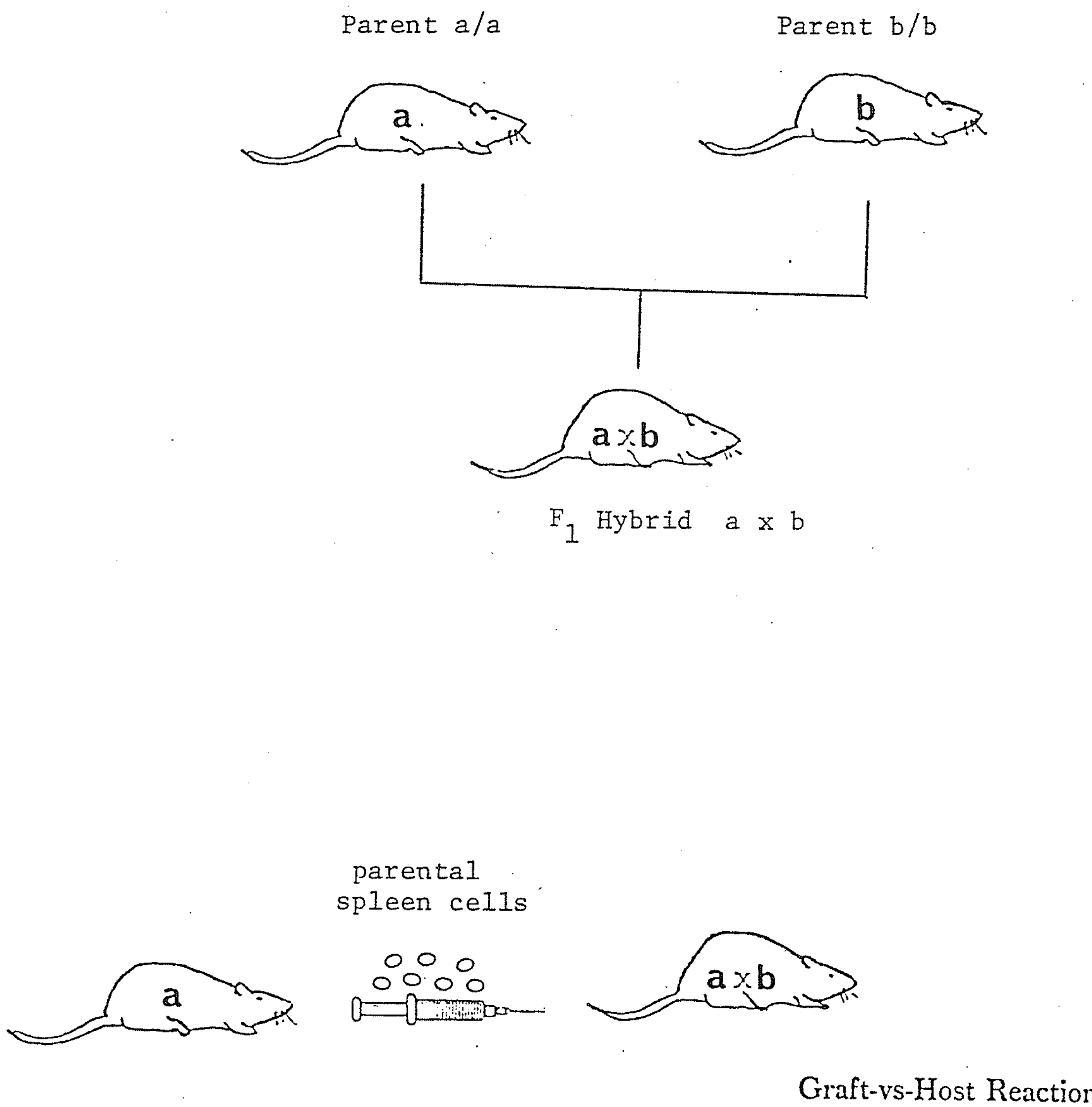


Figure 1 - Principle of Experimental GVH Reaction

the donor strain origionally used in the induction of neonatal tolerance (Billingham et al 1955; Billingham and Silvers,1961). In the case where the parabiotic surgical union of an animal (graft) with an immunologically non-responsive partner (host) through vascular anastomoses, the syndrome of parabiosis intoxication characterized by severe anemia, weight loss, and death of the non-responsive partner (host), had been identified to be one form of systemic GVH reactions (Eichwald et al,1959). Lethally irradiated animals, when transplanted with allogeneic bone marrow cells, usually recovered from the primary effect of irradiation, but they eventually developed "secondary diseases" due to the activities of the immunocompetent cells in the transplanted bone marrow inoculum (Trentin,1956).

Besides the systemic category, several localized forms of GVH reactions have been described. These included the intrarenal GVH reactions produced by inoculating parental lymphoid cells into the renal capsules of F_1 hosts (Elkins,1964), and the intracutaneous GVH reaction observed in hamsters and guinea pigs when parental spleen cells were injected intradermally (Brent and Medarwa,1966). When adult chicken leukocytes were distributed over the chorioallantoic membrane of a genetically unrelated embryo, white focal "pocks" developed. Using this type of local GVH reaction, the small lymphocytes were established as participants in such reactions (Simonsen,1967). Popliteal lymph node (PLN) hypertrophy, or enlargement, after the injection of parental lymphoid cells into the foot-pads of animals such as hamsters and rats, has also been used to quantitate local GVH reactivities (Grebe and Streilein,1974).

The importance of GVH reaction in immunology is mainly two-fold. From an academic point of view, GVH reaction has been and will continue to be a useful experimental approach in studying immunobiology, the immune response, and immunoregulation. In clinical investigations, GVH reactions have been important in situations of : intrauterine blood transfusion of Rh erythroblastosis fetalis (Naiman,1969), the transfusion of blood into congenitally immune deficient infants (Hathaway,1965), and bone marrow transplantation studies (Kretschner,1970). GVH reaction today remains the major stumbling block in the success of bone marrow tranplants. Approximately 70% of patients receiving bone marrow grafts develop GVH reactions (Thomas et al,1975); and about half of these die from GVH related infections, or from the GVH reaction itself (Bunn,1977; Rosen et al,1978). Granulocyte transplantations have recently been used with some success in acute leukemic patients (Graw et al,1970), but GVH reaction is significantly induced with platelet transfusions (Mathe et al,1974), presumably due to the simultaneous transfer of contaminating leokocytes (Cohen et al,1979).

Because of academic significance and extensive clinical applications, many research efforts have been devoted to study the phenomenon of graft-versus-host reaction. In the following review of literature, relevant significant findings are described.

REVIEW OF LITERATURE

An enormous amount of literature is available on the various aspects of the GVH reaction, and since the review of literature is not meant to be all-inclusive, only articles pertinent to the theme of the present thesis are presented. The review is divided into three main sections : Sequelae of graft-versus-host reactions, Immunology of GVH reactions, and Immunology of host-versus-graft (HVG) reactions. Within each section, significant observations will be described.

SEQUELAE OF GRAFT-VERSUS-HOST REACTION

In the animal model, graft-versus-host reactions can be induced by the injection of parental lymphoid cells into the appropriate F_1 hybrids, and in applying this technique to newborn mice, the GVH syndrome observed is historically known as "runting diseases". In clinical situations, GVH diseases in humans are usually the result of bone marrow transplantations. In the animal model, GVH reactions have been extensively investigated; its manifestations and sequelae of the phenomenon are described in the following.

Manifestations of Graft-versus-host Reaction

When parental lymphoid cells are injected intravenously or intraperitoneally into the appropriate F_1 hybrids, systemic GVH reactions can take either an acute or a chronic course, depending on the number of grafted parental cells, the age of the hybrid at the time of transplantation, and the extent of histoincompatibility involved (Brent and Medawar,1966).

In the animal model, anatomical manifestations of the GVH syndrome as initially described in "runting diseases" include the failure of normal somatic development of the newborn, splenomegaly, hepatomegaly, hypertrophy, then atrophy of the lymphoid organs, and lesions of the cutaneous tissues (Billingham,1968). The hallmark of the systemic process is significant hyperplasia of the splenic parenchyma. Early splenic enlargement had been attributed to the proliferation of the grafted donor cells, but in later periods, splenomegaly is mainly due to the proliferation of the host lymphoid cells (Auerbach and Globerson,1966; Hilgard,1970; Bonney and Feldbush,1973; Bennett and Hand,1978).

Histologically, the follicular structure of the spleen becomes obliterated and the majority of the organ is occupied by blast-like cells and necrotic foci (Simonsen,1957). This acute stage of lymphoid organomegalies is followed by a stage of pronounced hypoplasia, particularily of the thymus. The most important physiological changes are immunological, hematological, and hepatic dysfunctions. Decreased immune responsiveness results in increased susceptibility to bacterial and viral infections (Elkins,1971). In many murine strain combinations tested, hosts undergoing GVH reactions developed remarkably frequent, malignant reticuloendothelial tumors (McBride,1966).

The manifestations of GVH reactions in the human patients studied are usually secondary to bone marrow transplantations. The signs and symptoms of GVH disease appear from 10 to 30 days after grafting of the bone marrow. The earlier the manifestations appear, the more serious the reaction and prognosis (Cline et al,1975). The clinical picture in these patients consists of dermatitis with erythematous maculopapular eruptions spreading all over the body, hepatitis accompanied by jaundice, elevated hepatic enzymes, and gastrointestinal disturbances (Thomas et al, 1975). These synptoms will usually lead to generalized immunodeficiency states, complicated by severe infections, resulting in septic shock and death (Wells and Ries,1978).

Experimental GVH reactions in the animal model do not, as a rule, cause the death of the host. In fact, if the host can survive the initial GVH syndrome, spontaneous recovery is expected, and a type of secondary GVH reaction is not easily inducible, if not impossible. This type of natural resolution of GVH reaction manifestations observed in experimental animals unfortunately does not occur in humans because the simultaneous presence of intrinsic diseases in humans are absent in the experimental animals.

Remission of Graft-versus-host Reaction

If the dogma of transplantation immunology is correct in asserting that the F_1 hybrid immunocompetent cells are incapable of reacting against the parental lymphoid cells, it seems possible that when a single parental immunocompetent cell, injected into the F_1 host, and given sufficient period of incubation, would proliferate and produce a full blown picture of GVH syndrome; but this is not the case. In fact, many studies have shown that F_1 animals which survived the acute stage of GVH reaction usually recover (Gowans,1962).

Natural remissions of both systemic and localized GVH reactions in experimental animals have been extensively reported. When lethally irradiated (CB x MHA) F_1 hamsters were inoculated with parental MHA lymphoid cells, the severe cutaneous reaction of lethal epidermolysis (one form of systemic GVH reactions) were produced. But using normal (CB x MHA) F_1 hmasters as recipients of intravenously inoculated parental MHA lymphoid cells, the severe cutaneous reaction observed in lethally irradiated syngeneic F_1 hamsters was absent, and the F_1 hosts eventually returned to a normal state with no apparent pathological sequelae (Streilein and Billingham,1970a).

Studies on localized GVH reactions also show that the pathological manifestations resolve spontaneously. By injecting parental lymphoid cells into the renal subcapsular spaces of the F_1 recipients to demonstrate local GVH pathology, it was observed that after the initial reaction, diminishing inflammatory infiltrate appeared by the 14th day. At the end of the 40th day, there was little macroscopic evidence that GVH reaction had occured (Elkins,1964). Using (DA x Lewis) F_1 rats as hosts, parental Lewis lymphoid cells were inoculated intradermally to initiate a localized GVH reaction. After the initial skin manifestations, the reaction regressed to a stage where barely perceptible residual lesion, identified by a necrotic skin nodule remained on the F_1 hosts (Streilein and Billingham,1967). By injecting parental lymphoid cells into the foot-pads of the appropriate F_1 hybrids, local GVH reactions, quantitated by assaying popliteal lymph node weights consistently followed a self-limited course (Grebe and Streilein,1974).

The self-limited nature of GVH reaction has also been quantitated by delayed hypersensitivity reactions involving the measurement of the thickness of the foot-pads of F_1 hosts injected with parental lymphoid cells. When parental C57BL/Rij spleen cells were transplanted into (C57BL/Rij x CBC/Rij) F_1 mice, GVH reactivities were noted to be maximal from day 5 to day 8, and thereafter the reactivities decreased progressively (Wolters and Benner,1978).

Spontaneous remissions of GVH reactions seem to involve certain immunoregulatory mechanisms. Features of the GVH syndrome usually subside in experimental animals when the lymphoid organs are replenished by proliferating host lymphoid cells (Fox,1966). In the characteristic development of splenomegaly during GVH reactions, the weights of the spleens, after reaching peak values, decline progressively. This is followed by complete recovery (Simonsen and Jensen,1959). It has been suggested that the host spleen provides an immunoregulatory microenviroment in which cell-mediated immune response, including GVH reaction, are modulated or regulated (Grebe and Streilein,1976).

Absence of Secondary Graft-versus-host Reaction

In addition to the spontaneous remission of GVH reaction in the genetically tolerant F_1 hybrid host, certain mechanisms seem to produce in the host animal a state of refractoriness to subsequent GVH-inducing challenges, and no secondary GVH reaction parallel to the secondary antibody response can be initiated. This phenomenon was first made in experiments in which adult (CBA x C57BL/6) F_1 mice, previously injected with parental C57BL/6 spleen cells, failed to develop GVH reaction on subsequent injection of either strain of parental spleen cells (Fox and Howard,1963). The observation was later supported by the fact that rats which survived the first GVH reaction were subsequently found to be staunchly resistant to a second inoculation of lymphoid cells from the same parental strain (Field and Gibb,1966). Such resistance to a secondary challenge has also been demonstrated in parabiosis studies during the acute stage of the GVH syndrome where certain unidentified humoral factors were suggested to be responsible for the refractory state (Field and Cauchi,1967). In the case of F_1 hybrid hamsters that have survived the early phase of GVH disease, a subsequent challenge with lymphoid cells of the original donor genotype resulted in similar refractoriness as reported in other animals tested (Streilein,1972).

The absence of a secondary GVH reaction has been studied in its specificity, and both "specific refractoriness" as well as "non-specific refractoriness" have been identified. Using DA, Fischer (parental donors) and (DA x Fischer) F_1 hybrid rats (GVH recipients) as an example, the term "specific refractoriness" referred to a situation in which the parental strain used in both the primary (e.g.,DA) and secondary (e.g.,DA) challenges were identical, while "non-specific refractoriness" indicated the situation where the parental strain used in the primary (e.g.,DA) and the secondary (e.g.,Fischer) challenges were dissimilar. In both situations, the absence of a secondary GVH reaction was confirmed (Grebe and Streilein,1976).

These observations implicated a situation in which, parental immunocompetent cells, after exposure to semi-allogeneic F_1 host tissues, induced certain immunoregulatory mechanisms in the F_1 hybrids so that a secondary GVH-response is modulated or suppressed.

Failure of Adoptive Passage of Graft-versus-host Reaction

If it is true that the F_1 generation is genetically tolerant to parental immunological challenge, then it should be possible to produce the GVH reaction serially from the primary host to a syngeneic secondary host by adoptive transfer of lymphoid cells. It was however, impossible to elicit "adoptive runting diseases" in mice by transferring spleen cells from the first F_1 recipient to the second syngeneic recipient (Russel,1961). Moreover, evidence has been presented that within 24 hours after injection into the newborn F_1 hybrids, parental lymphoid cells lost their capacity to initiate GVH reaction in a secondary host (Simonsen and Jensen,1959). Using isogeneic rodents and inbred chicken strains, a few studies have only been able to achieve at best, one or two passages before the cell suspensions lost their capacity to incite GVH reactions (Ramseier and Billingham,1966; Steinmuller,1967).

Attempts to passage the GVH reaction serially in hamsters beyond the tertiary host by adoptive cellular transfer were unsuccessful (Streilein and Billingham,1970a). Using adult (Fischer x DA) F_1 rats as primary and secondary hosts in serial passage experiments, it was noted that there was only popliteal lymph node enlargement (local GVH) in the secondary host and none in the tertiary host (Grebe and Streilein,1976).

The failure to transfer serially a GVH reaction in syngeneic hosts can be explained by two possibilities. The first possibility is that the donor lymphoid cells were being serially diluted to such a level as to be ineffective in inducing GVH reaction when transferred to the secondary or tertiary hosts. The possibility of such a diluting effect on the donor cells seems unlikely because there is inadequate explanation to account for the inability of donor cells to proliferate so as to compensate for the diluting effect in a genetically tolerant host.

The second possibility is that certain immunoregulatory mechanisms take place during a GVH reaction within the primary host, thus rendering the donor cells incapable of initiating the reaction in the syngeneic secondary host. Evidence supporting this assumption has been reported in studies using T_6 chromosome markers in donor cells. It was observed that during GVH reaction, parental donor cells in active mitosis constituted only about 1% in the host spleen by the 14th day, indicating the suppression of proliferation of donor parental cells (Fox,1966). In a study using popliteal lymph node assay of local GVH reaction, it was noted that 75% of the cells in the lymph node were of host origin, again demonstrating the inability of donor cells to proliferate specifically in the spleen of the F_1 host (Grebe and Streilein,1974). In studies of systemic GVH reactions involving radioisotope labelling of parental cells with ^{3}H-thymidine, it was noted that the majority of labelled donor cells were found dead in the lymphoid organs of the host within one to two weeks after the induction of GVH reaction (Sprent,1976). Similar findings in local GVH reactions have been reported (Clancy and Adams,1973).

If it is true that the GVH reaction initiated certain immunoregulatory mechanisms leading to the immunosuppression of the grafted or passaged parental lymphoid cells resulting in the failure of adoptive transfer of GVH reactions serially, then one would expect that the effect of immunosuppression, not only will affect the transplanted parental cells, but will also exert its influence on the immunocompetent cells of the host as well. In fact, this seems to be the situation observed in many studies. Features of such immunosuppression or immunoincompetence have been reported in many experimental and clinical conditions.

Immunological Incompetency in Graft-versus-host Reaction

The immune response of F_1 hybrid animals undergoing GVH reactions has been shown to be profoundly suppressed. This type of functional immunoincompetence seems to involve both humoral antibody response (Lawrence and Simonsen,1967) as well as cell-mediated immune response (Lapp and Moller,1969).

The humoral immune response of mice which have survived the initial onslaught of GVH reaction after allogeneic bone marrow transplants proved to be defective against a variety of antigens (Gengozian and Owen, 1958; Gengozian and Congdon,1965; Gengozian and Toya,1971). Adult $B6AF_1$ mice that underwent GVH reactions were deficient in forming antibodies to an intraperitoneal challenge of T_2 bacteriophages. The basis of such immunoincompetence was attributed to the destruction of host lymphoid cells (Blaese and Good,1964). When parental donor spleen cells, previously immunized to sheep red blood cells (SRBCs), were used to induce GVH reactions in F_1 hybrids, and the specific antibody response were tested 7 days later, a marked suppression of anti-SRBC antibody synthesis was detected (Moller,1971);i.e., F_1 host suppressed the parental cells.

Studies on the ability of hamsters undergoing GVH reactions to make specific antibodies in response to tetanus toxoid revealed the absence of circulating antibodies for as long as 26 days after antigenic challenge (Streilein,1972). Even in the presence of repeated antigenic stimulations, GVH-induced F_1 hosts failed to produce any detectable level of anti-SRBC antibody (Treiber and Lapp,1973). Using the Salmonella flagellar antigen in long-term allogeneic chimera studies, it was noted that the level of antibody suppression was dependent upon the severity of GVH reaction (Gengozian and Congdon,1973). There is however recent evidence indicating the lack of correlation existing between the severity of GVH diseases and the immunological capacity of the T and B lymphocytes (Urso and Gengozian,1977).

Many studies have examined the suppression of cell-mediated immunity in GVH-induced animals. Marked prolongation of allograft survival on the ears of rabbits undergoing a GVH reaction suggested the depression of transplantation rejection mechanism (Vrubel,1961). Prolonged survival of allografts has also been reported in adult (C57BL/6 x CBA) F_1 hybrids injected with C57BL/6 parental spleen cells (Howard and Woodruff,1961). Dramatic immunosuppression, resulting from a GVH reaction, was shown by the finding that H-2 incompatible skin grafts from a third party donor could survive two to three times longer on the F_1 host undergoing the GVH reaction (Lapp and Moller,1969). Using skin allografts, suppression of cell-mediated immune responses have also been demonstrated in both hamsters and rats undergoing GVH reactions (Streilein,1972).

It has been suggested that GVH-induced suppression of the humoral immune response was more persistent than the suppression of the cell-mediated immune response. While repeated (SRBC) antigenic challenges in the suppressed host did not produce any detectable level of anti-SRBC antibody, the injection of a third party's bone marrow cells into a GVH-induced host caused a subsequent rejection of the skin allograft identical to the third party bone marrow cell genotype (Treiber and Lapp,1973). In addition, GVH-induced animals were able to produce cell-mediated responses to xenogeneic as well as allogeneic antigens, following appropriate stimulations with the specific antigens (Treiber and Lapp,1976).

Immune deficiency associated with GVH reaction has been shown to cause suppression of specific antibody responses to both thymic-dependent and thymic-independent antigens (Blaese and Good,1964; Lawrence and Simonsen 1967; Zaleski and Milgrom,1973; Byfield et al,1973). In the suppression of antibody responses to thymic-independent antigens, single challenges in GVH-induced mice with Escherichia coli lipopolysaccharide endotoxin or the pneumococcal polysaccharide Type III preparations, failed to elicit significant specific antibody responses (Moller,1971; Byfield et al,1973), but multiple antigenic challenges have been shown to relieve GVH-induced suppression of humoral responses to thymic-independent antigens (Treiber and Lapp,1978). In the situation of thymic-dependent antigen related suppression, even multiple challenges failed to induce significant antibody production in GVH-induced and suppressed mice (Treiber and Lapp,1976; Treiber and Lapp,1978).

Mechanisms of GVH-induced Immunosuppression

The exact nature of the immunosuppressed state in a GVH-induced host remains a point of contention. Different mechanisms have been proposed to explain the phenomenon, and these can be categorized into three or four main groups; deficiency of a T cell mediator, the induction of suppressor cells, interference of T and B cell interaction, and the possible regulatory role of the macrophages.

GVH-induced immunosuppression was initially suggested to be the result of certain defects in the thymic-derived cell population since the administration of thymic tissues was able to restore only partially the immunocompetence of GVH-induced mice in response to SRBC antigens (Lapp et al,1974). The explanation given was that even though T cells were present in the host, the intense immune response generated by the GVH reaction functionally depleted both host and donor T cells of a thymic mediator which is essential for immunological reactivity. To support this hypothesis, GVH-induced spleen cells and normal syngeneic spleen cells were cultured in a Marbrook culture system but were separated from cell to cell contact by a cell-impermeable membrane. The possibility that normal spleen cells restoring competence to GVH-induced spleen cells through soluble mediators was examined. The results suggested that a T cell mediator could be involved (Parthenais et al,1974). In order to identify the source of this depleted mediator, thymus, bone marrow, and lymph node cells from normal mice were individually supplied to the GVH-suppressed spleen cells. While significant plaque-forming-cell (PFC) responses were restored by the thymic and lymph node cells, normal bone marrow cells were found incapable of restoring immunocompetence to the GVH-suppressed cells (Elie and Lapp,1976a).

Anatomically, this type of T cell related immunosuppression, presumably induced by the GVH reaction, did not seem to occur in all the lymphoid organs of the GVH-induced host. It appeared to be confined to the host's spleen, since spleen cells from these GVH-induced F_1 animals produced very poor restoring results. In contrast, thymic and lymph node cells from the same GVH-induced animals were able to restore significant capacity to produce anti-SRBC antibodies when supplied to the GVH-induced and suppressed spleen cells (Elie and Lapp,1976b). Interestingly, while normal bone marrow cells could not restore the GVH-suppressed spleen cells to produce good PFC responses, bone marrow cells taken from mice three days after GVH induction, restored the PFC responses in the GVH-suppressed cells even though the proportion of theta-antigen bearing cells in these GVH-induced bone marrow cells was not elevated. This indicates that the possible restoring effect of GVH-induced bone marrow cells was not due to an increased level of T cells in the bone marrow cell population (Elie and Lapp,1976a).

The restoring capacity of T cells was shown to be regulated by a population of splenic accessory (A) cells present in the spleens of mice undergoing GVH reactions (Elie and Lapp,1977). It was also noted that the optimal ratio of splenic (A) cell to non-adherent (NA) cell for maximal reconstitution of GVH-suppressed PFC responses was 1 to 10. The proportions of splenic (A) cells in the range of 20% to 40% actually suppressed the PFC response instead of restoring it. This seems to indicate that the accessory (A) cells were responsible for GVH-induced suppression of T cell helper function in the inductive phase of the immune response (Elie and Lapp,1977).

The cellular level of GVH-induced immunosuppression seems to affect both T and B cells. Suppression of the humoral response to thymic-dependent antigens was suggested to be due to a defect in the activity of helper T cell and appeared to be mediated by soluble factors. It was postulated that large quantities of soluble factors were produced by the adherent cells and were released during a GVH reaction, resulting in the suppression of T cell helper function (Treiber and Lapp,1978).

GVH-induced immunosuppression at the T and B cell level was suggested by the fact that the antibody response to a single injection of *Escherichia coli* lipopolysaccharide antigenic preparation into a GVH-induced host was significantly reduced. It was postulated that the suppression of immmune responses to thymic-independent antigens at the B cell level, was caused by the binding of a large amount of splenic (A) cell factors to the mitogenic sites on the B cell, rendering them immunosuppressed. Multiple challenges of GVH-suppressed hosts with thymic-independent antigens would provide enough antigenic determinants to display these splenic (A) cell factors, resulting in the restoration of the humoral immune response (Treiber and Lapp,1978). Immunosuppression observed in murine allogeneic chimeras has also been suggested to be due to interference of the immune response at the B cell level possibly by humoral blocking factors (Urso and Gengozian,1977).

Apart from the mechanisms of depletion of T cell related mediator and interference at the T and B cell level, the involvement of suppressor cells which may include spleen cells, macrophages, or T cells have also been suggested for GVH-induced immunosuppression.

Spleen cells obtained from F_1 hybrids undergoing GVH reactions were shown to be inhibitory on the humoral response of normal syngeneic spleen cells (Elie and Lapp,1977). While some authors attributed the suppressor effect to the splenic (A) cells as described previously, the role of splenic macrophages as suppressor cells had also been implicated. It was noted that the inhibitory effect of these GVH-induced spleen cells was not abolished by anti-theta antisera and complement treatment, but was eliminated by treatment with iron powder and removal of the phagocytic cells (Sjoberg,1972). The immunosuppressive effect of adherent cells from GVH-induced hosts had also been reported by other investigators (Hoffman and Dutton,1971; Scott,1972; Treiber and Lapp,1976). Adherent cells which inhibited the PFC responses were detected in GVH-induced spleen cells 10 days after GVH induction and were found to be theta-negative. These theta-negative adherent cells were able to produce an immunosuppressive effect on normal spleen cells from either the donor or the host (Parthenais and Lapp,1978).

Suppressor T cells were initially demonstrated in GVH-induced F_1 hybrids using cell-fractionation analysis and selective deletion of the donor or host cells (Shand,1975). It had also been shown that the suppressor T cells induced in GVH reactions were derived from the donor (C57BL/6) spleen cells because of the following evidence. Suppression was abrogated after irradiation of the donor spleen cells, but not after irradiation of the (C57BL/6 x DBA/2) F_1 hybrids. Treatment of GVH-induced spleen cells with anti-H-2^d (anti-DBA/2) antisera and complement did not affect the immunosuppressive activity (Pickel and Hoffman,1977). Parental origin of the suppressor T cells had also been demonstrated in other mouse strains (Shand,1976). The phenotypic components of these GVH-induced suppressor T cells were identified to be Ly-1^+, 2^+, 3^+, Ia^+, and were distinct from those suppressor T cells induced by concanavalin A (Shand,1977).

It has recently been suggested that at least two different GVH-activated spleen cell populations could non-specifically suppress the immune response. A distinct third cell population could be involved in the specific suppression of the immune reaction. The data seems to indicate the presence of non-specific suppressor cells in both the adherent and non-adherent spleen cells from GVH-induced animals which were previously depleted of T lymphocytes. It has been suggested that the non-specific immunosuppressive effect of GVH-induced spleen cells was mediated by GVH-activated macrophages existing in the anti-theta treated adherent cell population, and possibly also mediated by the B lymphocytes existing in the non-adherent cell population (Parthenais and Lapp,1978).

In summary, this section has briefly reviewed the significant manifestations and sequelae of a graft-versus-host reaction. Experimental results from various investigations implicated the existence of certain immunoregulatory mechanisms during the confrontation between the grafted donor cells and the semi-syngeneic F_1 cells. The regulatory mechanisms manifested itself as the absence of a "secondary" form of GVH response, failure to serially passage the GVH reactions along syngeneic hosts, immunosuppression of both humoral as well as cell-mediated immunities, and eventually, the graft-versus-host reaction progressively subsides, terminating in complete as well as spontaneous resolution. The nature of the immunoregulatory mechanisms involved in these reactions remain to be clarified.

IMMUNOLOGY OF GRAFT-VERSUS-HOST REACTION

In this section, the GVH reaction will be examined from the perspective of the grafted donor cells reacting against the tissues of the host. The fate of the donor cells within the F_1 recipient will be described in terms of proliferation and generation of cytotoxic effector cells. In cell-mediated immunity reactions involvoing the grafted cells against the host cells, the possible role of certain soluble mediators and their characteristics will be considered. The genetic aspect of the GVH reaction will also be examined in terms of the H-2 complex and the Mls (M-locus) histocompatibility systems. Finally, the contributions of certain immunoregulatory mediators in GVH reactions will be described.

The Donor Lymphoid Cells in GVH Reaction

The various facets of the reactivities initiated by the grafted donor cells in a GVH reaction are poorly understood at present. The donor cells seem to proceed into two distinct phases : (1) the Proliferative phase, and (2) the Effector cytotoxicity phase. The proliferative phase seems to be the combination of two distinct sub-phases: (a) stimulation of the donor immunocompetent cells by the foreign host antigens, leading to "specific proliferation" of the transplanted donor cells (Gowans,1962), and (b) the "non-specific proliferation" of the host lymphoid cells, resulting in hypertrophy of the lymphoid organs of the host (Fox,1966). Using popliteal lymph node assay of local GVH reactions, it was noted that 75% of the cells in the lymph node of the recipient host were of host origin (Grebe and Streilein,1974). The proliferative phase can therefore be described more accurately as a continuum of an initial proliferation of the grafted donor cells, followed by the proliferation of the host lymphoid cells. The proliferative phase is presumably replaced by the Effector cytotoxicity phase which will be described later. Proliferation of the grafted parental lymphoid cells seems to occur after the donor cells "homed" to their respective lymphoid organs.

The "homing" phenomenon of the transplanted parental donor cells to the lymphoid organs of the recipient host has been documented in many studies. Parental thymus cells transplanted into heavily irradiated F_1 hybrid mice were noted to locate in the spleen as well as in the lymph nodes of the host (Sprent and Miller,1972a). Using a radioisotope labelling technique to study the sites of localization of the grafted parental lymph node cells in the recipient host, 20% of the circulating thoracic duct lymphocytes were identified to be of donor origin. When the thoracic duct was cannulated and the circulating lymphocytes were transferred to a syngeneic host, they "homed" predominantly to the small intestines, and almost 40% of these donor cells were found within the area of the Peyer's patches (Sprent,1976). The systemic distribution of grafted parental donor cells will therefore explain the systemic manifestations of the graft-versus-host syndrome.

The proliferation of the donor cells within the F_1 animals had also been studied by a genetic technique using an identifiable chromosomal marker found only in the parental donor cells. When CBA strain parental spleen cells carrying the T_6 chromosome markers were injected intravenously into the F_1 hosts, bursts of mitotic activities within the F_1 spleen and lymph nodes were documented (Fox,1962). Towards the 14th day after GVH induction, parental donor cells undergoing mitosis in the spleen of the F_1 host constituted less than 2% of the total splenic population (Fox,1966). Proliferation of the grafted donor cells seem to terminate spontaneously.

The fate of the ^{3}H-thymidine labelled parental donor spleen cells was followed after they were injected intravenously into the F_1 recipients. The majority of these labelled donor cells were found dead within the lymphoid organs of the host approximately two weeks after transplantation (Sprent,1976). The labelling of these donor cells with ^{3}H-thymidine radioisotopes did not contribute to the death of the donor cells. Similar findings of dead parental donor cells after transplantation into the F_1 hosts have also been reported in studies on localized types of GVH reactions (Clancy and Adams,1973).

The grafted donor lymphoid cells within the host animal, seems to go through a defined course of initial proliferation when encountered by the foreign antigens of the host. This is followed by the phases of differentiation and generation of cytotoxic effector cells detected experimentally by cell-mediated target cell lysis assays. The time-frame reference of the generation of cytotoxic effector cells and the decline of the grafted donor cells remains to be clarified.

Cytotoxic Reactivity in GVH Reaction

Although the target-tissue in a GVH reaction is generally regarded to be the host immunocompetent cells, pathological manifestations of a GVH reaction are also found in non-lymphoid tissues such as the skin, the gastrointestinal tract, the liver and many other sites (Billingham,1968). The principal cause of tissue destruction in a GVH reaction remains unclear.

For example, skin destruction has been postulated to involve various mechanisms such as anti-epidermal antibodies (Merritt *et al*,1970), and lymphotoxins capable of killing non-lymphoid as well as "innocent bystander" cells (Streilein and Billingham,1970a).

The generation of *in vivo* lesions, initiated presumably by the donor cytotoxic effector cells reacting against host antigens, seems to be dependent upon the route of grafting of the donor cells. In hamsters, the injection of 2 x 10^7 parental lymphoid cells intracutaneously would produce the epidermolytic syndrome presumably the result of cytotoxic effector cells. However, the injection of the same number of parental lymphoid cells intravenously would not produce any epidermolytic syndrome at all (Streilein and Billingham 1970b), indicating the complexity of the generation of cytotoxic effector cells *in vivo*. To further complicate the picture, the production of the GVH-induced skin lesions has been shown to be dependent upon the presence of host leukocytes within the dermis of the host animals (Ramseier and Billingham,1966; Streilein and Billingham,1967; Zakarian and Billingham,1972). The generation of *in vivo* cytotoxic effects would therefore seem to be the result of reactivities of both donor and host lymphoid cells.

The generation of *in vitro* cytotoxic reaction in experimental situations is less complicated. Cytotoxic T lymphocytes of donor origin, with specificity for host strain target cells, have been consistently detected in the spleens of mice undergoing GVH reactions especially in the early phase of the reaction (Cerottini,1971; Cheers and Sprent,1974), and in fact, these cytotoxic lymphocytes have long been used as effector cells in many transplantation reaction studies. While the Mixed-Lymphocyte-Reaction

(MLR) is considered as the *in vitro* correlate of the proliferation of donor cells after their encounter with host antigens (Adler *et al*,1970), the Cell-Mediated-Cytolysis (CMC) assay of labelled target cell lysis by the cytotoxic effector cells is considered to be the *in vitro* correlate of the grafted donor cells attacking the host tissues (Hodes and Anderson, 1970; Solliday and Bach,1970; Hayry and Anderson,1973). Many investigations have used the CMC assay to study allograft reactions as well as any related phenomenon involving cytotoxic T cells.

The involvement of T lymphocytes in cell-mediated immunity reactions is a well documented fact, especially in rejection of allografts. However, there are three paradoxes that separate the GVH reaction from the allograft rejection reaction involving the T lymphocytes. (1) Restriction of the type of stimulator cells: normal transplantation reaction, skin grafting, for example, involves the stimulation of T lymphocytes by histoincompatible antigens expressed on a variety of tissues. In contrast, GVH reactive T lymphocytes are stimulated by foreign antigens on lymphocytes. (2) Species specificity: GVH reactive lymphocytes are stimulated to a much greater degree by allogeneic than xenogeneic hosts (Lafferty *et al*,1972). (3) Low immunization ability: the type of "Second Set" accelerated rejection reaction normally seen in skin-grafting experiments involving pre-sensitized lymphocytes is not seen in GVH reaction, i.e., absence of "Secondary" GVH reaction. Immunization of a donor of GVH reactive lymphocytes, against strongly histoincompatible host cells, does not increase, amd may actually decrease the severity of a GVH reaction (Ford and Simonsen,1971). The immunological mechanisms of a GVH reaction and an allograft reaction may therefore be two distinct entities.

The generation of cytotoxic T lymphocytes seems to require a sequential interaction of two distinct types of syngeneic T cells: (1) Initiator T lymphocytes (ITL), and (2) Recruited T lymphocytes (RTL). The ITLs were found to reside mostly in the spleen and the thymus, and they were interestingly, absent in the lymph nodes. In contrast, the RTLs were absent in the thymus and were detected to be predominantly in the lymph nodes (Livrat and Cohen,1975). The properties of the ITLs include : the resistance to hydrocortisone as well as to irradiation treatments, and adherence to nylon-wool columns. The properties of the RTLs were exactly the reverse of the ITLs except that both possessed theta-antigens (Cohen and Livrat,1976). The roles of ITLs and RTLs in a GVH reaction have not been elucidated, but the distributions of the ITLs and the RTLs seem to reflect the distribution of the injected parental donor lymphoid cells described in the "homing" phenomenon.

The realization of cytolysis by the GVH-induced cytotoxic effector cells seems to involve three not mutually exclusive mechanisms : (1) contact cytotoxicity in which the killer T cell can act in the absence of other cells; (2) antibody-mediated cytotoxicity in which specific antibodies synthesized by the donor cells together with complement, induced lysis of the host cells, and (2) soluble mediators generated in cellular immunity reactions are responsible for, direct cytotoxicity or non-specific activation of the effector-killer cells. The first two mechanisms have been extensively studied and reported by many investigators, while the understanding of the involvement of soluble mediators in GVH reactions is only in a primitive stage. Two particular soluble mediators : Lymphotoxin and Lymphocyte-Activating-Factor deserve some attentions because of their possible roles in the cytotoxic reaction.

<u>Lymphotoxin</u>

Lymphotoxin (LT) is a lymphokine which has the ability to cause cytolysis when released from stimulated lymphocytes. Cytolytic mediators of cellular immunity reactions were initially demonstrated in supernatants from suspensions of sensitized lymph node cells incubated with the antigen (Ruddle and Waksman,1968; Granger and Kolb,1968). From the elution pattern of DEAE-cellulose chromatography, lymphotoxin appears to be a netural protein with molecular weight in the region of 90,000 daltons (Namba and Waksman,1975).

Lymphotoxin can cause non-specific cytolysis of many types of mammalian cells (Streilein and Billingham,1970a), and lymphotoxin associated cytolysis had been shown to be dependent upon the factors of temperature and concentration (Williams and Granger,1969). Studies on lymphotoxin-induced cytolysis indicated that with low concentrations of lymphotoxin, target cells continued to grow until the rate of cell-death exceeded the rate of cell-multiplication. This seems to suggest that cellular DNA synthesis was not affected by the lymphotoxins (Walker and Lucas,1972). When a small inoculum of parental spleen cells are used to induce a GVH reaction, the F_1 host recovers from the GVH syndrome more quickly. This could be interpreted as the result of a low concentration of lymphotoxin because of a small number of grafted donor cells.

Differential susceptibility of different target cells to the cytolytic effect of lymphotoxin had been reported in different sublines of L929 cells (Kramer and Granger,1975). This differential effect had been ascribed to explain the differential pathology of tissue destructions in GVH reactions (Grebe and Streilein,1976).

Human lymphotoxins released by activated lymphocytes can be fractionated into several major classes. The stable molecules are found in the alpha and beta classes (Hiserodt et al,1976), and a third gamma class consists mainly of unstable molecules (Lee and Lucas,1976). Using these purified subclasses of lymphotoxins to raise specific antisera, it has recently been shown that lymphotoxin molecules indeed exist *in vivo* and may very well represent a direct measurement of the *in vivo* cytotoxic reactivity (Granger et al,1978). With all these advances in lymphotoxin studies, the roles of such soluble mediators will be clarifed, and the involvement of lymphotoxins in GVH-induced cytotoxicity reaction will become more clear.

Lymphocyte-Activating-Factor

In the situation of humoral immune response, the role of macrophages has been identified as the "processing" of antigens, and soluble factors may be involved in the production of specific antibodies. Cell-free supernatants from macrophage cultures have been shown to be capable of restoring the *in vitro* response of mouse spleen cells to SRBC indicating the involvement of such a soluble factor (Hoffman and Dutton, 1971). In the situation of cell-mediated cytotoxicity reactions, the role of macrophages is not clear. Through soluble mediators, a parallel role for macrophages may exist in cellular immunity as in humoral immunity, and Lymphocyte-Activating-Factor seems to be the suitable candidate.

Lymphocyte-Activating-Factors (LAFs), produced by macrophages, are capable of activating lymphocytes into proliferation. In GVH reactions, LAFs may be involved in the stimulation of lymphocytes into proliferation and possibly the generation of cytotoxic effector cells.

The existence of LAFs was initially demonstrated in the supernatants of macrophage preparations (Bach et al,1970), and confirmed by sensitivity to anti-macrophage antisera (Shortman and Palmer,1971). The stimulating effect of LAFs have been shown to affect both thymic and circulating T lymphocytes (Grey et al,1972), and stimulating LAFs have been detected in several species : mouse, rat, rabbit, and human (Gery and Waksman,1972). Most recently, macrophage derived LAFs were shown to be capable of restoring the immune response in athymic mice (Koopman et al,1978). The molecular weights of murine and human LAFs were estimated to be between 5000 to 25,000 (Gery and Handschumacher,1974; Koopman et al,1977). LAF activity was noted to be sensitive to proteolytic enzymes (Calderon and Unanue,1975), and was suggested to be a peptide (Blyden and Handschumacher,1977).

The speculative role of LAFs in GVH reactions resides mostly in the activation of lymphocytes into cytotoxicity reactions. Evidence in support of such a contention although lacking in literature, will no doubt be forthcoming when the involvement of macrophages in the cellular immune response is clearly delineated.

Genetics of the GVH Reaction

The importance of the Histocomaptibility-2 (H-2) complex in allograft reactions has been described in many recent reviews (Shreffler and David,1975; Klein,1975), and the involvement of the H-2 complex in a GVH reaction will be described. Apart from the H-2 complex, the M-locus (Mls) (Festenstein,1973) in relation to the GVH reaction will be included.

The Histocompatibility-2 System

Transplantation reactions, including GVH reactions, involve the recognition of histoincompatible antigenic determinants coded by the genes in the Major Histocompatibility Complex (MHC). The MHC of the mouse, known as the H-2 complex, is located on the 17th chromosome. The role of the H-2 complex in allograft rejection are described in early reports (Simonsen and Jensen,1959; Simonsen,1962), and since then, the immunogenetics of transplantation reactions have become an area of research on its own. For a brief review, the H-2 complex in the mouse is divided into four main regions: K, I, S, and D regions. The K and D regions code for the serologically defined transplantation antigens, while the S region controls both qualtitative and quantitative expressions of a serum beta-globulin believed to be the C'4 component of the serum complement system.

The antigenic determinants coded by the K and D regions of the H-2 complex appear to be the most potent antigens in eliciting the cytotoxic effector cells. Differences in the K and D regions alone, between the host and donor immunocompetent cells, can lead to significant cell-mediated cytotoxicity reactions in the absence of any known H-2 central region differences (Schendel _et al_,1973; Nabholz _et al_,1974).

The I region, based on the immune responses against the Ia (I-region-associated-region) determinants, has been further divided into three subregions: Ir-1A, Ir-1B, and Ir-C. The humoral response to the : GVH reactivity, natural or synthetic antigens, and the MLRs are considered to be under the control of the I region. In mice, the antigens coded by the I region appear to be the most potent in stimulating the donor immunocompetent cells into proliferative MLRs (Klein and Park,1973; Shreffler and David,1975; Klein,1975).

In studying the mechanism responsible for the cytotoxicity reaction against "self-antigens" or "altered-self-antigens", an important role of the H-2 complex in the interaction between the cytotoxic effector cell and the target cell had been implicated. Using viral-infected or chemically-modified cells as target cells and syngeneic effector cells in cell-mediated cytotoxicity studies, homology between the stimulating cells (i.e., the cells used in sensitizing for induction of cytotoxicity) and the "modified" target cells, at the H-2 complex, particularily in the H-2K or H-2D regions, was found to be necessary in order to produce significant cytolysis of the "modified" target cells (Zinkernagel and Doherty,1975; Rehn et al,1976; Zinkernagel,1978a; 1978b).

Two mechanisms have been proposed to explain the requirement of the H-2 complex homology between the effector and target cells in the generation of a cytotoxicity reaction. The first mechanism, the "Dual-Recognition Hypothesis", suggested that T cells would possess two distinct recognition structures; (1) an H-2 coded recognition structure which binds complementary unmodified H-2 gene-products, and (2), a second recognition structure which binds the foreign antigen; e.g., the viral or chemical hapten moiety on the surface of the "modified" cell (Zinkernagel and Doherty,1974). The second mechanism, the "Altered-Self Hypothesis", suggested that only one recognition structure would be present on the T cell. This recognition structure is capable of recognizing gene-products coded by the H-2 complex as well as the foreign viral or chemical haptens (Bevan,1976). Future investigations will decide which hypothesis is correct, and in the mean time, the importance of the H-2 complex in the generation of cytotoxicity reactions cannot be over-emphasized.

The Mls System in GVH Reaction

In mice, although the genes encoding the lymphocyte-activating determinants (LADs) are apparently confined to the MHC, another system, which is not linked to the H-2 locus, also encodes LADs which in turn also stimulate very strong MLRs. This is the M-locus (Mls-locus), which has been mapped to a chromosome other than the 17th chromosome. In some cases, Mls encoded LADs were even stronger than the MHC encoded LADs (Festenstein, 1973). Mls determinants have been detected on the cell surfaces of ; the B lymphocytes, macrophages (Ahmed *et al*,1975), bone marrow cells (Pena-Martinez *et al*,1973), stem cells (Bartova,1975), but not on T lymphocytes (Schirrmacher *et al*,1975). The Mls determinants located on the macrophages have a much stronger lymphocyte activating potential than the H-2 encoded determinants (Schirrmacher *et al*,1975).

The injection of Mls incompatible but H-2 compatible lymphocytes into the host's popliteal lymph node produced significant lymphadenopathy resembling a local GVH reaction observed in a parent-F_1 combination (Huber *et al*,1973; Matossian-Rogers and Féstenstein,1978). Lethal Mls-induced alloimmune reactions have been reported (Rodey *et al*,1974).

Absence of a systemic "secondary reaction" parallel to that observed in H-2 incompatible GVH reactions had been reported similarily in Mls incompatible combinations (Matossian-Rogers,1976). In addition, absence of local "secondary reaction" e.g., the suppression of popliteal lymph node enlargement had also been described in Mls incompatible combinations (Jacobsson *et al*,1975; Matossian-Rogers,1977). The role of the Mls system in GVH reactions will no doubt require more clarification, but its involvement in GVH reaction cannot be disputed.

Immunoregulatory Mediators in GVH Reaction

Immunoregulatory mediators are soluble products generated, or released from lymphoid tissues during an immune reaction. There are many soluble products that can affect the various functions of the lymphoid cells. They may be alpha-globulins, mitogenic factors, specific antibodies, immunoglobulins, and soluble proteins. A general classification of the many soluble mediators of cellular immunities, according to the targets being acted upon, has been proposed recently to clarify the confusing terminology (Rocklin,1978). For example, the Migration-inhibitory-Factor (MIF) is classified as mediators whose targets are the macrophages. The Mitogenic Factors (MFs) acting on target T and B cells are classified into the group of mediators affecting the lymphocytes, etc.

Specific reports on the role of the soluble mediators within the context of GVH reactions are very deficient in literature. The aspect of GVH-induced immunodeficiency observed in animals undergoing GVH reactions has been suggested to be the result of depletion of a T cell mediator (Lapp et al,1974; Parthenais et al,1974). The suppression of the humoral responses to thymic-dependent antigens in animals undergoing GVH reactions has been postulated to be mediated indirectly by soluble mediators released from the adherent cells (Treiber and Lapp,1978).

The involvement of soluble mediators in cellular immunity has been reported in many studies. Mediators in the forms of thymic hormones have been shown to assist the development of lymphoid system throughout the life-span of an individual (Friedman,1975). The alpha-proteins were suggested to regulate the immune system in embryonic life (Yachnin,1975), and later on they are replaced by the phenomenon of tolerance as the immune system matures

(Katz and Benacerraf,1974). Mediators with specificities directed to targets of lymphoid cells have been suggested to assume an important role in cellular immunity reactions (David and David,1972). As a result of inflammatory destruction in GVH reactions, self-antigens may be exposed or released, followed by the onset of autoimmune reactions. Certain C-reactive proteins have been suggested to be responsible for the inhibition of this type of autoimmune reaction (Mortensen et al,1975), but the identification of C-reactive proteins in GVH-induced animals has not been studied.

Mitogenic Factors as Soluble Mediators

Mitogens for the stimulations of T and B lymphocytes have been extensively investigated in MLRs. In transplantation reactions, the formation of blast-cells in MLRs has been considered to be the *in vitro* correlate of the inductive phase of the GVH reaction. Splenomegaly noted in GVH reactive hosts has been suggested to be the result of the release of certain mitogenic factors during the intense inflammatory reaction.

The natural resolution of the GVH syndrome has also been hypothesized to be due to: (1) depletion of mitogenic factors, (2) the suppression of blastogenesis by adherent cells despite the presence of the mitogenic factors (Folch and Waksman,1973). Prostaglandins may also be involved in inhibiting the mitogenic factors leading to remission of the GVH reaction (Goodwin and Bankhurst,1977). This is supported by the recent finding that prostaglandins can induce differential effects on the proliferative responses of different types of lymphocytes, thus suggesting the inhibition of blastogenesis possibly at the molecular level of the mitogenic factors (Novogrodsky,1979). The immunoregulatory role of the mitogenic factors cannot be ignored in future studies.

Immunoglobulins and Antibodies as Soluble Mediators

The production of immunoglobulins and antibodies by the donor lymphoid cells during the course of GVH reaction has been clearly established (Elkins,1971). Specific antibodies are known to suppress the "secondary" immune response as demonstrated in the adoptive transfer experiments (Uhr and Moller,1968). The ratio of antigens to antibodies within the immune system may regulate a delicate balance between tolerance and immune response. This is shown by the fact that a certain concentration of antibodies to antigens would form complexes which can induce tolerance to subsequent antigenic challenges (Diener and Feldman,1970). In this context, the immunoglobulins and antibodies are prime candidates for the regulation of the GVH reaction.

GVH reaction-induced runting disease could be prevented by the injection of antibodies raised against the parental donor cells, into the F_1 hybrid recipients (Russell,1960; Siskind et al,1960). The donor immunocompetent cells, during a GVH reaction, recognize and synthesize specific antibodies against the foreign antigens on the host cells, leading to the masking of host antigens and subsequent induction of tolerance. Immunocompetent cells from human volunteers became tolerant in HLA typing experiments when the antigens on the target cells were masked by the appropriate anti-HLA antibodies which were obtained from women inadvertently immunized by previous pregnancies (Brochier et al,1974)

Inhibitory immunoglobulins can also be involved in the GVH reaction. Donor lymphoid cells can be suppressed by the feedback mechanism of the inhibitory immunoglobulins resulting in the resolution of the GVH reaction. When F_1 mice , which survived the initial GVH reaction, were later irradiated, the subsided GVH syndrome reappeared. This seems to suggest that irradiation had removed the inhibitory antibodies which were presumably synthesized by the radiosensitive lymphoid cells (Schwartz and Beldotti,1963). This hypothesis is reinforced by the fact that, while sensitized lymphoid cells failed to induce popliteal lymph node hypertrophy, the depletion of antibody-forming cells from these sensitized parental lymphocytes could lead to popliteal lymph node hyperplasia in F_1 recipient (Fink et al,1974).

The passive treatment of parental donor cells with the donor-produced anti-F_1 antibodies was noted to depress the GVH reactivity (Safford and Tokuda,1970). Along the same line of experiments, adult F_1 mice, when given parental IgG anti-F_1 antisera, and later induced with GVH reaction, would develop much reduced splenomegalies (Jose et al,1974).

Apart from immunoglobulins and antibodies, other immunoregulatory mediators probably also exist in the GVH reaction. The role of the idiotypic antibodies and its immunoregulatory function in the GVH reaction will be described in a later section.

In summary, this section has described the fate of the parental donor cells within the F_1 host, the GVH reaction in terms of the graft against the host, the generation of donor cytotoxic effector cells mediating cytotoxicity reactions under the influence of the H-2 complex as well as the Mls-locus, and the possible role of certain immunoregulatory mediators within the context of the graft-versus-host reaction.

IMMUNOLOGY OF HOST-VERSUS-GRAFT REACTION

In this section, the GVH reaction will be examined from the perspective of the host's immunocompetent cells reacting against the grafted donor cells; i.e., the host-versus-graft (HVG) reaction.

The grafting of parental immunocompetent cells into the F_1 hybrid recipients produces the classical adult GVH syndrome. According to the genetics of transplantation reactions, the F_1 hybrids, being genetically tolerant to parental antigens, are expected to be incapable of reacting against the grafted parental cells. Studies on the GVH reaction in past decades did not provide satisfactory explanations to the spontaneous resolution of the GVH reaction. The limited understanding to date is that the donor lymphoid cells set in motion an extremely complex sequence of events within the F_1 host which requires further comprehension. Evidence has been accumulating in the last few years to indicate that the F_1 host, not only actively participates in the GVH-HVG reactions, but the host may in fact terminate the GVH reaction through certain unidentified immunoregulatory mechanisms. Significant observations concerning the perspective of the host-versus-graft reaction are described in the following.

Proliferation Response of Host Lymphoid Tissues

The lymphoid tissue-megalies of the host, for example, splenomegaly observed during the early phase of the GVH reaction, was initially considered the result of the proliferation of the grafted donor cells because of antigenic stimulations. Many studies have since been advocating that *in vivo* hepatosplenomegaly was due to the proliferation of actually host's lymphoid tissues as a consequence of the inflammatory response to tissue destruction (Jandl and MacDonald,1965). Parental lymphocytes have also been shown to induce proliferative granulocytopoiesis in F_1 hybrid mouse spleen-explants in vitro (Auerbach and Globerson,1966). Using the T_6 chromosome marker technique, cytogenetic analyses of the proliferating cells in host's spleen revealed that donor cell divisions rapidly declined within a week and subsequently decreased to 1% of the total by the end of the second week (Fox,1966). These early reports clearly indicated that host lymphoid organomegalies are manifestations of the proliferative responses of the host's immunocompetent cells.

The severity of experimental GVH reactions can be measured by the comparisions of individual spleen indices (Simonsen,1959). In comparing the spleen indices between normal and irradiated F_1 hybrids undergoing GVH reactions, it was noted that splenomegaly in GVH-induced F_1 hybrid mice could be abolished by irradiation at a dosage of 500 Rads (Hilgard,1970). The spleen indices of the irradiated F_1 hosts were significantly lowered, suggesting that the proliferating host cells were radiosensitive (Singh et al,1972).

In the assessments of local cutaneous GVH reactions induced in irradiated guinea pigs, it has been demonstrated that as leukopenia of the host increased, the appearance of GVH skin lesions decreased (Zakarian and Billingham,1972). This is parallel to the situation where splenomegaly decreases with increasing irradiation of the F_1 recipients. Similar to hepatosplenomegaly, lymph node hypertrophy was noted to be due to the proliferation of host cells originated from the bone marrow. Furthermore, in lethally irradiated F_1 hosts, lymph node enlargements could be restored by syngeneic bone marrow grafts as late as 9 days after the induction of

GVH reaction (Bonney and Feldbush,1973). In similar experiments also using popliteal lymph node assays of local GVH reactions, 75% of the cells dissociable from the injected lymph nodes were identified as host-derived, and 50% of these cells were shown to migrate to the lymph node via a hematogenous route (Grebe and Streilein,1974).

Using specific cytotoxic alloantisera, the proportion of host cells in the popliteal lymph node in rats undergoing GVH reactions had been estimated to be as high as 90% of the total viable cells (Rolstad, 1976). The origin of the cells accumulating in the popliteal lymph nodes of mice and rats undergoing GVH reactions has also been studied by karyotype analyses, immunofluorescence and radioautography. It was shown that on the 7th day after GVH induction, the enlarged lymph nodes consist of, at the most, 2% donor cells (70% T lymphocytes, 30% B lymphocytes). During the period studied, the proliferating donor cells represented, at the most, 20% of the total cell population in the regional popliteal lymph node (Piguet and Vasalli,1977).

The proliferation of host lymphoid tissues, as manifested by hepatosplenomegaly, PLN hypertrophy, seems to provide certain immunoregulatory function for the GVH-induced F_1 host. This is evidenced by the fact that lethal GVH disease induced in irradiated mice (Trentin,1956) could be abolished by reconstituting the host with syngeneic spleen cells (Bennett and Hand,1978). Resolution of a GVH reaction in the F_1 host, therefore seems to be initiated as a very first step, by the proliferation of host's immunocompetent cells. As described in the following section, the proliferative F_1 lymphoid cells are indeed immunoresponsive.

The Allogeneic Effect in GVH Reaction

The GVH reaction-induced suppression of the humoral immune response has been described in a previous section (Gengozian and Owen,1958; Lawrence and Simonsen,1967; Lapp and Moller,1969). A phenomenon, known as the Allogeneic Effect, contrary to the immunodeficiency state also exists in the GVH-induced F_1 host. It is a situation in which the humoral immune response to an antigen, not only is not suppressed, but is actually elicited or enhanced by the grafting of parental lymphoid cells to the F_1 recipient.

This phenomenon was first described in (strain 2 x 13) F_1 guinea pigs previously primed with DNP-ovalbumin and subsequently grafted with normal parental (strain 2) lymphoid cells. Such a transfer of semi-allogeneic or semi-syngeneic lymphoid cells produced a striking secondary anti-DNP antibody response in the F_1 host when challenged with DNP coupled to another carrier protein unrelated to ovalbumin. The phenomenon has also been observed in rats (Katx and Benacerraf,1972), and in hmasters (Scott and Ornellas,1974).

Stimulation of the IgG antibody response associated with the allogeneic effect has been demonstrated in mice (Schimpl and Wecker,1973). The allogeneic effect, in addition to enhancing the IgG and IgM responses to the DNP hapten, also stimulates subpopulations of host B cells, as evidenced by the spectrum of antibody heterogeneity identified by the isoelectrofocusing studies of host sera (Klaus and McMichael,1974).

The allogeneic effect phenomenon was postulated to involve the activation of the F_1 host antibody-forming cells as a result of the immunological attack by the parental donor cells (Katx and Paul,1971). The explanation implies that parental T cells, in the process of reacting against the allogeneic F_1 hybrid antigens, is able to stimulate the F_1 hybrid B cells to produce specific anti-hapten antibodies. This hypothesis implicated the co-operation of semi-allogeneic T and B cells in the course of an immune response.

The appearance of autoantibodies in the course of GVH reaction in F_1 hamsters may be a reflection of the allogeneic effect (Streilein and Stone,1973). In the same context, detection of Coomb's antibodies of host origin and an increased level of anti-SRBC antibody in mice, neonatally induced with GVH reactions, suggested the stimulation of the F_1 host's B lymphocytes by the semi-allogeneic donor cells (Lindholm and Strannegard,1973).

In mice, lymphoid cells capable of inducing the allogeneic effect also proliferate in the mixed-leukocyte-reaction (Corley and Kindred, 1977). This imples that the parental T cell initiating the allogeneic effect and the parental T cell which responded to the foreign F_1 histo-compatibility antigens are in the same lymphocyte population. This implication has recently been confirmed by the fact that those T cells reactive to the F_1 alloantigens can also mediate the allogeneic effect (Corley and Lefkovits, 1978). Most recently, allogeneic effect associated MLRs were attributed to the "hybrid-specific antigens" associated with the I region of the H-2 complex (Fathman and Augustin,1978).

It has been hypothesized that the process of recognition by the parental T cell of the Ia antigenic determinants on the semi-allogeneic F_1 hybrid B cells activated the hybrid B cells to produce IgG antibodies against the challenging hapten (Delovitch and McDevitt,1977).

Many studies on the allogeneic effect have shown that soluble factors could be involved in the phenomenon. When allogeneic mouse spleen cells were mixed *in vitro*, the T lymphocytes secreted a product which guided the maturation of the B lymphocytes (Ekpaha-Mensah,1971; Britton, 1972). The soluble factor involved was named "Allogeneic Effect Factor" (AEF), and was later characterized to be a highly active protein with a molecular weight in the range of 30,000 to 40,000 daltons, exhibiting some strain-specific properties (Armerding and Katz,1974). The biological active moiety of the AEF was identified to bear Ia determinants and therefore, was probably the gene products of the I region of the H-2 complex (Armerding and Sachs,1974).

Experimental evidence in support of the allogeneic effect phenomenon also serves the important purpose of implicating the immuno-responsive capacity of GVH-induced F_1 lymphoid cell previously believed to be incapable of initiating an immune response. Such immunoresponsiveness in the F_1 host undergoing GVH reaction may also explain the autoimmune phenomenon observed in GVH reactions.

The Autoimmune Phenomenon in GVH Reaction

The manifestations of the autoimmune phenomenon in GVH reaction-induced hosts can be examined from the perspectives of : (1) the production of autoantibodies by the F_1 host, and (2) the autoimmune histo-pathology observed in the GVH-induced F_1 animals.

The general assumption is that in GVH reaction, helper T cell activity provided by the donor cells could stimulate the host's B cells into proliferation and production of autoantibodies (Katz and Paul,1971). This has been suggested by the allogeneic effect phenomenon described in F_1 animals undergoing GVH reactions (Lindholm and Strannegard,1973; Streilein and Stone,1973; Scott and Ornellas,1974).

Repeated injections of parental lymphoid cells into the F_1 hybrid mice have been shown to induce the formation of anti-nuclear antibodies, and using allotypic marker analyses, the source of these autoantibodies was identified to be the F_1 host (Fialkow et al,1973). The polyclonal autoantibodies detected were also found to be of the host's allotype and were reactive in other mouse strains.

Coomb's-positive autoimmune hemolytic anemias have been noted in mice undergoing GVH reactions (Gleichmann and Wilke,1972; Lindholm and Strannegard,1973). Hamsters undergoing GVH reactions were similarily prone to severe autoimmune hemolytic anemias (Streilein and Duncan,1975). The GVH process in hamsters seems to induce the production of a wide spectrum of autoantibodies which include autoantibodies against : immunoglobulins, lymphocytes, epidermal cells and erythrocytes (Streilein and Stone,1973). The anti-erythrocyte antibodies were noted to be true autoantibodies since they failed to discriminate strain specificities among different inbred lines of hamsters (Streilein and Duncan,1975).

A study has shown that during the interval in which autoantibodies were detectable, the ability of the GVH-induced F_1 host to respond to a new extrinsic antigenic challenge was severly suppressed (Streilein,1972). The significance of autoantibodies in relation to the immunodeficiency states observed in GVH-induced animals remains unclear. It has recently been noted that in normal mice, a high proportion of immunoglobulin-producing lymphoid cells were actually making autoantibodies constantly (Steele and Cunningham,1978). This may lead to the speculation that the GVH process inadvertently releases the normally operating autosuppressive mechanisms within the host to the extent that autoantibodies are synthesized and released.

Apart from the production of autoantibodies, host animals undergoing GVH reactions, in F_1 hybrid mice and rats for example, also display a propensity to disorders with heavy autoimmune implications. The kidneys in these GVH-induced hosts were found to contain immune complexes of host-IgG and erythrocytes, demonstrating truly autoimmune histopathology (Gleimann and Wilke,1972).

The IgG immunoglobulin "Long-acting-thyroid-stimulator" (LATS) is known to be associated with the disorder of autoimmune thyroiditis. The possible involvement of LATS in local GVH reaction has recently been reported in experiments in which the thyroid glands of the F_1 hybrid rats were injected intraparenchymally with parental lymphoid cells. On autopsy, the histopathological picture obtained was identical to that observed in a typical autoimmune thyroiditis reaction (Konetzki and Streilein,1978).

If GVH-induced F_1 hosts can produce the phenomena of the allogeneic effect and autoimmunity, they are expected to participate in the GVH reaction to the extent that "counter-antibodies" may be produced against specific antibodies synthesized as a consequence of the recognition of foreign histocompatibility antigens of the F_1 host by the transplanted parental immunocompetent cells. This in fact may actually happen and the concept of anti-idiotypic antibodies is described in the following section.

Anti-Idiotypic Antibodies in GVH Reaction

The quantity of literature related to idiotypes and the anti-idiotypic antibody is so enormous that it is impossible to cover every aspect in this brief review. The intention here is to construct a relationship between anti-idiotypic antibody and the graft-versus-host reaction, especially from the host-versus-graft perspective.

Idiotypes are considered to be antigenic markers for the antibody binding sites and were found to correlate with the primary structure of the antibody molecule (Glynn and Steward,1977). Idiotype appears to represent the antigenicity of the antigen-binding site of an antibody molecule. Idiotypic determinants are said to be located in the Variable region of molecule (Fudenberg,1976).

Idiotypic determinants, being antigenic, can induce the formation of anti-idiotypic antibodies. In many instances, the reaction between idiotypes and anti-idiotypic antibodies is inhibited by haptens against which the idiotypic antibodies were raised (Kabat,1969; Glynn and Steward,1977). Anti-idiotypic antibodies can be produced by the injection of certain specific antibodies into syngeneic animals (McKearn et.al.,1974a). Anti-idiotypic antibody demonstratable in the serum of F_1 hybrid rats after the injection of parental lymphoid cells may block the receptors on other parental strain cells which are specific against the serologically defined histocompatibility antigens of the host (McKearn et.al.,1974b).

In graft-versus-host reaction, idiotypes presumably located in the Varible region of an receptor antibody molecule on the surface of the injected parental strain cells, may be able to induce anti-idiotypic antibodies. Such anti-idiotypic antibodies must logically be produced by the F_1 host cells, otherwise parental cells would be producing antibodies against its own receptors. In the above context, anti-idiotypic antibodies may be speculated to be antibodies against the "recognition structure" or receptor on the parental cell.

Evidence supporting the involvement of anti-RS antibodies in GVH reactions was initially provided by the experiment in which, sera from adult F_1 hybrid mice, rats, and hamsters, containing antibodies against one of the parental strain as a result of immunization, specifically inhibited the recognition by immunocompetent cells of the immunizing genotype of transplantation antigens of the other parent (Ramseier and Lindenmann,1969). In other words, adult (A x B) F_1 hybrid, induced into GVH reaction by the parental strain A lymphoid cells bearing RS_B, produced anti-RS_B antibodies which inhibited the recognition of B antigens by the parental strain A cells. Similar results in many F_1-parent combinations in different species of animals have been reported (Ramseier,1973).

When anti-B antibodies were injected into the (A x B) F_1 mice, the resulting F_1 sera, presumably containing anti-RS_B antibodies, were noted to inhibit local GVH reactions (Binz and Lindenmann,1973; McKearn,1974). Using adoptive transfer of anti-RS antisera into F_1-newborns which were neonatally injected with parental lymphoid cells, significant reduction of neonatal mortality was noted (Joller,1972). Anti-RS antibodies, in the presence of complement, have been shown to be cytotoxic to GVH reactive parental spleen cells (Binz et al,1974), and anti-idiotypic antibodies were suggested to play an important role in immunoregulation (McKearn et al,1974).

Idiotypic markers have been found in the sera and urine of normal individuals (Binz and Wigzell,1975). When these idiotypic markers were presented to the same individuals in a concentrated form, the recipients were able to produce anti-idiotypic antibodies against these markers. The "Network Hypothesis" proposed that sequential antibody, anti-idiotypic-antibody responses would exert a negative feedback on the immune response itself (Jerne,1973). Suppression of specific antibody productions by the anti-idiotypic antibodies have been clearly established (Hart et al,1972; Cosenga and Kohler,1972; Eichman,1974). Animals injected with antibody to the idiotype of a clone of their own lymphocytes could become unresponsive to the homologous antigen (Eichmann,1975). The immunization of rats with their own lymphocytes which have been presensitized to the histocompatibility antigens of another strain of rat led to prolonged tolerance of kidney grafts from the sensitizing strain (Binz and Wigzell,1976). This type of immunosuppression by auto-produced anti-idiotypic antibodies were shown to be mediated by either antibody or thymus-derived lymphocytes (Aguet et al,1978).

To summarize briefly, host-versus-graft reaction has so far been examined mainly in the aspect of the humoral immune response through the description of allogeneic effect, autoimmune phenomenon, and idiotypic antibodies. The cellular immune response aspect of the host-versus-graft are described in the following sections.

In Vitro Non-specific Cytotoxicity in GVH Reaction

The term "non-specific cytotoxicity" refers to the situation where specific sensitization of immunocompetent cells against certain H-2 histoincompatible antigens, resulted in the lysis of at least two genetically different target cells. The lysis of target cells syngeneic to the cell used in sensitization is known as "specific cytotoxicity", while the lysis of target cells which are semi-syngeneic, allogeneic, and even xenogeneic to the cells used in sensitization is known as "non-specific cytotoxicity". In case of a GVH reaction, donor parental lymphoid cells are specifically sensitized against the histoincompatible but semi-syngeneic F_1 hybrid cells. The resultant lysis of target cells allogeneic to the donor cells is the classical "specific cytotoxicity", and the lysis of target cells syngeneic to the F_1 cells represents the "non-specific cytotoxicity".

Specificity has been the hallmark of immunological reactions, however, the simultaneous existence of both specific and non-specific cytotoxicity reactions has been documented in many reports. For example, in the presence of specific antigen, lymph node cells from inbred rats with delayed hypersensitivity to bovine gamma-globulins, produced destruction of monolayers of allogeneic fibroblasts (Ruddle and Waksman,1968). When human peripheral blood lymphocytes were sensitized to soluble antigens e.g., PPD, non-specific lysis of allogeneic and xenogeneic target cells have also been reported (Butterworth,1973).

Using fibroblasts as target cells, lymphoid cells previously sensitized <u>in vitro</u>, in addition to showing a specific cytotoxicity reaction against the sensitizing genotype, also demonstrated a weaker cytotoxic effect on fibroblasts carrying different H-2 antigens. Such a non-specific effect was observed even when the "bystander" cells were syngeneic to the cytotoxic lymphocytes (Svedmyr and Hodes,1970). In rats, lymph node cells sensitized <u>in vitro</u> against allogeneic or xenogeneic fibroblasts were shown to be cytotoxic to syngeneic target cells (Cohen and Feldman,1970).

The observation of <u>in vitro</u> non-specific cytotoxicity in a systemic GVH reaction was initially reported in F_1 hybrid mice. Lymphoid cells, taken from the spleens of the GVH-induced F_1 mice, were noted to exert a non-specific cytotoxic effect on syngeneic, allogeneic and even xenogeneic target cells (Singh <u>et al</u>,1972). This type of non-specific cytotoxicity was attributed to the F_1 host's lymphoid cells, and supporting evidence was provided by the following experiments. Strain B parental spleen cells were injected into (A x B) F_1 hybrids for the induction of GVH reactions, and non-specific cytotoxicity was detected by the lysis of the B genotype target cells. Antisera with specificity against the A genotype cells were raised in strain B animals. The pooled sera, in the presence of complement, were cytotoxic to any cells carrying the A genotype, including the (A x B) F_1 hybrid cells, but not toxic to cells carrying the B genotypes, i.e., the parental strain B cells. When the GVH-induced (A x B) F_1 lymphoid cells were treated with these anti-A sera plus complement, non-specific cytotoxic reaction on the B genotype target cells were abolished, thus indicating the contribution of non-specific cytotoxicity by the F_1 lymphoid cells (Singh et al,1972). Macrophage target cells were found to be resistant to the non-specific cytotoxicity reaction (Singh et al,1973). These observations were later confirmed in similar studies using the microcytotoxicity assay technique in different parent-F_1 combinations (Fung and Sabbadini,1976).

A non-specific cytotoxicity reaction had been implicated to involve a population of lymphoid cells which were found to be theta-negative (Grant and Alexander,1974). The reaction appeared to be mediated by a soluble factor which would be directly cytotoxic to the target cells even in the absence of the activated effector cells (Distasio et al,1978).

The detection of non-specific cytotoxicity in F_1 hosts induced with GVH reactions suggested the active participation of F_1 lymphoid cells during the course of the GVH reaction. Such F_1-host-versus-graft reaction are described more fully in the following sections.

The Hybrid Resistance Phenomenon in GVH Reaction

It has been generally accepted that antigens of the Major Histocompatibility Complex are co-dominantly expressed, and on the lymphoid cells of the F_1 generation, serologically detectable antigens of both parents are manifested. However, evidence has been accumulating to suggest that intra-allelic interactions at the gene level may be more common than generally assumed, and the inheritance of certain MHC components may not be strictly co-dominant. This would result in the loss of certain parental-specific gene-products and the appearance of hybrid-specific determinants on the heterozygous F_1 cells. Such an assumption was first made when the failure of proliferation of certain parental cells in supposedly tolerant F_1 hybrid mice was noted (Cudkowicz and Stimpfling,1964), and later echoed by other investigators (Goodman and Bosma,1967; Claman and Hayes,1969).

This non-acceptance of parental lymphoid cells by the F_1 hosts was termed "Hybrid Resistance", and the existence of a "Hybrid Histocompatibility" (Hh) locus within the H-2 region, linkage group IX of the mouse had been suggested (Cudkowicz,1968). The parental strain was postulated to possess an antigen determined by the homozygous Hh-1a/Hh-1a gene which was not expressed in the heterozygous F_1 hybrids. Hybrid resistance therefore, was attributed to the absence of the parental homozygous antigen(s) which are controlled by the MHC linked Hh-1 gene, in the F_1 hybrids (Cudkowicz and Bennett,1971). The Hh gene (currently = Hemopoietic histocompatibility) was designated as such because of its expression in the cells of the lympho-myeloid complex and also because of the barrier posed to hemopoietic cell transfers in parent-F_1 and allogeneic combinations. In mice, the host responded to the Hh gene-products in ways different from that of other MHC coded, co-dominantly inherited antigens (Shearer and Schmitt-Verhulst,1977).

The hybrid resistance phenomenon had been shown to be resistent to irradiation treatments (Cudkowicz,1971; Lotzova and Cudkowicz,1974). In genetic studies of bone marrow transplantations, it was noted that both irradiated (C57BL/6 x AKR) F_1 and non-irradiated (DBA/2 x AKR) F_1 hybrids were resistent to transplants of spontaneous lymphomas of AKR parental donor origin (Gallagher and Trentin,1976).

Hybrid resistance had also been demonstrated to be ; thymus independent (Cudkowicz and Bennett,1971); suppressible by anti-macrophage agents (Cudkowicz,1975); and genetically mapped in or near the D region (H-2D-Hh-1) of the murine MHC (Cudkowicz and Lotzova,1973). In the case of (129 x CBA) F_1 mice (strain 129 haplotype $H\text{-}2^b$ x strain CBA haplotype $H\text{-}2^k$) injected with parental CBA bone marrow cells, the F_1 recipients rejected the $H\text{-}2^k$ marrow graft in the usual manner and the genetic control of this hybrid resistance phenomenon had been mapped to the $H\text{-}2^k$ region in this particular situation (Cudkowicz and Warner,1979). It seems, therefore, both $H\text{-}2^d$ and $H\text{-}2^k$ regions may control the hybrid resistance phenomenon.

Observations resembling hybrid resistance in which hemopoietic cells from parental donors failed to grow in heavily irradiated hosts have also been described in the dogs (Rapaport et al,1972; Rapaport et al,1973), and possibly also in humans (Van Bekkum,1975; L'Esperance et al,1975).

The phenomenon of hybrid resistance postulated the activity of F_1 hybrid cells against the homozygous parental antigen. Such hypothesis implicated the existence of F_1-host-versus-graft reaction outside the GVH reaction, which in fact, gives strong evidence that the F_1 immunocompetent cells are not really genetically tolerant to the parental donor cells. In the following section, significant F_1 versus parent reactivities will be described.

Immunoreactivity of F_1 Hybrid against Parent

According to the laws of transplantation reactions, the F_1 lymphoid cells from inbred mice, are genetically incapable of reacting against either one of the two parental genotypes. This assumption is being challenged because recent evidence of F_1 immunocompetent cells active against parental histocompatibility differences have surfaced.

Parental cells grafted into F_1 hybrids were first noted to proliferate very poorly in the F_1 host (Cudkowicz and Bennett,1971). Prior injection with subclinical doses of parental strain (A) cells into (A/B) F_1 rats have shown to induce a state of specific resistance to local GVH reactions. Parental T-cells depleted of specific alloreactivity to the host alloantigens failed to induce specific local GVH resistance (Woodland and Wilson,1977).

Specific resistance to systemic GVH reaction had also been demonstrated by the injection of F_1 animals with subclinical doses of parental lymphocytes. This resistance is ; radioinsensitive, transferable to syngeneic F_1 hosts adoptively, and reflects a host-T-cell mediated immune response to αMHC receptors (AαB) on the donor strain (A) T cells. (Bellgrau and Wilson,1978). This immunity had also been shown to be effective for αB receptors on third party (e.g., C.D.E...) T cells (Bellgrau and Wilson,1979).

F_1 spleen cells, not only were found capable of inhibiting the growth of parental bone marrow grafts, but could also generate <u>in vitro</u> cytotoxic activity specifically against parental target cells (Shearer,1975). Similar studies showed that cells from (C57BL/6 x DBA/2) F_1 hybrids could develop a primary <u>in vitro</u> cytotoxic response to C57BL/6 target cells (Shearer et al,1976). When (AKR x DBA/2) F_1 lymphoid cells were mixed with parental AKR lymphocytes <u>in vitro</u>, Thy-1 positive effector cells specifically cytotoxic against parental AKR target cells were generated (Schmitt-Verhulst and Zata,1977).

The F_1 anti-parent cytotoxicity reaction was noted to develop later than the immune response against alloantigens. Selective abolition of the F_1 anti-parent cytotoxicity could be achieved without abrogation of the reactivity against alloantigens. This suggested that two different mechanisms may be responsible for the two reactions (Shearer et al,1976), and the F_1 anti-parent reaction was suggested to be mediated by the T cells (Ishikawa and Dutton,1979).

Involvement of the MHC in this type of F_1 anti-parent reaction has been investigated recently using heterozygous F_1 spleen cells cultured with homologous stimulator cells from the parent. Specific anti-parent cytolytic effects were noted to be coded for, or regulated by the H-2K-Hh-3 region of the MHC. The K end of the H-2 complex seems to control the F_1 anti-parental $H\text{-}2^k$ cell mediated lysis (CML), and the D end seems to control those of F_1 anti-parent $H\text{-}2^b$ CML (Warner and Cudkowicz,1979).

The second type of evidence demonstrating the F_1 anti-parent activity was derived from MLR studies. Adult (C3H x CBA) F_1 lymphoid cells were injected into the parental CBA hosts, and the lymphoid cells from these CBA animals were later found incapable of reacting against C3H cells in the one-way MLR reaction in which the stimulator cells were irradiated C3H cells. It was postulated that the F_1 donor lymphocytes inhibited the T cells of the CBA parent from proliferation during the one-way mixed-leukocyte-reaction (Lilliehook et al,1978).

In addition, parental CBA lymphocytes were found incapable of in vivo proliferation in (C3H x CBA) F_1 hosts which were previously injected with CBA spleen cells and later irradiated before the transfer of new CBA parental cells. This implied that the F_1 hybrids could become immunized against the parental CBA cells and the inhibitory mechanism was probably radioresistent (Lilliehook and Blomgren,1978).

The injection of (C3H x CBA) F_1 lymphocytes into irradiated parental CBA hosts resulted in rapid proliferation of the F_1 cells in the parental spleen. When these proliferating F_1 cells were transferred to new non-irradiated CBA parental hosts, they continued to proliferate. But when they were injected into syngeneic (C3H x CBA) F_1 hybrids, proliferation ceased abruptly (Blomgren and Lilliehook,1978). These observations may be interpreted as follows: the (C3H x CBA) F_1 lymphoid cells, during their transit through the irradiated CBA hosts, became immunized against the parental CBA antigens. When they were injected into new CBA hosts, they continued to proliferate as in a secondary immune response reaction, but when they were exposed to the syngeneic F_1 hosts, the parental CBA antigens on the F_1 cells were probably inaccessible, and proliferation arrested.

Recently, a syndrome known as the "Host-versus-graft disease" has been described. The HVG disease is obtained by the injection of F_1 hybrid spleen cells into parental newborn mice perinatally, resulting in a fatal complex of lesions. Principal features of the syndrome included: thrombocytopenia, intestinal hemorrhage (Hard and Kullgren,1970), hyper-fibrinogenemia (Smith et al,1977), and disseminated intravascular coagulation (Hard and Still,1975). Death from acute HVG disease was attributed to the rapid formation of immune complexes causing severe glomerulopathies (Hard and Moncure,1973). Lymphocyte depletions in spleen and lymph nodes have also been reported (Simpson et al,1974; Cornelius,1978).

Mice with HVG disease usually developed severe T cell mediated immunodeficiency by about 3 to 4 weeks of age, and sequential pathological studies revealed that the thymic dependent portions of lymphoid organs were severely depleted of small lymphocytes (Hard and Campbell,1979); and yet, splenomegaly is characteristic of HVG diseases (Hard and Kullgren,1970).

The type of lymphoid cells proliferating in the spleen accounting for the splenomegaly is unknown. It is tempting to speculate that the injected donor cells being reactive against parental antigens, proliferate in the host-parent to give splenomegaly. The significance of these reports is that the fatal syndrome induced by HVG disease is a reverse image of the GVH-induced runting disease in newborn mice.

In summary, this section has reviewed the many interesting aspects of the host participating in the GVH reaction; beginning with the observations of proliferation of host lymphoid cells in response to the injection of parental immunocompetent cells, the production of humoral immune responses by the F_1 hybrid cells manifested as the allogeneic effect, and the detection of autoimmune as well as anti-idiotypic antibodies.

In cellular immune responses, the host-versus-graft reaction is probably manifested by the phenomenon of non-specific cytotoxicity, hybrid resistance, and F_1 anti-parent reactivity. All these reactions may very well be the contributing parts of the total process of the GVH reaction.

RATIONALE

The injection of parental immunocompetent lymphoid cells into the F_1 hybrid animals results in the sensitization of the donor cells against the host's histoincompatible antigens. This initiates a series of complex interactions between the donor and host cells, and the manifestations of the GVH reaction include: the runting syndrome (Simonsen,1957); the proliferation of the reticuloendothelial tissues (Weiss et al.1957); the increased phagocytic activities (Howard et al,1961); and deficiencies in both humoral and cell-mediated immune responses (Lapp and Moller,1969).

The manifestations of GVH reaction are usually followed by spontaneous remission of the reaction (Streilein and Billingham,1970a; Grebe and Streilein,1974; Grebe and Streilein,1976; Wolters and Benner, 1978), and this interesting aspect of the GVH reaction has not been satisfactorily explained. To this end, different mechanisms have been proposed. These include: the induction of tolerance in the donor cells towards host antigens, the "allergic cell death" of the grafted donor cells overwhelmed by the exposure to the enormous amount of host antigens, the production of "blocking antibodies", "anti-idiotypic antibodies", and the involvement of the phenomenon of "hybrid resistance". But none of these even came close to suggest that the genetically tolerant F_1 host immunocompetent cells could become activated in a GVH reaction resulting in the mediation of a host-versus-graft reaction.

The pathological changes seen in the F_1 host animal in the GVH reaction is generally believed to be the result of immunological attacks of host tissues by the activated donor lymphoid cells, as evidenced by the production of donor effector cells mediating "specific cytotoxicity".

Cell-mediated cytotoxicity reactions have generally been observed to be immunologically specific (Allison,1971). However, immunologically "non-specific cytotoxicity" reactions have also been reported by many authors, in which, allogeneic, semi-syngeneic, and even xenogeneic target cells were also affected (Berke et al,1972; Binet et al,1962; Singh et al,1971). Experiments involving inhibition of cytotoxicity with specific alloantisera have indicated that this type of GVH-induced non-specific cytotoxicity was due to the F_1 host cells (Singh et al,1972). By contrast, effector cells from lethally irradiated F_1 animals undergoing GVH reactions lysed specifically only target cells of the genotype against which the donor cells have been sensitized, while those from non-irradiated hosts exhibited non-specific cytotoxicity reactions (Singh et al,1973; Greenberg et al,1973).

These investigations indicated that different populations of cells were responsible for the two types of cell-mediated cytotoxicity reactions. Specific cytotoxicity is due to the donor immunocompetent cells reacting against the host, and non-specific cytotoxicity reaction is due to immunocompetent cells of F_1 host origin. The cellular nature of the effector cells involved in this type of host cell mediated non-specific cytotoxicity have not been determined in previous studies.

The mechanisms operating in a GVH reaction remain a mystery to the immunologists. Despite a genetically tolerant environment, the parental donor cells somehow are regulated as a consequence of the inflammatory reaction, and the host animal eventually recovers from the immunological attack spontaneously. This is a significant challenge to the genetic dogma of transplantation tolerance. The various possible immunoregulatory mechanisms involved, no doubt are complex and multiple, and no coherent hypothesis which can explain the manifestations, as well as the termination of the GVH reaction, exists to date. To this end, the present study attempts to elucidate the underlying mechanisms of the semi-syngeneic cytotoxicity reaction and to relate it in total perspective to the understanding of the immunological phenomenon of graft-versus-host, as well as the host-versus-graft reactions.

The experimental designs and the results of the present thesis will be presented in three separate sections.

Experiments in the first section examined the various parameters involved in the GVH-induced semi-syngeneic cytotoxicity reaction; e.g., route of induction, anatomical distribution of the F_1 effector cells, kinetics of the cytotoxicity reaction, etc.

The second section consists of experiments designed to identify the nature of F_1 effector cells in semi-syngeneic cytotoxicity, the in vitro activation of the F_1 effector cells, the kinetics of the activation process.

In the last section, the in vivo aspect of the GVH reaction were examined from the perspectives of : in vivo activation of the F_1 cells, the role of the GVH activated F_1 cells in the in vivo GVH reaction, and the involvement of the F_1 immunocompetent cells in the host versus graft reaction.

EXPERIMENTAL DESIGNS AND RESULTS

The various experimental designs and results of this report are presented in three individual sections. The first section includes studies on the various parameters of the F_1 host cell mediated semi-syngeneic cytotoxicity reaction. The second section consists of experiments to identify the nature of the F_1 host effector cells as well as their *in vitro* roles in the cytotoxicity reaction. The last section describes the involvement of the F_1 host cells in the phenomenon of natural resolution of the GVH reaction. The underlying mechanism of such a phenomenon will be examined in the section of Discussion.

PARAMETERS OF SEMI-SYNGENEIC CYTOTOXICITY

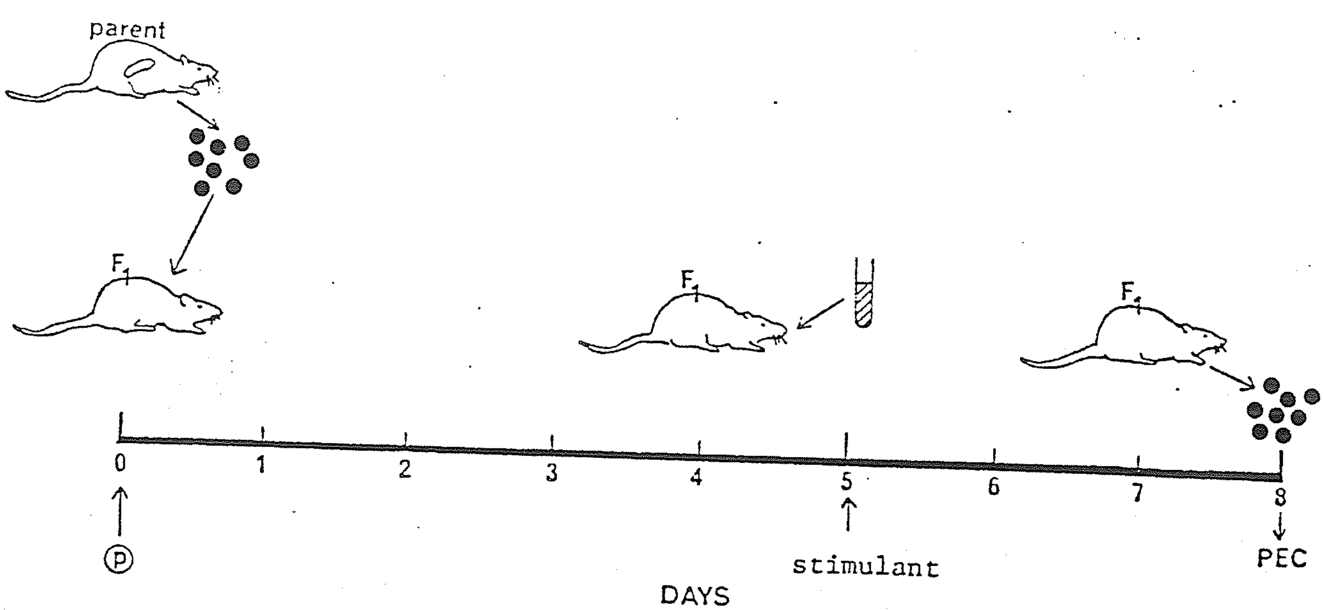
A GVH reaction classically occurs when immunocompetent cells are grafted into histoincompatible but genetically tolerant recipients. The F_1 hybrid generation is considered immunologically unresponsive to the histocompatibility antigens of both parents and is theoretically incapable of reacting against the transplanted parental donor lymphoid cells. The specificity of the cytotoxicity reaction however, is not absolute. In our laboratory, a "non-specific" cytotoxicity reaction observed in GVH-induced hosts, producing *in vitro* lysis of target cells bearing the parental H-2 genotype had been reported to be mediated by the F_1 host lymphoid cells (Singh *et al*,1972; 1973). In this first section of experiments, the many parameters involved in this F_1 host mediated semi-syngeneic cytotoxicity reaction will be examined.

Induction of GVH Reactions in Mice

Graft-versus-host reactions were induced in mice by injecting intraperitoneally 1.5 x 10^8 parental spleen cells into different groups of individual F_1 hybrids on day zero.

In order to obtain a high yield of peritoneal exudate cells (PEC), the animals were injected with 1.5 ml of a 6% sodium caseinate solution 3 days before harvesting the PECs from the peritoneal cavities using Alsever solution washings. Differential staining of these PECs demonstrated that they consisted mainly of macrophages and mononuclear cells. After centrifugation at 250 G for 3 minutes 1X, macrophages were found to constitute above 90% of total cells. The PECs when harvested on the ninth day from the peritoneal cavities of these GVH-induced animals would constitute the effector cell population.

Spleen cells from these GVH-induced F_1 recipients were also used as effector cells in some experiments. Control effector cells were obtained from F_1 syngeneic animals injected with syngeneic F_1 spleen cells replacing the parental spleen cells.



Cell Mediated Cytotoxicity Assay

This CMC test is done by mixing, in the wells of the Microcytotoxicity test-plates (Microntest II, Falcon Plastics), a 0.1 ml volume of a suspension of effector cells (1 to 2 x 10^6 cells/0.1 ml) with a 0.1 ml volume of a suspension of ^{51}Cr-labelled target cells (1 to 2 x 10^4 cells/0.1 ml). The test-plates were sealed with the plastic covers and were incubated at 37°C for 16 hours. After incubation, the plates were centrifuged at 250 G for 10 minutes to sediment the effector and the lysed target cells. A 0.1 ml volume of the cell-free supernatants were aspirated from each individual wells and transferred to separate test-tubes which were counted in an automatic gamma-ray counter (Chicago Nuclear). The counts per minute of each test-tubes were recorded.

The corrected percent lysis of the target cells was obtained according to the following formula :

$$\text{Corrected Percent Cytotoxicity} = \frac{CPM_{Exp.} - CPM_{Cont.}}{CPM_{Tot.} - CPM_{Cont.}} \times 100$$

where : CPM = counts per minute of ^{51}Chromium radioactivity

CPM_{Exp} = mean CPM in supernatants of the experimental wells

$CPM_{Cont.}$ = mean CPM in supernatants of the control wells

$CPM_{Tot.}$ = mean CPM of maximal or 100% target cell lysis

The experimental wells contained GVH-activated F_1 spleen or peritoneal effector cells obtained from the F_1 hosts previously injected with parental spleen cells for the induction of GVH reactions.

The control wells contained F_1 spleen or peritoneal exudate cells obtained from the F_1 animals previously injected with syngeneic F_1 spleen cells just as in the induction of GVH reactions. These syngeneic F_1 cells were used as controls in various experiments to eliminate the background or truly non-specific target cell lysis.

Approximately 3 ml of the ^{51}Cr-labelled target cells used in each individual experiments was transferred to a test-tube which was put through 3 cycles of alternate freezing anf thawing to obtain maximal or 100% lysis of the labelled target cells. A 0.1 ml volume of the cell-free supernatant was aspirated into different test-tubes, and the CPMs of 6 replicates were obtained for each experiment to give a mean CPM.

Various experimental conditions as applied in the CMC assays will be described accordingly in each individual experiments.

Radioisotope for Target Cell Labelling

Chromium-51 (^{51}Cr) isotope with specific activity between 80 to 150 uc/gm was obtained from Atomic Energy of Canada Ltd., Ottawa, Ontario, as Na_2Cr0_4 form in NaOH solution. The solution containing the isotope was neutralized with HCl and diluted to the concentration of 1.0 uc/ml. Radioactivity was determined by a well-type gamma-rays scintillation counter (Nuclear Chicago Corporation, Des Plaines, Illinois).

Labelling of Target Cells

Target cell suspensions in one ml volumes containing 1×10^7 target cells per ml were suspended in RPMI 1640 without fetal calf sera and incubated with 80 to 100 uc of radioactive ^{51}Cr isotope in 12 x 75 mm plastic tissue culture tubes (Falcon Plastics) at 37°C for 30 minutes agitated every 10 minute intervals. After incubation, the target cells were washed 3X in HBSS at $20^{o}C$, followed by 2X RMPI 1640 without fetal calf serum and antibiotics also at $20^{o}C$, and then resuspended in RPMI 1640 medium adjusted to 1×10^{5} taget cells per ml volume. These labelled cells were used as target cells in CMC assays.

Semi-syngeneic Cytotoxicity in GVH Reactions

Inbred strain mice of approximately six to eight weeks old from established lines were obtained from the Jackson Laboratory, Bar Harbor, Maine, U.S.A. Parental donors included female strains of A/J ($H\text{-}2^{a} = H\text{-}2^{k}/H\text{-}2^{d}$), C57BL/6($H\text{-}2^{b} = H\text{-}2^{b}/H\text{-}2^{b}$), DBA/2J($H\text{-}2^{d} = H\text{-}2^{d}/H\text{-}2^{d}$) and C3H/HeJ($H\text{-}2^{k} = H\text{-}2^{k}/H\text{-}2^{k}$). The F_1 hybrid recipients included male strains of B6AF$_1$(C57BL/6 x A/J = $H\text{-}2^{b} \times H\text{-}2^{k/d}$), B6D2F$_1$(C57BL/6 x DBA/2J = $H\text{-}2^{b} \times H\text{-}2^{d}$), and C3D2F$_1$(C3H/HeJ x DBA/2J = $H\text{-}2^{k} \times H\text{-}2^{d}$).

For the induction of GVH reactions in different strains of parent-F_1 combinations, parental spleen cells from various strains of the A/J, C3H/HeJ, and DBA/2 female mice were injected intraperitoneally into the appropriate F_1 hybrids of B6AF$_1$, C3D2F$_1$, and B6D2F$_1$ recipients respectively. Different strains of target cells bearing the respective parental donor H-2 genotypes which were in effect semi-syngeneic to the F_1 host cells, were used within each parent-F_1 combination in CMC assays. The results of the respective semi-syngeneic cytotoxicity reactions are shown in Table I .

Significant semi-syngeneic target cell lysis reactions were obtained with peritoneal exudate cells and spleen cells in all of the three parent-F_1 combinations tested. The F_1 peritoneal exudate cells were consistently more effective than the spleen cells in eliciting the semi-syngeneic cytotoxicity reaction.

Table I - Semi-syngeneic Cytotoxicity Reactions in Different Combinations of Parental Donors and F_1 Recipients

Donor	Recipient	Effector Cells	Target Cells	Corrected Lysis*
A/J	$B6AF_1$	Spleen	Sarcoma I	14.59 ± 0.33
		PEC	"	39.31 ± 2.12
C3H/HeJ	$C3D2F_1$	Spleen	L929	21.27 ± 1.62
		PEC	"	42.24 ± 1.04
DBA/2	$B6D2F_1$	Spleen	P815	18.25 ± 1.07
		PEC	"	37.74 ± 0.64

* Mean percent corrected lysis of target cells induced by pooled effector cells from groups of 10 animals ± SE in 4 replicates

Activation of F_1 Host Effector Cells

Parental donor mice were sacrificed by cervical dislocation and the spleens were removed, dissected into fragments then teased into cell suspension using two needles in tissue culture medium RPMI 1640 buffered with HEPES, supplemented with antibiotics as previously described. The cells in the suspension were filtered through a sterile (pressurized steam sterilization) stainless steel mesh (gauge 200) screen and collected in sterile plastic 12 x 75 mm plastic tissue culture tubes (Falcon Plastics). The cells were then washed 3X with HBSS or RPMI 1640 and refiltered through other unused stainless steel mesh screen twice. The spleen cells were resuspended in RPMI 1640 in various required concentration for later experimental uses.

Adult parental female A/J spleen cells (1 x 10^8/recipient) were injected intravenously and/or intraperitoneally into three separate groups of 25 male $B6AF_1$ recipients in the absence of peritoneal exudate cell stimulants. The *in vitro* cytotoxicity of these GVH-induced F_1 host lymphoid cells on parental donor H-2 genotype target cells were examined eight days after the induction of GVH reaction. The release of ^{51}Cr radioactive labels from the target cells was determined after incubating the F_1 peritoneal exudate effector cells with the parental genotype target cells at 37°C for 16 hours as previously described. The results as shown in TableII indicated that the intraperitoneal route of induction of GVH reactions in the F_1 hybrids was the most significant in determining the degree of *in vitro* semi-syngeneic cytotoxicity. The F_1 host peritoneal exudate cells were more efficient in producing target cell lysis than the spleen cells from the same group of GVH-induced animals.

Table II. Activation of Effector Cell Populations using various Routes of Induction of GVH Reaction

GVH Induction Routes	Effector Cells	Target Cells	Corrected Lysis ***
Intravenous	Spleen*	Sarcoma I	1.71 ± 0.77
	PEC**	"	9.31 ± 1.73
Intraperitoneal	Spleen	Sarcoma I	7.28 ± 0.35
	PEC	"	36.56 ± 1.95
Intravenous and intraperitoneal	Spleen	Sarcoma I	14.59 ± 1.02
	PEC	"	29.29 ± 2.67

* Spleen = Spleen cells from $B6AF_1$ 8 days after transplantation of A/J parental spleen cells.

** PEC = Peritoneal exudate cells from $B6AF_1$ 8 days after transplantation of A/J parental spleen cells.

*** Mean percent corrected lysis of target cells by pooled effector cells from groups of 25 animals ± SE in four replicates.

Sensitivity of Target Cells in Semi-syngeneic Cytotoxicity

Male $B6AF_1$ mice were grafted intraperitoneally with 1×10^8 of A/J parental spleen cells, and 5 days later injected with 1.5 ml of the 6% sterile sodium caseinate solution also intraperitoneally. Using the spleen cells and peritoneal exudate cells from these GVH-induced $B6AF_1$ hybrids as effector cells, semi-syngeneic cytotoxicity reactions were compared among the three different types of target cells possessing, at least the (H-2^k) genotype of the parental donor cells.

Four different target cells were used in the in vitro assay of host cell mediated semi-syngeneic cytotoxicity reactions.

(1) L-929 Cells of C3H origin were obtained from Microbiologic Associates, Bethesda, Maryland. The cells were grown in monolayers and incubated in tissue culture chambers at 37°C in the absence of carbon dioxide. Cell culture procedures were done in sterile chambers under the strict sterile procedure guide lines as in microbiological cultures. A treatment of the monolayer with a 0.25% trypsin solution in Madin-Darby's solution at 37°C for 10 to 20 minutes resulted in liberation of the cells into a suspension for target cells.

(2) P815x2 (P815) Mastocytoma) cells were grown in ascites forms in mice of strain DBA/2J (H-2^d/H-2^d) and they bear the same H-2^d genotype as the host of the tumor cells. The tumor cells were harvested intraperitoneally six to eight days after the injection of 1×10^6 cells per animal. After centrifugal separation of the tumor cells from the ascitic fluid, the cells were washed in HBSS 3X and then suspended in hypotonic solution (1 volume DS diluted with 6 volumes of double-distilled water) for lysis of the red blood cells. After 30 seconds in this

solution, isotonicity was immediately restored by adding the appropriate volume of a 5% (weight to volume) NaCl solution and the tumor cells removed from the solution by centrifugation, then suspended in tissue culture medium RPMI 1640 as described previously.

(3)Sarcoma I (SaI) tumor target cells were obtained from the Jackson laboratory and were grown in the ascitic fluid in peritoneal cavity of strain A/J mice. The tumor cells were harvested 4 to 6 days after the injection of 1×10^6 tumor cells per animal. The harvested tumor cells were prepared in the same procedure as described for the P815 tumor cells from strain DBA/2J animals. The Sarcoma I target cells bear the same $H\text{-}2^a(H\text{-}2^k/H\text{-}2^d)$ genotype as the host A/J recipients.

(4) Macrophage target cells were harvested from the peritoneal cavities of mice 3 days after intraperitoneal injection of 1.5ml of a 3.55 gram-percent autoclaved Dextran (molecular weight range of 5 to 40 $\times 10^6$) solution. The cells were collected in Alsever's solution, washed 3X in HBSS, 2X in RPMI without fetal calf sera. The cells were then suspended in the supplemented RPMI 1640 tissue culture medium to be used as target cells.

As shown in Table III, it can be seen that significant differences in the susceptibility of the same target cell to cytolysis by the spleen and peritoneal exudate cells from the same group of F_1 hosts were detected. Tissue-culture propagated L929 target cells were noted to be the most efficient target cells for such experimental CMC assays, while normal macrophages were noted to be the least efficient target cells. The A/J genotype Sarcoma I tumor cells were intermediate between the L929 cells and the A/J macrophages as target cells in the semi-syngeneic cytotoxicity assays.

Table III - Sensitivity of Taget Cells in Semi-syngeneic Cytotoxicity Reaction of F_1 Hybrids Undergoing GVH Reactions**

Target Cells	Effector Cells	Corrected Lysis *
L929 ($H-2^k/H-2^k$)	Spleen	13.50 ± 1.02
	PEC	39.28 ± 2.07
Sarcoma I ($H-2^a$) = ($H-2^k/H-2^d$)	Spleen	10.82 ± 1.77
	PEC	23.31 ± 3.12
Macrophage ($H-2^a$) = ($H-2^k/H-2^d$)	Spleen	-1.90 ± 0.79
	PEC	9.31 ± 1.73

* Mean percent corrected lysis of target cells by pooled effector cells from groups of 10 animals ± SE in four replicates.

** <u>GVH Reaction Combination</u>

Parental donor cell genotype = A/J ($H-2^a$) = ($H-2^k/H-2^d$)

F_1 hybrid genotype = $B6AF_1$ = ($H-2^k/H-2^d$) x ($H-2^b/H-2^b$)

Kinetics of Semi-syngeneic Cytotoxicity Reactions

The relationship between the degree of *in vitro* semi-syngeneic cytotoxicity and the interval of incubation of the effector and target cells was studied using two different effector to target cells ratios under identical experimental conditions.

Parental C3H/HeJ spleen cells were used to induced the GVH reactions in C3D2F_1 hybrids which were later stimulated by the 6% sodium caseinate solution. At different intervals of incubation in the CMC assays, the semi-syngeneic cytotoxicities were measured. As shown in Figure 2, the degree of semi-syngeneic cytotoxicity was directly proportional to; firstly, the quantity of effector cell present, and secondly, the interval of the *in vitro* incubation of the F_1 host effector cells with the semi-syngeneic target cells. These results demonstrated a dose-response type of relationship between the interval of incubation and the degree of cytotoxicity.

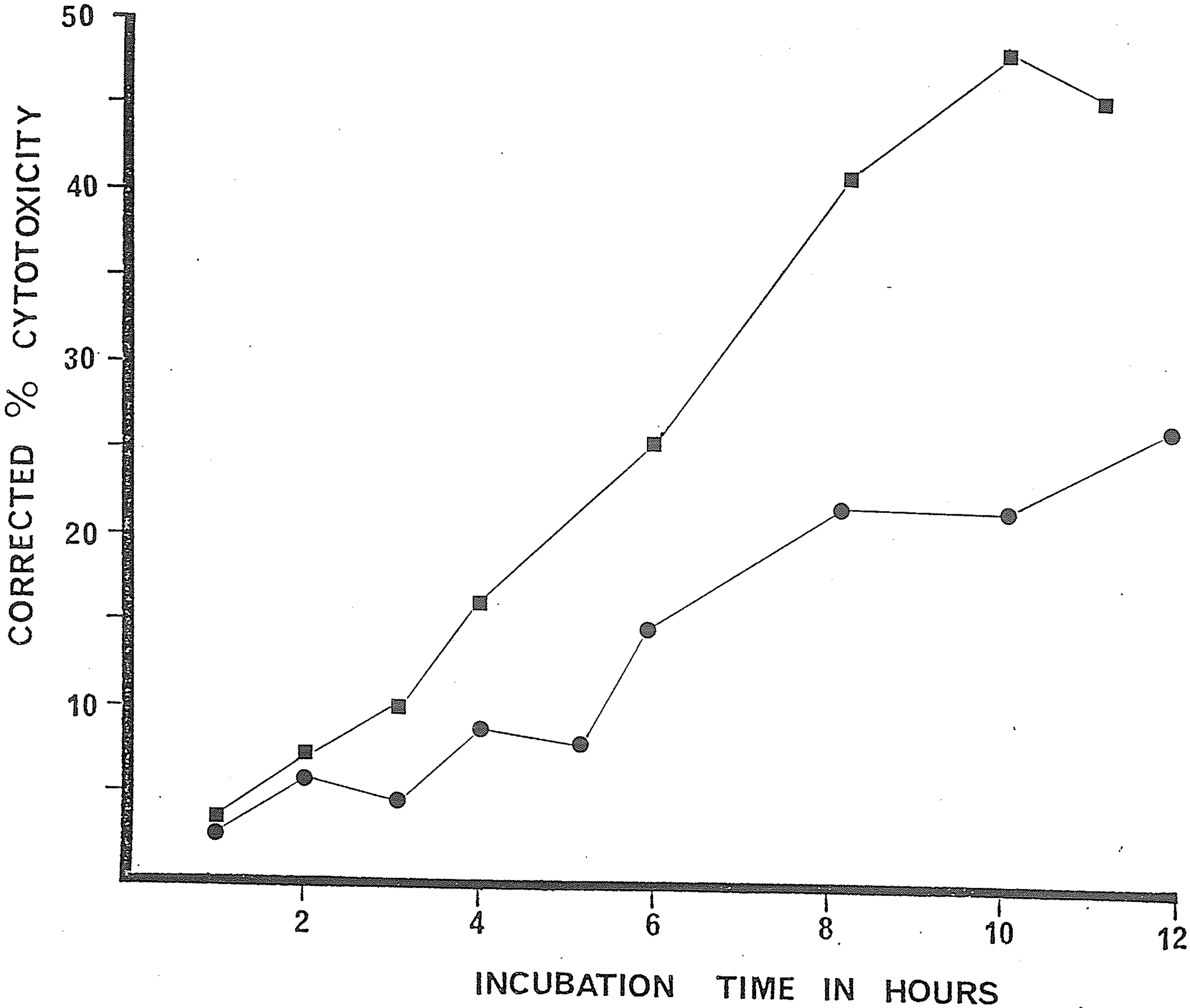


Figure 2 - Kinetics of Semi-syngeneic Cytotoxicity of Peritoneal Exudate Cells Induced by GVH Reaction

●———● Effector Cell to Target Cell Ratio of 100 to 1

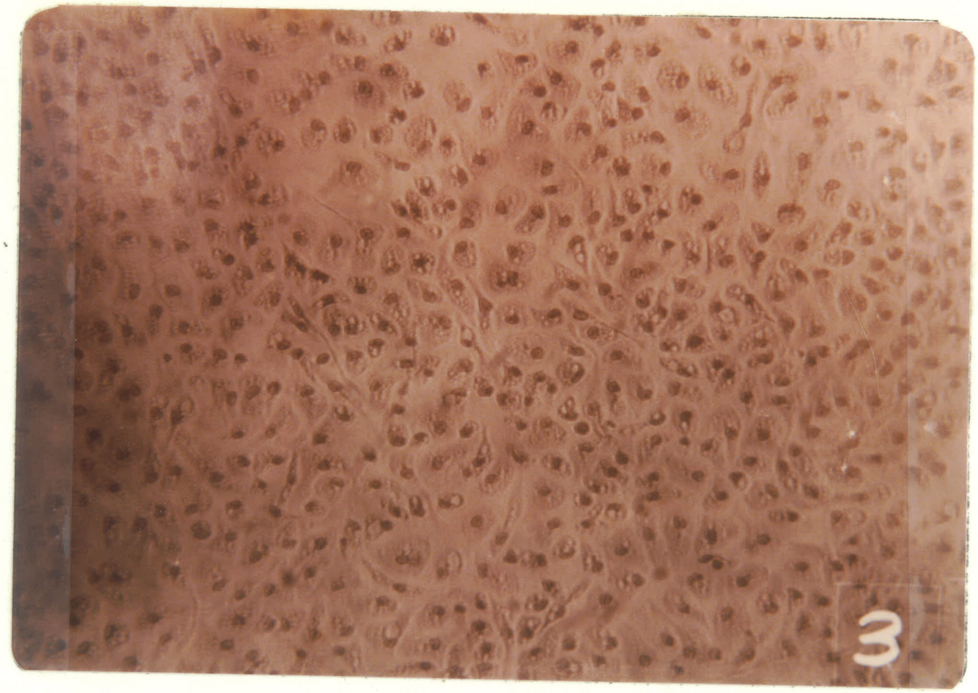
■———■ Effector Cell to Target Cell Ratio of 200 to 1

Cytological Study of Kinetics of Semi-syngeneic Cytotoxicity

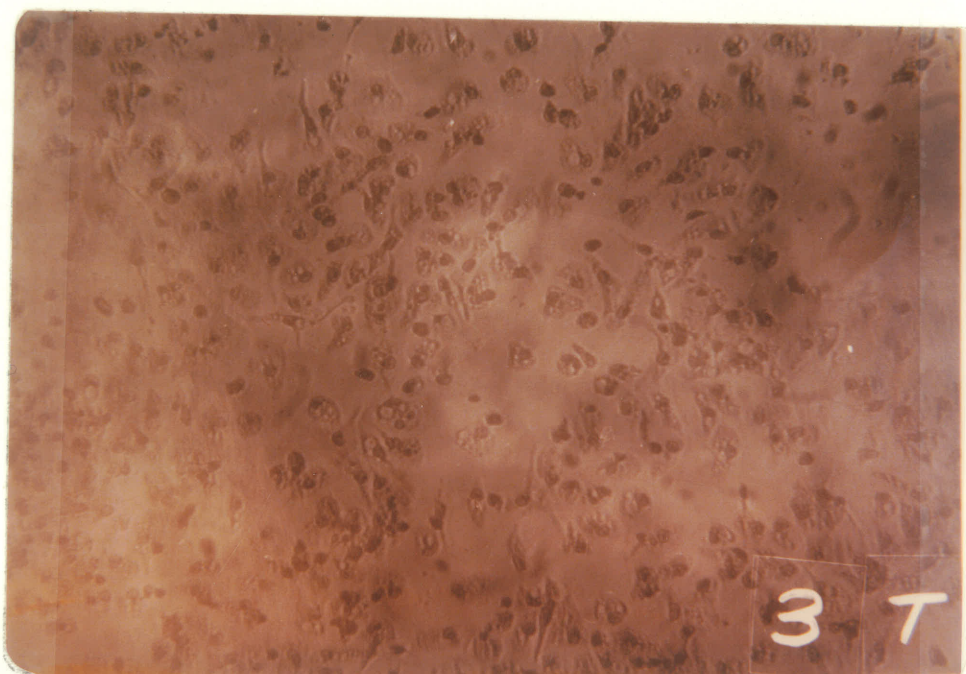
In vitro semi-syngeneic cytotoxicity in a GVH reaction has been demonstrated by the CMC assay in previous experiments. Evidence is presented here with microphotographs taken at different intervals during the process of *in vitro* cell mediated cytolysis to substantiate the observations in the CMC assays using ^{51}Cr-labelled target cells.

The L929 cells were transplanted from the tissue-culture bottles into the individual wells of the microcytotoxicity test plates as in the CMC assay. Each well contained 1×10^4 L929 target cells, and they were incubated at 37°C for 1 hour to allow the formation of a target cell monolayer. C3D2F_1 mice were injected with parental C3H/HeJ spleen cells for the induction of GVH reactions. The peritoneal exudate cells from these C3D2F_1 mice were added to the monolayer of target cells in the final ratio of 100 effector cells to 1 target cell. At three-hourly intervals, the plates were examined and microphotographs were taken with an Olympus microscope. The qualitative destruction of target cells are shown in the following pages. Target cells could be distinguished from the effector cells by their morphological appearances.

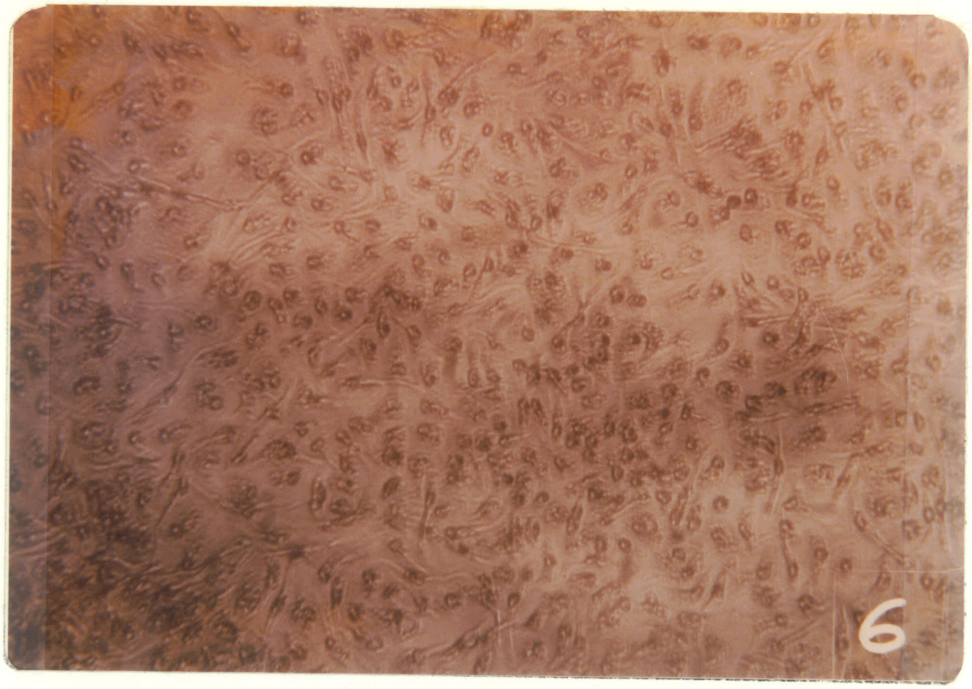
In each of the following pages, the top photograph is the control (i.e.,without F_1 effector cells), and the bottom photograph shows the destruction of the target cell monolayer by the F_1 effector cells. The intervals of incubations, at 3, 6, 9, and 12 hours, are indicated at the bottom right corner. The progression of increasing target cell lysis can be seen by comparing the control and the test photographs. The L929 target cell monolayer becomes more morbid as the incubation interval increased. The quantitative aspect of destruction of the monolayer of target cells is described in the following experiments.



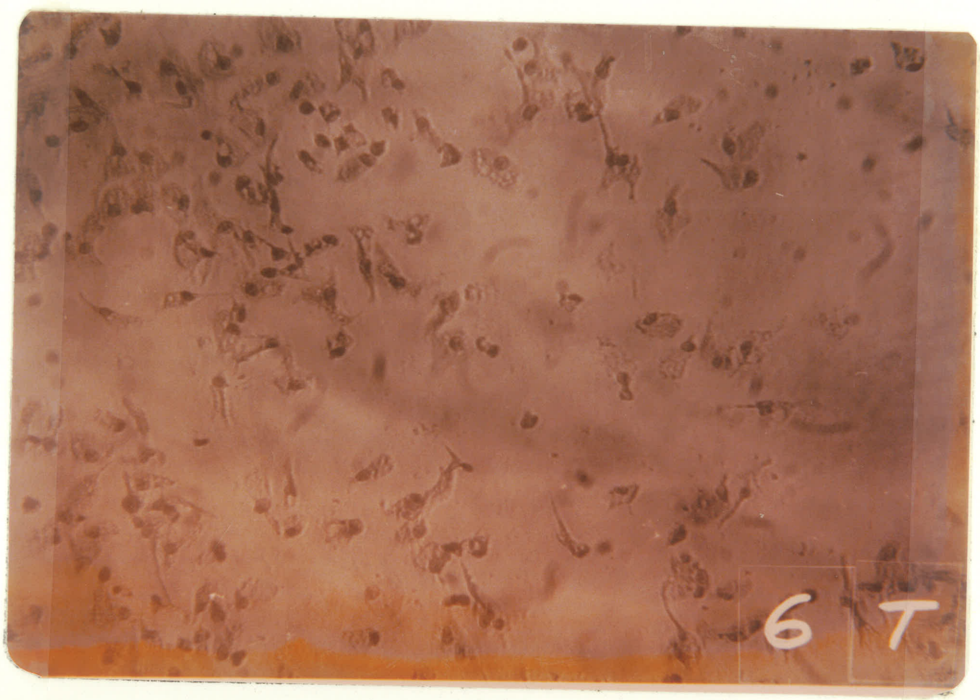
Control at 3 hours



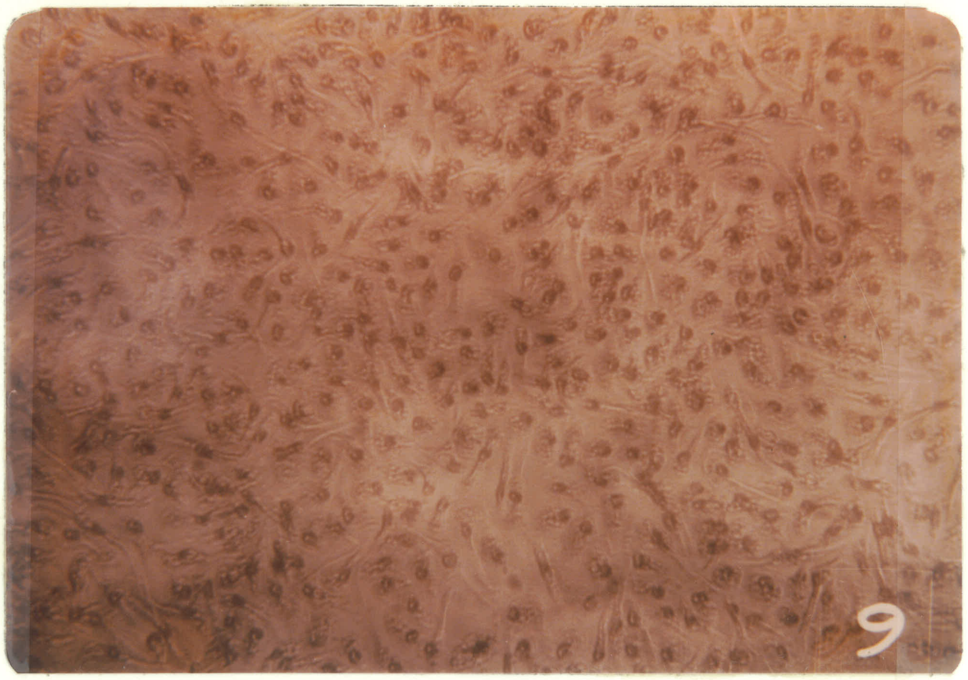
Test at 3 hours



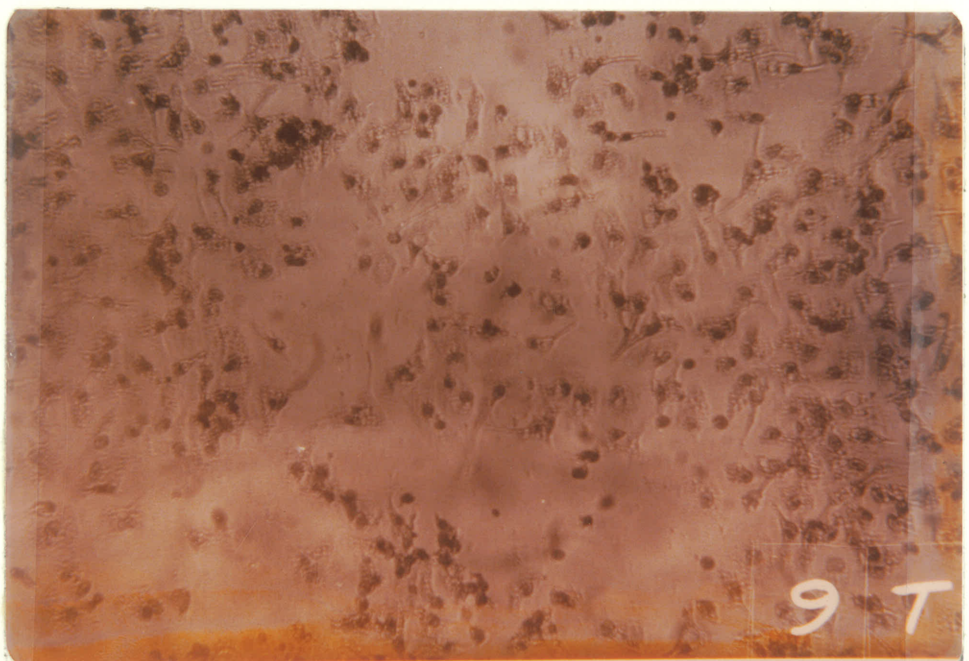
Control at 6 hours



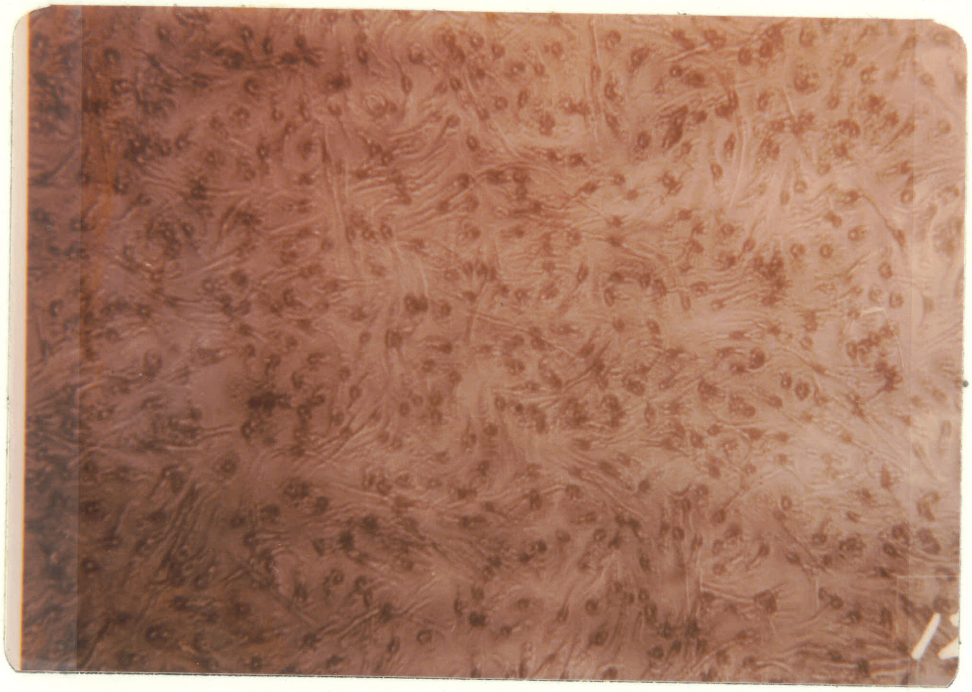
test at 6 hours



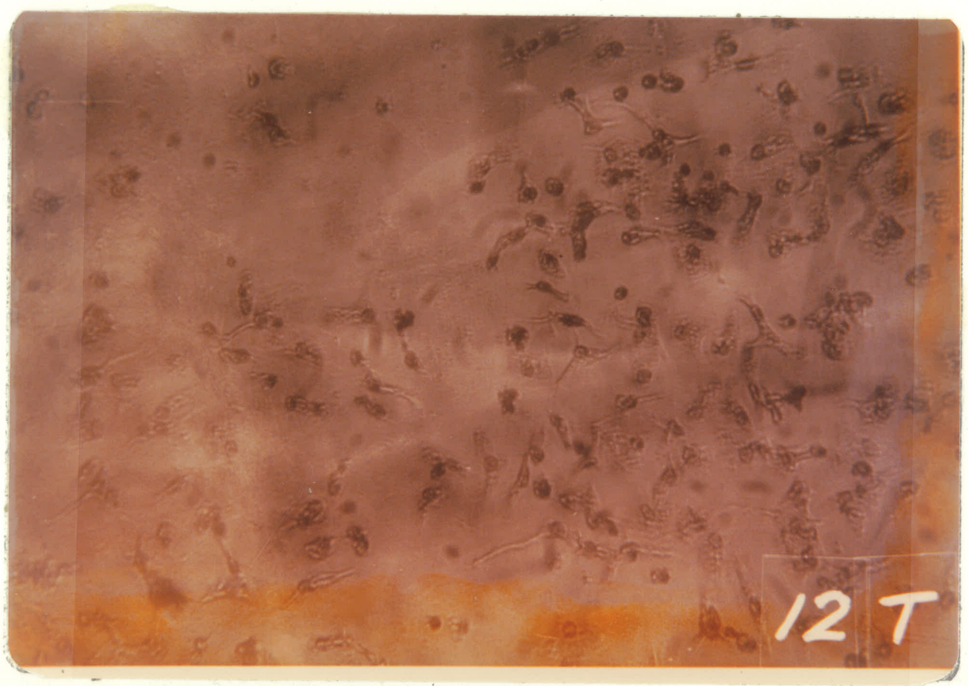
Control at 9 hours



Test at 9 hours



Control at 12 hours



Test at 12 hours

Assessment of Kinetics of Semi-syngeneic Cytotoxicity

This section compares the measurements of the *in vitro* semi-syngeneic cytotoxicity reactions by the ^{51}Cr-release method and the cell-counting method (Klein 1975) in relation to the kinetics of target cell lysis.

Parental C3H/HeJ spleen cells were injected into $C3D2F_1$ animals to induce GVH reactions and peritoneal exudate cell stimulants were injected as previosuly described. These F_1 PECs were used as effector cells in both assay methods. The quantitation of ^{51}Cr release-method has been described. The cell-counting method involved quantitating viable and non-viable target cells in standardized counting fields. The wells of the CMC plate were examined at 1:40 magnification and 5 standardized small squares were randomly selected for counting. Target cells were distinguished from effector PECs by their morphological appearance in the cell-cultured monolayer. Non-viable target cells were stained by trypan blue solution. The number of viable and dead target cells were counted and the percentage of cytotoxicity was calculated by the following formula :

$$\text{Percent Cytotoxicity} = \frac{\text{No. of dead target cells} \times 100}{\text{No. of dead taget cells + No. of viable target cells}}$$

The arithmetic means and standard errors were calculated and plotted in Figure 3. The results displayed a parallel relationship of kinetics of semi-syngeneic cytotoxicity between the two assaying methods. The ^{51}Cr release method is more sensitive at the early incubation period, while the cell-counting method is more sensitive at the later incubation interval. At 6 hours of incubation, the sensitivities of the two assay methods were noted to be approximately equal.

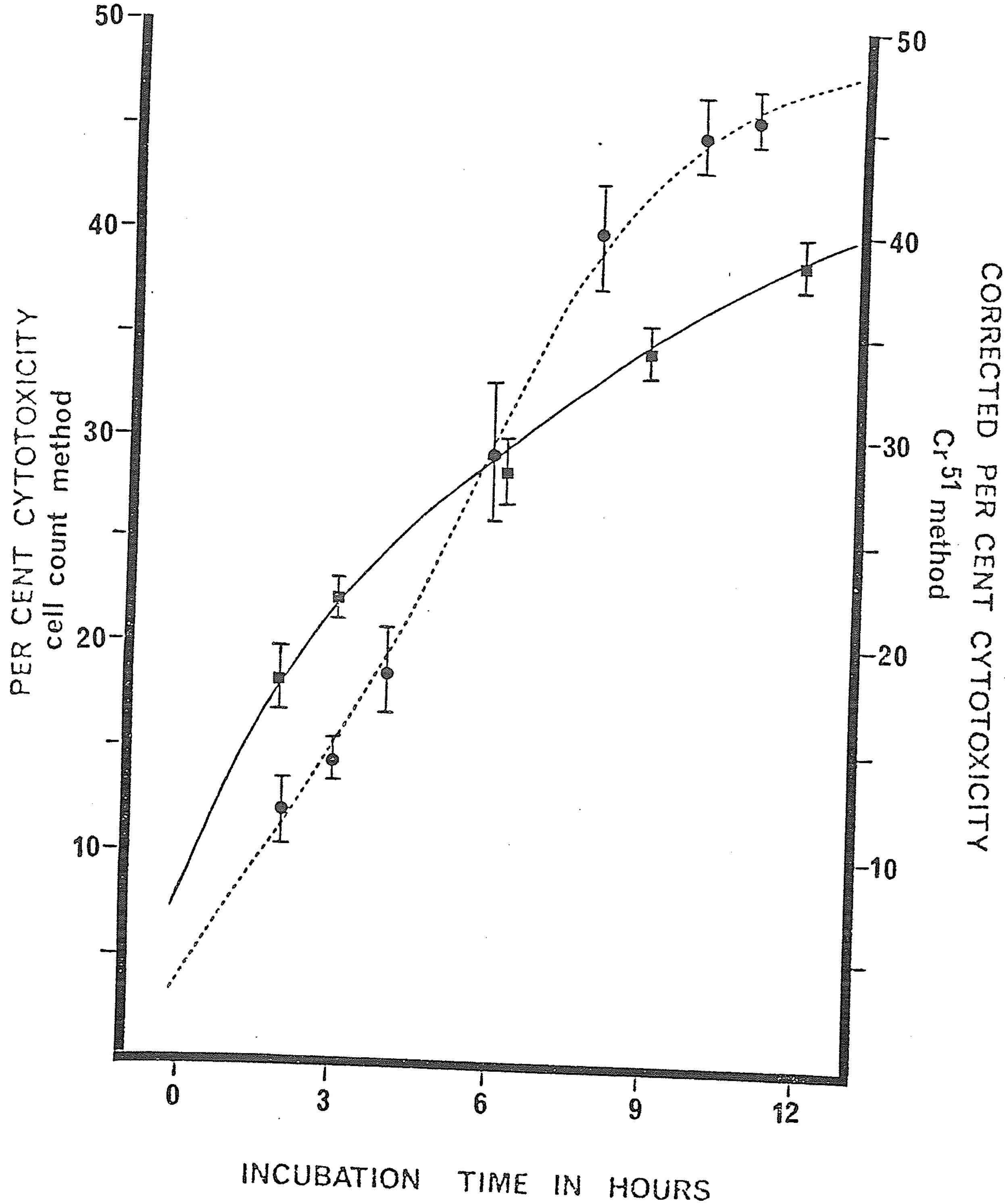


Figure 3 - Correlation between ^{51}Cr method and Cell Count method in assays of Semi-syngeneic Cytotoxicity

———— ^{51}Cr method of assay

- - - - - - - Cell Count method of assay

Statistical Analysis of Data

The arithmetic means and standard errors (SE) were calculated for all cytotoxicity assays using pooled materials. Analysis of regression for individual cytotoxicity reactions where applicable were performed by the following computer program ST 31 available at the Computer Department of Faculty of Medicine, University of Manitoba.

ST 31 (Simple Linear Regression and Correlation)

Function

This program performs a linear regression and correlation tests along with the tests of significance. There is an option to calculate the confidence limits for the mean of the dependent values and for a single dependent value given any independent value and also an option for a plot of the data about the regression line.

Output

- Mean and standard deviation of both variables.
- Simple correlation coefficient and its square.
- Intercept and regression coefficient.
- Standard error of estimate.
- Standard deviation of the regression coefficient.
- T-value for the regression coefficient.
- Analysis of variance table.
- Observed, expected, adjusted, and residual values.
- Plot of data about the regression line.
- Confidence limits for the mean of the dependent values and for a single dependent value given any independent value.

Details of the mathematical aspect of the ST 31 program are described in Appendix section.

Statistical Analyses of Kinetics of Semi-syngeneic Cytotoxicity

To verify that the kinetic relationship observed in above studies is statistically significant, the experimental data from the kinetic study using ^{51}Cr target cell cytolysis was analysed by the computer program ST 31 for comparison between the dependent variable (semi-syngeneic cytotoxicity) and the independent variable (incubation time) as described previously.

Tables IV and V are direct computer print-outs of data input, regression coefficient analyses, and tables of analysis of variances for the two different effector to target cell ratios studied.

In both tables, it can be seen clearly that the two calculated regression coefficients, namely 0.971 (effector to target cells 100 : 1), and 0.984 (effector to target cells 200 : 1) were very close to the ideal value of 1, indicating a linear correlation which is statistically significant. In the analysis of variance tables, the OBSERVED and EXPECTED values were very close together mathematically, with RESIDUAL values in the range of 0.004 (minimal) and 3.558 (maximal). These results again indicated the statistical significance of semi-syngeneic cytotoxicity reactions.

The direct plots of the two linear regression lines are shown in Figures 4 (effector to taget cells 100 : 1) and 5 (effector to target cells 200 : 1). The directly proportional relationship between the degree of semi-syngeneic cytotoxicity and incubation time interval was graphically demonstrated.

```
DATA...
1 2.06
2 5.79
3 4.13
4 8.53
6 14.6
8 21.55
10 19.74
11 24.71/


N =     8

 X          MEAN               SD


 1          5.625              3.739
 2         12.639              8.681

SELECTION... 2 1


R   = 0.971
RSQ = 0.943


 2 ON  1

INTERCEPT    =    -0.040
B            =     2.254
SD ESTIMATE  =     2.244
SDB          =     0.227
T            =     9.937

OP1.. Y
           ** ANALYSIS OF VARIANCE **

  SOURCE        DF       SS          MS          F

REGRESSION      1      497.282     497.282     98.754
DEVIATIONS      6       30.214       5.036
TOTAL           7      527.496

OP2.. Y

 NO.   OBSERVED    EXPECTED    ADJUSTED   RESIDUAL

   1     2.060       2.214      12.485     -0.154
   2     5.790       4.468      13.961      1.322
   3     4.130       6.722      10.047     -2.592
   4     8.530       8.976      12.193     -0.446
   5    14.600      13.484      13.755      1.116
   6    21.550      17.992      16.197      3.558
   7    19.740      22.500       9.878     -2.760
   8    24.710      24.754      12.594     -0.044
```

Table IV – Computer Print Out of Statistical Analysis of Semi-syngeneic Cytotoxicity Ratio of EC : TC of 100 to 1

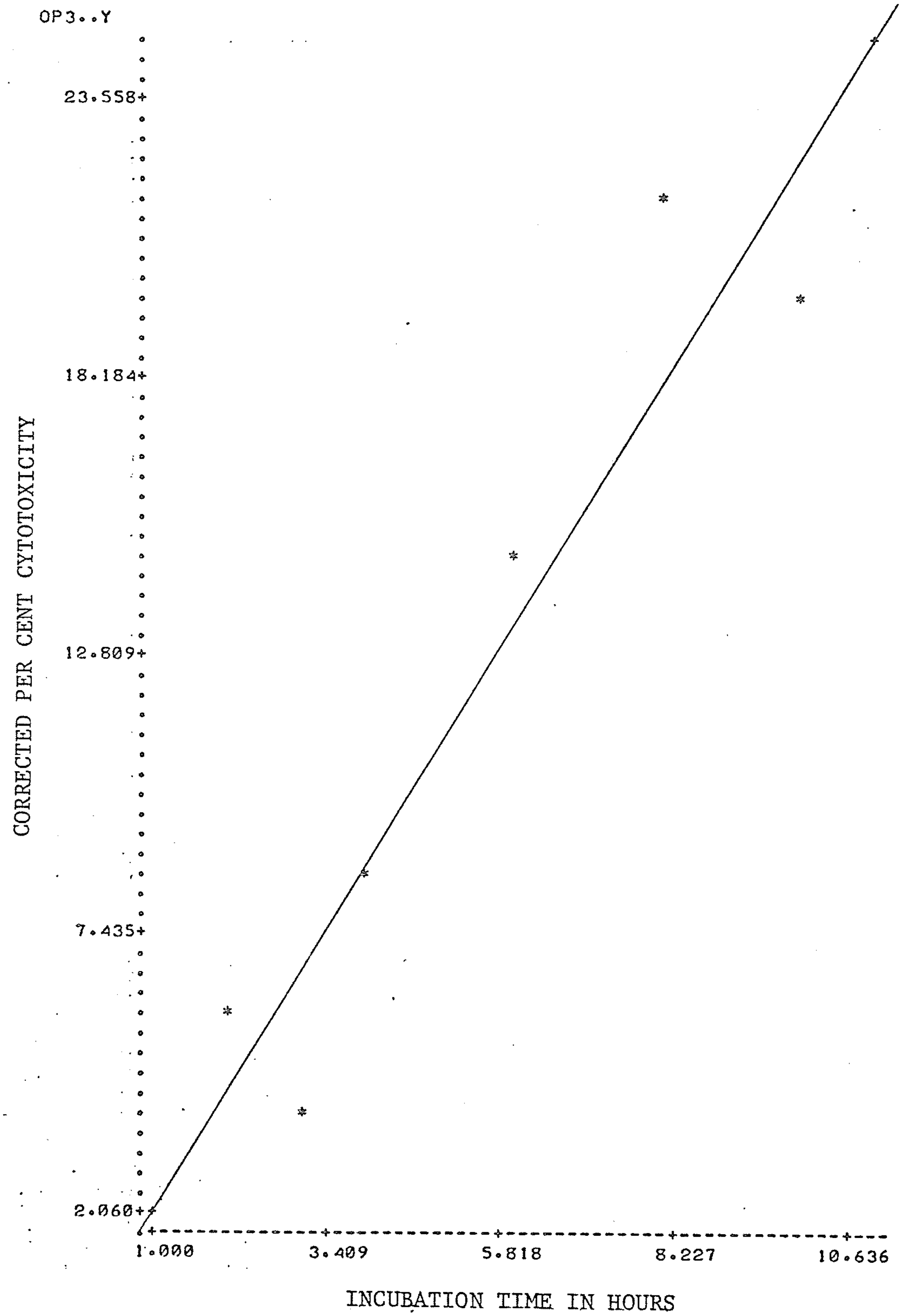


Figure 4 - Regression Coefficient Plot Between Corrected Per Cent Cytoxicity and Incubation Time In Semi-Syngeneic Cytotoxicity Ratio of EC : TC of 100 to 1

```
DATA...
1 4.07
2 7.63
3 10.79
4 16.27
6 25.63
8 41.62
10 47.51
11 44.94/


N =     8

 X         MEAN              SD


 1          5.625            3.739
 2         24.808           17.722

SELECTION... 2 1

R   = 0.984
RSQ = 0.969

 2 ON  1

INTERCEPT    =    -1.435
B            =     4.665
SD ESTIMATE  =     3.372
SDB          =     0.341
T            =    13.686

OP1.. Y
            ** ANALYSIS OF VARIANCE **

  SOURCE       DF        SS         MS          F

REGRESSION      1     2130.311   2130.311    187.315
DEVIATIONS      6       68.237     11.373
TOTAL           7     2198.548

OP2.. Y

 NO.   OBSERVED   EXPECTED   ADJUSTED   RESIDUAL

   1      4.070      3.230     25.647      0.840
   2      7.630      7.896     24.542     -0.266
   3     10.790     12.561     23.037     -1.771
   4     16.270     17.226     23.851     -0.956
   5     25.630     26.557     23.880     -0.927
   6     41.620     35.888     30.540      5.732
   7     47.510     45.218     27.099      2.292
   8     44.940     49.884     19.864     -4.944
```

Table V - Computer Print Out of Statistical Analysis of Semi-Syngeneic Cytotoxicity Ratio of EC : TC of 200 to 1

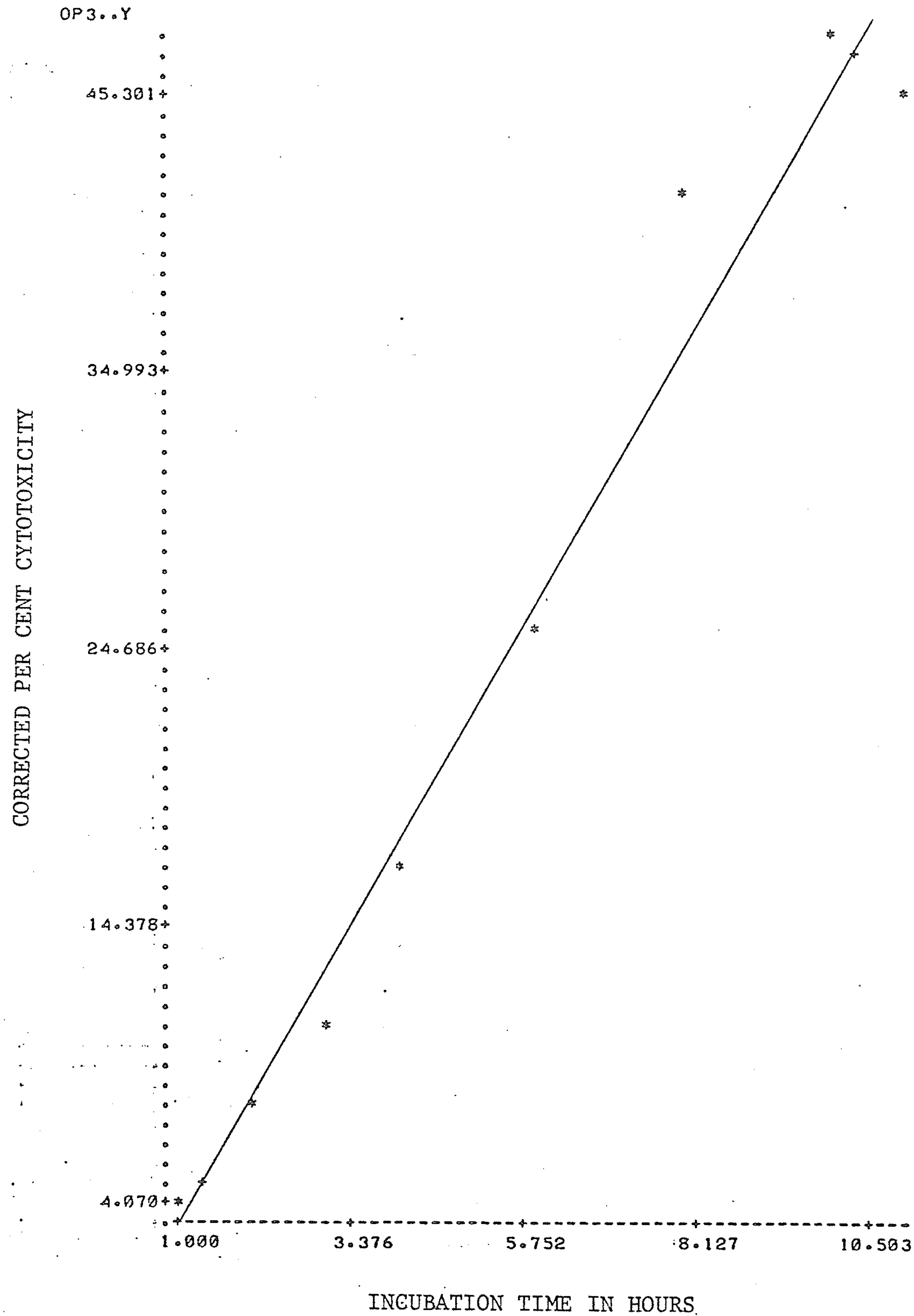


Figure 5 - Regression Coefficient Plot Between Corrected Per Cent Cytotoxicity And Incubation Time In Semi-Syngeneic Cytotoxicity

Ratio of EC : TC of 200 to 1

Abrogation of Semi-syngeneic Cytotoxicity by Irradiation of the GVH Activated F_1 Effector Cells

To prove that the F_1 immunocompetent cells were responsible for the effector mechanism in the in vitro semi-syngeneic cytotoxicity reaction, this experiment examined the situation in which the F_1 effector cells were lethally irradiated and its subsequent effect on the syngeneic target cell lysis.

GVH actiavted F_1 animals were produced by the injection of parental C3H/HeJ and DBA/2 spleen cells into B6C3F_1 and B6D2F_1 hosts respectively. On day 8 post induction of GVH reactions, some of the F_1 animals in these two groups were lethally irradiated.

The mice were caged inside ventilated plastic containers and were exposed to total body gamma-rays irradiation which was generated by a ^{60}Cobalt isotope source (Manitoba Cancer Treatment and Research Centre). The source to mid-body distance was approximately 100 cm, and the dose rate was approximately 80 rads per minute. The total body dose was 850 Rads.

Peritoneal exudate cells were collected from four groups of the GVH activated F_1 animals, i.e., irradiated B6C3F_1, non-irradiated B6C3F_1, irradiated B6D2F_1, and non-irradiated B6D2F_1. These effector cells were tested in the semi-syngeneic cytotoxicity assays.

The results as shown in Table VI demonstrated the abrogation of the in vitro semi-syngeneic cytotoxicity reaction as a result of rendering the F_1 effector cells immunoincompetent by irradiation. This experiment proved that viable F_1 peritoneal exudate cells were responsible for the phenomenon of in vitro semi-syngeneic cytotoxicity.

Table VI - Effect of Irradiation of the F_1 PEC on Semi-syngeneic Cytotoxicity

Parental Donor	GVH F_1 Recipient	Effector Cells	Target Cells	Corrected* % Lysis
DBA/2	B6D2F_1	PEC	P815	39.58 ± 1.35
"	"	"	"	1.64 ± 0.78
C3H/HeJ	B6C3F_1	PEC	L929	41.32 ± 1.67
"	"	"	"	2.29 ± 1.34

* Mean percent corrected lysis of target cells induced by pooled effector cells from groups of 10 animals ± SE in 4 replicates

Abrogation of Semi-syngeneic Cytotoxicity by Specific Antisera

GVH-activated F_1 host effector cells were obtained from C3D2F_1 mice which were injected with parental C3H/HeJ spleen cells as previously described. The peritoneal exudate cells from these F_1 hosts were used as effector cells against the parental $H\text{-}2^k$ genotype L929 target cells in CMC assays. Antisera with specificity against the $H\text{-}2^k$ genotype were prepared by multiple injections of C3H/HeJ spleen cells in Freund's complete adjuvant into the C57BL/6 mice. The hyperimmune sera collected were Complement inactivated at 37°C for 30 minutes. 0.1 ml of thess pooled sera preparation was added into each well in the cytotoxicity test plate containing the labelled L929 target cells and the C3D2F_1 host effector cells as in other CMC assays. The controls of the experiment are the assays in the presence of normal mouse sera and in the absence of any mouse sera. The results are shown in Table VII.

As shown in Table VII, antisera with specificity against the $H\text{-}2^k$ genotype, when mixed with the GVH-induced C3D2F_1 effector cells, significantly suppressed the semi-syngeneic cytotoxicity reaction. The degree of suppression of cytotoxicity was almost 90%.

The exact site of such a suppression is not clear because the specific anti-$H\text{-}2^k$ antibodies could either mask the k antigens on the surfaces of the L929 target cells, or they could interfer with the process of recognition of the k antigenic determinant by the "recognition structure" of the F_1 effector cells. In any event, the data confirmed that the H-2 antigenic determinants were invloved in the process of the semi-syngeneic cytotoxicity reaction.

Table VII - Suppression of Semi-syngeneic Cytotoxicity Reaction by Specific Anti-H-2 antisera

Parental donor	Effector cells	Target cells	Sera addition	Corrected Lysis *
C3H/HeJ	$C3D2F_1$-PECs	L929	none	44.16 ± 2.03
"	"	"	Normal[(1)]	37.40 ± 0.45
"	"	"	Anti-H-2^{k}[(2)]	4.07 ± 1.72

* Mean percent corrected lysis of target cells induced by pooled effector cells from groups of 6 animals ± SE in 4 replicates.

(1) Normal pooled C57BL/6 sera.

(2) Pooled sera from C57BL/6 hyperimmunized to the C3H/HeJ cells.

In recent studies, the F_1 anti-parent reactivity resembling the semi-syngeneic cytotoxicity reaction has also been reported in some laboratories (Shearer et al,1976; Schmitt-Verhulst 1977; Warner and Cudkowicz 1979; Ishikawa and Dutton 1979). In experiments in the previous sections, parental spleen cells were grafted to the F_1 hybrids, and the GVH-induced F_1 lymphoid cells were noted to produce lysis of target cells bearing the same H-2 genotype as the parental donor cells. The following experiments are designed to demonstrate that in the GVH phenomenon, the immunocompetent cells of the GVH-induced F_1 hosts are actively engaged in the host-versus-graft (HVG) reaction.

Preferential Cytotoxicity for Parental H-2 Genotype Target Cells by GVH-activated Host Effector Cells

Previous experiments have demonstrated the reactivity of the F_1 immunocompetent cells against semi-syngeneic target cells bearing the parental H-2 antigen. The question derived from these observations is as follows. When the GVH-activated F_1 effector cells are exposed to target cells of different H-2 genotypes, and one of the two target cells bear the same H-2 genotype as the parental donor cells used in induction of GVH reaction, should one expect the GVH-induced F_1 effector cells to "recognize" the parental H-2 genotype target cells more readily since the F_1 immunocompetent cells have been previously exposed to such parental H-2 antigens on the transplanted parental spleen cells ? The answer to this question is yes, and in fact the GVH-induced F_1 effector cells showed a preferential cytolysis of target cells bearing the parental H-2 genotype. The experimental approach of this experiment is described in Figure 6.

As shown in the figure, two strains of F_1 recipients, namely, B6C3F_1 (H-2^k x H-2^b) and B6D2F_1 (H-2^d x H-2^b) each possessing the H-2^b half were used as the F_1 hosts. Parental spleen cells of the genotype of the non-identical half between the two F_1 hosts, i.e.,H-2^k and H-2^d strains

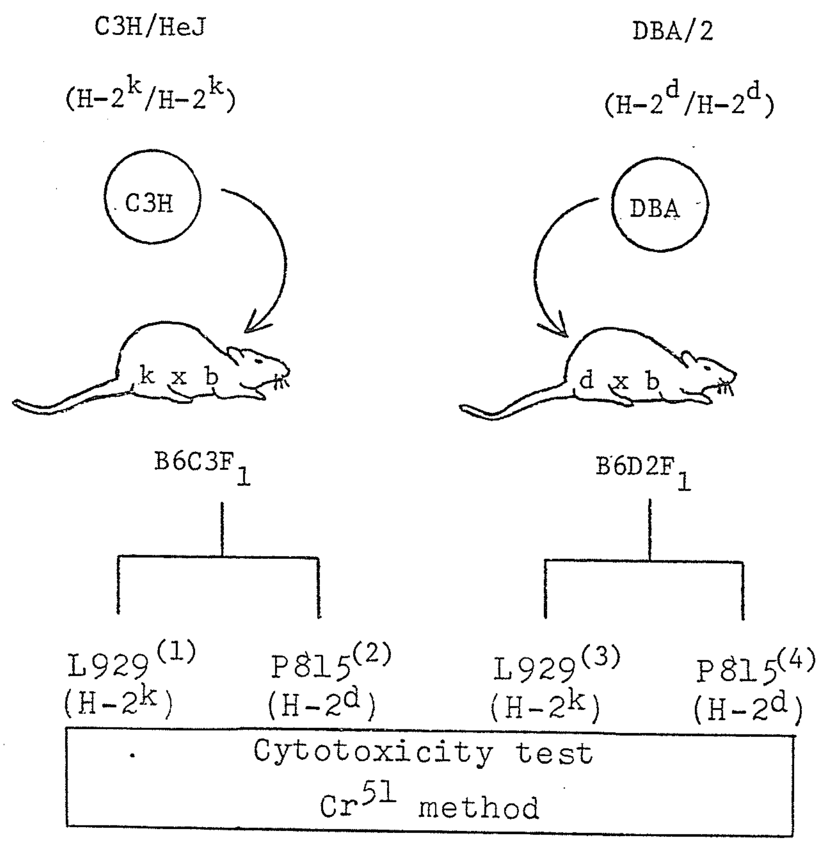


Figure 6 - Experimental protocol of inducing graft-versus-host reaction in two semi-allogeneic F_1 hybrids and comparing their relative cytotoxicity in identical genotype target cells.

(1) Target cell syngeneic to grafted parental donor cell

(2) Target cell allogeneic to grafted parental donor cell

(3) Target cell allogeneic to grafted parental donor cell

(4) Target cell syngeneic to grafted parental donor cell

were used as donor cells for the induction of GVH reactions in these hybrids. Parental C3H/HeJ ($H-2^k/H-2^k$) spleen cells were injected into the $B6C3F_1$ hybrids, and parental DBA/2 ($H-2^d/H-2^d$) spleen cells were injected into the $B6D2F_1$ hybrids respectively.

The peritoneal exudate cells from these two strains of F_1 hosts were used as effector cells in CMC assays as previously described. The genotypes of the two target cells used are identical with either one of the genotype of the parental donor spleen cells; i.e., L929 ($H-2^k$) target cell is syngeneic to the C3H/HeJ parental cells, and P815 ($H-2^d$) target cell is syngeneic to the DBA/2 parental donor cell. The cytotoxici activities of these two groups of GVH-induced F_1 hosts were compared in the CMC assays using the two H-2 different target cells.

The results in Table VIII showed that, when parental $H-2^k$ spleen cells were injected into the $H-2^k$ x $H-2^b$ ($B6C3F_1$) hosts to induce GVH reactions, the degree of semi-syngeneic cytotoxicity on the $H-2^k$ (L929) target cells (i.e. 36.67 $\pm$ 1.62) was significantly greater than that (i.e. 29.76 $\pm$ 2.27) by effector cells from the $H-2^d$ x $H-2^b$ ($B6D2F_1$) hosts induced into GVH reactions by the parental $H-2^d$ (DBA/2) spleen cells. Similar results were noted in the reverse situation. When the parental $H-2^d$ spleen cells were injected into the $H-2^d$ x $H-2^b$ ($B6D2F_1$) hosts, a significantly greater degree of semi-syngeneic target cell lysis was observed with the $H-2^d$ (P815) target cells (i.e. 46.30 $\pm$ 0.40) than that (i.e. 27.98 $\pm$ 1.73) by effector cells from the $H-2^k$ x $H-2^b$ ($B6C3F_1$) hosts induced into GVH reactions by the parental $H-2^k$ (C3H/HeJ) spleen cells.

The above results actually revealed the following situation. The cytotoxicity by the GVH-induced (k x b) F_1 host effector cells (the k parental cells injected into k x b F_1) on the k genotype target cell was significantly higher than the cytotoxicity by the GVH-induced (d x b) F_1 host effector cells (parental d cells injected into c x b F_1). In other words, GVH-activated F_1 effector cells, seem to possess a higher degree of

Table VIII - Preferential Lysis of Target Cells Bearing Parental Antigens by F_1 Hybrid Cells From Mice Undergoing Garft Versus Host Reaction.

Donor Cells	Parental H-2 Antigen	GVH Hosts	Target Cells	Corrected Lysis *
C3H/HeJ[(1)]	k/k	$B6C3F_1$[(2)]	L929 ($H\text{-}2^k$)	36.67 ± 1.62
DBA/2[(3)]	d/d	$B6D2F_1$[(4)]	"	29.76 ± 2.27
C3H/HeJ	k/k	$B6C3F_1$	P815 ($H\text{-}2^d$)	27.98 ± 1.73
DBA/2	d/d	$B6D2F_1$	"	46.30 ± 0.40

* Mean percent lysis of target cells induced by pooled effector cells from groups of 8 animals ± SE in 4 replicates.

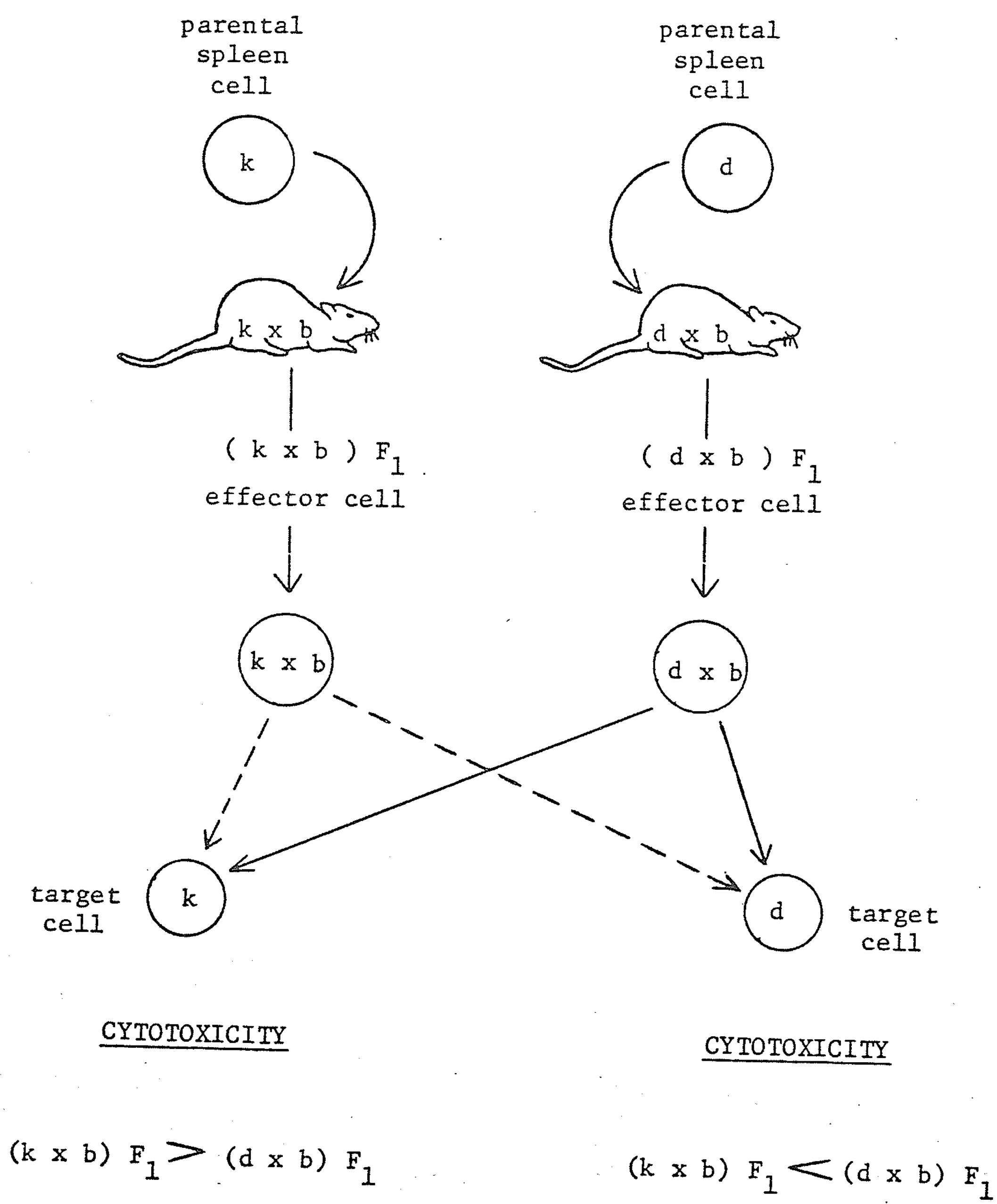
(1) C3H/HeJ = $H\text{-}2^k/H\text{-}2^k$

(2) $B6C3F_1$ = $H\text{-}2^k \times H\text{-}2^b$

(3) DBA/2 = $H\text{-}2^d/H\text{-}2^d$

(4) $B6D2F_1$ = $H\text{-}2^d \times H\text{-}2^b$

cytotoxicity on target cells which bear the same H-2 genotype as the parental cells used in the induction of GVH reactions than that by the effector cells from a different F_1-parent combination in which the H-2 genotype of the parental cells used in GVH induction is different from the H-2 genotype of the target cell. Graphically, it is as follows.



The demonstration of preferential cytotoxicity on target cells syngeneic to the transplanted parental donor cells have implicated the capacity of the F_1 immunocompetent cells reacting against the H-2 antigens of the invading parental lymphoid cells. The significance of such implication will be explored in the next experiment.

Cytotoxicity of F_1 PEC on Parental H-2 Genotype Target Cells

To investigate into the possibility that GVH-induced F_1 cells were reacting against the H-2 antigens of the other parent (the parent not used as donor), the F_1 immunocompetent cells can be grafted into lethally irradiated parental animals, and any semi-syngeneic cytotoxicity detected would be due to the grafted F_1 cells reacting against the parental antigens since the recipient parents were immunosuppressed by irradiation. In the following actual experiments, (A x B) F_1 cells were injected into irradiated (A x C) F_1 animals instead of the parental (A/A) animals because of the possibility that the homozygous (A/A) parents may express the "homozygous antigens" which the heterozygous (A x B) F_1 can react against as postulated by the "Hybrid Resistance" phenomenon.

In the first experiment, spleen cells from B6D2F_1 (H-2^d x H-2^b) were injected into lethally irradiated C3D2F_1 (H-2^k x H-2^d) recipients bearing the parental (in respect to B6D2F_1) H-2^d antigen. This has eliminated as discussed above, the possible involvement of the "Hybrid Resistance" phenomenon since both donor and recipient were heterozygous hybrids. The objective was to detect lysis of the parental H-2 genotype target cells by the B6D2F_1 PECs in CMC assays. The development of cytotoxicity by the C3D2F_1 immunocompetent cells against the H-2^b antigen expressed on the $B6D2F_1$ cells was eliminated because the $C3D2F_1$ recipients were lethally irradiated. The cytotoxic effects of the $B6D2F_1$ immunocompetent cells were tested with the L929 ($H\text{-}2^k$) and the P815 ($H\text{-}2^d$) target cells in CMC assays.

In the second experiment, the roles of the donor and the recipient in the above experiment were reversed. Spleen cells from the $C3D2F_1$ hybrids ($H\text{-}2^k$ x $H\text{-}2^d$) were grafted into lethally irradiated $B6D2F_1$ ($H\text{-}2^b$ x $H\text{-}2^d$) hosts bearing the parental (in respect to $C3D2F_1$) $H\text{-}2^d$ antigen expressed on the $B6D2F_1$ cells. The objective again was to detect the lysis of the parental H-2 genotype target cells by the $C3D2F_1$ PECs using CMC assays. The results are shown in Table IX.

As seen in the table, when the $B6D2F_1$ spleen cells were exposed to the irradiated $C3D2F_1$ cells <u>in vivo</u>, significant cytotoxicity reactions were observed on target cells bearing; (1) the parental $H\text{-}2^d$ antigen (target cell P815) and (2) the allogeneic $H\text{-}2^k$ antigen (target cell L929). The cytotoxic reaction on the $H\text{-}2^d$ genotype target cell (i.e., semi-syngeneic cytotoxicity) was significant because it indicated the situation of the ($H\text{-}2^b$ x $H\text{-}2^d$) F_1 lymphoid cells reacting against the parental $H\text{-}2^d$ antigen. The cytotoxicity observed with the L929 ($H\text{-}2^k$) target cells (i.e., classical specific cytotoxicity) was expected since the $H\text{-}2^k$ genotype and its antigenic determinants were foreign to the $B6D2F_1$ ($H\text{-}2^b$ x $H\text{-}2^d$) immunocompetent cells.

In the situation where the roles of donor and recipient was reversed, cytolysis of target cells bearing the parental $H\text{-}2^d$ antigen was again demonstrated. When $C3D2F_1$ spleen cells were transplanted into the lethally irradiated $B6D2F_1$ recipients ($H\text{-}2^b$ x $H\text{-}2^d$), significant cytotoxicity

Table IX - Cytotoxicity of F_1 Cells on Target Cells Bearing parental Histocompatibility antigen.

Donors Cells	Irradiated recipients	Target Cells	Corrected Lysis*
$B6D2F_1$ ($H-2^{bxd}$)	$C3D2F_1$ ($H-2^{kxd}$)	P815 ($H-2^d$)	35.56 ± 2.02 (1)
"	"	L929 ($H-2^k$)	45.93 ± 1.18 (2)
$C3D2F_1$ ($H-2^{kxd}$)	$B6D2F_1$ ($H-2^{bxd}$)	P815 ($H-2^d$)	43.97 ± 3.43 (3)
"	"	L929 ($H-2^k$)	-3.76 ± 2.90 (4)

* Mean percent corrected lysis of target cells induced by pooled effector cells from groups of ten animals ± SE in four replicates.

(1) Cytotoxicity here showed that F_1 cells lysed cells of parental $H-2^d$ genotype (semi-syngeneic cytotoxicity)

(2) Cytotoxicity is expected since the k antigen in $C3D2F_1$ induced sensitization in allogeneic $B6D2F_1$ cells (specific cytotoxicity)

(3) Cytotoxicity here again showed that F_1 cells can lyse target cells bearing parental $H-2^d$ antigen (semi-syngeneic cytotoxicity).

(4) Absence of cytotoxicity due to absence of sensitizing k antigen in $B6D2F_1$ recipients.

was observed with target cells bearing the parental (in respect to $C3D2F_1$) $H\text{-}2^d$ antigen (target cell P815) indicating that the $C3D2F_1$ ($H\text{-}2^k$ x $H\text{-}2^d$) immunocompetent cells could be induced to react against the parental $H\text{-}2^d$ antigen (i.e.,semi-syngeneic cytotoxicity reaction).

The absence of significant cytotoxicity by the $C3D2F_1$ effector cells on the L929 ($H\text{-}2^k$) target cells was also expected since the irradiated $B6D2F_1$ ($H\text{-}2^b$ x $H\text{-}2^d$) recipients which provided the antigenic stimulation to the $C3D2F_1$ immunocompetent cells did not possess any $H\text{-}2^k$ genotype and therefore logically no $H\text{-}2^k$ antigenic determinants to sensitize the $C3D2F_1$ lymphoid cells to become cytotoxic to the L929 ($H\text{-}2^k$) target cells in the CMC assays. The significance of the above observation is that this type of semi-syngeneic cytotoxicity on the parental H-2 genotype target cell by the transplanted F_1 cells is not a truly non-specific cytotoxicity reaction otherwise the $H\text{-}2^k$ (L929) target cells would be non-specifically attacked and lysed in the process.

The results of these experiments will be reviewed in more detail in the section of Discussion. It is suffice to reiterate here that the F_1 immunocompetent cells can become sensitized to the parental H-2 antigens resulting in cytotoxicity reactions.

THE HOST EFFECTOR CELL IN SEMI-SYNGENEIC CYTOTOXICITY

The experiments described in the previous section examined the various parameters related to the phenomenon of semi-syngeneic cytotoxicity observed in immunocompetent cells from GVH-induced F_1 hosts. In this section, the identity and nature of the host effector cell responsible for the semi-syngeneic cytotoxicity reaction will be examined. In addition, the *in vitro* and *in vivo* effects of the GVH-activated F_1 peritoneal exudate cells on syngeneic F_1 animals undergoing GVH reactions will be described.

Cytotoxicity of Host Peritoneal Exudate Cells

Peritoneal exudate cells are known to contain mainly the macrophages which adhere to plastic or glass surfaces and lymphocytes which are characteristically non-adherent. To investigate into the type of cell responsible for the semi-syngeneic cytotoxicity reaction, PECs from GVH-induced F_1 hosts were separated into adherent and non-adherent populations. Individual cell populations were tested in CMC assays for semi-syngeneic cytotoxicity reactions as previously described.

Peritoneal exudate cells from F_1 hybrid animals undergoing graft-versus-host reactions were suspended in supplemented RPMI 1640 medium at the concentration of 1×10^7 cells per ml. Aliquots of 10 mls. of this suspension were transferred into tissue culture dishes measuring 60 x 15 mm (Falcon Plastics, Oxnard, California) which were incubated at 37°C for one hour and then washed with HBSS three times. The washings were pooled together, centrifuged, and the non-adherent cells were resuspended at the concentration of 1 x 10^7 cells per ml. in supplemented RPMI 1640 culture medium. The adherent cells were washed off the surface of the tissue dishes by a jet stream produced by forcing HBSS through a syringe with a 25 gauge needle. Adherent cells were also resuspended in RPMI culture medium at concentration of 1 x 10^7 cells per ml.

B6D2F_1 hybrids were injected with 1 x 10^8 parental DBA/2 spleen cells 8 days previously and challenged with 1.5 ml of the 6% sterile sodium caseinate solution 5 days later. The immunocompetent cells collected from the peritoneal cavities of these GVH-induced F_1 animals were pooled together and incubated at 37°C in tissue-culture dishes (Falcon Plastic Petri Dish) to allow separation of the adherent and non-adherent lymphoid cells as previously described. Individual cell populations were tested against the P815 target cells for semi-syngeneic cytotoxicity reactions.

As shown in table X ,the adherent cells from GVH-induced F_1 animals, just as the unseparated population, produced a significant degree of cytotoxicity reaction, while the non-adherent cell population from the same GVH-induced peritoneal exudate cell pool was not cytotoxic to the parental H-2 genotype target cells. These results indicated that peritoneal exudate cells, previously quantitated to comprise more than 90% macrophages were involved as the end-effector mechanism in the production of semi-syngeneic target cell cytolysis by the GVH-induced F_1 immunocompetent cells. Quantitatively, the proportion of semi-syngeneic cytotoxicity due to the adherent cells was approximately 90% of the total cytotoxicity seen in the original unseparated peritoneal exudate cell population (i.e.,28.26/33.74 approximately 90%).

Table X - Semi-syngeneic Cytotoxic Activities of Individual Populations of Effector Cells from GVH-induced F_1 PECs

Effector Cells*	Target Cells	Corrected Lysis**
Unseparated PECs	P815	33.74 ± 0.64
Adherent PECs	"	28.26 ± 1.72
Non-adherent PECs	"	-0.15 ± 1.77

* Effector cells from $B6D2F_1$ hybrids injected with parental DBA/2 spleen cells as previously described.

** Mean percent corrected lysis of target cells induced by the pooled peritoneal exudate cells from 20 animals ± SE in groups of 6 replicates.

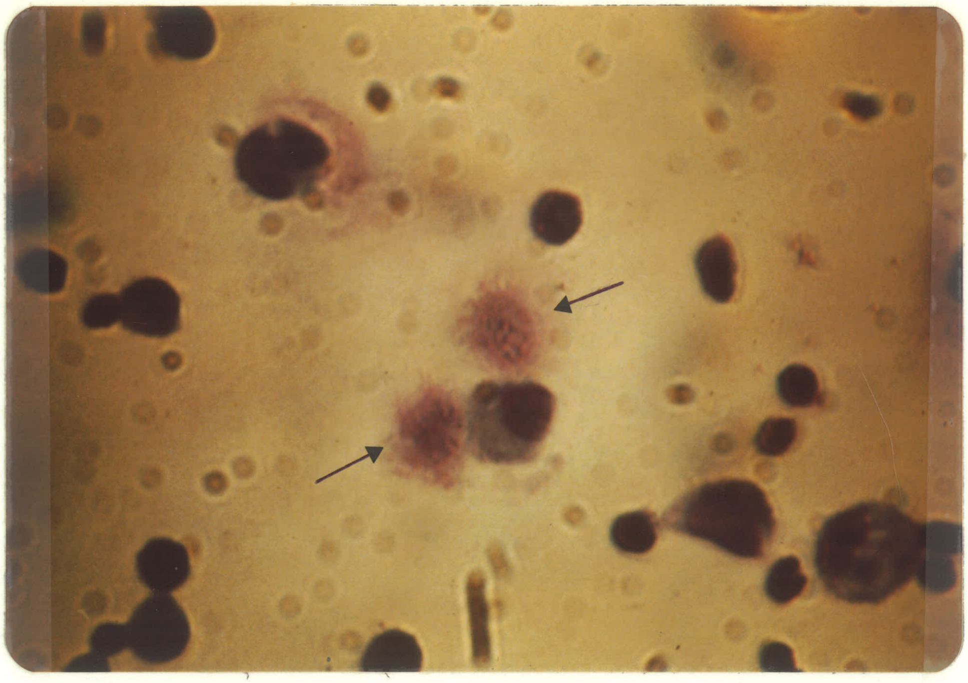
Microscopic Study of Cytolysis by GVH-activated Macrophages

Having demonstrated by the *in vitro* CMC assays that the F_1 peritoneal macrophages were responsible for the phenomenon of semi-syngeneic cytotoxicity reaction, the actual process of semi-syngeneic target cell lysis by the F_1 host macrophages was investigated by the microscope. The results are reported in the following pages.

Peritoneal macrophages were obtained, by procedures previously described, from the GVH-induced $B6D2F_1$ hosts, and P815 cells were used as target cells for the qualitative cytological assessment of the CMC process.

The next three pages contain microphotographs depicting the process of target cell destruction by the GVH-activated macrophages. The dark blue cells are identified as the semi-syngeneic target cells with the abnormal nuclei which were distinguished from the normal nuclei of the pinkish peritoneal macrophages. The cytoplasmic anatomy of the two cells are also characteristically distinguishable.

In photograph 1, two macrophages were seen approaching the target cells near centre. In photograph 2, one macrophage was seen extending its cytoplasmic edges around the surface of the target cell on the left. In photograph 3, the macrophage seemed to engulf the target cell completely. In photograph 4, 5, and 6, the macrophages evolved to a stage where intra-cellular granules were discretely seen inside the cytoplasm. In photograph 4, the granules were near the edge of the cytoplasmic border, while in photograph 5, the granules were released and completely surrounded the target cell including the nucleus. The plasma membrane of the target cell was lysed. In photograph 6, even the nucleus of the target cell was attacked and fragmented. Total destruction is almost complete at this stage.



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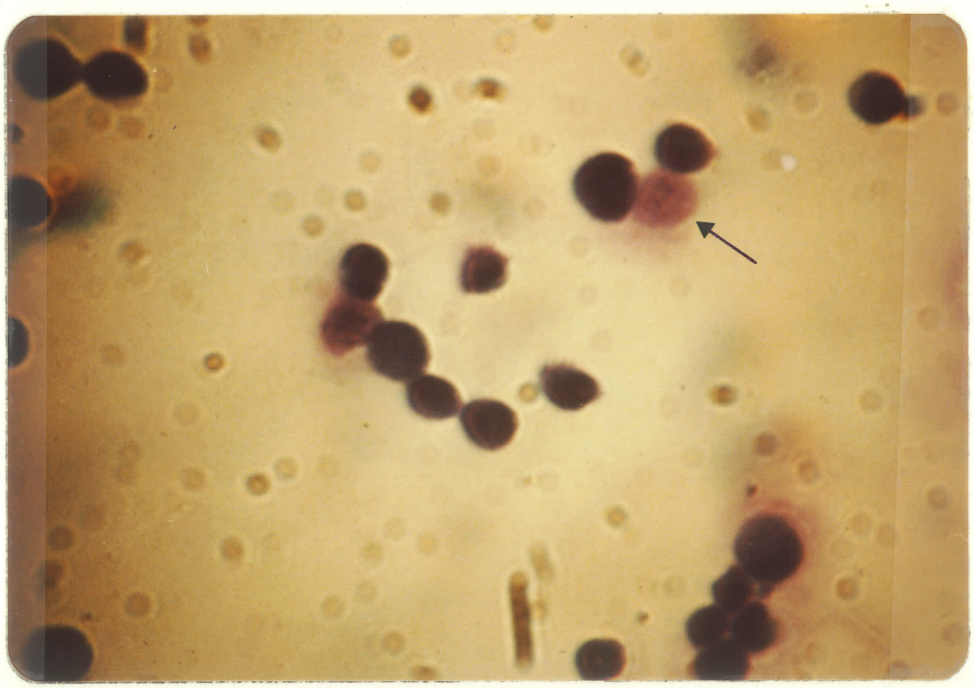

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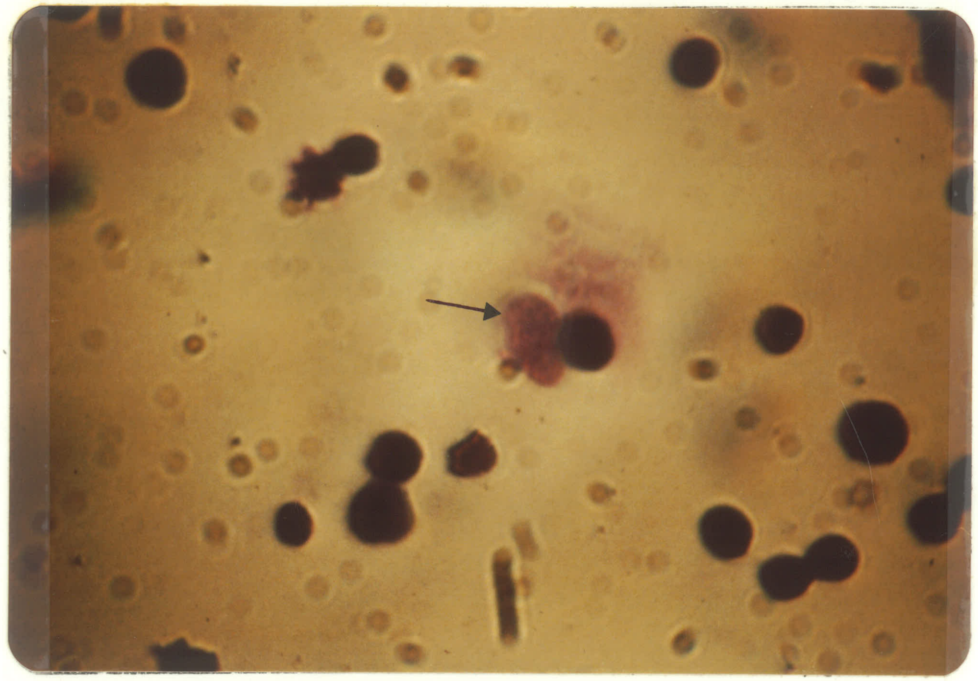

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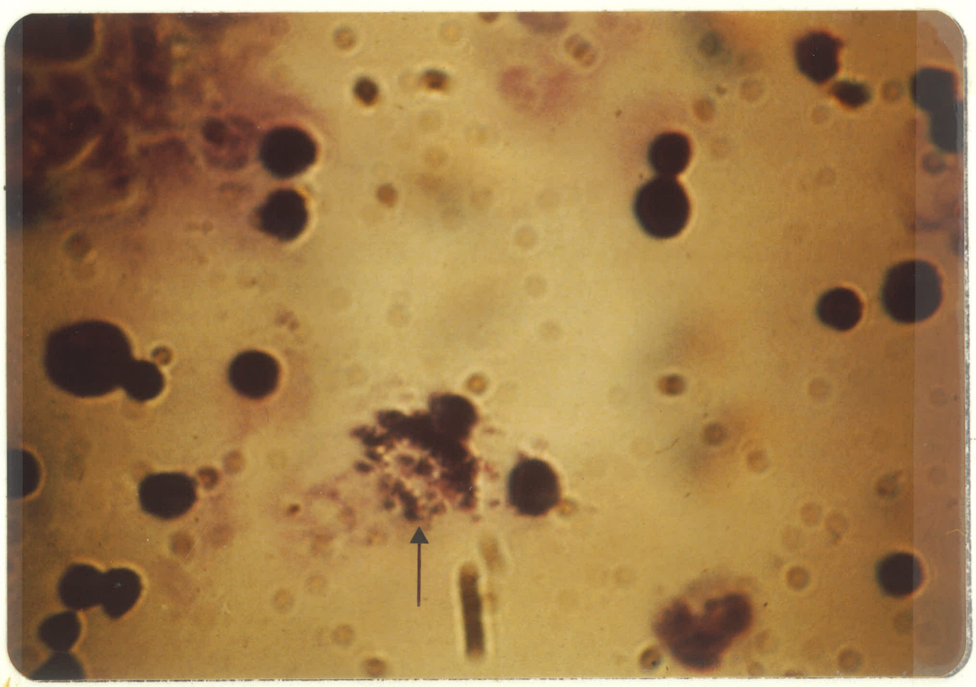

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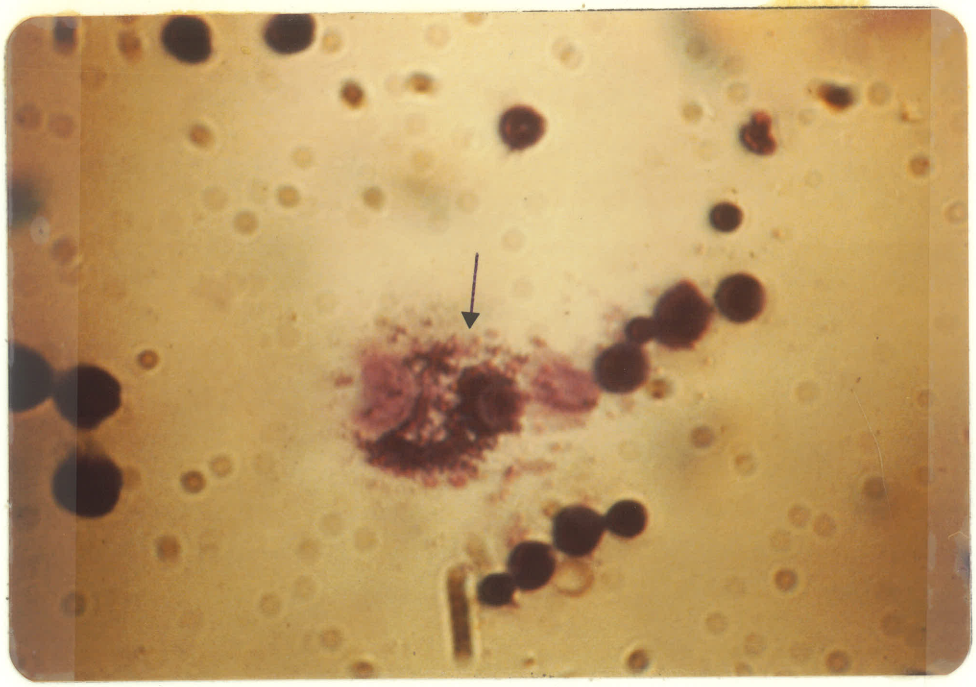

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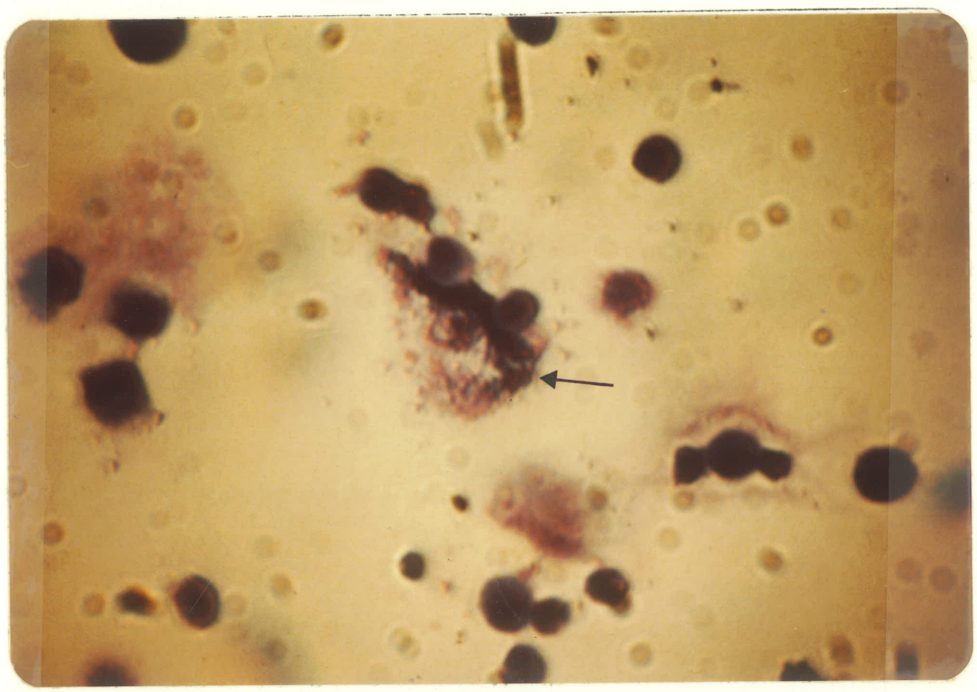

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To substantitate the above indication that the GVH-induced F_1 host peritoneal macrophages were invloved in the semi-syngeneic cytotoxicity reaction, the following experiments will present further evidence confirming the role of the F_1 macrophages mediating the lysis of parental H-2 genotype target cells.

Specific Macrophage-cytocidal Effect of Silica Particles

Crystalline silica particles are known to be highly toxic for the macrophages while possessing little cytocidal effect for other cells for example, lymphocytes. This particular aspect of cytotoxicity was studied in this experiment with the aim of establishing the optimal dose concentration of silica particles required for the maximal lysis of macrophages.

Macrophages from the peritoneal exudates were partially purified by exposing 3 mls. of PEC suspensions to three brief cycles of centrifugations (30 seconds each, maximum g = 100) and discarding between each cycle the supernatant containing the small and large lymphocytes. The cell suspension obtained in this manner comprised of more than 99 per cent mononuclear cells which were identified to be macrophages on the basis of morphological criteria and their ability to adhere to plastic surfaces and to phagocytose collodial carbon particles.

Silica particles of average size 5u were obtained through the courtesy of Dr. K. Robock, Steinkohlenberg-Bauverein, 43 Essen-Krey, West Germany. The particles were sterilized in pressurized steam (120°C) chamber and suspended in supplemented RPMI 1640

Crystalline silica particles in varying quantities, were added to a constant number (10^4 cells per well in CMC assay plates) of ^{51}Cr-labelled L929 target cells, C3H/HeJ spleen cells, $B6C3F_1$ peritoneal macrophages purified as previously described, from PECs, and a mixture of labelled L929 target cells plus unlabelled $B6C3F_1$ macrophages. The amount of the ^{51}Cr-isotope released due to the cytocidal effect of silica on these different cell populations were measured after incubation at 37°C for 14 hours. The results are shown in Figure 7.

As demonstrated in the figure, a significantly specific cytocidal effect of silica particles on peritoneal macrophages has been observed. Such a cytocidal effect was particularly evident at the dose concentration of 10 ug of silica particles per 10^4 cells. On the other hand, silica induced a very low cytocidal effect on the parental C3H/HeJ spleen cells presumably due to the small amount of macrophages present in the population of spleen cells. Furthermore, such insignificantly low cytocidal effect for the parental C3H/HeJ spleen cells has been noted with silica concentration as high as 100 ug per 10^4 cells (not reported in the figure). These results indicated the non-cytocidal effect of silica particles on the parental spleen cells. This particular fact will become significant in the following experiment in which, it will demonstrate that silica can abrogate the <u>in vitro</u> semi-syngeneic cytotoxicity reaction. It will be seen later that, if silica, is cytocidal to the F_1 macrophages but not cytocidal to the parental spleen cells, the abrogation of semi-syngeneic cytotoxicity by silica will necessarily indicate the conclusion that

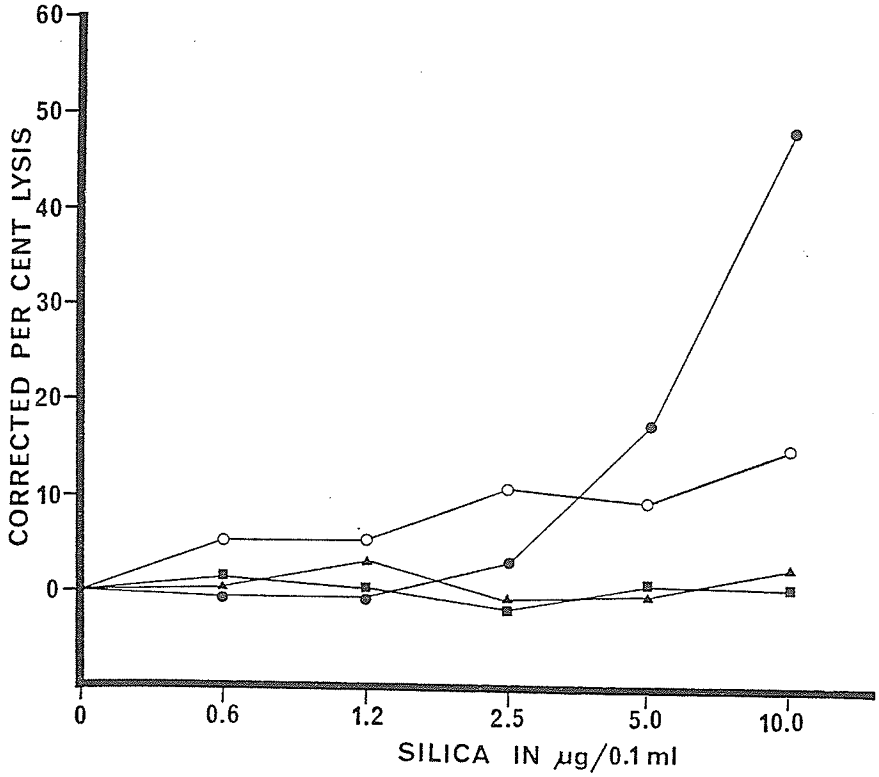


Figure 7 - Cytotoxic Effect of Silica Particles on Peritoneal Macrophages.

●——● $B6C3F_1$ peritoneal macrophages

○——○ Parental C3H/HeJ spleen cells

■——■ L929 parental H-2 genotype target cells

▲——▲ L929 target cells + unlabelled macrophages semi-syngeneic cytotoxicity is mediated by the F_1 macrophages.

The results in Figure 7 also demonstrated that silica has no cytocidal effect on the L929 target cells, and therefore the treatment of the F_1 peritoneal exudate cells with silica particles will not affect the outcome of the CMC assays. Moreover, the presence of macrophages which were destroyed by silica, did not induce any significant lysis of the labelled L929 target cells. This indicated that the lysed macrophages did not in turn induce any non-specific lytic effect on the "innocent bystanding" L929 target cells, and therefore accordingly, the results of the CMC assays involving treatments, even of cells susceptible to silica toxicity, will not be complicated. The following experiment involves such a treatment of the F_1 effector cells with silica particles.

Suppression of Semi-syngeneic Cytotoxicity with Silica

GVH reactions were induced in B6C3F_1 ($H\text{-}2^k$ x $H\text{-}2^b$) and B6D2F_1 ($H\text{-}2^d$ x $H\text{-}2^b$) hybrids by the intraperitoneal injection of 1 x 10^8 parental C3H/HeJ and DBA/2 spleen cells respectively. The 6% sterile sodium caseinate peritoneal exudate cell stimulant was injected into these GVH-induced F_1 hosts 5 days later as previously described. The peritoneal exudate cells from these different strains of GVH-induced F_1 animals were used as effector cells in the CMC assays against the corresponding L929 and P815 parental H-2 genotype target cells.

For demonstrating the contributory role the F_1 macrophages in semi-syngeneic cytotoxicity reactions, 100 ug of crystalline silica particles were added to a mixture of 1 x 10^4 of ^{51}Cr-labelled target cells and 1 x 10^6 peritoneal exudate effector cells from the GVH-induced F_1 hosts.

Table XI - Abrogation of Semi-syngeneic Cytotoxicity Reaction by Treatment of F_1 Effector Cells with Silica

Parental Donor	GVH-host Recipient	Treatment of Effector Cells	Target Cells	Corrected Lysis*
C3H/HeJ	$B6C3F_1$	No treatment	L929	36.67 ± 1.62
"	"	Silica **	"	3.76 ± 1.01
DBA/2	$B6D2F_1$	No treatment	P815	42.30 ± 0.40
"	"	Silica	"	8.60 ± 0.63

* Mean percent corrected lysis of target cells induced by pooled PEC from groups of 6 animals ± SE in 6 replicates

** 100 ug of silica particles were added into the culture and incubated together with effector and target cells.

The controls in this experiment are the GVH-induced F_1 peritoneal exudate cells in the absence of silica particles. The results are shown in Table XI.

As shown in the table, the addition of silica particles to the F_1 host effector cells significantly suppressed the semi-syngeneic cytotoxicity reaction in the two F_1-parent combinations tested, indicating the involvement of the F_1 peritoneal macrophages in the cytolysis of the parental H-2 genotype target cells.

The involvement of the parental spleen cells in the semi-syngeneic cytotoxicity reaction in a truly non-specific fashion can be excluded on the basis that; parental spleen cells have been shown not susceptible to the toxic effect of silica particles, and if parental spleen cells were responsible for the semi-syngeneic cytotoxicity reaction, then the lysis of the parental genotype target cells should not be suppressed by the addition of silica. But semi-syngeneic cytotoxicity was shown to be significantly suppressed by the treatment of silica, and macrophages were demonstrated to be highly susceptible to the cytocidal effect of the silica particles, therefore, semi-syngeneic cytotoxicity reaction must be contributed by the F_1 macrophages.

The involvement of the F_1 immunocompetent cells in semi-syngeneic cytotoxicity reactions has been implicated in previous studies in our laboratory. When one of the two parents was used to induce GVH reactions in the F_1 hybrids, the treatment of the GVH-induced effector cells with antisera specifically against the other non-donor parent will destroy only the F_1 and not the parental donor immunocompetent cells. Such a treatment had been shown to result in the abrogation of semi-syngeneic cytotoxicity, and the F_1 cells were concluded to be involved in the reaction.

In Vitro Activation of Macrophages in Semi-syngeneic Cytotoxicity

The effect of mixing GVH reaction activated F_1 peritoneal macrophages with normal syngeneic lymphoid cells was investigated by the *in vitro* incubation of macrophages from F_1 hybrids undergoing GVH reactions with normal syngeneic F_1 macrophages. The cytotoxicity effect on semi-syngeneic target cell by these *in vitro* incubated cells were measured by the CMC assays as described previously.

A constant number of (2×10^6) peritoneal macrophages purified from PECs of $B6D2F_1$ hybrids previously induced into GVH reactions by the injection of 1×10^8 parental spleen cells, were pipetted into the wells of a microcytotoxicity test plate as in other CMC experiments. Into each of these wells already containing the 2×10^6 GVH activated macrophages, additional : (1) normal syngeneic F_1 macrophages purified from normal PECs, and (2) normal syngeneic F_1 spleen cells, were added in increasing concentrations of 0.5×10^6, 1.0×10^6, 1.5×10^6, and 2.0×10^6 cells per well in a series. A third group of wells contained 2×10^6 normal spleen cells in place of the GVH-induced macrophages plus the increasing concentration of normal spleen cells as described above. The cytotoxicity among the three groups on semi-syngeneic target cells were compared.

As shown in Figure 8 , GVH-activated peritoneal macrophages seem to activate normal syngeneic macrophages into cytotoxic activities resulting in an increasing level of semi-syngeneic target cell lysis when incubated together *in vitro*. In contrast, GVH-actiavted macrophages were not able to render normal syngeneic spleen cells into cytotoxicity probably due to the small amount of macrophages present in spleen cells. In addition, the spleen cells may exert certain immunoregulatory effect on the GVH-induced macrophages since cytotoxicity of that combination was noted to be reduced.

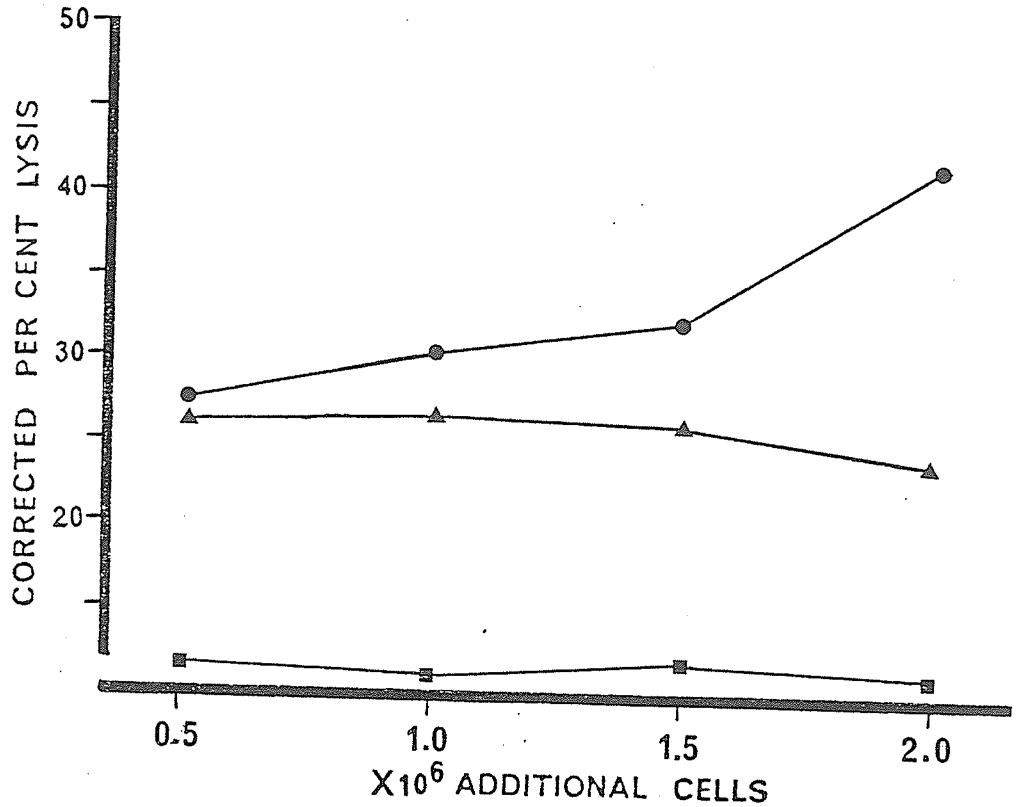


Figure 8 - Effect of GVH-activated Macrophages on Normal Syngeneic Peritoneal Macrophages in Semi-syngeneic Cytotoxicity

	2×10^6 cells		$0.5, 1.0, 1.5, 2.0 \times 10^6$ cells
●——●	GVH-macrophages	+	Normal macrophages
▲——▲	GVH-macrophages	+	Normal spleen cells
■——■	Normal spleen cells	+	Normal spleen cells

THE IN VIVO ROLES OF GVH ACTIVATED F_1 MACROPHAGES

In Vivo Activation of Macrophages in Semi-syngeneic Cytotoxicity

The role of graft-versus-host reaction activated F_1 peritoneal macrophages on in vivo activation of other normal syngeneic macrophages was studied by injecting (1) normal peritoneal syngeneic F_1 macrophages purified from normal PECs, and (2) normal syngeneic F_1 spleen cells, into different groups of syngeneic F_1 hosts previously induced into GVH reactions as described in other experiments.

Equal numbers of 1×10^7 peritoneal $C3D2F_1$ macrophages purified from normal syngeneic PECs or spleen cells were injected separately into two groups of syngeneic $C3D2F_1$ hosts five days after the induction of graft-versus-host reaction by transplanting parental C3H/HeJ spleen cells intraperitoneally. 48 hours after the transfer of the exogenous syngeneic lymphoid cells into these GVH-induced hosts, semi-syngeneic target cell cytolysis were measured with PECs from these hosts using the CMC assays as described previously.

As shown in Table XII, the injection of exogenous syngeneic F_1 macrophages into the F_1 animals already undergoing GVH reaction resulted in a marked increase in semi-syngeneic cytotoxicity. In contrast, the injection of exogenous syngeneic spleen cells, not only did not enhance the degree of semi-syngeneic cytotoxicity, but also produced certain inhibitory effect instead. This reflects the similar observation in the in vitro activation experiment and an earlier experiment also involving syngeneic normal spleen cells. However, the significance of both in vitro and in vivo activation of normal syngeneic macrophages by GVH-induced macrophages have been demonstrated.

Table XII- The *In Vivo* Activation of Syngeneic F_1 Immunocompetent Cells in Semi-syngeneic Cytotoxicity Reaction

Parental Donor	F_1 Hybrid Recipient	F_1 Cells Transplanted	Target Cell	Corrected Lysis*
C3H/HeJ	C3D2F_1	none	L929	35.25 ± 2.56
"	"	F_1 spleen cells	"	21.56 ± 2.06
"	"	F_1 macrophages	"	64.21 ± 1.60

* The F_1 hosts were injected with 1.5×10^8 parental spleen cells and 5 days later, 1×10^7 F_1 syngeneic spleen cells or purified macropahges were injected. 3 days later, their PECs were used as the effectro cells in CMC assays.

** Mean percent corrected lysis of target cells induced by pooled PECs from groups of 6 animals ± SE in 6 replicates.

Kinetics of In Vivo Activation of F_1 Macrophages

The time interval required for the *in vivo* activation of syngeneic normal F_1 peritoneal macrophages by the GVH-activated syngeneic F_1 macrophages was studied by transferring 1 x 10^7 normal syngeneic F_1 macrophages intraperitoneally into groups of F_1 hosts previously induced into graft-versus-host reactions. These normal macrophages were injected at 72, 48, and 24 hours prior to the measurement of semi-syngeneic target cell cytolysis using the PECs from these injected syngeneic F_1 hosts.

The results, as shown in Table XIII indicated that increasing level of semi-synegenic cytotoxicity was obtained with increasing time of residence of the exogenously transferred normal syngeneic macrophages in these syngeneic F_1 hosts undergoing graft-versus-host reactions, suggesting an *in vivo* activation mechanism within the peritoneal cavities of the GVH reaction induced F_1 host animals. The activation process rendered the normal syngeneic macrophages to become cytotoxic towards semi-syngeneic target cells detected in CMC assays.

In comparing the kinetics of *in vitro* and *in vivo* activations of normal syngeneic F_1 macrophages into semi-synegenic target cell cytolysis, it was noted that the *in vivo* activation mechanism required a much longer time interval of incubation than that of the *in vitro* situation. In both activation studies, syngeneic spleen cells were noted to possess certain regulatory effect on the activation of normal macrophages into cytotoxic effector cells, and because of the presence of a larger number of spleen cells in the *in vivo* situation, the extended time of incubation for *in vivo* activation could be due to the presence of regulatory spleen cells.

Table XIII- Effect of In Vivo Incubation Interval of Syngeneic Macrophages in GVH-Induced F_1 Hosts on Semi-syngeneic Cytotoxicity Reaction

Transplant of cells into GVH-induced $C3D2F_1$ *	Time of transplant of F_1 macrophages before assays	Target Cells	Corrected Lysis **
none	N/A	L929	38.98 ± 2.15
$C3D2F_1$ macrophages	24 hours	L929	57.71 ± 0.98
" "	48 hours	L929	66.90 ± 4.19
" "	72 hours	L929	70.56 ± 3.68

* $C3D2F_1$ hybrids were injected with 1.5×10^8 C3H/HeJ spleen cells and PEC stimulants as previously described. At the time indicated in the second column, groups of 6 animals received the injection of 1×10^8 $C3D2F_1$ macrophages per animal. CMC assays were done 8 days after the induction of GVH reactions.

** Mean percent corrected lysis of target cells by pooled effector cells from each group of 6 animals ± SE in 6 replicates.

Kinetics of Semisyngeneic Cytotoxicity of In Vivo Activated F_1 Macrophages

The kinetics of semi-syngeneic cytotoxicity noted in the in vivo activation of syngeneic F_1 macrophages was studied in relation to the time of incubation in CMC assays involving two different incubation periods of the exogenously transferred macrophages. Parental C3H/HeJ spleen cells were used to induce GVH reactions in $C3D2F_1$ hybrids which later received 1×10^7 syngeneic macrophages at 48 and 72 hours prior to CMC assays using the PECs from these GVH-induced F_1 hosts. The results as shown in Figure 8, showed that a directly proportional relationship detween percent semi-syngeneic cytotoxicity and the time of incubation in the CMC assays.

To verify that the directly proportional dose-response relationship observed is statistically significant, the experimental data were analysed by the computer program ST 31 previously described.

In Tables XIV , and XV , it can be seen that the two regression coefficients, namely, 0.991 (in vivo activation period of 48 hours) and 0.995 (in vivo activation period of 72 hours) were very close to the ideal value of 1, indicating a linear correlation which is statistically significant. In the analysis of variance tables, the OBSERVED and EXPECTED values very close together mathematically, with RESIDUAL values in the range of 0.142 to 3.931 in the case of 48 hours of in vivo incubation, and 0.080 to 4.018 in the case of 72 hours of in vivo incubation of adoptively transferred macrophages.

The direct plots of the two linear regression lines are shown in Figures 10 (48 hours of in vivo incubation) and 11 (72 hours of in vivo incubation). The directly proportional relationship between the degree of semi-syngeneic cytotoxicity and in vivo incubation time of adoptively transferred syngeneic macrophages was graphically depicted.

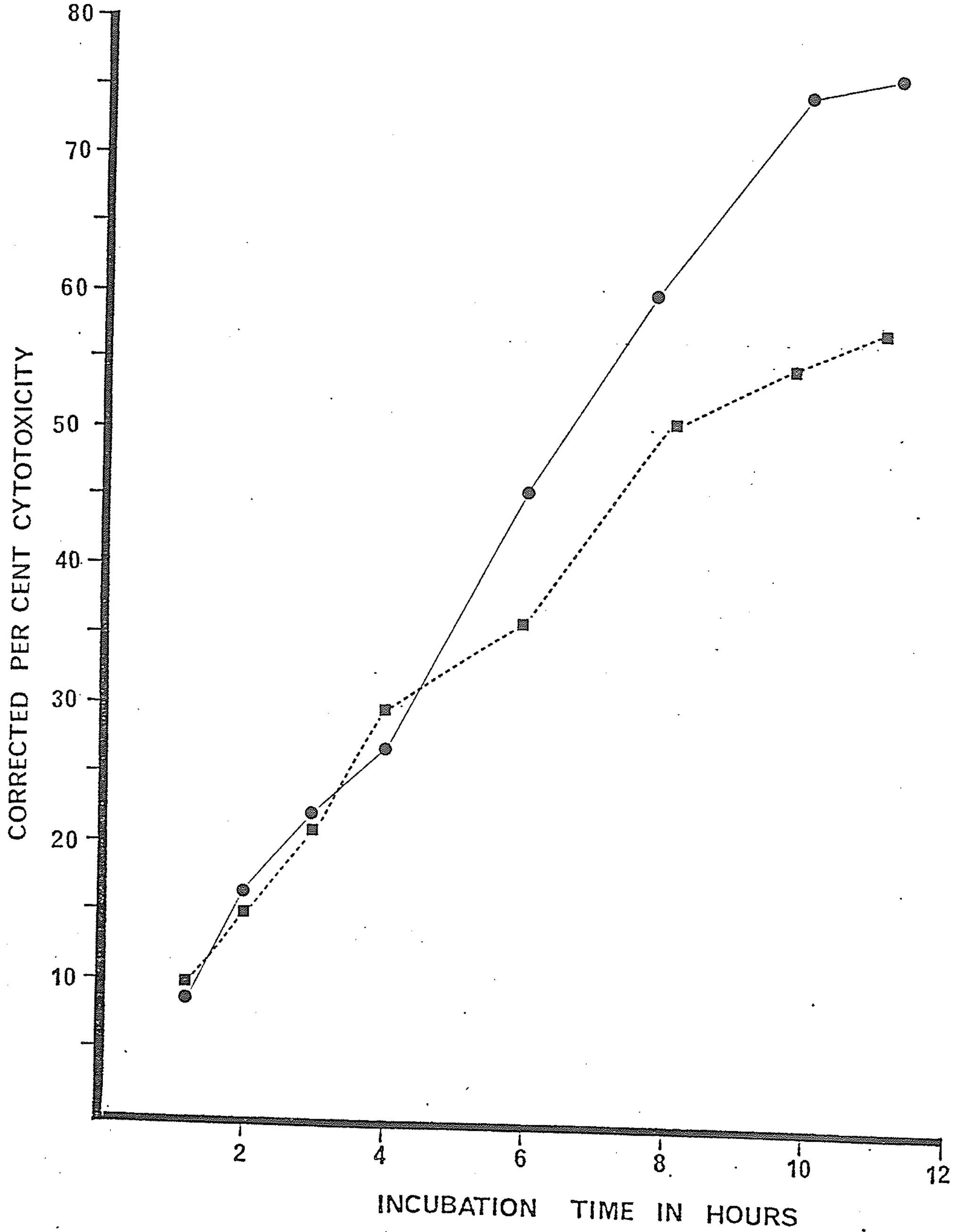


Figure 9 - Kinetics of in vivo activated peritoneal macrophages in Semi-syngeneic Cytotoxicity

●———● 72 hrs. of in vivo activation of F_1 macrophages

■------■ 48 hrs. of in vivo activation of F_1 macrophages

```
DATA...
1 10.9
2 15.28
3 23.88
4 30.09
6 35.3
8 50.84
10 56.6
11 58.97/


N =     8

 X          MEAN              SD

 1           5.625            3.739
 2          35.233           18.552

SELECTION... 2 1

R   = 0.991
RSQ = 0.982

 2 ON  1

INTERCEPT    =    7.578
B            =    4.916
SD ESTIMATE  =    2.691
SDB          =    0.272
T            =   18.075

OP1.. Y
          ** ANALYSIS OF VARIANCE **

 SOURCE        DF        SS          MS           F

REGRESSION      1     2365.687    2365.687     326.712
DEVIATIONS      6       43.445       7.241
TOTAL           7     2409.132

OP2.. Y

NO.   OBSERVED    EXPECTED    ADJUSTED   RESIDUAL

 1     10.900      12.494      33.638     -1.594
 2     15.280      17.411      33.102     -2.131
 3     23.880      22.327      36.785      1.553
 4     30.090      27.243      38.079      2.847
 5     35.300      37.076      33.456     -1.776
 6     50.840      46.909      39.164      3.931
 7     56.600      56.742      35.091     -0.142
 8     58.970      61.658      32.545     -2.688
```

Table XIV - Computer Print Out of Statistical Analysis of In Vivo Activated Macrophages In Semi-syngeneic CMC Assays 48 Hours After Injection Into Syngeneic F_1 Undergoing GVH Reactions

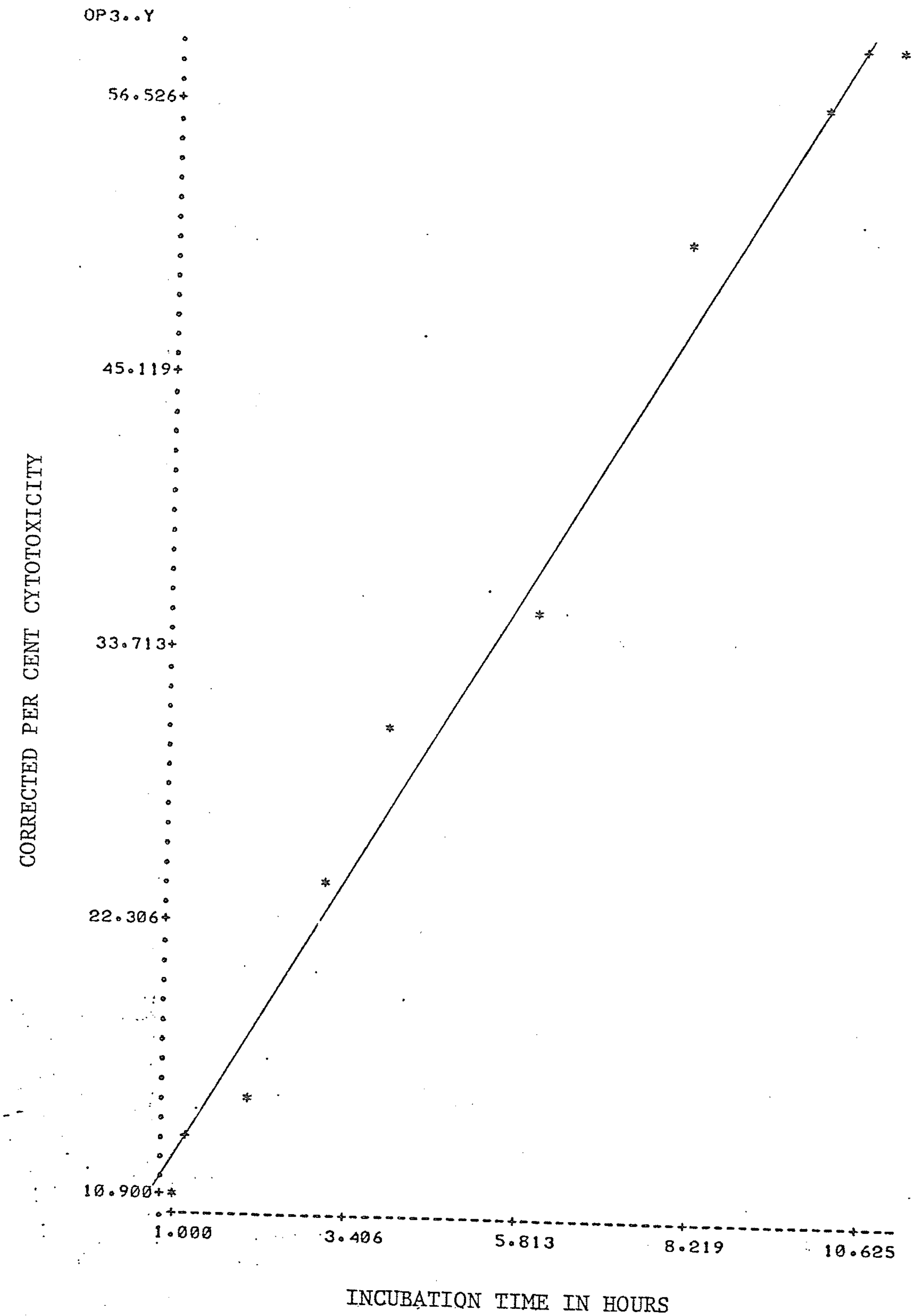


Figure 10 - Regression Coefficient Plot Between Corrected Per Cent Cytotoxicity And Incubation Time In Semi-Syngeneic Cytotoxicity 48 Hours After In Vivo Activation of Syngeneic Macrophages

```
DATA...
1 8.34
2 16.69
3 22.61
4 26.44
6 46.11
8 62.50
10 75.77
11 76.81/


N =     8

 X         MEAN                SD


 1         5.625             3.739
 2        41.909            27.205

SELECTION... 2 1

R    = 0.995
RSQ  = 0.990


 2 ON  1

INTERCEPT    =     1.180
B            =     7.241
SD ESTIMATE  =     2.872
SDB          =     0.290
T            =    24.939

OP1.. 2 1
TE 1

OP1.. Y
```

** ANALYSIS OF VARIANCE **

SOURCE	DF	SS	MS	F
REGRESSION	1	5131.403	5131.403	621.976
DEVIATIONS	6	49.501	8.250	
TOTAL	7	5180.904		

OP2.. Y

NO.	OBSERVED	EXPECTED	ADJUSTED	RESIDUAL
1	8.340	8.420	41.828	-0.080
2	16.690	15.661	42.938	1.029
3	22.610	22.902	41.617	-0.292
4	26.440	30.143	38.206	-3.703
5	46.110	44.624	43.395	1.486
6	62.500	59.105	45.303	3.395
7	75.770	73.587	44.092	2.183
8	76.810	80.828	37.891	-4.018

Table XV - Computer Print Out of Statistical Analysis of In Vivo Activated Macrophages In Semi-syngeneic CMC Assays 72 Hours After Injection Into Syngeneic F_1 Undergoing GVH Reactions

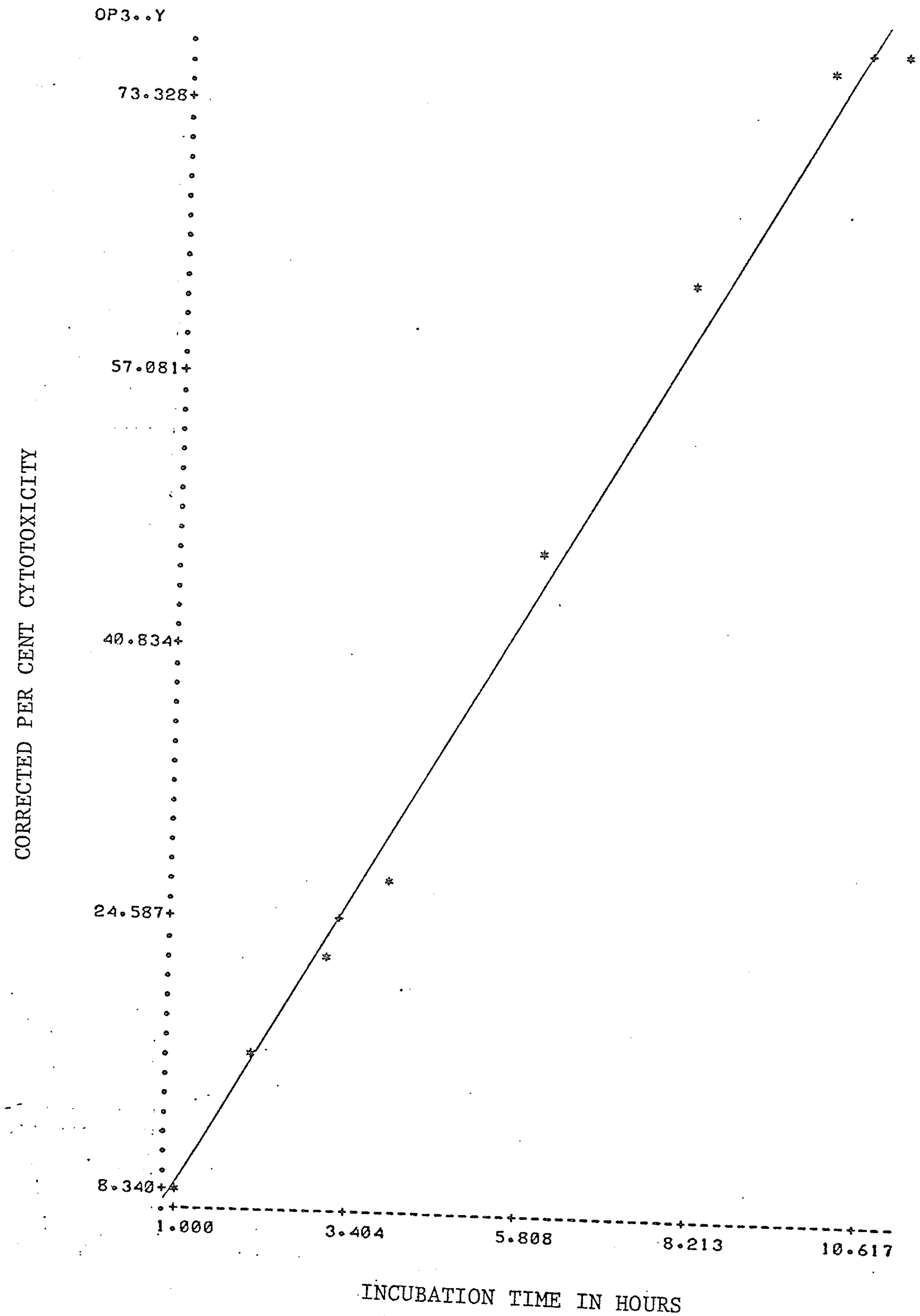


Figure 11 – Regression Coefficient Plot Between Corrected Per Cent Cytotoxicity And Incubation Time In Semi-Syngeneic Cytotoxicity 72 Hours After In Vivo Activation of Syngeneic Macrophages

In previous experiments, GVH activated macrophages were shown to be responsible for the *in vitro* semi-syngeneic cytotoxicity reaction. The following experiments were designed to demonstrate that these GVH activated macrophages were also involved in the *in vivo* aspect of the spontaneous resolution of the GVH reaction. Two experiments, one using the Spleen Index, the other using the reduction of mortality were performed.

Effect of GVH Activated PECs on GVH Reaction Assayed by Spleen Indices

The spleen index is considered as the *in vivo* indicator of the GVH reaction in experimental animals (Simonsen 1962). To obtain splenomegaly parental spleen cells were injected into F_1 hybrids, and on day 9 post induction, the body and spleen weights of eacg animal were measured. For the controls, syngeneic spleen cells were injected into a separate group of F_1s. The body and spleen weights of these animals were also measured on day 9. The spleen index was calculated by the following formula :

$$\text{Spleen Index} = \frac{\dfrac{\text{Spleen Weight of GVH Animal}}{\text{Body Weight of GVH Animal}}}{\dfrac{\text{Spleen Weight of Control Animal}}{\text{Body Weight of Control Animal}}}$$

Normal B6C3F_1 recipients were injected intraperitoneally with C3H/HeJ parental spleen cells in increasing numbers : $5x10^6$, $1x10^7$, $5x10^7$, and $1x10^8$ cells per F_1 recipients. On day 9 post GVH induction, the F_1 recipients were sacrificed and spleen indices were obtained for these animals representing the classical GVH reaction.

In order to study the *in vivo* effect of the GVH activated F_1 PECs on the GVH process, these GVH activated F_1 immunocompetent cells were obtained from $B6C3F_1$ hybrids previously injected with 1 x 10^8 parental C3H/HeJ spleen cells. These PECs were coolected 8 days post GVH induction and were injected intravenously (in increasing numbers of : $0.5x10^6$, $1.0x10^6$, $5x10^6$, and $1x10^7$ cells per recipient) into four groups of syngeneic $B6C3F_1$ hosts. Each group of these new syngeneic F_1 hosts were induced into GVH reaction by the injection of increasing number ($5x10^6$, $1x10^7$, $5x10^7$ and $1x10^8$ cells per recipient) of parental spleen cells. The spleen indices of each of these new F_1 hosts were obtained on day 9 post injection.

As shown in Figure 12, the spleen indices of the classical GVH reaction followed a linear pattern with increasing number of parental spleen cells injected, i.e., in vivo GVH reaction is directly proportional to the quantity of parental immunocompetent cells transferred.

The spleen indices of $B6C3F_1$ receiving only syngeneic spleen cells without any parental spleen cells indicated a non-GVH reaction. This normal control level represented the absence of in vivo GVH reaction.

The spleen indices of GVH induced F_1 recipients which have received the additional GVH activated syngeneic PECs at the time of induction the GVH reactions showed an inversely proportional relationship to the quantity of GVH activated PECs adoptively transferred.

Increasing number of GVH activated PECs pa-sively transferred into syngeneic F_1 recipients at the beginning of the induction of GVH reaction, produced a decreasing level of spleen indices in these GVH induced syngeneic F_1 animals. This indicated a suppressive effect of the in vivo GVH reaction by the syngeneic GVH activated F_1 immunocompetent cells.

The following experiment examined another in vivo parameter of the GVH reaction in the presence of GVH activated syngeneic F_1 immune cells.

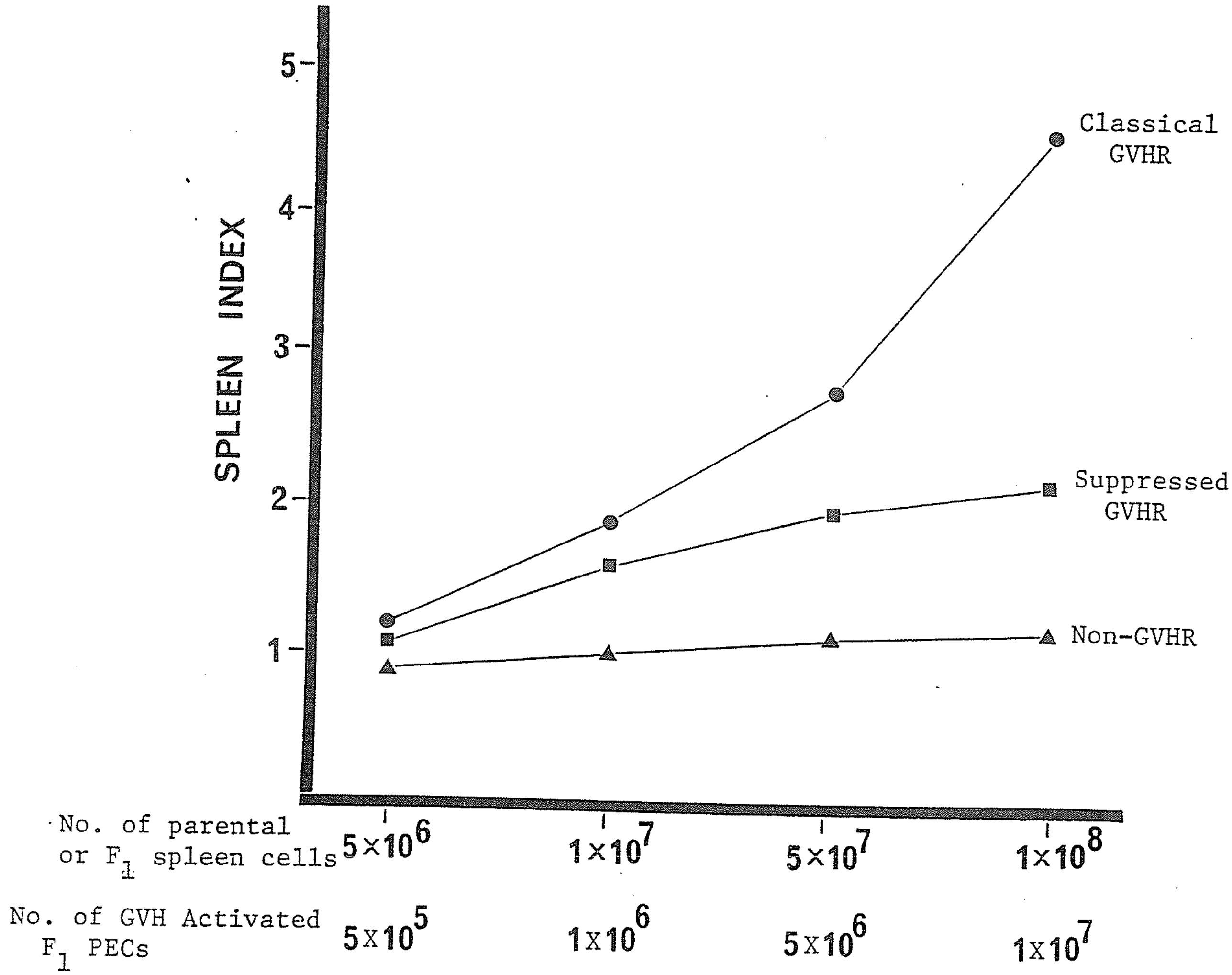


Figure 12 - Effect of Graft-versus-Host Reaction Activated Macrophages on Spleen Indices of F_1 Hosts Undergoing Graft-versus-Host Reaction

●——● Increasing No.of Parental Spleen Cells injected into Normal F_1 (Normal Graft-versus-host Reaction)

▲——▲ Increasing No.of Syngeneic Spleen Cells injected into Normal F_1 (Syngeneic Cellular Transfer Reaction)

■——■ GVH-activated PEC injected into F_1 undergoing GVH Reaction These F_1 animals received :

(1) Increasing No.of Parental Spleen Cells

(2) Increasing No. of (0.5, 1.0, 5.0, 10.0 x 10^6 cells per recipient) GVH-activated syngeneic macrophages

In Vivo Rescue Effect of GVH Activated F_1 Immunocompetent Cells

This experiment investigated into the *in vivo* effect of GVH activated peritoneal macrophages on reducing the mortality of lethally irradiated syngeneic F_1 hosts undergoing GVH reactions.

The mice were caged inside ventilated plastic containers and were exposed to total body gamma-rays irradiation which was generated by a ^{60}Cobalt isotope source (Eldorado Unit, Atomic Energy of Canada) at the Manitoba Cancer Treatment and Research Foundation centre. The source to mid-body distance was approximately 100 cm, and the dose rate was approximately 80 Rads per minute.

Fifty normal male B6C3F_1 hybrids were lethally irradiated. 1×10^7 parental C3H/HeJ spleen cells were injected intravenously into these F_1 hybrids for the induction of GVH reactions. The number of F_1 recipients that died on certain pre-selected days were recorded. This group of animals served as the control group indicating the mortality rate of lethally irradiated and GVH reaction induced F_1 hybrids in the absence of any subsequent external intervention on the GVH process.

Another fifty male B6C3F_1 hybrids were also lethally irradiated and injected with parental spleen cells as the control animals. In addition, 1×10^7 of GVH-activated syngeneic F_1 macrophages were injected into each of these F_1 hybrids intravenously. The number of animals that died on the same pre-selected days as in the control group was recorded. The results between these two groups were compared.

As shown in Table XVI, the cumulative number of deaths and the cumulative total percentage of deaths of these two groups of animals within the observation period were described. The results showed that passively transferred syngeneic F_1 macrophages which were activated by previous GVH

Table XVI - Percentage Deaths of F_1 Hybrids previously Irradiated and Induced into GVH Reactions by Parental Spleen cells

GVH Induction =	1×10^7 Parental Spleen cells		1×10^7 Parental Spleen cells	
Treatment of GVH-induced Recipients =	none		1×10^7 GVH-activated F_1 macrophages	
Days after GVH induction	x/50	Total %	x/50	Total %
0	0	0	0	0
4	1	2	0	0
7	9	18	0	0
10	16	32	0	0
14	29	58	1	2
17	46	92	3	6
21	48	96	3	6
Median Survival time (in days)	12		>> 12	

note : x = cumulative number of dead experimental animals

50 = total number of experimental animals in the group

reaction, were capable of prolonging the median survival time of the animals lethally irradiated and induced with GVH reactions as compared to those F_1 hybrids which did not receive any additional cells i.e., the control group of F_1 hybrids.

To obtain a better representation of the data, the percentage deaths of both groups of F_1 animals were plotted against the time interval of observation in the experiment. The results are shown in a PROBIT analysis graph as shown in Figure 13.

The cumulative percentage deaths of the lethally irradiated F_1 animals induced into GVH reactions and received no additional cellular transfer, followed a directly proportional relationship with the number of days after the induction of GVH reactions. The Median (50% survival) survival time of this group of F_1 hosts was estimated to be approximately 12 days after GVH induction.

The other group of lethally irradiated and GVH-induced F_1 hosts which have also received additional GVH-activated syngeneic F_1 macrophages demonstrated a prolonged survival interval indicating the passively transferred syngeneic GVH-activated macrophages exerted a rescuing effect on those animals probably as a result of the adoptive transfer of such GVH-activated macrophages. The Median (50% survival) survival time of this latter group of GVH-induced F_1 hosts was extended to beyond 12 days and extrapolated to approximately 21 days after GVH induction.

These experimental results are significant because they have demonstrated the capacity of GVH-activated macrophages in the prolongation of the survival of lethally irradiated and GVH-induced F_1 hosts. They also implicated the possible involvement of the F_1 immunocompetent cells in mediating the recovery or resolution of the GVH reaction observed in the non-irradiated F_1 hosts as described previously in literature.

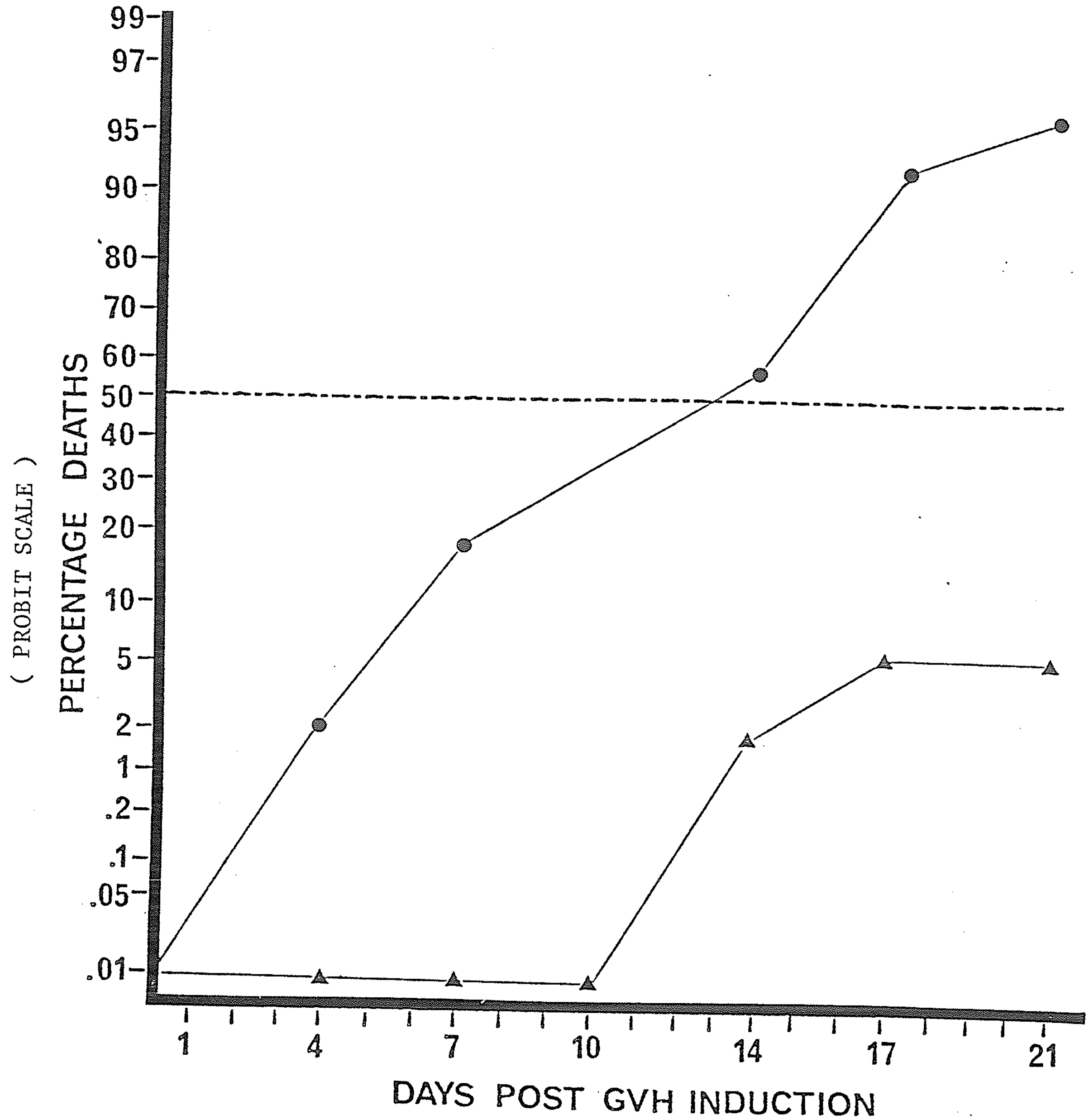


Figure 13 – Effect of GVH Activated Macrophages on Mortality of Lethally Irradiated F_1 Hosts Undergoing Graft versus Host Reaction

●——● Irradiated GVH F_1

▲——▲ Irradiated GVH F_1 + GVH Activated F_1 Macrophages

Abrogation of Spontaneous Resolution of GVHR

To prove that GVH activated F_1 cells are responsible for the spontaneous resolution of the GVH reaction, the following two experiments were performed.

In both experiments, some of the GVH activated F_1 animals were lethally irradiated, and the immunoincompetent F_1 PECs were collected and transferred into new syngeneic F_1 hosts. These new F_1 hosts were injected with parental spleen cells from the same parental strain used in the induction of GVH activated F_1 donor PECs.

In the first experiment, the degree of the in vivo GVH reaction in the presence of GVH activated irradiated, and GVH activated non-irradiated F_1 donor PECs were compared by the spleen index assays.

In the second experiment, the degree of in vivo protection of irradiated new F_1 hosts by syngeneic GVH activated irradiated and GVH activated non-irradiated F_1 donor PECs were compared by the 50% mortality assay which was previously described.

In the first experiment, GVH activated F_1 PECs were obtained from B6D2F_1 previously induced with GVH reaction by the injection of parental DBA/2 spleen cells. In one group of these GVH activated F_1s, the PECs were collected 8 days post GVH induction and were injected intravenously (in increasing numbers of ; $0.5x10^6$, $1x10^6$, $5x10^6$, and $1x10^7$ cells per new F_1 recipient) into groups (6 animals each group) of syngeneic B6D2F_1 hosts. GVH reactions were also induced in these new hosts by the injection of $5x10^6$, $1x10^7$, $5x10^7$ and $1x10^8$ parental spleen cells in each respective group of F_1s.

Another group of GVH induced B6D2F_1 animals were lethally irradiated. The F_1 PECs from these GVH activated hosts were collected and injected also

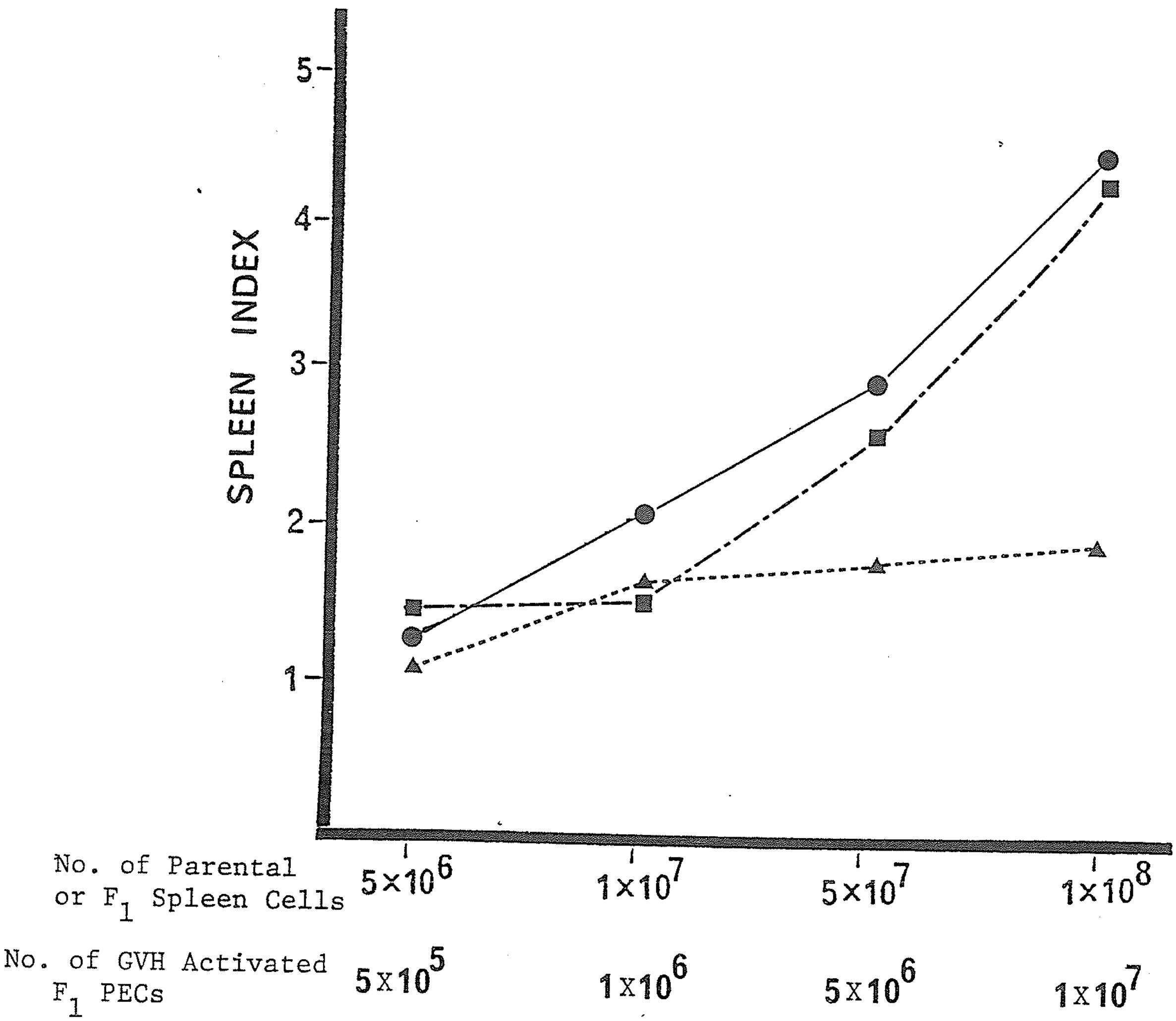


Figure 14 - Effect of Irradiation on GVH Activated F_1 Cells in the Reduction of Spleen Indices of Syngeneic F_1 Hosts Undergoing GVH Reactions

●——● Increasing No. of Parental Spleen Cells injected into Normal F_1 (Normal GVH Reaction)

▲——▲ GVH Activated F_1 PEC injected into Syngeneic F_1 recipients undergoing GVH Reactions (*)

■——■ GVH Activated and Irradiated F_1 PEC injected into Syngeneic F_1 undergoing GVH Reactions (*)

(*) These animals received :

1. increasing No. of parental spleen cells
2. increasing No. of GVH activated syngeneic F_1 PECs which were either irradiated or not irradiated

intravenously (in increasing numbers of 0.5×10^6, 1×10^6, 5×10^6, and 1×10^7 cells per new F_1 recipient) into groups of syngeneic B6D2F_1 hosts. Parental DBA/2 spleen cells, (in increasing numbers also ; 5×10^6, 1×10^7, 5×10^7, and 1×10^8 cells per recipient) were injected into these groups of new F_1 hosts for the induction of GVH reactions.

The results as shown in Figure 14 , demonstrated that when GVH activated F_1 donor PECs were not irradiated, the spleen indices of the syngeneic new hosts were decreased indicating the suppression of the *in vivo* GVH reaction by the exogenous GVH activated syngeneic F_1 effector cells.

However, when GVH activated F_1 donors were lethally irradiated and the F_1 donor PECs transferred, the spleen indices of the syngeneic F_1 recipients followed the same pattern as the classical GVH reaction with close correlation. In other words, no suppression of the ongoing GVH reactions by the irradiated F_1 donor PECs were observed.

The results in this experiment demonstrated that in order to bring about the suppression of the *in vivo* GVH reaction as measured by the spleen indices, the adoptively transferred GVH activated F_1 immunocompetent cells must be viable or able to proliferate in the new F_1 recipient. This experiment also demonstrated that GVH activated F_1 immunocompetent cells were responsible for the reduction of the degree of *in vivo* GVH reaction.

In a previous experiment, it had been shown that GVH activated F_1 PECs, when adoptively transferred into lethally irradiated syngeneic F_1 hosts undergoing GVH reactions, were able to reduce significantly the 50% mortality rate of these new F_1 recipients. The following second experiment examined the situation of irradiating the GVH activated F_1 donor PECs and the resultant effect if any, on the reduction of the mortality rate of the new F_1 hosts undergoing GVH reactions.

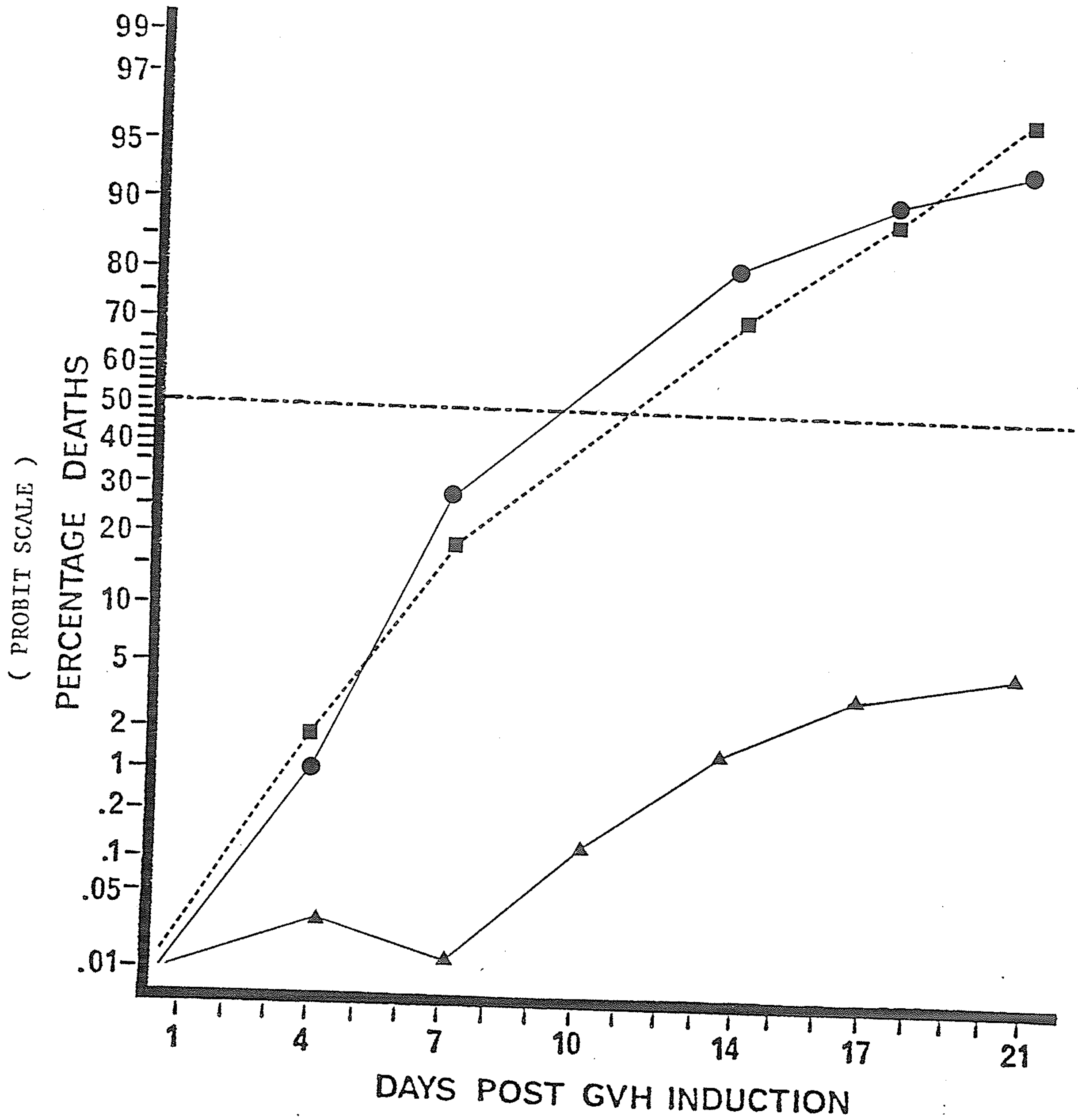


Figure 15 – Abrogation of the Rescuing Effect of GVH Activated F_1 Donor PECs in Irradiated F_1 Hosts undergoing GVH Reactions

●——● Irradiated GVH-induced F_1

▲——▲ Irradiated GVH-induced F_1 + GVH-activated F_1 Donor PECs

■——■ Irradiated GVH-induced F_1 + Irradiated GVH-activated F_1 Donor PECs

GVH activated F_1 donor PECs were obtained by the induction of GVH reactions in B6D2F_1 hybrids with DBA/2 parental spleen cells. Some of these F_1 animals were lethally irradiated so as to produce irradiated GVH activated F_1 donor PECs. Other non-irradiated F_1 animals were used as source of GVH activated F_1 donor PECs.

Three groups of thirty normal male B6D2F_1 hybrids were lethally irradiated and injected with 1×10^7 DBA/2 parental spleen cells for the induction of GVH reactions. One group of these GVH induced syngeneic F_1 hosts received 1×10^7 GVH activated F_1 donor PECs. The second group received 1×10^7 GVH actiavted but irradiated F_1 donor PECs. The last group received no additional F_1 cells and served as the control indicator of the in vivo GVH reaction. The number of animals that died on certain pre-selected days were recorded. The percentage deaths for each individual group on those pre-selected days were calculated.

The results as shown in Figure 15, demonstrated the phenomenon that GVH activated F_1 PECs when adoptively transferred into syngeneic F_1 hosts undergoing GVH reactions, were able to suppress the 50% mortality rate very significantly. On the other hand, when the GVH activated F_1 donor PECs were rendered immunoincompetent by irradiation and transferred into new syngeneic F_1 recipients undergoing GVH reactions, the phenomenon of reduction of the 50% mortality rate was not observed. This clearly indicated the role of the GVH activated F_1 donor PECs in rescuing the syngeneic F_1 recipients and also implied the involvement of F_1 immunocompeten- cells in bringing about the spontaneous resolution of the in vivo GVH reaction.

Specificity of Suppression of in vivo GVH Reaction

In previous experiments, it have been shown that GVH activated F_1 effector cells seem to possess a higher degree of preferential in vitro cytotoxicity on target cells bearing the same H-2 genotype as the parental cells used in the induction of GVH reactions. This experiment investiagted into the in vivo situation to see if there is any specificity in the suppression of the in vivo GVH reaction by the adoptively transferred GVH activated F_1 immunocompetent cells.

In order to study this particular aspect, GVH reactions were induced in two groups of the F_1 animals. One group was injected with one parental strain spleen cells. The other group was injected with the other parental strain spleen cells. The PECs from these GVH activated F_1s were transferred into lethally irradiated syngeneic F_1 recipients. These new F_1 recipients were induced into GVH reactions by either one of the two strains of parental spleen cells. The degree of suppression of the in vivo GVH reaction bewteen these two groups was compared by the 50% mortality assay. In other words, the GVH activated F_1 PECs encountered either one of the two parental strain spleen cells in the lethally irradiated new F_1 recipients. One of the GVH inducing parental strain cells had been exposed to the GVH activated F_1 PECs previously, while the other parental strain cell had not been exposed to the GVH activated F_1 PECs before. The control for this experiment was provided by a group of lethally irradiated F_1 hosts not receiving any GVH activated syngeneic F_1 donor PECs. The results are shown in Figure 16 and Figure 17.

In the first experiment, GVH activated F_1 donor PECs were obtained from $B6D2F_1$ injected with parental DBA/2 spleen cells. These GVH

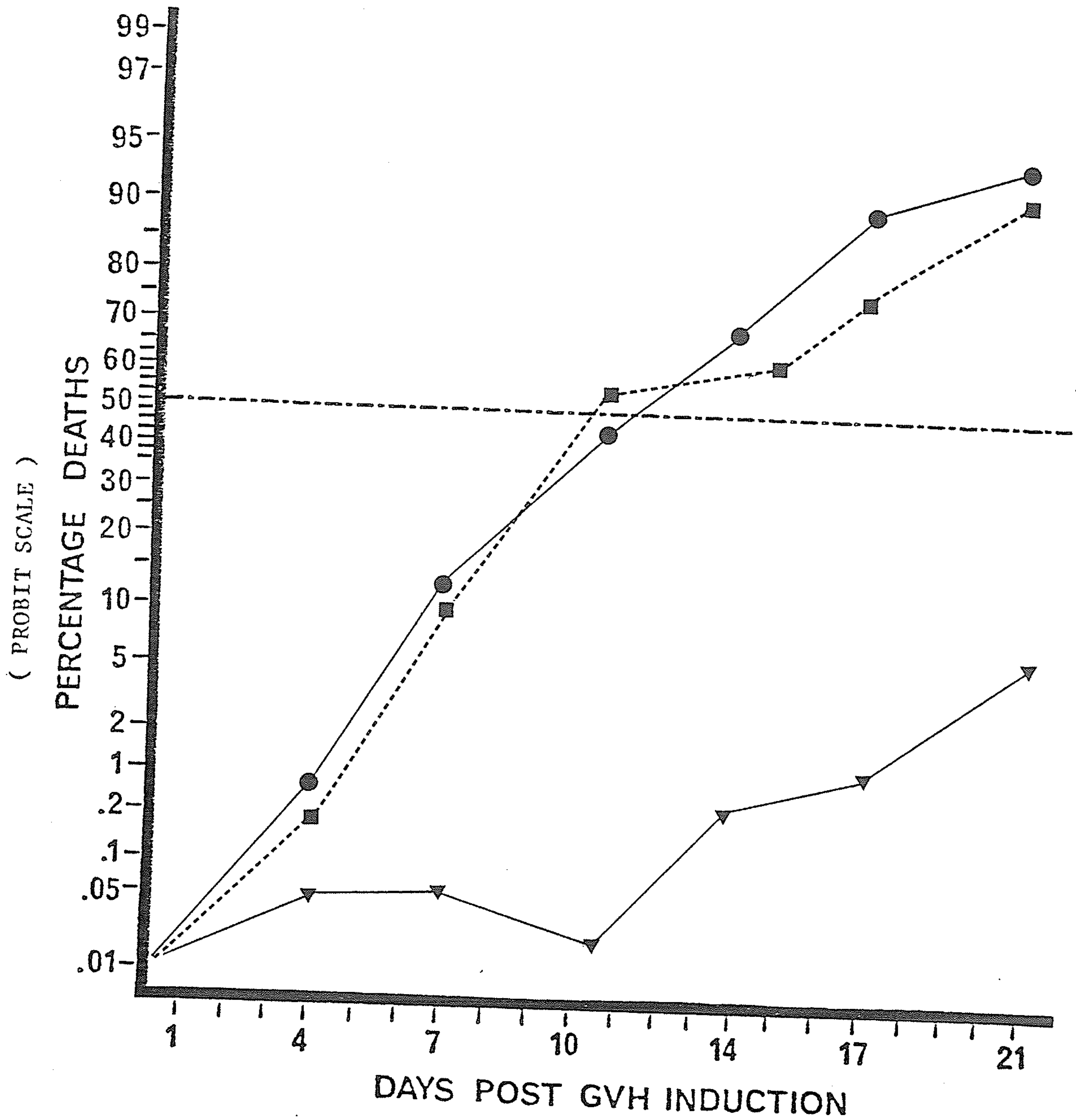


Figure 16 - Specificity of Suppression of <u>in vivo</u> GVH Reaction

●———● Irradiated $B6D2F_1$ undergoing GVHR (DBA/2 parental donor)

▼———▼ Irradiated $B6D2F_1$ undergoing GVHR + $B6D2F_1$ PEC (GVH activated by parental DBA/2 cells)

■———■ Irradiated $B6D2F_1$ undergoing GVHR + $B6D2F_1$ PEC (GVH activated by parental C57BL/6 cells) activated F_1 PECs were transferred into two groups of lethally irradiated syngeneic B6D2F_1 recipients. One group of the new F_1 recipients were induced into GVH reactions by the injection of parental DBA/2 spleen cells. The other group of the new F_1 recipients were injected with parental C57BL/6 spleen cells. Remember that the GVH activated F_1 donor PECs have only been exposed to DBA/2 spleen cells previously and never to C57BL/6 parental spleen cells before.

As shown in Figure 16 , GVH activated B6D2F_1 donor cells previously activated by the exposure to DBA/2 parental spleen cells were able to suppress the in vivo GVH reaction induced by the same parental DBA/2 spleen cells. When these GVH activated B6D2F_1 PECs encountered the other C57BL/6 parental spleen cells (Which they have not been exposed to before) the DBA/2 activated F_1 cells were not able to suppress the in vivo GVH reaction induced by the C57BL/6 parental cells. The results indicated a specificity reaction in which one can state that GVH activated F_1 immunocompetent cells were only able to suppress an in vivo GVH reaction if the parental strain spleen cells used in producing the GVH activated F_1 donor cells was identical to the parental strain used in inducing the second in vivo GVH reaction.

In the following second experiment, another combination of F_1 and parental strains was used to prove the same point. C3D2F_1 were injected with C3H/HeJ spleen cells to produce GVH activated F_1 donor cells. These activated F_1 PECs were injected into two groups of lethally irradiated syngeneic C3D2F_1 recipients. One group was induced into GVH reactions by the parental C3H/HeJ spleen cells. The other group was induced by the other parental C57BL/6 strain spleen cells. The degree of suppression of the in

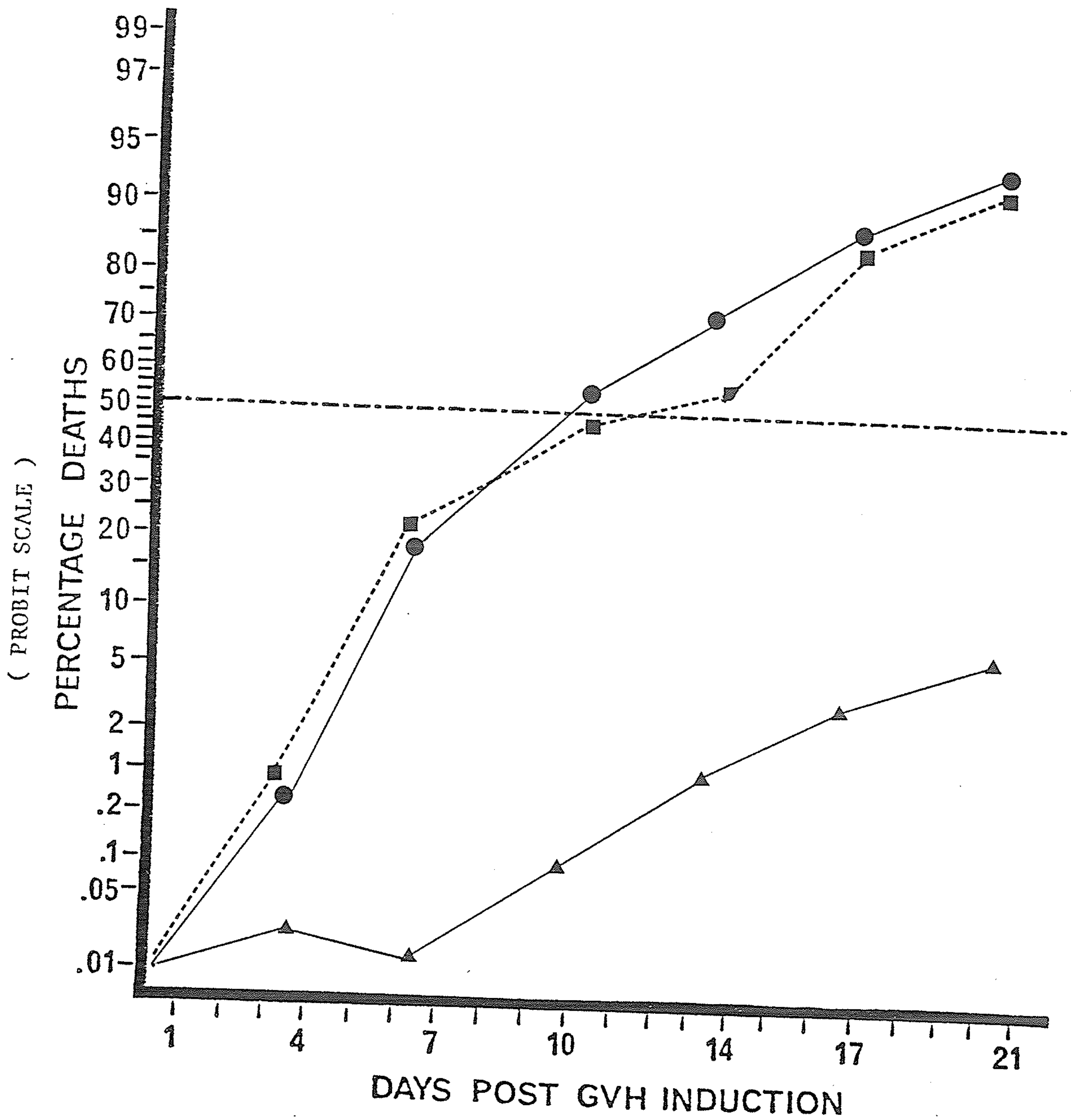


Figure 17 - Specificity of Suppression of in vivo GVH Reaction

●——● Irradiated C3D2F$_1$ undergoing GVHR (C3H/HeJ Parental donor)

▲——▲ Irradiated C3D2F$_1$ undergoing GVHR + C3D2F$_1$ PEC (GVH activated by parental C3H/HeJ spleen cells)

■——■ Irradiated C3D2F$_1$ undergoing GVHR + C3D2F$_1$ PEC (GVH activated by parental C57BL/6 spleen cells) vivo GVH reaction was compared between these two groups by the 50% median mortality assay. The control group received no GVH activated F_1 PECs.

The results as shown in Figure 17 revealed the same type of previous findings ; i.e. suppression of in vivo GVH reaction by the GVH activated F_1 PECs could only be achieved if the parental strain used in the production of GVH activated F_1 PECs was identical to that used in inducing the in vivo GVH reaction.

DISCUSSION

The garft-versus-host reaction was initially introduced as an experimental tool in studying the transplantation reactions and the complex interactions between various immunocompetent cells. To date, there is emerging realization that, because of intrinsic immunological regulations, a GVH reaction goes through a programmed course of syndromes and eventually results in complete resolution. The interactions between the grafted parental cells and the immunocompetent cells of the F_1 host can be examined from two perspectives. The classical perspective is the study of the cytotoxic activities of the grafted parental cells against the foreign antigens of the F_1 host, and the non-conventional perspective is the study of the paradox of the immunocompetent cells of the presumably genetically tolerant F_1 host reacting against the grafted parental cells. The first perspective has been extensively investigated in the past, while the second perspective is currently under initial investigations and seem to be gaining more importance.

During a GVH reaction, the pathological features occuring in the F_1 host and the subsequent demise of the F_1 recipient have classically been attributed to the direct attack of the host tissues by the parental donor lymphocytes. It has been suggested that the interactions between the parental lymphoid cells and the tissues of the F_1 host resulted in a non-specific activation of the host's lymphoid cells into cytotoxic cells as demonstrated by the in vitro CMC assays. While such an indiscriminate destructive event can occur in vivo, there have not been any satisfactory explanation as to why, in almost all situations studied, the attacking donor cells disappeared early during the GVH reaction, and the F_1 hosts, provided that they were not rendered immunodeficient, eventually all recovered from the pathological reactions.

Previous studies in this laboratory demonstrated that the "non-specific cytotoxicity reaction" observed in GVH-induced F_1 cells was mediated by the immunocompetent cells of the F_1 host (Singh et al,1972). The cytotoxic reaction is in effect, the lysis of H-2 semi-syngeneic target cells by the F_1 immunocompetent cells. This reaction is presently referred to as the semi-syngeneic cytotoxicity reaction which encompasses the concept of the F_1 anti-parent reactivity.

In the present study, various aspects of the semi-syngeneic cytotoxicity reaction were investigated. These included; the activation of the F_1 effector cells, the effects of stimulants on the F_1 peritoneal exudate cells, the types of H-2 semi-syngeneic target cells best suited for the CMC assays, the kinetics of the cytotoxic reactions in terms of the effector to target cell ratios, and the in vitro histological process of the lysis of the semi-syngeneic target cells.

Data in the present study revealed that the optimal route to induce the semi-syngeneic cytotoxicity was the intraperitoneal injection of the parental spleen cells into the appropriate F_1 recipients. Moreover, the F_1 peritoneal exudate cells, regardless of the route of induction of the GVH reactions, were more effective than the F_1 spleen cells in producing the cell-mediated cytotoxicity reactions. The tissue-cultured L929 cell-line was noted to be the most efficient target cells for such in vitro CMC assays. The kinetics of the semi-syngeneic cytotoxicity reactions were analysed by the pre-tested computer programs designed for computing the regression coefficients and the analysis of variances. The experimental results were found to be statistically significant.

Apart from studying the various experimental aspects of the semi-syngeneic cytotoxicity reaction, the identity and the nature of the F_1 effector cells were investiagted. The classical assumption is that the peritoneal lymphocytes of donor origin, when obtained from alloimmuned F_1 animals, were cytotoxic to the tissues of the F_1 host (Berke et al,1972). However, in GVH reactions, parental donor lymphocytes have been shown to die and disappear very early in the course of the GVH reaction, and the semi-syngeneic cytotoxicity reaction was still detectable long after the disappearance of the grafted donor cells. In addition, if the parental donor lymphocytes were responsible for the semi-syngeneic cytotoxicity reaction, they have to be truly autocytotoxic, the mechanism of which is difficult to understand. Further more, previous experiments have clearly established the F_1 cells were responsible for the semi-syngeneic cytotoxicity reaction (Singh et al,1972).

Experimental evidence in the present study indicated that the F_1 peritoneal macrephages were responsible for the semi-syngeneic cytotoxic reactivities. Indirect evidence in support of this conclusion derived from the fact that the F_1 cytotoxic effector cells possessed the properties of surface adherence. Direct evidence indicating the F_1 macrophages to be the cytotoxic effector cells was provided by the experiments in which the in vitro semi-syngeneic cytotoxicity reaction was suppressed by irradiating the F_1 effector cell or by adding silica particles which were shown to be cytotoxic only to the F_1 peritoneal macrophages and not the lymphocytes regardless of whether F_1 or parental origin.

The demonstration of the F_1 peritoneal macrophages being the cytotoxic effector cells is supported by similar reports in literature. The phenomenon of *in vitro* generation of a F_1 anti-parent cytotoxicity reaction has also been reported to be easily abrogated by a single small dose of silica particles (Yung and Cudkowicz 1977). Cytotoxic macrophages from human sources were also noted to be specifically inhibited by silica particles (Howitz *et al*,1979). A very significant observation reported recently was that, the *in vitro* generated F_1 anti-parent cytotoxicity reaction was inhibited by silica treatment, while the development of the anti-allogeneic cytotoxic reactivity was not affected by silica particles (Shearer *et al*,1978). This report confirmed our observation that the F_1 macrophages were the cytotoxic effector cells.

The present study also demonstrated that normal F_1 macrophages could be activated by the GVH-induced syngeneic F_1 macrophages through both *in vitro* and *in vivo* mechanisms and together with the GVH-induced F_1 effector macrophages, produced an enhanced semi-syngeneic cytotoxicity reaction in the CMC assays. The kinetics of such *in vivo* activation of the normal F_1 macrophages was analysed by the ST-31 computer program, and the results were found to be statistically significant. The anatomical progression of the F_1 effector macrophages in attacking the semi-syngeneic target cells were reported in the present study. Histological evidence of cytotoxic effector macrophages in other experimental situations as reported in literature was confirmatory to our present findings (Piessens 1978).

The *in vivo* effects of the GVH-activated F_1 macrophages on syngeneic F_1 animals were investigated. The data showed that such GVH-activated F_1 effector macrophages, when adoptively transferred into other syngeneic F_1 hosts undergoing GVH reactions, were capable of reducing the severity of the in vivo GVH reactions as reflected by the reduction of the spleen indices in these adoptively grafted animals. In addition, the adoptively injected GVH-activated F_1 effector macrophages were capable of exerting an extrinsic rescuing effect on other syngeneic F_1 animals which were lethally irradiated and injected with parental spleen cells. A significant increase of the median (50%) survival time of these GVH crippled recipients was obtained, implicating the GVH-activated effector macrophages to be an intergral part of the cytotoxic mechanism in the natural resolution of the GVH reactions.

The spontaneous resolution og the in vivo GVH reaction secondary to the adoptive transfer of syngeneic F_1 PECs could be abrogated by rendering the GVH activated F_1 donor PECs immunoincompetent. This had been demonstrated clearly in the experiments in which the GVH activated F_1 donor PECs, when irradiated and transferred into syngeneic F_1 hosts undergoing GVH reactions were not ablt to either reduce the spleen indices nor increase the median survival rate of the new F_1 recipients. It could be concluded that the F_1 immunocompetent cells were responsible for the active host-versus-graft reaction in bringing about the resolution of the in vivo graft-versus-host reaction.

In addition, it had been clearly demonstrated that there was certain degree of specificity in the capacity of the GVH activated F_1 PECs to suppress or interfer with an in vivo GVH reaction. Immunocompetent cells from F_1 donors GVH activated by one parental strain were capable of suppressing an in vivo GVH reaction if the GVH inducing parental cells injected into the irradiated new syngeneic F_1 recipients were from the same parental strain.

However, if one parental strain was used to to produce the GVH activated F_1 donor PECs and another parental strain was used to induce the in vivo GVH reaction in the new syngeneic F_1 recipients, suppression of the in vivo GVH reaction secondary to the adoptive transfer of the GVH activated F_1 PECs could not be achieved. These in vivo results complimented the previously described in vitro phenomenon of preferential lysis of target cells bearing parental antigens by the GVH activated F_1 PECs. A hypothesis to explain such host-versus-graft reaction will be presented later.

Implication of the active host-versus-graft role of the F_1 macrophages had been reported in recent literature. For example, the anti-tumor effect of the purified immune macrophages had been tested in the experiments in which, normal mice were injected with the L5178Y tumor cells, and two days later, 5×10^5 syngeneic immune macrophages were also injected into these animals. Those mice which received the immune macrophages survived 4 to 8 weeks more than the group which received the non-immune macrophages (Alexander et al,1973). The ability of macrophages from C57BL/6 mice bearing the B16 melanoma to inhibit pulmonary metastases in vivo had been reported. In this experiment, macrophages cultured in vitro with the B16 melanoma tumor cells, when adoptively transferred, could significantly reduce the number of pulmonary nodules. Moreover, those macrophages cultured in vitro with sodium caseinate or thioglycollate without the exposure to the B16 melanoma tumor cells, could not reduce the number of pulmonary metastases (Hibbs et al,19720. Data from certain immunotherapeutic trials in pulmonary carcinoma indicated that macrophages stimulated by BCG vaccine, when injected intravenously, could migrate to the lung and arrested the growth of the existing pulmonary nodules (Hopper and Pimm 1976). All these reports in literature supported the contention that macrophages are effective in vivo against syngeneic cells.

Apart from the capacity of reacting against homologous tumor cells, some activated macrophages could reportedly inhibit certain functions of the lymphocytes. For example, the suppression of the generation of cytotoxic T lymphocytes have been shown to be present in the spleens of normal mice (Hodes and Hathcock 1976) and the cells responsible for such a suppressive activity were identified as macrophages (Weiss and Fitch 1977; Kung et al,1977). Indirect evidence implicating the splenic macrophages to be responsible for the regression of a GVH reaction had been reported in studies on the Mls histocompatibility system in which, local peripheral lymph node enlargements were suppressed by the activated macrophages (Jacobsson et al,1975; Matossian-Rogers 1977).

Perhaps the best evidence implicating the F_1 macrophages as the effector cells in rescuing the host from a GVH reaction as reported in recent literature was the demonstration that a local GVH reaction could be abrogated by collodial carbon particles which possess similar toxic activities as the silica particles specifically against the macrophages (Hanna and Watson 1965). As reported in that study, a single prior injection of colloidal carbon particles could augment the local GVH reaction of peripheral lymph node hypertrophy in the footpads of the F_1 recipient rats. The colloidal carbon particles, when injected alone, did not produce any lymph node enlargement or runting disease (Yamashita et al, 1978). The colloidal carbon presumably caused the suppression of the F_1 macrophages resulting in the augmentation of the local GVH reactions.

Apart from establishing the F_1 macrophages as the effector cells in the semi-syngeneic cytotoxicity, the present study also explored into the underlying mechanism of such reactivities within the context of the GVH reaction. In the investigation of the immunological aspect of the semi-syngeneic cytotoxicity reaction, it was revealed that a significantly higher level of target cell lysis was obtained when the parental spleen cells (used in GVH induction) and the target cells (used in CMC assays) were of the same H-2 genotype as compared to the situation in which the genotype of the parental donor spleen cells (used in GVH induction) and the genotype of the target cells (used in CMC assays) were different at the H-2 level. This was designated as the preferential cytotoxic effect exhibited by the GVH-induced F_1 effector cells in CMC assays.

The observation of such a preferential cytotoxic effect by the F_1 effector cells necessitates a consideration of three hypothetical situations which could explain the underlying mechanism. They are : (1) the F_1 immunocompetent cells are not really genetically tolerant to the parental cells but can react against the histocompatibility antigens of the parental spleen cells during a GVH reaction; (2) the GVH reaction somehow activated the different clones of the F_1 immunocompetent cells one of which can become autoimmune against self-antigens present on the parental spleen cells, and (3) the cytotoxic reaction is the result of the activation of the F_1 immunocompetent cells by certain non-specific mechanism during the GVH reaction.

The experimental data from the present study seems to favor the first hypothesis. Before going into the details of such experimental evidence, the suggestion that the F_1 macrophages were non-specifically activated by the GVH reaction can be classified as a lesser possibility if one considers the following facts. It is a well known fact that GVH reactions can not be serially transferred. If the parental spleen cells could non-specifically activate the F_1 cells in the first injection, there is no reason why they could not non-specifcally activate the F_1 cells in the second host. It had been reported that the injection of the F_1 lymphocytes into irradiated parental hosts resulted in proliferation of the F_1 cells in the recipient parental spleen. This indicated that the F_1 cells were activated into proliferation in the absence of any active proliferation of the parental lymphoid cells (Blomgren and Lilliehook 1978). The time course of events during a GVH reaction could not support the suggestion that the parental spleen cells non-specifically activated the F_1 lymphoid cells. The F_1 host macrophages were shown to be cytotoxic long after the disappearance of the donor spleen cells, and the grafted parental spleen cells died and disappeared very early after transplantation into the F_1 hosts. Moreover, macrophages capable of cytotoxicity against certain antigens in the absence of GVH reactions have been reported in literature; in mice (Fink 1976) and in human adherent cells (Horwitz et al,1979). Further more, as reported in the mouse, the induction of an inflammatory exudate in the peritoneal cavities with agents such as sodium caseinate, thioglycollate, starch, mineral oils and peptones have been shown to be an insufficient stimulus to convert macrophages into cytotoxic cells specifically against syngeneic normal or tumor cells (Hibbs et al,1972; Hibbs 1974; Cleveland et al,1974). With all the above facts on hand, one can consider the activation of the F_1 effector cells in the GVH reaction to be a specific event, an intergral part of the entire reaction.

To explain the underlying mechanism of the semi-syngeneic cytotoxicity reaction, one can postulate that, under normal conditions, the F_1 immunocompetent cells reactive against the self-MHC antigens are only suppressed, not eliminated as some reports suggested, by certain immunoregulatory mechanisms, so that autoreactivity would not normally take place. The suppressive mechanisms could involve any regulatory mediators such as suppressor cells, anti-recognition structure molecules, etc. However, when an external stimulus is presented to upset the delicate balance between self-suppression and self-sensitization, for example, the GVH reaction, the suppressive activity would become inoperative. The release from such normal self-suppression may proceed to the situation in which the F_1 immunocompetent cells become capable of "recognizing" the parental histocompatibility antigens expressed on the surfaces of the parental lymphoid cells transplanted previously in the induction of GVH reactions. The outcome of such a recognition process is the proliferation of the F_1 immunocompetent cells resulting in the lysis of the target cells bearing the parental H-2 antigenic determinants. This hypothesis was investigated in the present study, and supportive results were obtained.

Evidence implicating the existence on the immunocompetent cells of specific antigen-binding receptors capable of recognizing the self-MHC-antigens producing autosensitization and auto-cytotoxicity, but are normally immunosuppressed, has been described in literature (Binz and Wigzell 1978). The lack of such "Horror Autotoxicus" reactivity in the normal F_1 murine spleen cells is likely due to the concomitant presence of both the suppressor cells and the autoreactive immunocompetent cells, and the latter cells are inhibited by the first one. However, if the suppressor cells are selectively removed from the population, the auto-cytotoxic immunocompetent cells can then be detected. This assumption in support of the presently proposed hypothesis of self-suppression in the F_1 hybrid animals has been illustrated in certain experiments reported recently using bovine serum albumin discontinuous gradients to separate the mouse splenic population of cells into different density fractions. After separation of the fractions of the spleen cells, a medium density cell population was shown to be auto-cytotoxic (Osband and Parkman 1978). The presence of suppressor cells in many immunological reactions is well known. The involvement of suppressor cells in transplantation reactions and tolerance have been described in literature (Argyris 1966; Dorsch and Roser 1977; Rieger and Hilgert 1977; Holan _et al_,1978). The existence of suppressor cells in the F_1 hybrid mice is a logical proposition, and the existence of the capacity of the F_1 immunocompetent cells to react against self-MHC antigens is discussed in the following.

In the present study, in order to demonstrate that the F_1 immunocompetent cells possess the potential to react against the parental histocompatibility antigens, spleen cells from the (A x B) F_1 hybrids were injected into lethally irradiated (B x C) F_1 hybrids and cytotoxicity reactions against the H-2^b genotype target cells were measured. This particular experimental design was used in experiments described previously and a detailed explanation in the interpretation as well as the implications of the results in reference to the present hypothesis are discussed in the following paragraphs.

Referring to Figure 18 Part (I); when parental spleen cells are injected into the F_1 hybrids, two types of immunological reactions could exist between the donor cells and the host cells : (1) the GVH type of reaction ; manifested as specific cytotoxicity (parent immunocompetent cells against foreign F_1 antigens), the hypothetical truly non-specific cytotoxicity in which, syngeneic, allogeneic, and even xenogeneic target cells are destroyed. (2) the HVG type of reaction ; manifested as, the phenomenon of semi-syngeneic cytotoxicity, hybrid resistance, in vitro generated F_1 anti-parent cytotoxicity, and autoimmune reactions. These HVG reactions, according to the classical concept of self-tolerance, should not exist.

In reference to Part (II) of Figure 18 when the roles of donor and host are reversed and the recipient hosts are lethally irradiated, i.e., the F_1 cells are injected into lethally irradaited parental recipients, only the graft against host type of reaction can occur. This reaction, in name, is a GVH reaction, but in fact, is equivalent to the HVG type of reactions described in Part (I), which is manifested therefore as ; the phenomenon of semi-syngeneic cytotoxicity, hybrid resistance, in vitro generated F_1 anti-parent cytotoxicity, and autoimmune reactions. The other type of reaction in Part (II), i.e., the HVG reaction which is

PART (I)

GVH

(1) Specific Cytotoxicity
EC genotype = A
TC genotype = B or A/B

(2) Truly Non-specific Cytotoxicity
EC genotyoe = A
TC genotype = A,B,A/B, or xenogeneic

HVG

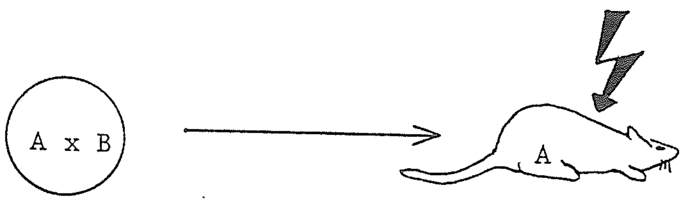
(1) Semi-syngeneic Cytotoxicity
EC genotype = A/B
TC genotype = A

(2) Hybrid Resistance

(3) F_1-antiparent Cytotoxicity

(4) Autoimmune reactions

PART (II)



GVH

(1) Semi-syngeneic Cytotoxicity

(2) Hybrid Resistance

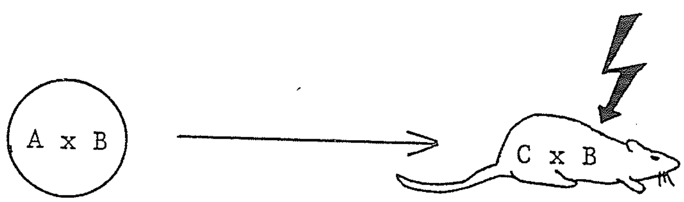
(3) F_1-antiparent cytotoxicity

(4) Autoimmune reactions

HVG

absence of specific cytotoxicity (radiation chimera)

PART (III)



GVH

(1) Semi-syngeneic Cytotoxicity
EC genotype = A/B
TC genotype = B

(2) Specific Cytotoxicity
EC genotype = A/B
TC genotype = C

HVG

absence of cytotoxicity

EC = Effector Cell
TC = Target Cell

Figure 18 - Possible Types of Reactions Involving the Graft and the Host in GVH and HVG directions

equivalent to the GVH reaction in Part (I) would not be manifested since the parental recipient hosts were lethally irradiated. The situation in Part (II) could allow the manifestation of the hypothetical phenomenon of Hybrid Resistance since certain recessive antigens are hypothetically expressed only in the homozygous genotype (i.e., the parental A/A host in the present situation) which the heterozygous F_1 cells do not possess and are therefore foreign. Because of the lack of the hypothetical homozygously expressed antigens, the F_1 cells can react against these antigens resulting in the production of in vitro cytotoxicity reactions.

To avoid the misinterpretation of cytotoxicity due to the hypothetical Hybrid Resistance phenomenon as the semi-syngeneic F_1 anti-parent cytotoxicity, the F_1 immunocompetent cells should be injected into lethally irradiated heterozygous and semi-syngeneic F_1 hosts possessing one half of the genotype common to both the donor F_1 and the irradiated recipient F_1 animals; i.e., donor F_1 genotype would be (A x B), and the irradiated recipient F_1 genotype would be (C x B), with $H\text{-}2^b$ as the common genotype. In this combination, the stimulating recipient which were lethally irradiated, would also be heterozygous. This experimental approach is described in Part (III) of Figure 18. As shown in Part (III) of the figure, two types of immunological reactions can occur. The first one is the semi-syngeneic cytotoxicity reaction in which the (A x B) F_1 immunocompetent cells would react against the parental B antigens on the (C x B) F_1 stimulating cells. The second type of reaction is the classical version of specific cytotoxicity which is known to exist.

Using the experimental approach as described in Part (III) of Figure 18, the F_1 immunocompetent cells have been shown to be capable of producing the lysis of target cells bearing the parental H-2 genotype. The results were presented previously in Table IX.

To explain the mechanics of such a reactivity involving the sensitization of the F_1 cells by the surrogate parental H-2 antigens, one can postulate that, on the surface of the $B6D2F_1$ immunocompetent cells, there exist : (1) the B and D antigens inherited from the two parental genotypes, and (2) the "recognition structures" (RS) recognizing the B and D antigens. The action of recognition by the recognition structures are normally suppressed as indicated earlier. In addition, there are other recognition structures recognizing other antigens such as the K antigen of the $H-2^k$ genitype. The H-2 antigens and the recognition structures are illustrated in Figures 19 and 20 by different symbols.

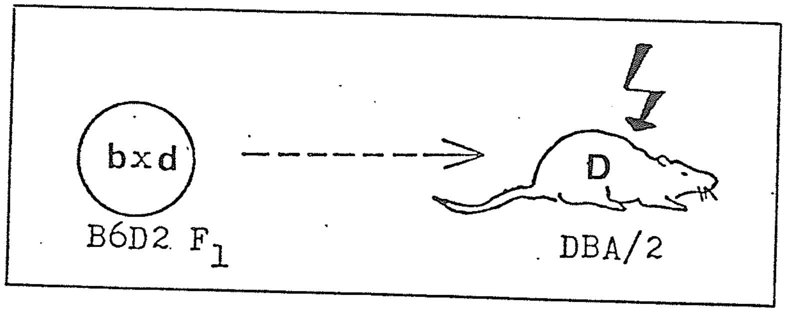
When the $B6D2F_1$ spleen cells are injected into the lethally irradiated $C3D2F_1$ recipients, the (B x D) F_1 immunocompetent cells are exposed to the H-2 antigens of the (K x D) $C3D2F_1$ cells, and three transplantation reactions of relevance can occur. As described diagrammatically in Figure 19, they are : <u>$B6D2F_1$ (B x D)</u> <u>$C3D2F_1$ (K x D)</u>

RS_d reacting against the D antigen

RS_k reacting against the K antigen

RS_b not reacting against the K and D antigens

The most significant reaction here is the reaction of the RS_d reacting against the D antigen on the $C3D2F_1$ cells. The occurence of such a reaction would lead to the lysis of the parental H-2 genotype target cell detectable in the CMC assays. In fact, the results in Table IX demonstrated the existence of such a reaction.



Hypothetical experimental situation of F_1 immunocompetent cells reacting against parental antigens in an one-way reaction.

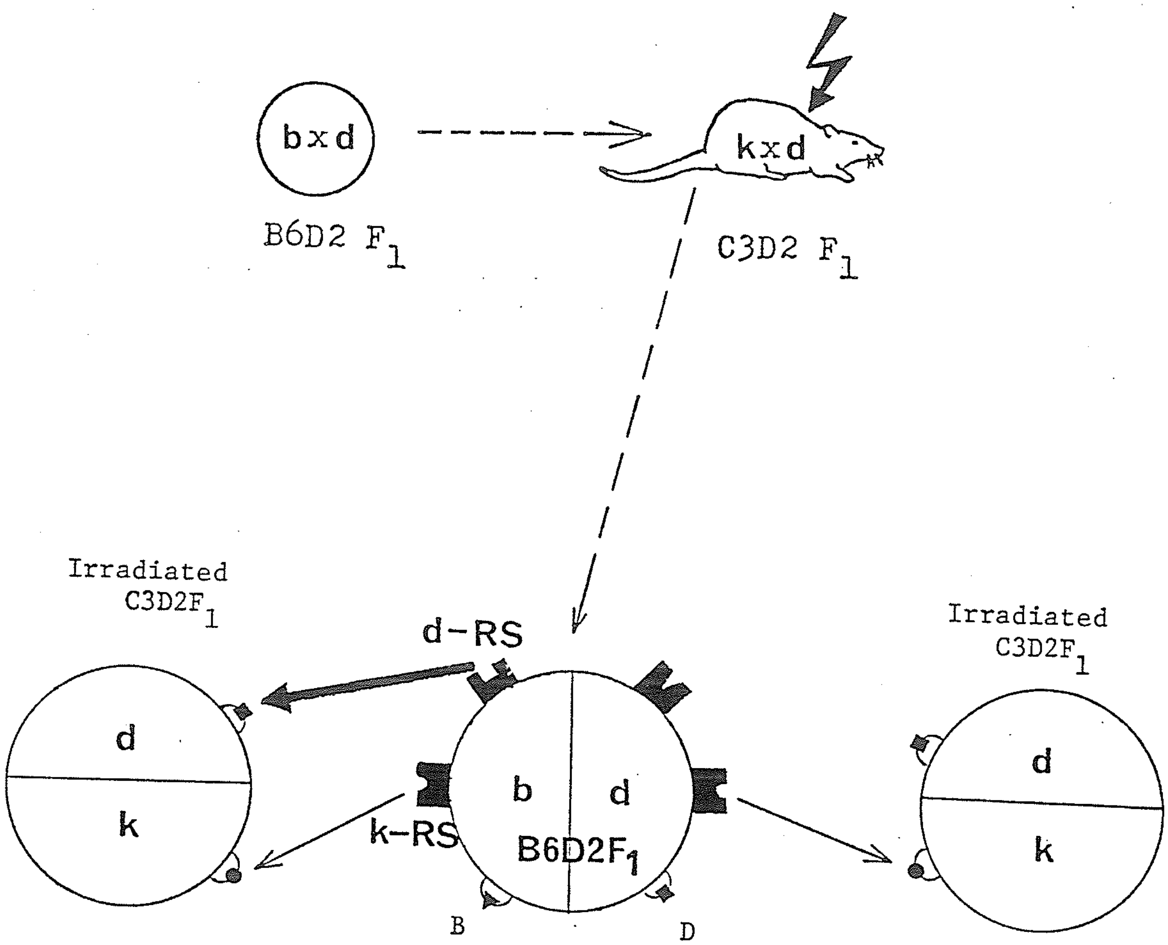


Figure 19 - Experimental situation of F_1 cells reacting against Parental Antigens (see text)

d - RS = RS_d (Recognition Structure for D antigen)

k - RS = RS_k (Recognition Structure for K antigen)

The similar experimental approach using the same two F_1 mice strains in reversed roles is depicted in Figure 20. The $B6D2F_1$ mice were used as the lethally irradiated recipients, into which, spleen cells from the $C3D2F_1$ mice were injected. The (K x D) $C3D2F_1$ cells were exposed to the antigens of the (B x D) $B6D2F_1$ recipients. The three transplantation reactions of relevance are described in Figure 23 diagrammatically.

The exposure of the $C3D2F_1$ immunocompetent cells to the H-2 antigens of the $B6D2F_1$ cells could result in the following transplantation reactions :

$C3D2F_1$ (K x D)	$B6D2F_1$ (B x D)

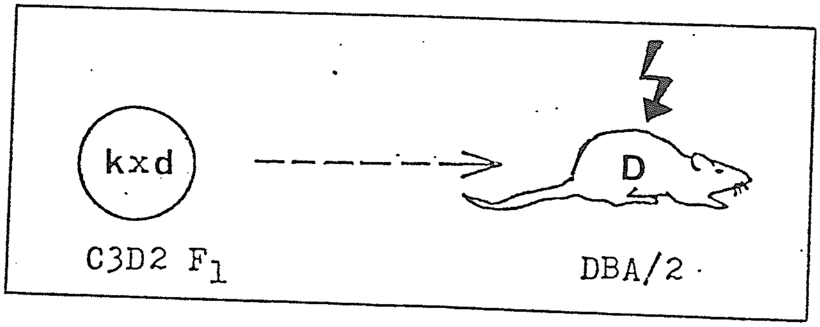
RS_d reacting against the D antigen

RS_b reacting against the B antigen

RS_k not reacting against the B and D antigens

The most significant reaction here again is the reaction of the RS_d against the D antigen on the $B6D2F_1$ cells. The existence of such a reaction was detected and reported previously in Table IX, in which, the F_1 cells were noted to be capable of causing the lysis of the surrogate parental H-2 semi-syngeneic target cells after the exposure to the parental H-2 antigens on the lethally irradiated semi-syngeneic F_1 host possessing the common parental H-2 antigen. Together with the evidence that the lysis of the parental H-2 semi-syngeneic target cells by the GVH-activated F_1 effector cells was inhibited by the H-2 specific antibodies, the underlying mechanism of the semi-syngeneic cytotoxicity reaction was interpreted to involve the antigenic determinants of the H-2 complex, thorugh which, the GVH-activated F_1 immunocompetent cells mediated the F_1 anti-parent cytotoxicity reaction.

The above hypothesis and interpretations of the experimental results are significant because a concept as well as data opposite to the



Hypothetical experimental situation of F_1 immunocompetent cells reacting against parental antigens in an one-way reaction.

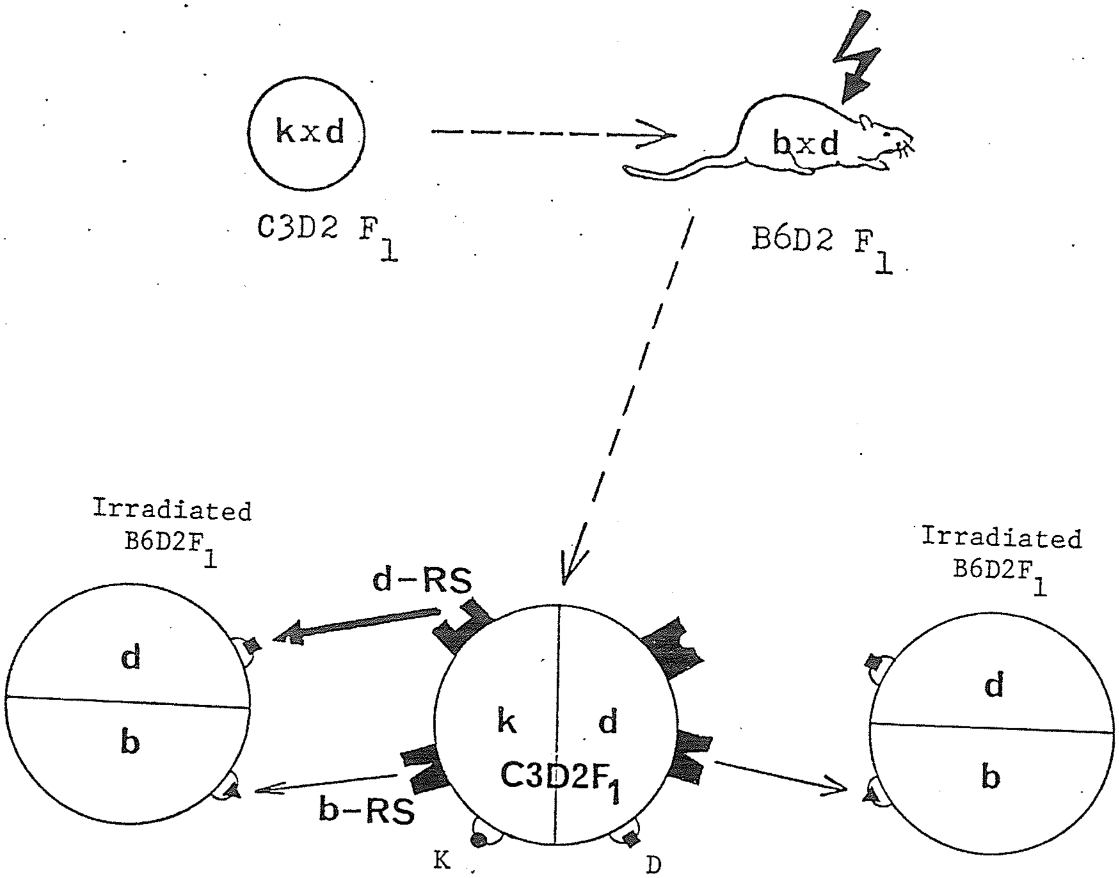


Figure 20 - Experimental situation of F_1 cells reacting against Parental Antigens (see text)

d - RS = RS_d (Recognition Structure for D antigen)

b - RS = RS_b (Recognition Structure for B antigen) classical "Clonal Deletion Theory" in transplantation immunology has been presented. The implications would undoubtedly be of importance in future clinical applications of bone marrow transplantations amd the studies on the autoimmune diseases.

Evidence demonstrating the proliferative response of the lymphoid cells against syngeneic and/or autologous stimulator cells in the mixed-lymphocyte-reactions have been reported in literature. They tend to support the contention of self-reactivity as proposed in the present thesis. Neonatal thymus cells were found capable of responding vigorously when cultured or exposed to mitomycin-treated syngeneic adult spleen cells (Howe et al,1970). In addition, adult lymph node cells were noted to proliferate in response to mitomycin-treated syngeneic or autologous adult spleen cells (Ponzio et al,1975; Finke et al,1976). This type of auto-responsiveness or self-sensitization have also been reported in different subpopulations of human peripheral blood lymphoid cells (Opelz et al,1975; Kuntz et al,1976).

The observation of the presently reported cytotoxic effect by the F_1 effector cells on target cells bearing the parental H-2 antigens is also supported indirectly by a number of recent studies. For example, under certain in vitro conditions, the F_1 immunocompetent cells were noted to react against target cells bearing the parental genotype, the situations of which, were not GVH related (Warner and Cudkowicz 1979). This type of in vitro generation of F_1 anti-parent cytotoxic cells has also been observed by other investigators (Verhulst and Zata 1977; Lilliehook and Blomgren 1978). The cytotoxic effect was also implicated to involve the MHC antigens on the parental cells (Botzenhardt et al,1978; Ishikawa and Dutton 1979). In vivo evidence of the F_1 immunocompetent cells reacting against the parental H-2 genotype target cells has been provided by a recent report in which GVH-activated F_1 spleen cells were shown to be cytotoxic to the parental genotype (methylcholanthrene induced sarcoma) target cells by the in vivo neutralization test (Nagino et al,1978).

The in vivo induction of the semi-syngeneic cytotoxicity reaction reported in this thesis and the parallel situation of in vitro generation of F_1 anti-parent cytotoxicity reported by another investigator are strongly supported by the recently described "Host-versus-Graft" disease. This HVG phenomenon occurs when adult F_1 spleen cells were injected into the parental newborn mice, producing a complex syndrome similar to the GVH-induced "Runting Disease" in the newborn F_1 mice (Smith et al,1977; Cornelius 1978; Hard and Campbell 1979). The present study however, is the first report to describe the in vivo generation of the F_1 cytotoxic effector cells reactive against the parental H-2 associated antigenic determinants which paradoxically, the F_1 cells themselves also possess.

While self-sensitization by the F_1 immunocompetent cells against the parental histocompatibility antigens occurs as a result of the removal of the naturally occuring immunosuppressive mechanisms by the GVH reaction, the transplanted parental immunocompetent cells actually have a head start in reacting against the histoincompatible antigens on the F_1 host cells producing the pathological syndromes observed in the early part of the course. As a result of the initiation of the GVH reaction, the F_1 immunocompetent cells are activated to react against the invading parental cells. This type of F_1 anti-parent reactivity can be detected experimentally as the semi-syngeneic cytotoxicity reaction. The effect of the manifestation of such a reaction in the F_1 host is the natural resolution of the GVH reaction in an immunocompetent host animal. Evidence in support of this contention has been reported in recent literature. It is known that GVH diseases occur in approximately 70% of human patients after bone marrow transplantations. Patients suffering from the acute GVH disease were noted to lack in their peripheral blood, the TH_2^+ leukocytes. Reappearance of the TH_2^+ leukocytes in the blood samples signaled the subsidence of the acute GVH disease (Reinherz et al.1979). This clinical observation closely resembles the experimental demonstration that the adoptive transfer of GVH-activated macrophages could reduce the severity of the in vivo GVH reaction as evidenced by the reduction of the spleen indices described previously.

In conclusion, the present thesis represents a pioneering work on the understanding of the graft-versus-host phenomenon in the context of the host-versus-graft reaction. A mechanism explaining the activation and the cytolytic process of the GVH-induced F_1 immunocompetent cells in the F_1 anti-parent semi-syngeneic cytotoxicity reaction has been proposed. It is suggested that the F_1 immunocompetent cells possess the capacity to react against the syngeneic antigens on the parental cells, but such reactions are normally immunosuppressed. During a GVH reaction, the balance of self-suppression and self-sensitization was disturbed, and the F_1 immunocompetent cells, in defence of the viability of the F_1 host, became activated and acquired cytotoxicity against the invading parental cells. These host-versus-graft reactivities eventually lead to the resolution of the graft-versus-host reaction. When the F_1 host was made immunodeficient by lethal irradiation prior to the induction of GVH reaction, the depletion of the F_1 immunocompetent cells resulted in the continuous progression of the graft against host reaction, ending in the death of the F_1 host.

Phosphate Buffered Saline Solution (DULBECO) :

Reference : J. Exp. Med., (1954), 99 : 167

Components -

NaCl	8000.0 mg/L
KCl	200.0 "
Na_2HPO_4	1150.0 "
KH_2PO_4	200.0 "
$CaCl_2$ (anhydrous)	100.0 "
$MgCl_2.6H_2O$	100.0 "

Hank's Balanced Salt Solution (DULBECO) :

Reference : Proc. Soc. Exp. Biol. Med., (1949), 71 : 196

Components -

NaCl	8000.0 mg/L
KCl	400.0 "
$Na_2HPO_4.2H_2O$	60.0 "
KH_2PO_4	60.0 "
$MgSO_4.7H_2O$	100.0 "
$CaCl_2$ (anhydrous)	140.0 "
Glucose	1000.0 "
$MgCl_2.6H_2O$	100.0 "
$NaHCO_3$	350.0 "
Phenol Red	10.0 "

Tissue Culture Media and Buffers

Tissue culture medium RPMI 1640(Rosewell Park Memorial Institute media series 1640, Buffalo New York) without bicarbonate was obtained from commercial source (GIBCO, Grand Island New York). Cell suspending solution and transport media of Hank's Balanced Salt Solution (HBSS) and Dulbecco Solution (DS) were obtained from DIFCO Laboratories, Detroit, Michigan,U.S. All solutions were buffered by a 0.04 Molar HEPES buffered solution. The HEPES(N-2 hydroxyethylpiperazine-N-2, ethanesulphonic acid) powder was obtained from CALBIOCHEM CO.LTD., La Jolla, California, U.S.A. The tissue culture medium RPMI 1640, a 1.043 grams-percent solution, buffered by 40 mls. of HEPES buffered solution per liter of medium was adjusted to pH 7.2 by a stock solution of 1N NaOH(sodium hydroxide) and 1N HCl(hydrochloric acid), and supplemented with 10% fetal calf sera(complement inactivated at 56°C for 30 minutes), 100 units/ml penicillin; 100 ug/ml streptomycin. The medium was sterilized by Millipore filters under constant negative pressure and stored at 4°C before use.

Trypan Blue Exclusion Test

This test of cell viability was applied in all experiments to ascertain the degree of viability of various cell suspensions just prior for use in various tests and experiments. A 0.2% aqueous solution of trypan blue was made isotonic by the addition of appropriate amount of a 5% NaCl(sodium chloride) solution before use and was used to dilute the cell suspension in a 1:10 ratio. After 10 minutes incubation at 37°C the number of stained and unstained cells were counted in a hemocytometer and the percentage of viability calculated.

RPMI MEDIA SERIES 1640

Ref.:[1] Iwakata, S., Grace, J. T. Jr.; N. Y. J. of Med., 64/18:2279-2282 (September 15, 1964).

[2] Moore, G. E., Sandberg, A. A. and Ulrich, K.; J. Nat. Can. Inst., 36/3:405 (March 1966).

[3] Published with the kind permission of George E. Moore, M.D., Ph.D., Roswell Park Memorial Institute, Buffalo, New York.

[4] Ibid.

The RPMI media series, developed at Roswell Park Memorial Institute, were designed specifically for growing human and mouse leukemia cells.

COMPONENT	RPMI[3] 1603 mg/L	RPMI[1] 1629 mg/L	RPMI[2] 1630 mg/L	RPMI[3] 1634 mg/L	RPMI[4] 1640 mg/L
Ferrous Sulfate • $7H_2O$	1.0	—	—	—	—
$CaCL_2$	—	100.0	—	—	—
$Ca(NO_3)_2 \cdot 4H_2O$	200.0	—	100.0	100.0	100.0
Glucose	2500.0	3000.0	2500.0	2000.0	2000.0
$MgSO_4 \cdot 7H_2O$	200.0	200.0	100.0	100.0	100.0
KCl	400.0	400.0	400.0	400.0	400.0
$Na_2HPO_4 \cdot 7H_2O$	1512.0	—	2835.0	2835.0	1512.0
$NaH_2PO_4 \cdot H_2O$	230.0	580.0	—	—	—
NaCl	6000.0	6460.0	6000.0	6000.0	6000.0
L-Alanine	—	13.4	—	—	—
L-Arginine (free base)	200.0	42.1 (HCl)	200.0	100.0	200.0
L-Asparagine	50.0	45.0	30.0	30.0	50.0
L-Aspartic acid	—	19.9	30.0	30.0	20.0
L-Cysteine	—	31.5	—	—	—
L-Cystine	50.0	—	100.0	100.0	50.0
L-Glutamic acid	15.0	22.1	80.0	80.0	20.0
L-Glutamine	500.0	219.2	300.0	300.0	300.0
Glutathione (reduced)	—	0.5	10.0	10.0	1.0
Glycine	15.0	7.5	15.0	15.0	10.0
L-Histidine (free base)	20.0	20.9 ($HCl \cdot H_2O$)	35.0	35.0	15.0
L-Hydroxyproline	—	19.7	—	—	20.0
L-Isoleucine (Allo free)	80.0	33.3	50.0	50.0	50.0
L-Leucine (Methionine free)	80.0	39.3	50.0	50.0	50.0
L-Lysine HCl	25.0	36.5	60.0	75.0	40.0
L-Methionine	30.0	14.9	15.0	15.0	15.0
L-Phenylalanine	20.0	16.5	30.0	30.0	15.0
L-Proline (Hydroxy L-Proline free)	10.0	17.3	30.0	30.0	20.0
L-Serine	100.0	26.3	50.0	50.0	30.0
L-Threonine (Allo free)	35.0	17.9	50.0	50.0	20.0
L-Tryptophane	20.0	3.1	10.0	10.0	5.0
L-Tyrosine	20.0	18.1	30.0	30.0	20.0
L-Valine	10.0	17.6	40.0	40.0	20.0
Ascorbic acid	—	0.5	—	—	—
Biotin	0.05	0.2	0.2	0.1	0.2
Vitamin B_{12}	0.05	2.0	0.05	0.1	0.005
D-Ca pantothenate	0.25	0.2	3.0	0.25	0.25
Choline Cl	2.0	5.0	3.0	3.0	3.0
Folic acid	0.01	10.0	2.0	1.0	1.0
Folinic acid	0.01	—	—	—	—
i-Inositol	5.0	36.0	5.0	15.0	35.0
Manganese Sulfate • H_2O	1.0	—	—	—	—
Niacin	—	0.5	—	—	—
Nicotinamide	0.2	0.5	2.5	2.5	1.0
Nicotinic acid	0.01	—	—	—	—

ST 31 PROGRAM

Definition of Output

Let N be the number of observations,
Y be the dependent variable,
X be the independent variable.

- MEAN = mean
- SD = standard deviation
- R and RSQ = simple correlation coefficient and its square
- INTERCEPT and B = Y-axis intercept and regression coefficient

The estimated regression equation is :

$$\hat{Y} = \text{INTERCEPT} + BX$$

where $$B = \frac{\sum (X_i - \bar{X})(Y_i - \bar{Y})}{\sum (X_i - \bar{X})^2}$$

$$\text{INTERCEPT} = \bar{Y} - B\bar{X}$$

- SD ESTIMATE = standard error of estimate

$$\text{SD ESTIMATE} = \sqrt{\frac{\text{SSDEV}}{N - 2}} \quad \text{where SSDEV} = (Y - \hat{Y})^2$$

- SDB = standard deviation of the estimated regression coefficient

$$\text{SDB} = \frac{\text{SD ESTIMATE}}{\left(\sum (X_i - \bar{X})^2 \right)^{\frac{1}{2}}}$$

- T = calculated T - value for the estimated regression coefficient

$$T = \frac{B}{\text{SDB}}$$

- ANALYSIS OF VARIANCE TABLE   Define : $\hat{Y}$ = INTERCEPT + BK

$$SSREG = \sum(\hat{Y} - \bar{Y})^2$$
$$SSDEV = \sum(Y - \hat{Y})^2$$
$$SSTOT = \sum(Y - \bar{Y})^2$$

note : (SSREG + SSDEV = SSTOS)

SOURCE	DF	SS	MS	F
REGRESSION	1	SSREG	SSREG	$\frac{SSREG}{SSDEV/(N-2)}$
DEVIATIONS	N-2	SSDEV	SSDEV/(N-2)	
TOTAL	N-1	SSTOT		

- OBSERVED Y was input by the user
- EXPECTED Y = $\hat{Y}$
- RESIDUAL = Y - $\hat{Y}$
- ADJUSTED = $\bar{Y}$ + RESIDUAL
- PLOT The regression line may be drawn by joining the two +s
  The data are indicated by asterisks (*)
- LIMITS = confidence limits

  LOWMEAN UPPMEAN LOWY UPPY

  X_o (X - value specified for confidence limits)
  THET T (theoretical T - value) are input by the user

$$LOWMEAN = \hat{Y}_o - (THET\ T)\ (SD\ ESTIMATE)\sqrt{\frac{1}{N} + \frac{(X_o - \bar{X})^2}{\sum(X_i - \bar{X})^2}}$$

$$UPPMEAN = \hat{Y}_o + (THET\ T)\ (SD\ ESTIMATE)\sqrt{\frac{1}{N} + \frac{(X_o - \bar{X})^2}{\sum(X_i - \bar{X})^2}}$$

LOW Y =
$$\hat{Y}_o - (THET\ T)\ (SD\ ESTIMATE)\sqrt{1 + \frac{1}{N} + \frac{(X_o - \bar{X})^2}{\sum(X_i - \bar{X})^2}}$$

UPP Y =
$$\hat{Y}_o + (THET\ T)\ (SD\ ESTIMATE)\sqrt{1 + \frac{1}{N} + \frac{(X_o - \bar{X})^2}{\sum(X_i - \bar{X})^2}}$$

LITERATURE CITED

Adler W.H., Takiguchi T., Smith R.T., (1970) J. Immunol. 105:984

Aguet M., Anderson L.C., Wigzell H., (1978) J. Exp. Med. 147:50

Ahmed A., Sell K.W., Knudsen R.C., (1975) Trans. Proc. 7 Suppl. 1:213

Alexander P., and Evans R., (1971) Nature (New Biol) 232:76

Alexander P., Evans., Mikulska Z.B., (1973) "Chemotherapy of Cancer Dissemination and Metastases" Eds. Garattini and Franchi, Raven Press, New York, 1973.

Allison A.C., (1971) Arch. Int. Med. 128:131

Argyris B., (1966) J. Immunol. 96:273

Armerding D., and Katz D.H., (1974) J. Exp. Med. 140:19

Armerding D., Sachs D.H., (1974) J. Exp. Med. 140:1717

Auerbach R., Globerson A., (1966) Exp. Cell. Res. 42:31

Bach F.H., Alter B.J., Janis M., (1970) Cell. Immunol. 1:219

Bartova J., Ivanyi D., (1975) J. Immunogenetics 2:365

Bast R.S., Cleveland R.P., Littman B.H., (1974) Cell. Immunol. 10:248

Bennett M., and Hand A.S., (1978) Transplantation 26:199

Bellgrau, D., and Wilson, D.B., (1978) J. Exp. Med. 148:103

Bellgrau, D., and Wilson, D.B., (1979) J. Exp. Med. 149:234

Berke G., Sullivan K.A., Amos B., (1972) J. Exp. Med. 135:1334

Bevan M.J., (1976) Cold Spring Harbor Symp. 41:519

Billingham R.E., Brent L., Medawar P.B., (1955) Ann.N.Y.Acad.Sci. 59:409

Billingham R.E., and Silvers W.K., (1961) Trans. Bull. 28:113

Billingham R.E., (1968) Harvey Lect. 62:21

Binet J., Mathe G. (1962) Ann. N.Y. Acad. Sci. 99:426

Binz H., and Lindemann J., (1973) Nature (Lond.) 246:146

Binz H., Lindenmann J., Wigzell H., (1974) J. Exp. Med. 140:731

Binz H., and Wigzell H., (1975) Scand. J. Immunol. 4:591

Binz H., and Wigzell H., (1976) Nature 262:294

Binz H., Ramseier H., Lindenmann J., (1973) J. Immunol. 111:1108

Binz H., and Wigzell H., (1978) Fed. Proc. 37:2365

Blaese R.M., Good R.A., (1964) J. Exp. Med. 119:211

Blomgren H., and Lilliehook B., (1978) Scand. J. Immunol. 8:231

Blyden G., and Handschumacher R., (1977) J. Immunol. 118:1631

Boehmer M.V., (1974) J. Immunol. 112:70

Bonney W.W., and Feldbush T.L. (1973) Transplantation 15:215

Botzenhardt U., Klein J., Ziff M., (1978) J. Exp. Med. 147:1435

Brent L., and Medawar P.B., (1966) Proc.Roy.Soc.(Biol) 165:413

Britton S., (1972) Scand. J. Immunol. 1:89

Brochier J., Roitt I., Festenstein H., (1974) Eur. J. Immunol. 4:709

Bucker C.D., Epstein R.B., (1970) Blood 35:741

Bunn H.F., (1977) " Harrison Principle of Internal Medicine " 2:1644

Byfield P., Christie G., Howard J.G., (1973) J. Immunol. 111:72

Calderon J., and Unanue E., (1975) Nature 253(5490):359

Cerottini J.C., Nordin A.A., Brunner K.T., (1971) J. Exp. Med. 134:553

Cheers C., and Sprent J., (1973) Transplantation 15:336

Cheers C., and Sprent J., (1974) Cell. Immunol. 10:57

Claman H.N., and Hayes L.L. (1969) Transplantation 7:87

Cleveland R., Meltzer M., Zbar B., (1974) J. Nat. Can. Inst. 52:1887

Clancy J., and Adams B.J. (1973) Transplantation 15:52

Cline M.J., Gale R.P., Fahey J.L., (1975) Ann. Intern. Med. 83:691

Cohen D., Weinstein H., Mihm M., (1979) Blood 53:1053

Cohen I.R., and Feldman M., (1970) Cell. Immunol. 1:521

Cohen I.R., and Livnat S., (1976) Transp. Proced. 8:393

Cooper G.N., and Howard J.G. (1961) Brit. J. Exp. Pathol. 42:558

Corley R.B., and Kindred B., (1977) Scand. J. Immunol. 6:923

Corley R.B., and Lefkovits I., (1978) J. Immunol. 121:1082

Cornelius E., (1978) Am. J. Pathol. 90:675

Cosenza H., and Kohler H., (1972) Proc. Nat. Acad. Sci. 69:2701

Cudkowicz G., (1968) "The Proliferation and Spread of Neoplastic Cells" page 661. Willams and Wilkinson Co. Baltimore.

Cudkowicz G., and Bennett M., (1971) J. Exp. Med. 134:83

Cudkowicz G., and Stimpfling J.H., (1964) Nature(Lond.) 204:450

Cudkowicz G., and Lotzova E., (1973) Transp. Proced. 4:1399

Cudkowicz G., (1975) Transp. Proced. 7:155

Cudkowicz G., and Warner J.F., (1979) Immunogenetics

Currie G.A., Basham C., (1975) J. Exp. Med. 142:1600

David J.R., and David R.A., (1972) Prog. Allergy 16:300

Delovitch T.L., and McDevitt H.O., (1977) J. Exp. Med. 146:1019

Diener E., and Feldman M., (1970) J. Exp. Med. 132:31

Distasio J.A., Lopez D., Silverman M., (1978) J. Retic. Soc. 24:161

Dorsch S., and Roser B., (1977) J. Exp. Med. 145:1144

Eichmann K. (1974) Eur. J. Immunol. 4:296

Eichmann K. (1975) J. Immunol. 5:511

Eichwald E., Lustgraaf E., Strainer M., (1959) J. Natl. Canc. Inst. 23:1193

Ekpaha-Mensah A., (1971) Nature(New Biol.) 233:174

Elie R., and Lapp W.S., (1976a) Cell. Immunol. 21:185

Elie R., and Lapp W.S., (1976b) Cell. Immunol. 21:31

Elie R., and Lapp W.S., (1977) Cell. Immunol. 34:38

Elkins W.L., (1964) J. Exp. Med. 120:329

Elkins W.L., (1971) Prog. Allergy 15:78

Fathman C.G., and Augustin A., (1978) J. Immunol. 121:259

Festenstein H., (1973) Transp. Rev. 15:62

Fialkow P.J., Gilchrist C., Allison A.C. (1973) Clin. Exp. Immunol. 13:479

Fidler I.J., (1974) Cancer Res. 34:1047

Field E.O., and Gibbs J.E., (1966) Clin. Exp. Immunol. 1:195

Field E.O., and Cauchi M.N., (1967) Transplantation 5:241

Fink M., Cloud C.L., McCarthy K.F., (1974) Cell. Immunol. 11:74

Fink M., (1976) "Macrophage in Neoplasia" Acad. Press. N.Y.

Finke J.H., Ponzio N.M., Battisto J., (1976) Cell. Immunol. 26:284

Folch H., and Waksman B.H., (1973) Cell. Immunol. 9:12

Ford W.L., and Simonsen M., (1971) J. Exp. Med. 133:938

Fox M., (1962) Immunology 5:489

Fox M., (1966) Transplantation 4:11

Fox M., and Howard J.G., (1963) Transplantation 1:1

Friedman H., (1975) "Thymus Factors in Immunity" Ann. N.Y. Acad. Sci. 249:1

Fudenberg, H.H., (1976) "Basic and Clinical Immunology" p.619

Fung K.Y., and Sabbadini E., (1976) Transplantation 22:449

Fyfe D.A. and Finke J.H., (1979) J. Immunol. 122:1156

Gallagher M.T., and Trentin J.J. (1976) Biomedicine 25:1

Gengozian N.T., and Owen R.D., (1958) Proc. Natl. Acad. Sci. 44:560

Gengozian N.T., and Congdon C.C., (1965) Science 149:645

Gengozian N.T., and Toya R.E., (1971) Transp. Proced. 3:434

Gengozian N.T., and Congdon C.C., (1973) Transplantation 16:32

Gery I., and Handschumacher R., (1974) Cell. Immunol. 11:162

Gery I., and Waksman B.H., (1972) J. Exp. Med. 136:143

Gery I., Gershon K., Waksman B.H., (1972) J. Exp. Med. 136:128

Gleichmann E., Wilke W.W., (1972) Transpl. Rev. 13:156

Glynn, L.E. and Steward, M.W., (1977) "Immunochemistry : An Advanced Textbook", p. 114

Goodman J.W., and Bosma G.C., (1967) Immunology 13:125

Goodwin J.S., and Bankhurst A.D., (1977) J. Exp. Med. 146:1719

Gowans J.L., (1962) Ann. N.Y. Acad. Sci. 99:432

Granger G.A., and Kolb W.P., (1968) J. Immunol. 101:111

Granger G.A., Shimizu L., Horn P., (1978) Clin. Immun. Pathol. 10:104

Grant C.K., and Alexander P., (1974) Cell. Immunol. 14:46

Graw R.G., Buckne C.D., Henderson E.S., (1970) Lancet 2:338

Grebe S.C., and Streilein J.W., (1974) Fed. Proced. 33:644

Grebe S.C., and Streilein J.W., (1976) Transplantation 22:245

Greenberg A.H., Hudson L., Roitt I., (1973) Nature 242:111

Hanna E.E., and Watson D.W., (1965) Proc. Soc. Exp. Biol. Med. 118:865

Hard R.C., and Kullgren B., (1970) Amer. J. Pathol. 59:203

Hard R.C., and Moncure C.W., (1973) Lab. Invest. 28:468

Hard R.C., and Still W.J., (1975) Amer. J. Pathol. 79:131

Hard R.C., and Campbell R.C., (1979) Cell. Immunol. 43:225

Hart D.A., Wang A.L., Nisonoff A., (1972) J. Exp. Med. 135:1239

Hathaway W.E., Fulginiti V.A., (1967) J. Am. Med. Assoc. 201:1015

Hayry R., and Anderson L.C., (1973) Transpl. Proced. 5:1697

Heise E.R., and Weiser R.S., (1966) J. Immunol. 103:570

Hibbs J.B., Lambert L.H., Remington J.S., (1972) Proc. Exp. Biol. Med. 139:1049

Hibbs J.B., Lambert L.H., Remington J.S., (1972) Nature(New Biol.) 235:48

Hibbs J.B., (1974) J. Natl. Cancer Inst. 53:1487

Hilgard H., (1970) Transplantation 10:396

Hirserodt J.C., Prieur A., Granger G.A., (1976) Cell. Immunol. 24:277

Hodes R.J., and Svedmyr E.A., (1970) Transplantation 9:470

Hodes R.J., and Hathcock K.S., (1976) J. Immunol. 116:167

Hoffman M., and Dutton R.W., (1971) Science 172:1047

Holan V., Hasek M., Chutna J., (1978) Transplantation 25:27

Hopper D.G., Pimm M.V., (1976) Lancet II:255

Horwitz D., Kight N., Allison A.C., (1979) Immunology 36:221

Howard J.G., and Woodruff M.A., (1961) Proc. Roy. Soc. Ser. 154:532

Howe M.L., Goldstein A.L., Battisto J., (1970) Proc.Natl.Acad.Sci.67:613

Huber B., Pena-Martinez J., Festenstein H., (1973) Transpl. Proced. 5:1373

Ishikawa H., and Dutton R.W., (1979) J. Immunol. 122:529

Jacobsson H., Lilliehook B., Blomgren H., (1975) Scand. J. Immunol. 4:181

Jandl J.H., MacDonald R.A., (1965) J. Exp. Med. 122:299

Jerne N.K., (1973) Science 229:52

Joller P.W., (1972) Nature(New Biol.) 240:214

Jose D.G., Stutman O., Good R.A., (1974) Transplantation 17:211

Kabat, E.A., (1969) "Structural Concepts in Immunology and Immunochemistry" page 238

Katz D.H., and Paul W.E., (1971) J. Immunol. 107:1319

Katz D.H., and Benacerraf B., (1972) Advan. Immunol. 15:1

Katz D.H., and Benacerraf B., (1974) "Immunological Tolerance" Acad.Press

Klaus G.B., and McMichael A.J. (1974) Eur. J. Immunol. 4:505

Klein J., and Park J.M., (1973) J. Exp. Med. 137:1213

Klein J., (1975) "Biology of the Mouse H-2 Complex" Springer-Verlag N.Y.

Konetzki W.S., and Streilein J.W., (1978) Transplantation 25:240

Koopman W.J., Farrar J., Oppenheim J., (1977) J. Immunol. 119:55

Koopman W.J., Farrar J., Fuller-Bonar (1978) Cell. Immunol. 35:92

Kramer J.J., and Grabger G.A., (1972) Cell. Immunol. 3:88

Kramer S.L., and Granger G.A., (1975) Cell. Immunol. 15:57

Kretschmer R., (1969) Pediatric Res. 3:34

Kung J.T., Brooks S., Talmage D., (1977) J. Exp. Med. 146:665

Kuntz M.M., Innes J., Weksler M., (1976) J.Exp. Med. 142:1042

Laffertz K.J., Walter K.Z., Scolly R.G., (1972) Transp. Rev. 12:198

Lapp W.S., and Moller G., (1969) Immunology 17:339

Lapp W.S., Wechsler A., Kongshaun P.A., (1974) Cell. Immuno-. 11:419

Lawrence W., and Simonsen M., (1967) Transplantation 5:1304

Lee S.C., and Lucas Z., (1976) J. Immunol. 117:283

Leonard E., Ruco L., Meltzer M., (1978) Cell. Immunol. 41:347

L'Esperance P., Hansen J.A., Jersild C., (1975) Transp. Proced. 7:823

Lilliehook B., Jacobsson H., (1978) Int. Arch.Aller.Appl.Immun. 56:238

Lilliehook B., Blomgren H., (1978) Scand. J. Immunol. 8:223

Lindholm L.L., and Strannegard O., (1973) Eur. J. Immunol. 3:511

Livnat S., and Cohen I.R., (1975) Eur. J. Immunol. 5:357

Lotzova E., and Cudkowicz G., (1974) J. Immunol. 113:798

Mathe G., Kiger N., Florentin I., (1974) Clin. Immunolbiol. 2:33

Matossian-Rogers and Festenstein H., (1976) J. Exp. Med. 143:456

Matossian-Rogers (1977) Transplantation 23:316

Matossian-Rogers and Festenstein H., (1978) Transpl. proced. 10:11

McBride R.A., (1966) Cancer Res. 26:1135

McKearn T.J., (1974) Science 183:94

McKearn T.J., Stuart F.P., Fitch F.W., (1974a) J Immunol. 113:1876

McKearn T.J., Hamada Y., Fitch F.W., (1974b) Nature(Lond.) 251:648

Merritt C.B., Mann D.L., Rogentine G.N., (1970) J. Exp. Med. 132:181

Miyazaki S., Nomoto K., Takeya K., (1976) Jap. J. Micro. 20:493

Moller G., (1971) Immunology 20:597

Mortensen R.F., Osmand A., Gewurz H., (1975) J. Exp. Med. 141:821

Muchmore A., Decker J., Blaese R., (1979a) J. Immunol. 122:1146

Muchmore A., Decker J., Blaese R., (1979b) J. Immunol. 122:1152

Nabholz M., Young H., Shreffler P., (1974) Eur. J. Immunol. 4:373

Naiman J.L., and Punnett H., (1969) New Eng. J. Med. 281:697

Nagino H., Nomoto K., Takeya (1978) Int. Arch. Aller. Appl. Immu. 56:48

Namba Y., and Eaksman B.H., (1975) J. Immunol. 115:1018

Novogrodsky A., Rubin A.L., Stenzel K., (1979) J. Immunol. 122:1

Oliner H., Schwartz R.S., Dameshek w., (1961) Blood 17:20

Opelz G., Kiuchi M., Terasaki P., (1975) J. Exp. Med. 142:1327

Osband M., and Parkman R., (1978) J. Immunol. 121:179

Parthenais E., Elie R., Lapp W.S., (1974) Cell. Immunol. 13:164

Parthenais E., and Lapp W.S., (1978) Scand. J. Immunol. 7:215

Pena-Martinez J., Huber B., Festenstein H., (1979) Transpl. Proced. 5:1393

Pickel K., and Hoffman M.K., (1977) J. Immunol. 118:653

Piessens W.F., (1978) Cell. Immunol. 35:306

Piguet P.F., Vassalli P., (1977) Cell. Immunol. 31:242

Ponzio N.M., Finke J.H., Battisto J., (1975) J. Immunol. 114:971

Ptak W., Hanczakowska M., Skowron-Cendrzak, A., (1975) Transplantation 19:12

Raff M.C., (1969) Nature (Lond.) 224:378

Ramseier H., (1973) Curr. Topic Micro. Immunol. 60:31

Ramseier H., and Billingham R.E., (1966) J. Exp. Med. 123:629

Ramseier H., and Lindenmann J., (1969) Pathol Micro. 34:379

Rapaport F.T., Watanaba K., Cannon F.D., (1972) J Exp. Med. 136:1080

Rapaport F.T., Ozaki A., Cannon F.D., (1973) Transpl. Proced. 5:845

Rehn T.G., Shearer G.M., Inman K., (1976) J. Exp. Med. 143:127

Reinherz E.L., Parkman R., Rapperport J. (1979) New Engl.J.Med. 300:1061

Riger M., and Hilgert, (1977) J. Immunogenetics 4:61

Rocklin R.E., (1978) "Basic and Clinical Immunology" LANGE series

Rodey G.E., Bortin M.M., Bach F.H., (1974) Transplantation 17:84

Rolstad B., (1976) Transplantation 21:117

Rosen R.C., Huestis D.W., (1978) J. Pediatrics 93:268

Ruddle N.H., and Waksman B.H., (1968) J. Exp. Med. 128:1267

Russel P.S., (1960) Ann. N.Y. Acad. Sci. 87:445

Safford J.W., and Tokuda S., (1970) Proc. Sco. Exp. Biol. Med. 133:651

Schendel D.J., Atler B., Bach F., (1973) Transpl. Proced. 5:1651

Schimpl A., and Wecker E., (1973) J. Exp. Med. 137:547

Schirrmacher V., Pena-Martinez J., Festenstein H., (1975) Nature 255:155

Schmitt-Verhulst A., and Zatz M.M., (1977) J. Immunol. 118:330

Schwartz R.S., and Beldothi L., (1963) Science 140:171

Scott D.W., and Ornellas E.P., (1974) Cell. Immunol. 11:116

Scott M.T., (1972) Cell. Immunol. 5:469

Sethi K.K., Teschner M., Brandis H., (1974) Infect. Immun. 10:960

Sethi K.K., and Brandis H., (1975) J. Natl. Cancer Inst. 55:393

Shand F.L., (1975) Immunology 29:953

Shand F.L., (1976) Immunology 31:943

Shand F.L., (1977) Eur. J. Immunol. 7:746

Shearer G.M., Yung Y.P., Cudkowicz G., (1978) Cell. Immunol. 39:61

Shearer G.M., and Schmitt-Verhulst T.G., (1977) Cold Spring Harbor Symp. Quant. Biol. 41:511

Shearer G.M., (1975) Science 190:890

Shearer G.M., Waksal H., Cudkowicz G., (1976) Transpl. Proced. 8:469

Shortman K., and Palmer J., (1971) Cell. Immunol. 2:399

Shreffler D.C., and David C.S., (1975) Adv. Immunol. 20:125

Simonsen M., and Jensen E., (1959) "Biological Problems of Grafting" Ed. Medawar, Blackwell, Oxford.

Simonsen M., (1957) Acta Pathol. Micro. Scand. 40:480

Simonsen M., (1962) Prog. Allergy 6:349

Simonsen M., (1967) Cold Spring Harbor Symp. 32:517

Simpson E., Mosier D., Cantor H., (1974) Immunology 27:989

Smith D.E., Fischer L., Hard R.C., (1977) Fed. Proced. 36:1082

Singh J.N., Sabbadini E., Sehon A.H., (1971) Transplantation 11:475

Singh J.N., Sabbadini E., Sehon A.H., (1972) J. Exp. Med. 136:39

Singh J.N., Sabbadini E., Sehon A.H., (1973) Cell. Immunol. 8:280

Siskind G., Leonard L., Thomas L., (1960) Ann. N.Y. Acad. Sci. 87:452

Sjoberg O., (1972) Clin. Exp. Immunol. 12:365

Solliday S., and Bach F.H., (1970) Science 170:1406

Sprent J., and Miller J.F., (1972a) Cell. Immunol. 3:385

Sprent J., and Miller J.F., (1972b) Cell. Immunol. 3:213

Sprent J., (1976) Cell. Immunol. 21:278

Steele E.J., and Cunningham A.J., (1978) Nature 274(5670):483

Steinmuller D., (1967) Science 158:127

Streilein J.W., (1972) J. Exp. Med. 135:567

Streilein J.W., and Billingham R.E., (1967) J Exp. Med. 126:455

Streilein J.W., and Billingham R.E., (1970a) J Exp. Med. 132:181

Streilein J.W., and Billingham R.E., (1970b) J Exp. Med. 132:163

Streilein J.W., and Stone M.J., (1973) Transpl. Proced. 5:861

Streilein J.W., and Duncan W.R., (1975) J. Immunol. 111:952

Svedmyr E.A., and Hodes R.J., (1970) Cell. Immunol. 1:644

Thomas E.D., Storb R., Buckner C.D., (1975) N. Eng. J. Med. 292:832

Treiber W., and Lapp W.S., (1973) Transplantation 16:211

Treiber W., and Lapp W.S., (1976) Transplantation 21:391

Treiber W., and Lapp W.S., (1978) Cell. Immunol. 37:118

Trentin J.J., (1956) Proc. Soc. Exp. Biol. Med. 92:688

Uhr J.W., and Finkelstein M.S., (1967) Prog. Aller. 10:37

Uhr J.W., and Moller G., (1968) Advan. Immunol. 8:81

Urso P., and Gengozian N.T., (1977) J Immunol. 118:657

Van Beckum D.W., (1975) Transpl. Proced. 7:805

Vrubei J., (1961) Nature(Lond.) 189:853

Waksman B.H., and Namba Y., (1976) Cell. Immunol. 21:164

Walker S.M., and Lucas Z.J., (1972) J. Immunol. 109:1233

Warner J.F., and Cudkowicz G., (1979) J. Immunol. 122:575

Weiss L., Aisenberg A.C., (1965) J. Cell. Biol. 25:149

Weiss A., and Fitch F.W., (1977) J. Immunol. 119:510

Wells J.V., and Ries C.A., (1978) "Basic & Clinical Immunology" LANGES Series, page 390

Williams T.W., and Granger G.A., (1969) J. Immunol. 102:911

Woodland,W.T., and Wilson, D.B., (1977) Eur.J. Immunol. 7:136

Wolters E.A., and Benner R., (1978) Transplantation 26:40

Yachnin S., (1975) J. Exp. Med. 141:242

Yamashita A., Miyamoto M., Kitawaki Y., (1978) Transplantation 26:56

Yung Y.P., and Cudkowicz G., (1977) J. Immunol. 119:1310

Zakarian S., and Billingham R.E., (1972) J. Exp. Med. 136:1545

Zaleski M., and Milgrom F., (1973) Cell. Immunol. 7:268

Zinkernagel R., and Doherty P., (1974) Nature (Lond) 248:701

Zinkernagel R., and Doherty P., (1975) J. Exp. Med. 141:1427

Zinkernagel R., (1978) Arch. Intern. Med. 138:1326

Zinkernagel R., (1978) Fed. Proced. 37:2379