THE EFFECT OF SILICA ON THE IMMUNE RESPONSE

by

Albert Falk, B.A.

A Thesis submitted to the Faculty of Graduate Studies, University of Manitoba, in partial fulfillment of the requirements for the degree Master of Science.

Department of Immunology, Faculty of Medicine.

August 1978

THE EFFECT OF SILICA ON THE IMMUNE RESPONSE

BY

ALBERT WILHELM FALK

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Acknowledgements:

I am indebted to Dr. Sabbadini whose patience, understanding and guidance was "above and beyond the call of duty".

I would like to thank Dr. Martin Davies for constructively criticizing my results.

Special thanks to Cindy for assisting with the typing.

This project was funded by grants from the Medical Research Council awarded to Dr. E. Sabbadini.

TABLE OF CONTENTS

TABLE OF CONTENTS Cont.

LIST OF ABBREVIATIONS USED IN THE TEXT

CMC	- Cell mediated cytotoxicity
T cells	- Thymus derived cells
B cells	- Bone marrow derived cells
ACT	- Tris buffered - isotonic ammonium chloride
GVH	- Graft versus host
PFC	- Plaque forming cell
SRBC	- Sheep erythrocyte cells
PUPNO	- Poly vinyl pyridine - N - oxide
FUdR	- 5 - fluoro - 2' deoxyuridine
^{125}IUdR	- ^{125}I labelled 5 - Iodo - 2' deoxyuridine
N.D.	- Not determined
PPD	- Purified protein derivative
DNP-GPA	- Dinitrophenyl guinea pig albumin
DNP-OVA	- Dinitrophenyl ovalbumin
^{3}H-IdR	- Tritiated thymidine
PEC	- Peritoneal exudate cells
PEL	- Peritoneal exudate lymphocytes
GRF	- Genetically related macrophage factor
NMF	- Non specific macrophage factor
RES	- Reticulo endothelial system
ALS	- Anti lymphocyte serum
LU	- Lytic Unit

LIST OF FIGURES

List of Figures cont.

LIST OF TABLES

INTRODUCTION

Involved in the immune response to an antigen, are several types of cells which, although different in ontogenetic origins, all arise from the same basic precursor: a bone marrow stem cell. These immunocompetent cells are specifically localized anatomically yet they are thought to participate co-operatively in the response to an antigen or antigens. T cells, or thymus derived cells are lymphocytes that originate in the bone marrow and pass through the thymus where they mature. B cells are also lymphocytes arising from the bone marrow. They were named "B" cells because in the chicken, where they were studied extensively, these cells pass through the Bursa of Fabricius where they mature into precursors of antibody forming cells. A mammalian organ homologous to the Bursa of Fabricius has not been found, thus B cells are generally thought of as bone marrow derived. The thymus and bone marrow are considered to be the primary lymphoid organs; the lymphocytes upon maturation move into the "secondary lymphoid organs". T cells localize in the paracortical regions of the lymph nodes and around the arterioles of the splenic white pulp. B cells are in the cortex and germinal centers of the lymph nodes and surround the areas inhabited by the T cells in the spleen. B cells form antibodies in response to foreign antigens; to do this they often require not only the presence of T cells but a third cell as well; a glass adherent cell. B cells mediate humoral responses while T cells mediate cellular immunity.

The adherent cell is thought to be of the monocyte series which also arise from the bone marrow and consist largely of phagocytic monocytes and macrophages. These cells remove debris and foreign matter and are found in most tissues of the body. Higher concentrations are found in the lungs, the

liver, spleen and peritoneum.

Considerable evidence for the three cell system involving the macrophage and lymphocytes exists, particularly in humoral immunity. Macrophages have also been implicated in cell mediated reactions involving the synergistic interaction of T cell sub-populations. A more detailed breakdown of T cell ontogeny will therefore be given, as well as the functions of the various sub-populations. B cell and macrophage ontogeny will also be covered but in less detail.

The answer to the question, of which are the respective functions of the different cells involved in the immune response might be more readily accessible if one could selectively remove a given cell type. The present thesis deals with the influence of host macrophages in two different immune reactions involving the transfer of cells into lethally irradiated hosts, i.e. the graft-versus-host reaction induced by parental spleen cells and the plaque forming cell (PFC) response developed by syngeneic spleen cells injected together with sheep red blood cells. To study this influence, it was considered necessary to remove or inactivate host macrophages. A review of the literature revealed that removal of macrophages in in vitro experiments has been successful, but in vivo evidence is still inconclusive and suggests that only partial inactivation of macrophages can be obtained. The effects of various macrophage toxins are discussed in the introduction and in particular the effects of silica are noted. Silica has been implicated as a macrophage toxin in a variety of systems, the first indication deriving from studies on human silicosis and thus is known to be effective in vitro and, at least to some degree, in vivo. Because of these promising features, its effect was studied in this thesis. It was realized, however,

that the isolated investigation of the effects of silica on macrophage function was not sufficient, since silica may affect the immune response through other mechanisms. This was the reason for studying, in addition to macrophage functions, as measured by the phagocytic index, the capacity of lymphocytes to migrate and proliferate in the spleen of silica treated hosts.

The toxic effects of silica on macrophages were confirmed by this investigation. However, the effect of silica on cell mediated cytotoxicity and on the PFC response turned out to be more complex than expected, inasmuch as cell mediated cytotoxicity was not significantly affected by this treatment and the PFC response was actually stimulated.

T CELLS

Thymus lymphocyte (T cell) maturation, location and general function is fairly complex. As was indicated, thymus lymphocytes originate from stem cells in the yolk sac and liver in embryonic life (Moore & Owen 1967, Owen & Ritter 1969), and from the bone marrow in adult life (Ford, et al, 1971). Stem cells or their progeny (pre-thymic lymphocytes) migrate to the thymus where the influence of the thymus epithelium causes them to proliferate and differentiate into thymus lymphocytes. Immature stem cells lack the normal surface antigens or markers of mature lymphocytes such as theta or TL; these are acquired during maturation from stem cell to thymic lymphocyte (Owen et al, 1971).

T cells are classified according to different criteria. One of the first classifications proposed was based on their recirculation properties (T_1-T_2). This classification is being abandoned because it does not always correspond to the functions of the cells. More recently T cells have been classified according to antigenic markers like Ly 1,2,3, etc. or to their immunological functions (T_H= helper etc.). The various classifications will be discussed in some detail.

T_1-T_2 classification:

Lymphocytes after leaving the thymus are broadly classified into two groups; a more mature long lived cell that recirculates in the periphery (T_2), and a non-recirculating cell which is generally thought to be short lived and localizes in the secondary lymphoid organs, the T_1 cell. The entire

maturational picture, from stem cell to semi-mature and mature thymus lymphocyte remains somewhat in doubt. Since within the thymus are found immature cells, as well as a small population of lymphocytes with characteristics very similar to those of peripheral T lymphocytes, it is tempting to propose that complete maturation from stem cell to mature T cell occurs within the thymus. However, many of the cells leaving the thymus do not become a part of the recirculating pool of lymphocytes (Davies et al 1971). It thus is possible that two lines of differentiation from stem cell to thymic lymphocyte and to mature T cell respectively, exist within the thymus. Although it may be difficult to conclusively show whether two separate lines exist, or whether the different thymocyte types are at different maturational levels, in this presentation the two major T cell types of the thymus are referred to as "mature" and "immature", respectively, according to Raff and Cantor (1970). The cells are most easily distinguished on the basis of antigenic markers on the surfaces of the cell membranes. The table shown below gives a brief summary of some of the characteristics of the immature and mature T lymphocytes as found within the thymus.

Table I

Properties of Thymic Lymphocytes

Property	Immature Thymus Cell	Mature Thymus and Peripheral T cells	Reference
Surface Antigens:			
TL	+	-	1 & 2
theta	++	+	3
H-2	+	++	4
MPLA	-	+	5

MPLA = mouse specific peripheral lymphocyte antigen.
References: 1 - Leckband 1970; 2 - Raff 1970 (a); 3 - Raff & Owen 1970; 4 - Raff 1971 (a); 5 - Raff & Cantor 1971.

(The table was compiled by Raff & Cantor 1971).

In addition to the above, immature thymocytes are very sensitive to corticosteroids (Schlesinger 1965), do not possess immunological competence (Blomgren & Anderson 1969, Leckband 1970, Anderson & Blomgren 1970), nor do they bind antigen (Raff & Cantor 1971).

Along with the knowledge that T cells are involved in cell mediated as well as humoral immune responses has come evidence that several T cell subpopulations may exist. This has become expecially apparent in studies of the graft versus host response. Cantor & Asofsky (1970), showed that two types of lymphoid cells obtained from parental donors responded synergistically in the F1 host. The graft versus host activity of spleen cells could be reduced considerably by small doses of anti lymphocyte serum (ALS) given several days prior to testing. However, addition of only a small number of peripheral blood lymphocytes restored the activity to a much greater degree than would have been expected on the basis of the number of cells added alone (Cantor & Asofsky 1971 (b)). Further indication that both cell types are T cells is given by the fact that both types of cells are diminished after neonatal thymectomy, (Cantor & Asofsky, 1971 (b), 1970), and are inhibited by anti theta serum (Cantor 1971 (a)).

Additional support for the above postulates come from Cantor and Simpson (1975) who showed that <u>in vitro</u> production of cytotoxic effector cells from peripheral T cells involves cells that differentiate to killer cells (pre-killer) and a second peripheral T cell population that regulates the degree of differentiation of pre-killer cells. Pre-killer cells were removed by anti thymocyte serum (ATS) and this left the ATS resistant (Thy 1 2^{+}, to be discussed later) spleen cell population. Some of the properties of the two different peripheral populations (referred to as T1 and T2) are shown below. (Table II).

Table II

Peripheral T cell Subpopulations

Property	T_1	T_2
Surface antigen		
TL	-	-
theta	++	+
MPLA	+	+
Peripheral lymphoid tissue of highest concentration.	spleen	lymph nodes, blood, thoracic duct
Recirculation	No	Yes
Sensitivity to ALS *in vivo*	+	+ + + +
Removal by thoracic duct drainage.	No	Yes
Effect of adult thymectomy	in 2-6 weeks	after 30 weeks
Migration *in vivo*	to spleen lymph nodes	to LN spleen

(Raff & Cantor 1970)

T_1 cells are generally thought to be less mature than T_2 and mature to become T_2 cells in response to specific antigen. T_1 cells do not respond actively to antigen except to mature T_2 cells. They also appear to be generally shorter lived; they may be continuously replaced from the thymus, or dependent on a thymus humoral factor (Raff & Cantor 1970).

The TL - Ly Series:

It appears that at the earliest stage of maturation all thymus lymphocytes possess at least the TL (Thymus leukemia) antigen, as is shown in

Table I.* They also possess, (initially at least) a marker of the Ly series ie. (TL^+ Ly 123^+). The Ly series consists of several systems. However only Ly-1, 2, and 3 are discussed here, because only these three antigenic markers have been clearly related to immunological functions of T cells. Ly-2 and Ly-3 are under closely linked genetic control and are always found together on the cell surface. Therefore, they behave as a single antigen. The resulting possible phenotypes are Ly 123^+, $Ly1^+$ (i.e. Ly $2,3^-$) and Ly 23^+ (Ly 1^-), respectively. Ly 123^+ appears to be an immature cell type appearing in early neonatal life. This type if fairly short lived and is selectively depleted shortly after adult thymectomy. The other two subclasses, Ly 1^+ and Ly 23^+ develop in later life and are not depleted shortly after adult thymectomy (Cantor & Boyse 1975a).

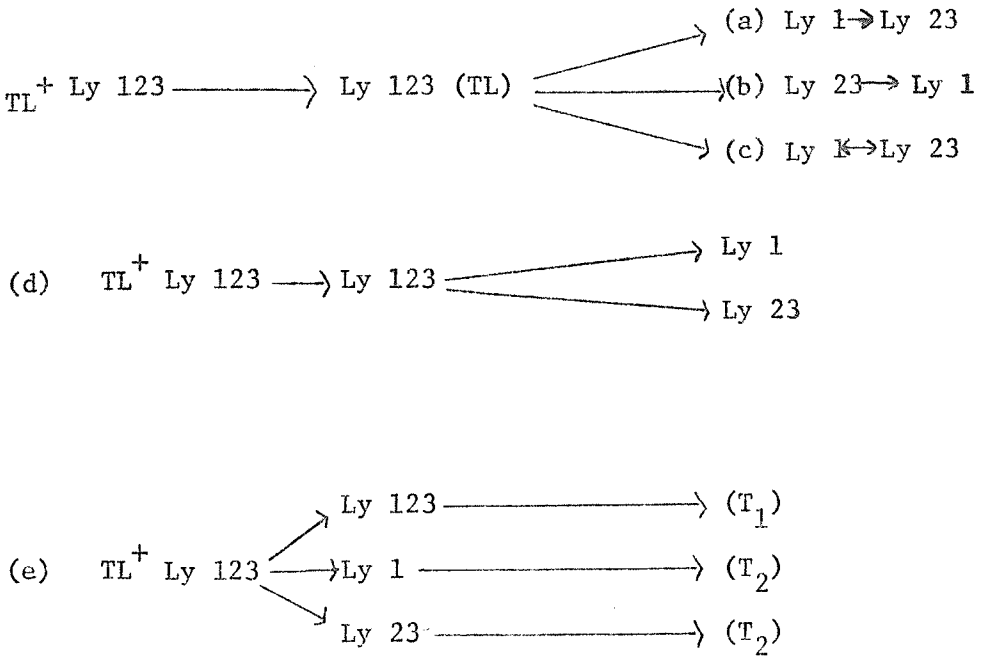
With respect to immunological functions, Ly 1^+ and Ly 23^+ both recognize alloantigens of the major histocompatibility complex, but only Ly 23^+ cells develop killer activity (Cantor & Simpson 1975). It appears now, that the development of killer activity by Ly 23^+ cells is amplified by Ly 1^+ cells, but that the presence of Ly 1^+ is not essential. Ly 1^+ cells also provide helper activity during primary antibody responses (Cantor & Boyse 1975 a). Further differences between Ly 1^+ and Ly 23^+ are: Ly 1^+ (amplifier) cells respond to Ia determinants, whereas Ly 23^+ cells respond to K and D determinants of the H-2 or major histocompatibility complex. TL + Ly 123^+ cells may generate three (TL^-) subclasses of T cells, Ly 1^+, Ly 23^+ and Ly 123^+ as a normal differentiative process independent of exposure to antigen (Cantor & Boyse 1975b).

*This statement is valid only for TL^+ strains. Other mouse strains do not express the TL antigens on normal thymocytes. Such antigens, in this case, appear only on leukemic cells (TL^- strains).

The Theta Antigen:

The theta antigen (Thy 1) is an additional marker of T cells which may be used in identification of T cell subclasses. Fluorescent anti Thy-1 antibody binds to the cells expressing that antigen on the cell membrane and the subclasses are identified by the degree of fluorescence which is determined by the fluorescence activated cell sorter (FACS). Cells are classified as "bright" or "dull" depending on the amount of fluorescence. Bright T cells and dull T cells have differing functional properties similar to those ascribed to T_2 and T_1 cells. The bright T cells appear to be analogous to T_1 cells, being found in greater numbers in the spleen rather than the lymph nodes; they are also relatively insensitive to anti-lymphocyte serum (ALS), home preferentially to the spleen in irradiated hosts and are lost more rapidly after thymectomy in adult life. Dull T cells on the other hand localize primarily in the lymph node, are anti lymphocyte serum sensitize, migrate preferentially to the lymph nodes, and are not substantially affected in the first several months after adult thymectomy (Cantor et al, 1975). In neonatal life the high proportion of bright T cells lends support to the theory that T_1 differentiate to T_2 cells. The two cell types (bright and dull) also act synergistically in cytotoxic reactions.

Huber et al, (1976), have developed several models of T cell maturation and differentiation. The early models proposed a simple three step pattern using only the "T" nomenclature, where T_0 is the initial immature cell which matures to T_1 and T_2, i.e. $T_0 \longrightarrow T_1 \longrightarrow T_2$. The more recent models assumes that TL^+ Ly 123 is the precursor of the various T cells found at any given time. The following five models are possible.



Model "d" is favoured since Ly 123 cells when antigenically stimulated appear to mature to Ly 23 cells and also because animals reconstituted with Ly 1 and Ly 23 respectively after irradiation, do not acquire any other Ly T cell types. This suggests separate lines of differentiation and not sequential stages of a single differentiative pathway (Huber et al 1976).

A more recent classification of T cells separates the various groups on the basis of immunological function; four major categories being cited (Snell 1978). The initiator lymphocytes (T_I) are defined as those cells from the thymus or spleen which, after having been sensitized to alloantigen, recruit other isogenic T cells to produce cell mediated lysis of appropriate targets. This is considered to be the initial act in the chain of responses touched off after exposure to antigen (Snell 1978). These cells resemble the T_1 cells of Raff and Cantor (1970). Helper lymphocytes (T_H) participate in most immune responses, cellular

and humoral. They participate in antibody formation by B cells, in the generation of cytotoxic T cells, and are also responsible for most of the activity in the mixed leukocyte response. They most likely are part of the T_2 population described earlier. The nature of cells responsible for delayed type hypersensitivity (T_{DTH}) is not completely clarified. The cytotoxic lymphocyte (T_C) is a lymphocyte with the capacity to cause *in vitro* cell mediated lysis. This is probably the active cell in graft rejection. The suppressor lymphocyte (T_S) can block specifically the humoral and cellular immune response (Zembala & Asherson 1973). These cells are found in mice rendered neonatally unresponsive to skin grafts and may be an agent in classical allograft tolerance (Dorsch & Roser 1977). A summary of the various properties of the above T cell categories is given in Table III (Snell 1978).

TABLE III

Classification of T Cells

Cell	Symbol	Thy-1	ALS	Ly-1	Ly-2,3	Ia[2]	FcR	Life span
Initiator	T_I	High	R[1]	+	+	+	+?	Short
B cell helper	T_{HB}	Low	S	+	-	+	-	Long
T_C cell helper	T_{HC}	Low	S	+	-	+	+	Long
Delayed hypersensitivity	T_{DTH}			+	-	-?		
Effector		Low	S					Long
MHC allogeneic?	T_C			-	+			
Non-MHC?	T_E			+	+	+		
Suppressor	T_S							
Specific?			S	-	+	$1\text{-}J^+$		
Nonspecific?				+	+			

[1] R = Resistant S = Sensitive

Synergy Among T Cell Subpopulations:

The existence of synergism among T cell subpopulations has already been mentioned. Synergy is expecially demonstrable among lymphoid cells mediating the graft versus host response.

It was shown by Cantor, Asofsky & Talal (1970), that spleen cells from three month old, and one year old NZB mice were capable of inducing graft versus host reactions in newborn C57BL/6N mice. Young NZB cells however, were five times as reactive as those from the older mice. Old cells combined with a small number of young cells produced the same effect as that of a large number of the young cells alone; and even mixtures of young and old cells, which on their own produced no detectable reactions, produced good reactions, when injected together. Both cell populations had to be live viable cells. Thus some form of synergy between the young and the old cells must be occurring in these graft versus host type reactions.

Since NZB mice develop a Lupus type of autoimmune disease, their lymphocytes may have abnormal attributes. The experiments shown above were repeated with Balb/C and C57BL/6 normal mice (Cantor & Asofsky 1970). Graft versus host activity was determined by spleen index measurement. The system involved Balb/C parental cells injected into (C57BL/6N Balb/C) F_1 recipients.

Of the two cell types cooperating in the GvH reactions, one is extremely sensitive to anti-thymocyte serum (ATS) in vivo and is probably part of the recirculating lymphoid cell pool whereas the second type, which is found in highest concentrations in the spleen and thymus, is

non recirculating and is insensitive to ATS. (Cantor and Asofsky, 1972). The former appears to be the T_2 cell and the latter is the T_1. The T_1 cell is considered to be the helper cell which supplies the activation of the T_2 to become the killer cell in cytotoxic reactions. The T_2 cell is also most probably the T cell sub-population involved in interacting with B cells in antibody production.

Evidence for distinct localization of the two T cell subclasses has already been given (Cantor and Asofsky 1970 b, Cantor and Asofsky 1972). However this is demonstrated more conclusively by Tigelaar and Asofsky (1973). Lethally irradiated mice were injected with syngeneic normal adult spleen cells. These cells were then recovered from the peripheral lymph nodes and the spleens of the recipients, and tested for the ability to elicit GvH reactions in other hosts. Only when the cells recovered from the spleen were combined in appropriate ratios with those recovered from the lymph nodes, was the normal level of the GvH reaction seen. Since both cell types were sensitive to anti theta serum, it was concluded that normal GvH expression involves interaction of two sub-populations of thymus derived cells with distinctive migratory patterns.

Positive synergism in T cell mediated reactions has been aptly demonstrated. However, of late it has become evident that suppressor mechanisms also operate in T cell mediated immune responses. Evidence for control and regulation of T cell mediated cytotoxicity responses is demonstrated by Sabbadini (1974). That particular system involved the use of tumor cells and skin allografts which were shown to induce the development of strong cell mediated cytotoxicity responses, while intra-

venous injection of allogeneic lymphoid cells stimulated a lower and shorter lived cell mediated cytotoxic reaction. The response in tumor and skin allografted mice was reduced by sublethal doses of radiation while the response in animals intravenously injected with allogeneic lymphoid cells was stimulated by the same radiation doses. This shows that different methods of regulation exist in these two situations but also that the precursors of the cytotoxic lymphocytes are radioresistant, and suggests that some radiosensitive suppressor cells are responsible for the low responses induced by allogeneic lymphoid cells. When irradiated animals were reconstituted with syngeneic thymus or spleen cells (after treatment with allogeneic cells), the level of cell mediated cytotoxicity was again reduced. Precursors of cytotoxic lymphocytes were shown to resist radiation of up to 700 rads while 500 rads were sufficient to knock out the suppressor cell population.

The same doses of radiation reduced the CMC response in animals treated with skin or tumor allografts. However, in this case response was restored with small numbers of spleen or lymphoid cells from syngeneic or semi allogeneic F_1 hybrid donors. The majority of effector cells generated in this manner were shown to be of host origin. This indicates that radiosensitive helper or amplifier cells were necessary for a full response against a skin or tumor allograft.

Further evidence for T cell synergy comes from Babu and Sabbadini (1977), who demonstrated synergism between hydrocortisone resistant and anti-thymocyte serum (ATS) resistant T cells. Lethally irradiated F_1 hybrid mice intravenously reconstituted with parental spleen cells yielded a good

in vitro cell mediated cytotoxicity response against target cells sharing some of the host H-2 antigens. Treatment of parental donors with hydrocortisone resulted in a considerable decrease in their ability to produce the cell mediated cytotoxicity reaction while treatment with anti thymocyte serum almost completely abolished this activity. However, a mixture of the hydrocortisone and ATS resistant cells not only restored the cytotoxic response but amplified it considerably. This synergism was completely abolished by anti theta treatment of either the hydrocortisone resistant or the ATS resistant cell, indicating T cell synergism.

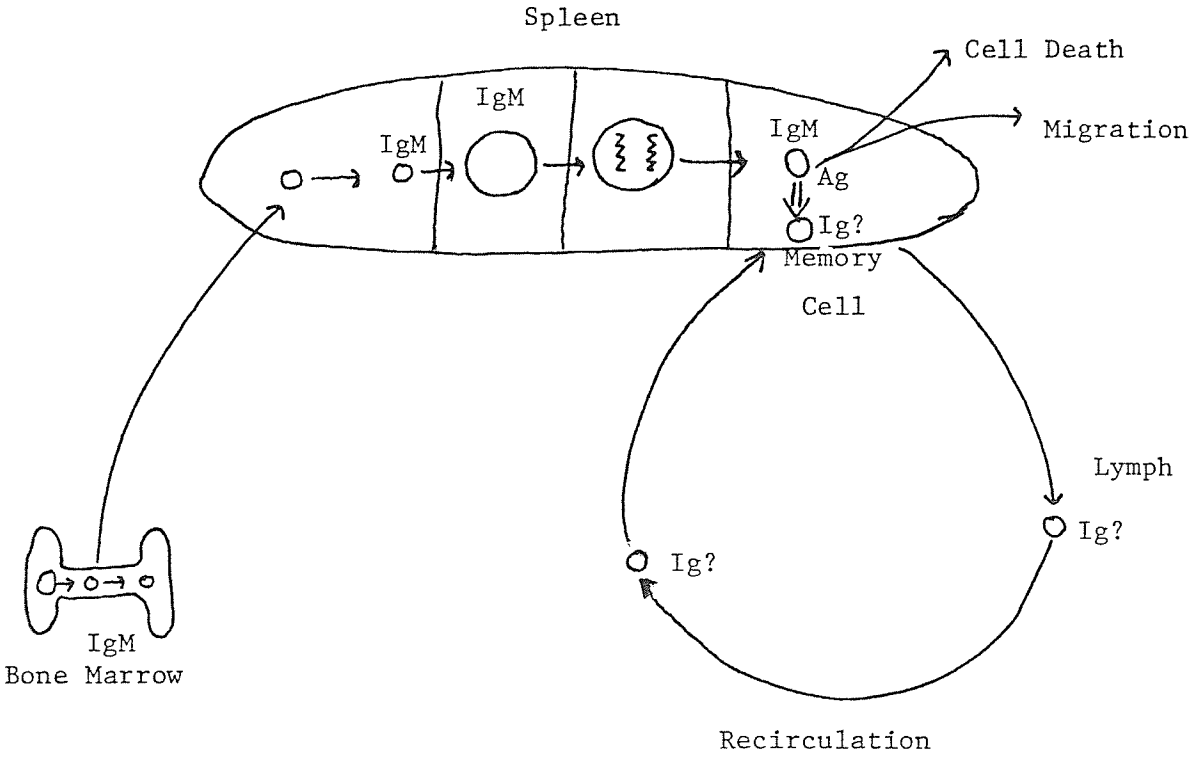
Specifically, the cells involved probably were the T_1 and T_2 cells. The ATS resistant cell probably corresponds to the T_1 population of Raff and Cantor (1971) consisting of non-circulating lymphocytes, while the corticoresistant population likely consisted mostly of precursors of cytotoxic lymphocytes.

B CELLS

Bursa-equivalent (B) lymphocytes are defined as those cells which interact with antigen and differentiate into antibody-secreting cells. The B cell lineage is thought to be derived from hematopoietic stem cells independently of thymic influence (Miller & Mitchell, 1969). These cells differentiate independently of antigen, from the hematopoietic stem cell to the virgin B cell i.e. a cell that has not yet seen antigen.

Virgin B cells are rapidly and continually renewed (about every 48 hours) from a large rapidly dividing bone marrow cell (Osmond and Nossal 1974) that contains no surface immunoglobulin, are associated with the solid lymphoid tissues and do not recirculate from the blood to the lymph, (Strober 1973). These daughter cells develop surface Ig within 36 hours and are thought to be intermediate cells on their way to functionally mature virgin B cells. Functional maturation is thought to occur in the spleen, after which these cells appear as large IgM bearing B cells. Subsequent interactions with antigen results in the production of antibody secreting cells and memory B cells. The turnover rate of these cells is considerably slower than that of the virgin B cells. In addition to the quantity of surface immunoglobulin increasing during maturation, the charge on the cell surface becomes more negative and the adherence of the cells to glass decreases.

A model of B cell development as proposed by Strober (1975) is given below.



Both Ig and non Ig bearing cells in the marrow can migrate to the spleen where they differentiate into large IgM bearing virgin B cells. Antigen independent clonal expansion then occurs producing small virgin B cells with surface IgM and perhaps an additional immunoglobulin class (Ig?). Interaction with antigen results in the production of recirculating memory B cells possessing an additional class of surface immunoglobulin (Ig?).

Triggering, or stimulation of B cells to produce antibody necessarily involves the specific antigen, but the actual interaction of B cells with the antigen or presentation of the antigen by other cells in the presence of various factors to the B cell is still far from being understood. It is accepted, though, that at least one other cell, the T cell is involved in most instances except in some cases where multiple identical determinants on the antigen enable direct B cell stimulation. Even this situation may involve T cell factors (Bretscher 1975). Involvement of an additional cell, the accessory cell or the macrophage, is also likely.

The question that remains to be answered then is how B cells are triggered to synthesize antibody. All the different hypotheses advanced to explain B cell triggering share the one basic assumption; that the Ig receptors are directly responsible for cell activation and deliver the initial triggering signal to the cell. This signal, generated by the combination of the Ig receptor and the antigen is according to some theories insufficient for B cell activation, and alone may result in paralysis. Thus, antigen sensitive B cells do not become active after binding thymus dependent antigens without the participation of several "helper mechanisms". It is these mechanisms which are the bone of contention in the matter of B cell triggering.

A model for B cell induction that fits these general guidelines has been proposed by Bretscher (1975). This model has a built in explanation for B cell tolerance to some antigens - specifically self antigens. It involves two signals to the B cell; the first signal is the result of antigen binding or being presented to receptors on the B cell. This signal alone leads to paralysis, or inactivation of the B cell. When

this signal (1) is followed by binding of associative antibody, (signal 2, which recognizes a different site on the antigen than does the B cell receptor), the B cell is stimulated to produce specific antibody. The associative antibody is of T cell origin but need not necessarily be attached to the T cell for stimulation to occur.

This hypothesis is a continuation of an earlier proposal by Cohn (1973) in which thymus independent antigens do not exist, hence a dominating role in induction of immune responses is ascribed to T cells. Other two-signal hypotheses are similar in design and differ mainly in the nature of the postulated second signal. i.e. Whether it is specific or non specific, but do not clearly propose a mechanism for tolerance induction. A criticism of these models states that they do not explain B cell activation by non specific ligands like mitogens.

Another group of hypotheses instead of postulating two signals for activations, suggests that B cell triggering is dependent on the pattern of antigenic presentation to the cell surface receptors. Macro molecules with repeated epitopes would trigger B cells directly because they would be capable of cross-linking the specific Ig receptors. Molecules that did not possess such a repeated structure would then only be capable of activating B cells if presented in a "locally concentrated" form. These hypotheses (Mitchison, 1971, Möller 1970) all suggest that T cells are active in triggering the B cells because they concentrate the thymus-dependent antigen either directly or via the macrophage surface. The actual triggering signal would be delivered by the Ig receptors when they are sufficiently aggregated or cross-linked.

Theories for non specific triggering of B lymphocytes also exist, the

chief proponents being Coutinho (1975) and Möller (1975). This postulate suggests that a single signal acting at a non specific site is able to stimulate B cells and tolerance is brought about by an excess of activating signal. These theories are at best questionable in the light of the considerable evidence suggesting B cells stimulation requires additional help or factors for most antigens.

MACROPHAGES

Macrophages can generally be classified as large mononuclear cells with a specially developed capacity to phagocytose. They also possess large numbers of lysosomes containing acid hydrolases presumably for use in digestion of phagocytosed material. They are the body's "garburators" i.e. clean up debris and dead cells, and are also important in recognizing foreign particulate antigens. Their main function, however, is protecting the body against foreign microorganisms. Macrophages are present in the blood as monocytes; in the liver, lining hepatic sinusoids (Kupfer cells), in the red pulp of the spleen lining the sinusoids and within lymphoid follicles of the white pulp. In the lymph nodes they line the sinuses and are found within the lymphoid follicles of the cortex; they line the sinusoids of the bone marrow and are also scattered in numerous other tissues like the peritoneum and in inflammatory exudates.

Macrophages, like lymphocytes and red blood cells are ultimately derived from a primitive stem cell within the bone marrow. They appear quite early in the development of vertebrates; much earlier than plasma cells and other elements of the classical antibody system. Maturation and development of the macrophage occurs between the bone marrow, circulation within the blood, and localization in the respective tissues.

Evidence for bone marrow origin of macrophages comes from studies where irradiated animals were injected with allogeneic bone marrow cells. Subsequent examination of the peritoneal macrophages of such chimaeras showed macrophages to be of donor origin (Balner 1963). Other experiments involving culturing of bone marrow cells showed immature cells with a

basophilic cytoplasm and a large folded nucleus termed promonocytes (Van Furth et al 1970) and presumed to be the immediate precursors of the blood monocytes, which do not label and are found in the same culture. Both these cell types and the blood monocytes adhere to glass and are able to phagocytose.

The possible origin of macrophages from lymphocytes, as proposed by Maximow in the middle 1920's, was at one time fairly controversial; however, the purity of the lymphocyte population was at times in doubt and in any case this would account for a very low percentage of the total macrophage population.

Monocytes typically are young cells on their way from their place of origin, the bone marrow, to their ultimate location, the tissues (Van Furth 1970). These cells are essentially non dividing and spend an average time of 32 hours in th e blood (in the mouse) (Van Furth and Cohn 1968) and then migrate to the tissues to become liver macrophages (Kupfer cells), peritoneal macrophages, lung macrophages or skin macrophages. Macrophages generally appear to be non-dividing long lived cells, but they are capable of dividing under certain conditions. Alveolar, skin, and liver macrophages divide very rarely but peritoneal macrophages are known to undergo extensive mitosis in response to reinjection of antigen in immunized mice. Up to 50% or more of peritoneal macrophages immunized with bovine serum albumin or ovalbumin in adjuvant incorporated tritiated thymidine 20 to 30 hours after a second injection of the same antigen (Forbes & Mackaness 1963). This indicates that although the macrophage may be at the end of a cell line, it is still capable of self replication. Macrophages are also capable of fusing to become giant cells in wound healing and chronic inflammation (Gillman & Wright 1966 a).

A simple model of macrophage origin and maturation is given below.

Bone Marrow	Peripheral Blood	Tissues
Precursor - promonocyte - monocyte ——→	monocyte ——→	peritoneal macrophage
	——→	Alveolar macrophage
	——→	Kupfer cell
	——→	skin macrophage

Macrophage - Lymphocyte Interaction:

The macrophage has been implicated in many varied reactions in the immune system. Aside from being a phagocytic cell removing debris, it is a cytotoxic cell and probably involved in the presentation of antigen which has been phagocytosed to lymphocytes in order to stimulate them. Evidence for macrophage influence in lymphocyte maturation, and in lymphocyte viability also exists. Following, is a short summary of macrophage - lymphocyte interaction, expecially with reference to immune response induction, in particular cell mediated reactions involving thymus lymphocytes.

Evidence for physical interaction between lymphocytes and macrophages or at least physical approximation during cultivation of these cells is given by Lipsky and Rosenthal (1973) and has been shown by numerous other authors as well (Cline and Swett, 1968; Mosier 1969; Siegel 1970). Examination of fixed preparations of lymphoid organs has supported the above (Andre-Schwartz 1964; Miller & Avrameas 1971).

This interaction can be demonstrated in the intact animal undergoing immunization, where blasts and lymphocytes are seen clustering about macrophages (Miller and Avrameas 1971), as well as in vitro induction of primary responses (Mosier 1969; Siegel 1970; Miller & Avrameas 1971). It is demonstrable even in in vitro proliferation of immune lymphocytes (Bartfeld and Kelly 1968, Cline and Swett 1968; Hanifin and Cline 1970), where antigen laden macrophages appear to be in direct contact with the immune lymphocytes. Specificity of this interaction is demonstrated by Lipscomb et al (1977). They obtained peritoneal exudate lymphocytes from immune guinea pigs; and cultured them one week on antigen pulsed autologous macrophages. When tested later for the ability to bind autologous macrophages which were freshly antigen pulsed, or macrophages pulsed with an irrelevant antigen, 30% bound relevant antigen pulsed macrophages whereas only 2-5% bound the irrelevant macrophages. This specific binding occurs after one hour. Lymphocyte-macrophage binding in the presence of antigenic stimulation is usually shown as cell clusters, with a central macrophage, and numerous lymphocytes clustered around it. This has been shown in the mouse (Miller & Avrameas 1971; Mosier 1971) and in rabbits. (Sulitzeanu & Kleinman 1971).

It has also been proposed by Siegel (1970) that non specific binding, i.e. in the absence of antigenic stimulation, also occurs and may be involved in maintaining lymphocyte viability (Chen and Hirsch 1972; Lipsky and Rosenthal 1975a) or promoting maturation of thymocytes (Mosier and Pierce 1972).

Binding between macrophages and lymphocytes requires viable macrophage populations, but not necessarily live lymphocytes, (Lopez et al. 1977).

Formalin treated thymocytes were bound by macrophages but irreversibly and eventually were phagocytosed. Living thymocytes bound reversibly and species specifically; maximum binding occurred at about one hour, then gradually diminished. Thymocytes when released do not bind again whereas macrophages will. Attachment of lymphocytes occurs in two stages, one a non-specific and quickly reversible stage; the other an antigen dependent more prolonged stage (Lipsky & Rosenthal 1975 a,b). The work of Lopez et al (1977) supports the earlier observation of Mosier and Pierce (1972), that binding of thymocytes to macrophages may assist in thymocyte maturation. Macrophages thus appear to possess a unique ability to recognize and bind lymphocytes.

After its uptake by macrophages, antigen is mostly degraded, but some few molecules remain associated with the surface membrane for a finite period in a form resembling the native molecules (Unanue et al 1969; Unanue & Cerrotini 1970). These surface molecules are considered to be immunogenic (Unanue and Cerrotini 1970; Schmidtke and Unanue 1971). This was shown by radiolabelled antigen cultured with macrophages where 3-7% of the protein antigen was associated with live macrophages (Calderon & Unanue 1974). Some of this bound antigen was immunologically similar to the original antigen. The antigen was probably internalized as trypsinization did not affect it. Further evidence for internalization of antigen by the macrophage comes from Ellner et al (1977) who showed that brief exposure of guinea pig macrophages to antigen at 37^{o} was sufficient to trigger proliferation and lymphokine production on subsequent co-culture with primed T cells. Again trypsinization had no effect nor did a high concentration of antibody directed against the antigen mediating the interaction.

Thus, it would appear that this macrophage associated antigen relevant to T lymphocyte activation is not located on the macrophage cell surface but in a restricted compartment accessible to the T lymphocyte and cannot be blocked by specific antibody or removed by trypsin. According to Calderon and Unanue (1974), this small pool of intracellular antigen is slowly catabolized and small amounts are slowly released into the cell exterior.

Waldron et al (1974), showed that macrophages incubated at 4^{o} with antigen and then trypsinized had a lower immunogenicity than did macrophages incubated at 37^{o} before trypsinization or incubated at 4^{o}, then at 37^{o} and then trypsinized. The degree of immunogenicity became progressively greater as the length of incubation at 37^{o} before trypsinization was increased. This suggests that macrophage processing of antigen involves an initial step of membrane binding, then subsequent metabolic dependent sequestration of bound antigen (Waldron et al. 1974). Waldron et al.(1973), also showed that for antigen induced lymphoproliferative responses in vitro, direct interaction of thymus derived lymphocytes with antigen does not occur to any significant extent. Using tritiated thymidine (^{3}H TdR) incorporation as a measure of antigenic stimulation, immune lymph node lymphocytes were incubated with the immunogen, purified protein derivitive (PPD). This did not elicit any response, while phytohemagglutinin induced cell proliferation. Addition of macrophages to lymphocytes which had been preincubated with PPD also resulted in little activation. When macrophages were preincubated with PPD and then added to the lymphocytes, the lymphocytes responded to the same extent as lymphocytes in continuous culture with PPD and macrophages. Macrophages thus seem to play an obligatory role in presentation of antigen to an immunospecific T cell. Additional evidence

for the role of the macrophage in stimulating the immune reactions is shown by Cohen (1973a). Guinea pigs were sensitized to the hapten carrier conjugate, dinitrophenyl guinea pig albumin (DNP-GPA). Peritoneal exudate cells (PEC) consisting largely of macrophages (80% macrophages, 10% lymphocytes and 10% polymorphonuclear leucocytes), and peritoneal exudate lymphocytes (PEL (95-98% lymphocytes)) were then cultured in vitro, restimulated with DNP-GPA and the incorporation of ^{3}H-TdR was used as an index of the immunological activity produced by PEL. Stimulation of the PEL cells could be inhibited by preincubation of the PEC population (treated with mitomycin) with DNP-OVA. This suggests the inhibition is due to competition for a hapten specific cytophilic antibody on the macrophage surface, and not to competition for a hapten specific receptor on T lymphocytes as was once thought (Cohen 1973a).

Antigen binding by macrophages is accomplished by one of two mechanisms; (1) sites of low avidity for antigen exist which are effective only at relatively high antigen concentrations and are not immunoglubulin. These sites dominate at high antigen concentration for both immune and non-immune peritoneal exudate cell populations. (2) Cytophilic antibody, which in hapten carrier systems is hapten specific and of high avidity; is present on peritoneal exudate cells from immune animals and is the primary means by which small amounts of antigen are concentrated by immune macrophages to stimulate lymphocytes (Cohen, Rosenthal & Paul 1973).

In the response of B lymphocytes to antigen, affinity of the B cell receptors for the antigen varies considerably. During a prolonged response the relative size of the subpopulations with higher affinity receptors increases, which results in a general increase in the average affinity of the responding B lymphocytes (Davie and Paul 1972). This is brought

about as a result of selective stimulation of percursor cells with high affinity receptors (Davie and Paul 1973). These high affinity cells producing high affinity antibody cause a general progressive increase in the affinity of serum antibody (Eisen & Siskind 1964; Siskind & Benacerraf 1969). A similar situation possibly exists in T lymphocytes but since T cell receptors are very hard to isolate, this has been impossible to demonstrate conclusively. It has been proposed by Möller et al. (1973) that minor changes in the affinity of T cell receptors do occur in the course of the immune response. Bast (1971) and Benezra (1971) suggest an increase in sensitivity to low level antigen concentration occurs. However, Cohen and Paul (1974) claimed that the increase in sensitivity occurs in the macrophage population as opposed to the T cell receptor. They showed that purified peritoneal exudate lymphocytes from animals sensitized to antigen 21 days prior to culture developed a greater response to antigen than did lymphocytes immunized 7 days prior to culture. This was especially evident at low levels of antigen. This increase in efficiency of the "21 day" lymphocytes appears to be the result of an increase in the efficiency of macrophages in presenting antigen to T lymphocytes. The increase in macrophage efficiency as suggested by Cohen et al. (1974) is the result of an increase in number and/or affinity of macrophage associated cytophilic antibody. This allows the macrophage to bind larger amounts of antigen when antigen levels are low, which then allows more efficient T lymphocyte stimulation.

Macrophage involvement in T lymphocyte activation has been considered in some detail above; macrophages may also be involved in activation of unprimed T cells by soluble protein antigens, to become helper cells (Erb & Feldmann 1975 a&b). However, certain genetic restrictions exist

for effective T cell macrophage interaction. More specifically macrophage T cell interaction in helper cell induction using soluble antigens is controlled by the I-A region of the H-2 complex i.e. the major histocompatibility complex of the mouse (Erb & Feldmann 1975a). Yamashita & Shevach (1977) indicated that macrophages in fact carried Ia antigens, by showing that 15-25% of oil induced macrophages could be lysed by anti Ia serum and complement. The remaining subpopulations remained matabolically intact but could not present antigen to T lymphocytes efficiently and was unable to function as stimulator cells when mixed with allogeneic T cells in a mixed leukocyte reaction. This "Ia" carrying subpopulation of macrophages thus appears important in T cell stimulation.

Additional information on the role of macrophages in the generation of T helper cells is shown by Erb et al. (1977). Two factors, genetically related macrophage factor (GRF) and a non-specific macrophage factor (NMF) appear to be involved. The T cell interacting with I region products in induction of mouse helper cells in vitro is a short lived T cell with the Ly $1^+2^+3^+$ membrane phenotype. This cell is not a helper cell precursor, but amplifies the induction of helper cell precursors (Feldmann et al. 1977, Feldmann & Erb 1977). As was mentioned earlier, a difference exists between T helper cell induction with soluble and particulate antigens. Soluble antigens require I region compatibility between the T cell and the macrophage (Erb & Feldmann 1975a) whereas particulate antigens have no similar genetic restrictions (Erb & Feldmann 1975 b&c).

Helper cell induction also does not require physical interaction between the macrophage and T Cell. The NMF factor released when macrophages are incubated without the presence of antigen causes T helper

cell induction with particulate antigens in culture. The GRF obtained from macrophages incubated with antigen induced T helper cells only when the macrophage source of GRF and T cells are similar. The GRF appears to be a complex of the IA antigen and a fragment of the immunogen, about 50,000 or 60,000 daltons in size. GRF acts by binding to a specific receptor found only on syngeneic thymocytes and T cells but not B cells, macrophages or fibroblasts (Erb, Meir & Feldmann 1976).

The helper cell precursors were found in the T_2 pool. T_1 cells are activated by GRF and soluble antigen to amplify T_2 cell induction. This T_1-T_2 cooperation did not require cell contact (Feldmann & Erb 1977). NMF and particulate antigens directly activate the helper cell precursor, T_2. T_1-T_2 cell cooperation or histocompatible macrophages are not required.

IN VIVO INHIBITION OF MACROPHAGE FUNCTION

The importance of a given cell or the requirement of that cell type in a given immunological reaction can best be shown by removing that particular cell and then monitoring the effect on the reaction in question. The effects of some common macrophage depressing agents will be discussed below.

An obvious anti macrophage agent is anti macrophage serum antibodies developed against macrophages, usually in a different animal species. Unanue (1968) described the properties of antimacrophage antibodies with respect to specificity, *in vitro* and *in vivo* effects. The anti serum was prepared against mouse macrophages in rabbits, and was specific for macrophages but not mast cells or lymphocytes. (Specificity was assured by using only adherent cells with which to sensitize the rabbits). *In vitro* effects were macrophage death as indicated by the Trypan blue exclusion test and reduction in phagocytosis, while *in vivo* results showed cytological aberrations of macrophages. Histological changes in the spleen were noticeable, in that a scarcity of cells in the sinuses with marked hemorrhage was evident. No impairment in antibody response was observed.

The effectiveness of antimacrophage serum is further shown by Hirsch et al. (1969). Peritoneal exudate cells were incubated for four days in culture dishes. At this time, the cells consisted of over 95% macrophages. Rabbits were immunized with these cells and the sera recovered. Antimacrophage serum treatment of macrophages *in vitro* reduced their adherence to plastic dishes and interfered with their capacity to phagocytose zymosan particles. Clearance of colloidal carbon from peripheral blood of mice was reduced by intraperitoneal

and intravenous injections of antimacrophage serum. Treatment of mice with antimacrophage serum also increased their mortality rate after infection with vesicular stomatitis virus.

A similar study by Loew, et al. (1969), resulted in effects similar to the studies described above, by Unanue, (1969) and by Hirsch et al. (1968). An impaired carbon clearance was shown, but the anti sheep erythrocyte antibody response was not altered. The sera prepared by Loew et al. was absorbed against mouse erythrocytes, whereas in the previous two studies the sera had not been absorbed. In all instances, the purified anti macrophage serum was shown not to affect lymphocytes.

Other antimacrophage agents operate mainly on a suicide principle where the phagocytosis of particulate antigens or substances such as silica, asbestos, or trace metals and their soluble salts such as Cd^{2+}, Ni^{2+}, Mn^{2+}, Cr^{3+}, and VO_3^-, results in the inactivation or death of the macrophage. Most of these agents have been described or implicated in human "occupational" diseases mainly in miners, eg. asbestosis, silicosis or coal miners lung. These particles are all air borne and consequently are breathed in, and picked up by alveolar macrophages in the lung. An in vitro study by Waters et al. (1975) on the metals, cadmium (Cd^{2+}), vanadate (VO_3^-), nickel (Ni^{2+}), manganese (Mn^{2+}) and chromium (Cr^{3+}) yielded the following results. Cell viability after a 20 hour exposure of alveolar macrophages to the compounds showed cadmium and vanadate to be the most toxic (32-67 times as toxic as nickel, manganese or chromium) followed by nickel, manganese and chromium. All these metals except cadmium caused cell lysis as well as cell death. Similar concentrations of the salt NaCl had no effect on cell viability.

The mechanism of cell death appears to involve alterations of the cell membrane. All five aforementioned metals cause reduction in acid phosphatase specific activity suggesting lysosomal or plasma membrane alterations.

Silica is composed of tetrahedra with a central silicon atom and four oxygen atoms at the corners, each of which is shared with a neighbouring atom of silicon. Different physical forms of silica exist as the result of differences in the spatial relationships of the tetrahedra. If the pattern is irregular as in quartz glass, the silica is termed amorphous.

Asbestos is a name given to minerals which break down into long fibres when processed. This results from being bonded together most strongly in one direction. Various fibrous mineral silicates differing in chemical composition show the properties of asbestos. Phagocytosis by alveolar macrophages, or deposition in the pleural mesothelium depends on the size and shape of these particles.

Lysis of macrophages which invariably follows silica and asbestos uptake, appears to involve increased permeability of the lysosomal membrane, resulting in leakage and cell destruction (see below for more specific discussion). After macrophage lysis, the particles are usually recycled, resulting in additional macrophage kill. Generally speaking, silica is more potent than asbestos, although some forms of asbestos, specifically chrysotile, may approach silica in cytotoxicity.

As was earlier stated, particles such as silica are initially ingested by the macrophage in the normal fashion. Engulfed particles normally are then sequestered in the phagosomes. Lysosomes then attach to the phagosomes and discharge their enzymes into the phagosomes. These enzymes

generally bring about the degradation of the engulfed material. In the case of silica after about 18 hours of incubation the engulfed silica as well as the enzymes are found in the cytoplasm (Allison A.C. 1971), indicating that silica may somehow affect the lysosomes resulting in the release of their contents. This enzyme release precedes cell death.

The actual mechanism of action of silica appears to involve hydrogen bonding. Phenols and silicic acids are examples of "hydrogen donors" while ketones and ethers are hydrogen acceptors by virtue of active lone pair electrons on oxygen or nitrogen. Thus the hydrogen bonding of phenolic hydroxyl groups, of the type present in silicic acid with secondary amide groups of proteins can lead to protein denaturation. This however, is less prevalent than the stronger interaction with phospholipids which disrupts biological membranes. These reactions then, are not specific to lysosomal membranes but can occur with cell membranes in general. Silicic acid in solution generally is coated with serum proteins, preventing the above reactions from taking place. It thus appears that the rigid structure of the quartz (silica) with the many hydrogen bonding groups arranged in a regular and immovable order on its surface must be responsible for membrane damage formation of multiple bonds may distort membrane structure badly enough to break it down.

A compound, polyvinyl pyridine-N-oxide (PVPNO) when added in combination with or soon after silica has been shown to markedly reduce the amount of fibrous tissue, in vivo. PVPNO is taken up by pinocytosis and concentrated in secondary lysosomes (Allison 1971). PVPNO has oxygen atoms which form hydrogen bonds with phenolic hydroxyl groups and thus appears to protect the lysosomal membrane by preferentially interacting with silica particles before membrane damage occurs.

The properties of silica with respect to phagocytic cells was described by Allison (1966 & 1971). Silica has been used by numerous investigators as a means of reducing the macrophage populations *in vitro* and *in vivo* and observing the ensuing effect on the immune response. Pearsall and Weiser (1968), demonstrated the *in vivo* effect on allograft survival time, in mice given intraperitoneal injections of silica. This effect was shown in mice treated 3 days to 4 hours before grafting, and in mice treated 5 days or more after grafting. It was suggested that these times corresponded to the afferent and efferent phases of allograft rejections respectively. Passive transfer studies with inocula of tumor cells mixed with immune macrophages containing freshly ingested silica provided direct evidence that silica abrogates the effector activity of transferred macrophages. Silica treated immune macrophages injected intraperitoneally along with tumor cells resulted in palpable tumor growths in susceptible mice while suspensions of immune macrophages and tumor cells alone did not.

Lotzova and Cudkowicz (1974) showed the involvement of macrophages in bone marrow rejection by using silica and PVPNO. F_1 hybrid mice are known to resist parental bone marrow grafts, even when lethally irradiated. It was shown that silica injected intravenously could abrogate this resistance considerably, as shown by ^{125}IUdR uptake of injected cells. Silica was effective 2 days prior to 2 days after bone marrow transplantation with decreasing effectiveness after that. PVPNO, when injected 1 day before silica prevented suppression of this hybrid resistance.

Silica was shown to increase the susceptibility to parasite in-

fections in mice (Kierszenbaum et al. 1974). Mice injected intravenously with silica were shown to support the growth of the parasite Trypanosoma Cruzi better and also had a higher mortality rate due to the infections. Proliferation of the parasite was shown by counting under a microscope, the number of parasites contained in silica and non silica treated macrophages. Depression of reticulo endothelial clearance in silica treated animals was further shown by examining the rate of intravascular clearance of colloidal carbon. Silica treated mice showed a significantly reduced capacity to clear carbon from the circulation four hours after the intravenous injections of silica.

Macrophages were shown to play an active role in the graft versus host reaction of parental cells in F_1 hybrids (Fung and Sabbadini 1976). GVH reactions were induced by injecting parental spleen cells into F_1 hybrids. In this reaction macrophages of sensitized and non-sensitized syngeneic animals may be induced to lyse the target cells. Only adherent cells (containing the macrophage population) when cultured with the target cells showed any lysis of target cells and this lysis was almost completely abolished by the addition of silica to the cultures. Specificity for macrophages of the silica was also demonstrated with very low toxicity shown for spleen or target cells.

Quartz is the stable silica crystal at atmospheric pressure (tetrahedron). Except at high concentrations, silica affects mainly the phagocytic cell population; the macrophages. Other amorphous silica forms exist which may be more toxic. An example may be the type described by Van Loveren, Snoek, and Den Otter (1977), which not only decreased the macrophage population <u>in vivo</u> (when injected intraperitoneally), but also

appeared to be toxic to lymphocytes. The size of silica particles - obtained from Sigma - was reported to be 0.012 microns which is in contrast to the size 0.1-10.0 microns described by Allison et al. (1966), or the silica obtained from Dr. K. Robock (1973) of a size less than 5 microns, used in the present study. Van Loveren et al. (1977) showed a 50% drop in peritoneal lymphocyte population within 14 days after intraperitoneal silica injection. A corresponding drop in thymus weight also was noted.

An agent which has effects similar to silica but is much slower in producing macrophage damage is carrageenan, a sulfated polygalactan, which is derived from marine algae. (Allison 1966). Carrageenan causes a marked and consistent depression of the immune response to an antigen injected by the same route but this decrease may not be due to macrophage depression (Asheim & Raffel 1971). Bice et al. (1972a) showed a similar suppression using sheep erythrocytes as antigen. This suppression was lost when carrageenan was injected intraperitoneally and the antigen was injected intravenously. Instead of depression, there was an increase in peritoneal macrophage populations. These cells appeared to be morphologically abnormal and not able to adhere to glass. Turner et al. (1977), however suggest that glass adherence and phagocytic ability of these macrophages is readily demonstrable. They also showed an adjuvant effect of carrageenan when injected with the antigen, or suppression when injected before the antigen. It was suggested by these authors that since carrageenan is a recognized inflammatory agent, suppression of the immune response to intraperitoneally injected antigen, may in fact be due to depressed drainage, resulting in low antigen availability for the spleen. Carrageenan also initiates the release of vasoactive mediators such as

histamine, so the early effects may be an increase in vascular permeability and antigen transport of intraperitoneally injected antigen to the spleen which might explain the adjuvant effect.

Suppression by carrageenan of antibody response to sheep erythrocytes is also shown by Ishizaka et al. (1977), who injected carrageenan intraperitoneally and the antigen intravenously.

As previously mentioned, abrogation of resistance to foreign bone marrow grafts by silica was shown by Lotzova & Cudkowicz (1974). Cudkowicz & Yung (1977) compared this effect with that of carrageenan which induced the same abrogation of hybrid resistance. Neither antimacrophage agent affects the homing to the spleen and the subsequent proliferation of donor cells significantly. This was important since in order to show that silica or carrageenan abrogate resistance to bone marrow grafts it became essential to show that these agents did not interfere with the viability or localization of cells used for repopulation of lymphoid organs. The carrageenan effect, however, was not sensitive to PVPNO treatment as was silica. Furthermore, silica and carrageenan used simultaneously produced an additive effect. It thus seems probably that carrageenan, though toxic for macrophages, may either operate via a different mechanism, e.g. on immature macrophages, or it may affect other cells such as lymphocytes (Cudkowicz & Yung 1977). This may help in explaining the varied effects with carrageenan described earlier.

Other means of macrophage suppression or inactivation include reticuloendothelial (RES) blockade, for example by carbon particles or trypan blue. RES blockade may simply involve overloading of the phagocytic system by the large doses of particles injected or may be due to the removal from the serum of opsonins. (Loegering & Carr 1977).

A single injection of colloidal carbon sufficient to produce RES

blockade also appears to produce an immunosuppressive effect. It was shown that RES blockade with carbon followed by immunization with sheep erythrocytes (SRBC) resulted in a dramatic decrease of antibody forming cells. The PFC method, (Jerne 1963) , described elsewhere, was used to demonstrate a decrease in antibody plaque forming cells of the direct (19S IgM) and of the indirect (7 S IgG) types when animals were pre-treated with carbon. Pretreatment was most effective 24 or 48 hours before immunization resulting in a 90% drop in the immune response. Carbon uptake in the spleen was largely limited to follicular areas. (Sabet 1972).

Much the same results were obtained, Straus et al. (1977) using trypan blue. However in this case it is not certain that RES blockade is involved since maximal suppression occurred when trypan blue was injected 6 hours after the antigen.

Rationale for the present study.

This thesis is a part of a larger study on the role of macrophages and T cells in the immune response. In the course of these studies, it became necessary to establish the role of host macrophages in a cell transfer system involving the injection of immunocompetent cells into irradiated recipients. As previously discussed, an approach to this end would be the elimination or the inactivation of macrophages from the host and the study of the effects of such elimination on the immune response. A review of the literature revealed that, while a total elimination of macrophages under *in vivo* conditions may be impossible, the use of crystalline silica may produce a marked reduction of these cells and, thus, may be useful to obtain an indication on their role in the immune response. Therefore, I began my work with the objectives of confirming the claim (Cudkowicz, 1974) that silica particles affect macrophage activity *in vivo*, and of establishing the effects of this treatment on two immune responses currently used in this laboratory, i.e. (i) the CMC which is developed by parental cells during GVH reactions in the spleens of lethally irradiated F_1 hybrids, and (ii) the PFC response produced in the spleen upon the injection of immunocompetent spleen cells and SRBC in lethally irradiated hosts.

Since it was planned to study only immune reactions taking place in the spleen, it was considered necessary for the silica particles to reach the spleen in sufficient amounts. For this reason the intravenous route of injection was selected and used throughout the present studies. As it was likely that only very high doses of silica could produce the desired

marked depression of macrophage activity, the initial experiments were aimed at establishing the maximal dose tolerated by the animals.

Once this was established, the effects of the treatment on macrophage activity were started. To this end, the organs which contain large numbers of macrophages such as the spleen, liver and lungs were studied histologically with and without the injection of carbon particles which are normally taken up by macrophages and can be easily detected histologically. However, such histological studies were not expected to yield clear-cut results, and more specific information about macrophage functions in silica-treated mice was obtained with the measurement of the phagocytosis of intravenously injected carbon particles (Biozzi, et al., 1953). Originally, these functional studies on phagocytosis were planned both in irradiated and non-irradiated animals. However, the difficulty of obtaining enough blood from irradiated animals made it impossible to perform statistically valid experiments on these animals. Therefore, studies on phagocytosis in irradiated mice would have required the use of a different method which did not involve repeated bleedings. However, these studies were not considered necessary, since the experiments in non-irradiated mice demonstrated a marked depression of phagocytic activity. It was reasoned that such a depression would have been even more marked in irradiated mice from which radiosensitive precursors of macrophages are eliminated, thus preventing any regeneration of these cells. Sporadic results obtained in irradiated mice, although not statistically valid, support this conclusion.

Further studies were performed to establish if the treatment with silica affected in any way the capacity of spleen cells to migrate to the

host spleen. This was considered necessary since any study of an immune reaction in the spleen would be meaningless if not enough immunocompetent cells migrate to this organ. In spite of some variability of results (which should be expected in animals receiving the harsh combined treatment of lethal irradiation and a nearly lethal silica injection these experiments clearly demonstrated that silica treatment does not significantly affect either the migration or the capacity of spleen cells to proliferate in the spleens of silica-treated mice.

After all these preliminary experiments had been completed, the treatment with silica was considered to fulfill the basic requirement for a study on the role of host macrophages in the experimental model introduced above, i.e., silica can be injected into lethally irradiated mice in amounts sufficient to produce a marked depression of macrophage activity without significantly altering the histology of the spleen or affecting the capacity of this organ to accept sufficient numbers of immunocompetent cells and to support their proliferation. Therefore, the studies on the GVH reaction and on the PFC reaction were performed as originally planned. The results of these studies were more complex than expected and cannot be interpreted according to the simple (and perhaps naive) assumption that since silica eliminates most macrophages, its effects on the immune response are solely due to the elimination of these cells. However, these results have shown interesting phenomena consisting of a decrease of CMC and a marked increase of the PFC response under similar experimental conditions. Further studies of these phenomena may shed some light on the mechanisms of regulation of cell-mediated and antibody mediated immune responses.

MATERIALS AND METHODS

Animals: Mice used in these studies were obtained from the Jackson Laboratory, Bar Harbor, Maine. The strains, along with their respective H-2 genotypes are as follows: C57BL/6J ($H\text{-}2^b$), B6AF1 ($H\text{-}2^b/H\text{-}2^d$), the offspring of C57BL/6J and DBA/2J. Animals were shipped at six to eight weeks of age and were kept at 70 - 76° centigrade with food and water ad libitum. B6AF1 and C57BL/6J mice were also obtained from the North American Laboratory Supply, a local supplier.

Chemicals:

Radioactive Chemicals: ^{51}Cr was supplied in the form of Na_2CrO_4 by Amersham Searle, Oakville, Ontario, Canada. ^{125}I-labelled 5-Iodo-2' deoxyuridine ($^{125}IUdR$) was also supplied by Amersham. Radioactive material was counted in a well type gamma scintillation counter (Nuclear Chicago Corp., Des Plaines, Illinois).

FUdR: 5-fluoro-2' deoxyuridine (FUdR) was supplied by the Sigma Chemical Co.

Agarose: Sea Kem agarose was obtained from Marine Colloid Inc. Rockland, Maine, U.S.A.

Carbon: A shellac free (Pelikan C-11-1431a), carbon suspension was obtained from the Günther-Wagner Company in Germany. Carbon particles varied in size from 250 to 500 angstroms.

ACT: Tris buffered - isotonic ammonium chloride (ACT) pH 7.2 was prepared according to the method of Boyle (1968).

Silica: Silica particles of average size 5 microns, were obtained as a gift from Dr. K. Robock, Stein Kohlenberg-Bauverein, 43 Essen-Krey, Germany, and from Mr. W. Whittaker, Institute of Occupational Medicine, Roxburgh Place, Edinburgh.

Additional Materials:

Tissue Culture Media: Hanks balanced salt solution (HBSS) (Hanks and Wallace 1949) was obtained from the Grand Island Biological Company Grand Island, N.Y. HEPES (Shipman 1969) (0.01 M N-2-Hydroxyethyl piperazine-N-2 ethane Sulfonic acid) was supplied by Calbiochem, Los Angeles, California. The HBSS was reconstituted using HEPES 0.01M instead of bicarbonate as the buffering agent, and the pH was adjusted to 7.2. This solution was termed Hanks-HEPES. RPMI-1640 medium obtained from Grand Island Biological Co. was buffered at pH 7.2 with 0.04 M HEPES supplemented with 5% Fetal Calf Serum, 100 microgram streptomycin, and 100 units of penacillin per ml. (Difco Laboratories Inc., Detroit, Michigan). This is referred to as supplemented medium.

Tissue Culture Plates: Microtest II tissue culture plates were supplied by Cooke Engineering Co., Virginia, U.S.A.

Petri Dishes: Sterile plastic Petri dishes (60 x 15 mm) were obtained from Falcon Plastics, Los Angeles, California.

Complement: Guinea Pig Serum used as a source of complement was supplied by North American Laboratory Supplies.

Sheep Red Blood Cells (SRBC): SRBC were obtained from the North American Laboratory Supply Co.

Micropipets: 25 lambda Yankee disposable Micropets - used for eye and tail bleeds were obtained from Clay Adams, Becton, Dickinson and Co. Parsippany, N.Y.

METHODS

Radiation

Animals received from 800-900 rads whole body radiation (100% lethal) from a cobalt source delivering between 100 to 200 rads per minute at a distance of 75 cm. The dose was varied because of animal mortality. Initially, at 900 rads this did not present a problem over the six days most experiments were run. However, due to various reasons (see silica mortality dose curve) the animals became more susceptible to the radiation dose, so this was decreased, first to 850 rads, then to 800 rads.

Injections

All injections except radioactive iodine (^{125}IUdR) and FUdR were given intravenously into the lateral caudal vein. Mice were first anaesthetised with ether, the tail was then dipped into warm water to dilate the veins and the injection given while the mouse was still under anaesthetic. ^{125}IUdR and FUdR injections were given intraperitoneally.

Preparation of Cell suspensions

Spleen cells were used in all assays and reconstitutions. After the organ was removed, cells were teased between two needles and aspirated in Hanks-Hepes medium to make up a single cell suspension. The suspension was then passed through a fine mesh screen and let stand for five minutes in a test tube to let the clumps settle. The remaining suspension was then drawn off with Pasteur pipette. Cells were then washed at least twice in Hanks-Hepes medium and resuspended in this medium for injections. For the CMC assay, cells were suspended in supplemented medium (RPMI-1640). Where desirable, erythrocytes were

removed by incubating in ACT at 37°C for 5 minutes, then washing in physiological saline (until the reddish color disappeared). Cells were then resuspended in complete medium.

Silica Preparation and Injections

Silica was suspended in 0.85% NaCl, sonicated to disperse clumps, and autoclaved. The suspension was made up to 10 mg/ml and needed shaking immediately before injection as it settled out very quickly.

Silica Dose-Mortality Curve

Increasing doses of silica were injected intravenously into the lateral caudal vein of B_6AF_1 mice. The silica was suspended in physiological saline as described above and was given as a single injection. The stock silica was shaken frequently to prevent settling out of the silica.

Histology

After animals were sacrificed, the respective organs were removed, and placed in a 10% formalin solution. Tissue sections and slides were prepared by Mrs. Susan Pylypas from the Anatomy Department here at the Basic Medical Sciences Complex. Tissues were embedded in paraffin and stained with hematoxalin and eosin. To determine the effect of silica on phagocytosis, carbon particles were injected 24 hours before the animals were sacrificed. Photographs of the microscopic slides were prepared with the assistance of Dr. F.H. Lee and Mr. H.G. Sanderson from the Immunology Department.

Carbon Clearance

Immediately prior to carbon injection, a 25 lambda sample of blood was taken from the animal by eye bleeding, from the retro orbital venous plexus. The mice were injected with a carbon suspension (0.036 mg/gm weight of the mouse) through a lateral caudal vein, and blood samples

were taken every minute thereafter for six minutes to determine the rate of carbon clearance from the blood. All bleedings except the initial one were taken from the tail; the very tip having been snipped off with a pair of scissors. The tail was stroked between the thumb and forefinger in a milking-like fashion to increase the rate of bleeding, when necessary. Animals were also preheated with a "heat" bulb suspended above the cage to increase blood flow. Blood was collected in 25 lambda Yankee disposable Micropets. All blood samples were placed in test tubes containing one milliliter of 0.1% Na_2CO_3. These samples were read in a spectrophotometer at 675 mu. Corrections for individual samples were then made by subtracting the optical density of the blood sample taken before carbon injections. Spectrophotometric readings were converted to micrograms of carbon per 100 lambda of blood by multiplying these corrected readings at 675 mu by a constant 8.62 (Figure 1). This constant was obtained by plotting a standard curve prepared by Dr. Martin Davies, who was employing the same assay and graciously allowed me its use. The constant is derived from the formula $A=\varepsilon bc$, (see below and Figure 1). According to the Beer-Lambert Law where:

A= absorbance - or optical density.

ε= molar extinction coefficient - a constant for a given compound.

b= distance light travels through the medium - cells were 1.0 cm in width.

c= concentration of the absorbing compound.

Since ε and b are constant and A can be measured, the concentration c of a given compound may be determined.

Phagocytic indices (K) were calculated by linear regression analysis of a plot of amount of carbon per 100 lambda of blood versus time over

the 6 minutes readings were taken. The values corresponded to the ones obtained by using the method of Biozzi, Halpern and Benacerraf (1953), using the formula:

$$\frac{\log Co - \log C}{T} = K$$

where C = concentration of carbon in the blood at time T.

Co = the blood concentration of carbon just after the injection.

K = a constant which characterizes the rate of clearance of carbon from the blood - (Granulopectic Index).

An example of these calculations is given graphically below and in Table IV.

Three to five animals were used per group, and the clearance assays were done at varying times after silica injections, i.e. 6 hours, 24 hours, and 72 hours.

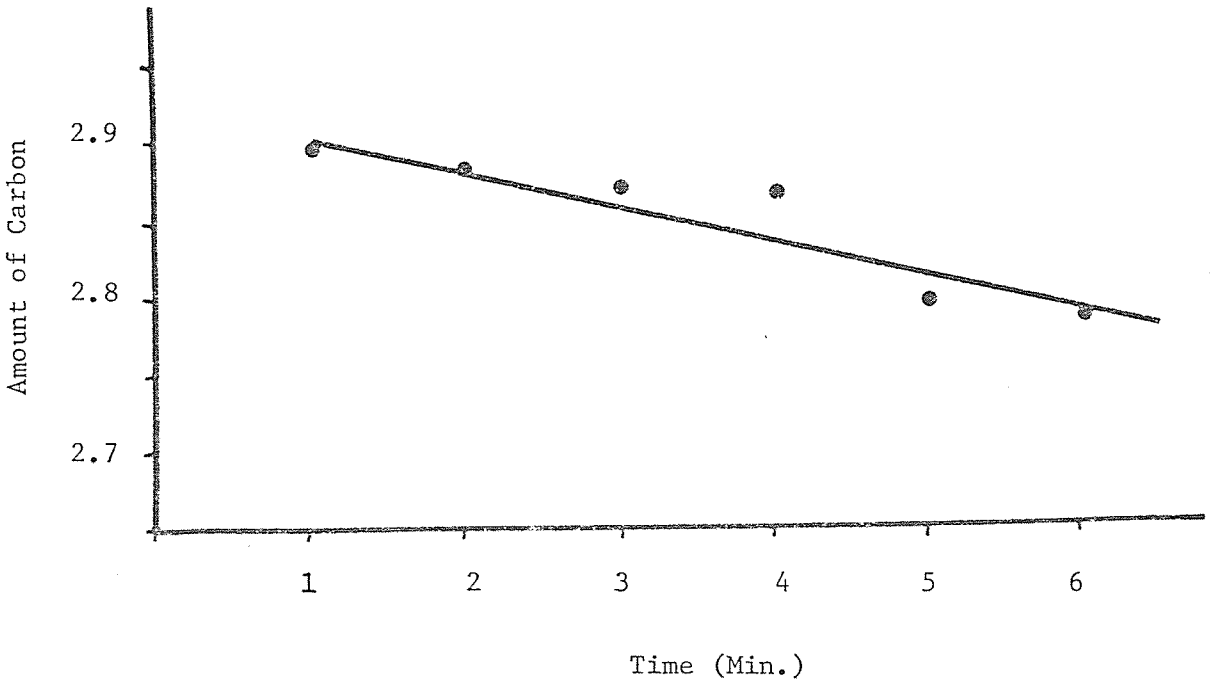


TABLE IV

Treatment of Carbon Clearance Data:

Raw Data: spectrophotometric readings before and after carbon injections to determine the rate of carbon removal from the blood.

Time: Minutes after carbon injections, 0 - time = eye bleed before carbon injection.

	Time							
	1	2	3	4	5	6	0	
(a)	.162	.158	.153	.153	.140	.138	.067	--raw data
(b)	.095	.091	.086	.086	.073	.071	--	--corrected raw data; i.e. 0 readings subtracted.

(c) multiply the (b) values by 8.62 to obtain ugm. carbon/100 lambda blood.

(d) multiply by 1000 and determine the log values which are;

(e) 2.9086 2.8945 2.8700 2.8700 2.7988 2.7868

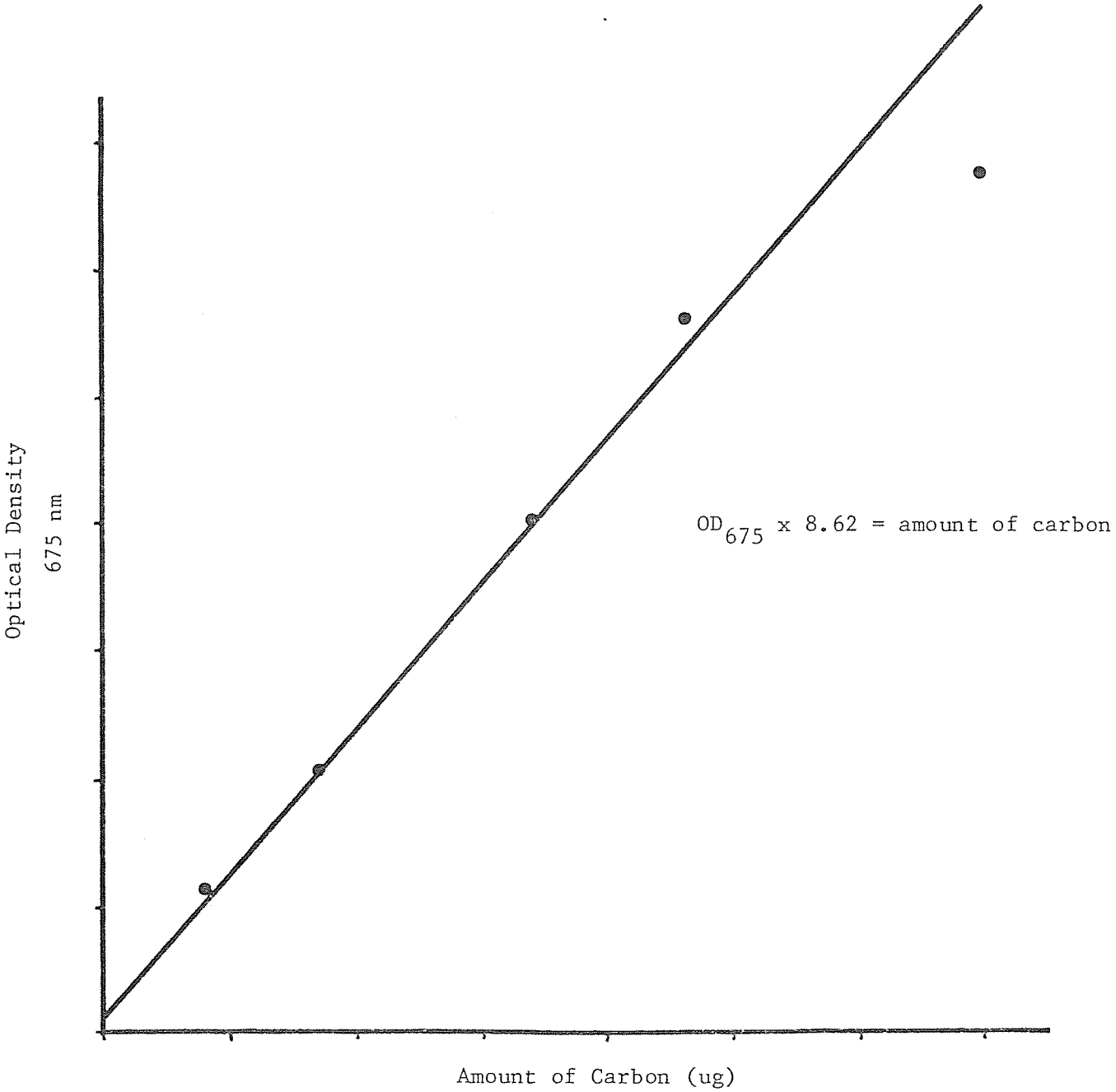
(f) The slope K = -0.0256 (obtained by plotting the ugm. carbon/100 lambda blood vs. time) is then computed through linear regression analysis, or may be obtained graphically as illustrated on the previous page.

(g) Statistical significance is determined by the analysis of variance method by comparing:

(i) control vs. experimental.

(ii) the various experimental times i.e. 6 hours. vs. 24 hours.

FIGURE 1



Standard Curve for conversion of spectrophotometer readings to concentration of carbon.

Homing

Animals were injected with ^{51}Cr labelled syngeneic or semi-allogeneic spleen cells. They were then sacrificed at various time intervals and the radioactivity localized in different organs was measured. This gave an indication of the percentage of injected cells that lodged in the organ in question.

Cells were labelled by incubating about $5x10^7$ to $1x10^8$ cells with 300 microcuries ^{51}Cr (as sodium chromate) in 1.0 ml supplemented medium at 37^oC for 45 minutes. The cells were then washed at least four times to remove radioactivity not incorporated into the cells. Injections into hosts were via the lateral caudal vein at $3x10^6$ cells per recipient and the animals were then sacrificed at pre-determined intervals by cervical dislocation. Blood (0.1 ml) the liver, lung, spleen, thymus and axillary, brachial and inguinal lymph nodes of each mouse were then checked for relative activity.

Readings were taken in a well type gamma counter. Some organs (lung-blood) were only examined for the first day as a preliminary experiment had demonstrated that activity levelled off after 24 hours. The degree of uptake per organ was calculated as a percentage of the total amount of radioactivity injected. This was done by placing an amount of radioactivity identical to that injected into the animal, into a tube and counting in the gamma counter on the day of the assay. This also eliminated the need to calculate radioactive decay.

(This series of experiments was begun together with Dr. U.M. Babu).

Cellular Proliferation Studies:

B6AF1 female mice were irradiated as in the homing study above, at about five o'clock in the afternoon and injected with silica between seven and nine o'clock the following morning. Animals received a standard dose of 0.2 mg/gm weight mouse, except in the dose-response assay, where the amount was varied. Injections were intravenous, into the lateral caudal vein. Donor cells, either syngeneic (B6AF1) or parental (C57BL/6J) were injected in a similar fashion; $5x10^7$ cells per recipient. 25 hours prior to measurement of proliferation the mice received 10^{-7} moles FUdR intraperitoneally, and an hour later received 0.5 microcuries ^{125}IUdR intraperitoneally (Hughes 1964). The uptake of radioactivity in the spleen and liver was measured 24 hours later, in a well type gamma counter. The uptake per organ was calculated as a percentage of the total amount of radioactivity injected.

Cell Mediated Cytotoxicity (CMC):

For the CMC assay hybrid female mice (8-12 weeks old) were given 850 rads (later changed to 800) whole body radiation (100% lethal from a Cobalt source). Animals were irradiated in the later afternoon, (5 o'clock). Silica injections, 0.2 to 0.3 mg/gm weight mouse followed between 8:00 to 10:00 am the next morning.

Sensitization: Parental cells were sensitized against the F1 mice by injecting the irradiated F1 with $5x10^7$ to $7.5x10^7$ C57BL/6J cells. This resulted in the donor cells being sensitized to the "foreign" antigens inherited by the F1 hybrid from the other parent (A/J). i.e. the parental cells developed a graft versus host reaction. Control groups received similar numbers of syngeneic F1 cells. Cells were injected after silica injections to avoid mixing of the silica and the cells. Cells were injected between 11:00 am and 1:00 o'clock. The CMC assay was carried out on the sixth day after reconstitution, since previous experiments (U.M. Babu) had demonstrated that this is the time of maximal CMC activity in this experimental model.

Animals were killed by cervical dislocation and the spleens removed and placed in Petri dishes containing about 3 ml of Hanks-Hepes medium. (Spleens were not pooled within groups, unless cell number was insufficient). The spleens were made up into single cell suspensions as described earlier. The cells were then washed and spun down three times and made up to the desired concentration. These sensitized cells are referred to as the effector cells.

Target Cells: Mastocytoma, P815-X2 maintained by serial intraperitoneal passage in DBA/2 mice was used as the target for the sensitized parental cells as they share common H-2 antigens. Cells were removed from the peritoneum of the DBA/2 mice by injecting medium (Hanks-Hepes) into the peritoneum, kneading the abdomen of the mouse gently with the fingers and drawing fluid back out with the syringe. This fluid contained the tumor cells

as a single cell suspension. Cells were then washed once in supplemented medium and then labelled for 45 minutes at 37^{o} celcius with 200-300 microcuries sodium chromate, the concentration being about $1x10^{7}$ cells per ml. Residual chromium not taken up by the cells was then removed by washing the cells five times in supplemented medium. The cells were then stored at room temperature. Since P815-X2 cells derive from DBA/2 mice, they carry $H-2^{d}$ antigens. Therefore, they share some antigenic determinants with the $H-2^{a*}$ antigens to which C57BL/6 cells become sensitized upon injection into B6AF1 hybrids.

Assay: The actual assay involves mixing varying effector cell:target cell ratios and incubating for 16 hours in a CO_2 incubator at 37^{o} celsius. The cells were mixed in the wells of round bottomed tissue culture plates; the target cell number was kept constant at $2x10^{4}$ cells in 0.05 ml and the effector cell number was varied to give ratios from 6:1 to 100:1 in a total volume of 0.25 ml. Before incubation the plates were centrifuged briefly (2 minutes at 1300-1500 rpm) to promote cell to cell contact. After incubation the plates were again spun down at 250g for ten minutes and 0.10 ml of the cell free supernatant taken off the top for measurement of the released radioactivity in a well type gamma counter. Percent corrected lysis was then calculated, using the equation $\frac{E-C}{Ft-C} \times 100$, where E is the counts per minute obtained in the 0.1 ml supernate experimental sample, C is the counts per minute in control wells where target cells were incubated with normal F1 cells, (again 0.1 ml), and Ft is the counts per minute released in a sample of target cells ($2x10^{4}$) which had been frozen and then thawed four times. This was considered to be the maximum amount of chromium

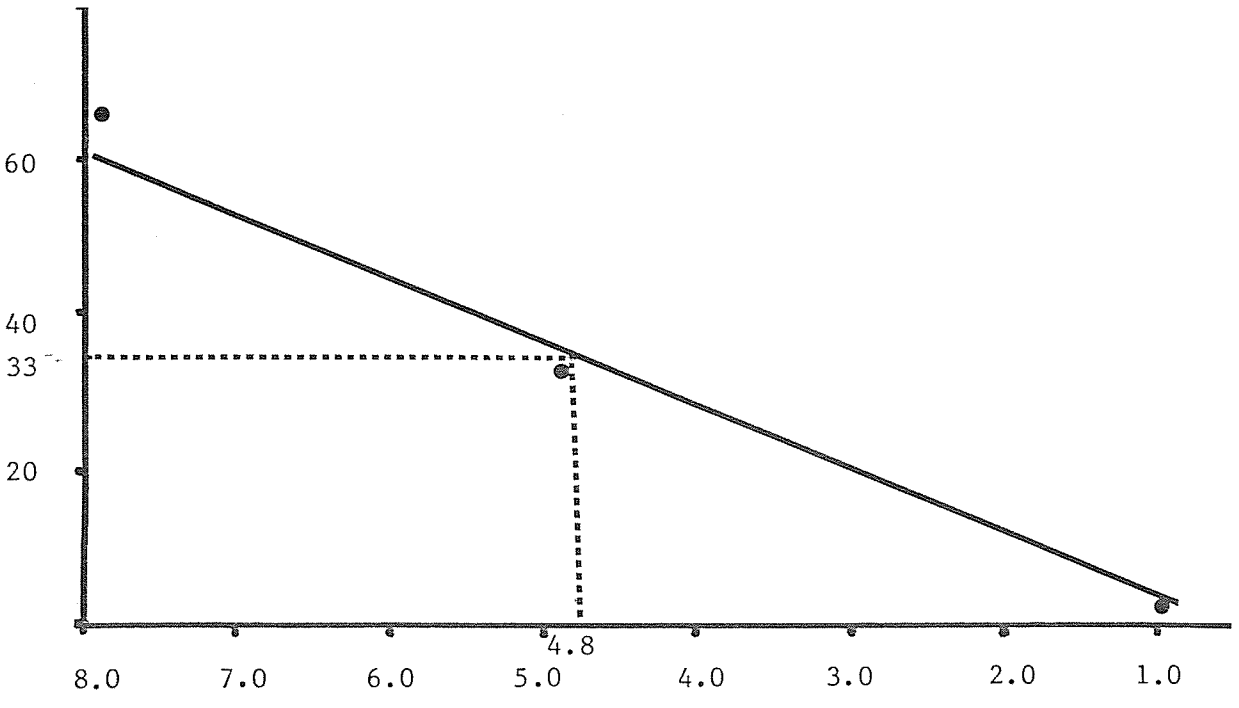
* The $H-2^{a}$ haplotype derives from the recombination of $H-2^{k}$ and $H-2^{d}$.

releasable when all the cells were disrupted. From the dose response regression line fitted to the experimental points, the number of cells required to lyse 33% of the target cells was calculated and defined as one lytic unit (LU). The total lytic units per spleen could then be determined, as the cell number per spleen was also recorded.

A typical example of the treatment of data is the following, taken from Table XIII, Experiment II, the fourth animal. (C_4).

The percent corrected lysis valued induced with the 30:1, 15:1 and 6:1 attacking to target cell ratios (corresponding to $6x10^5$, $3x10^5$ and $1x10^5$ attacking cells) were 69%, 30% and 5% respectively.

From the dose response regression line fitted to the experimental points, the number of cells required to lyse 33% of the target cells was determined to be 3.18×10^5 (1 Lytic unit). (This corresponds closely to the value obtained from the graph) i.e. Log 3.18×10^5. The Lytic units per spleen are then calculated from the cell yield value per spleen of 350×10^5; a value of 110.06.



Plaque Forming Assays:

Sensitization: B6AF1 hybrid female mice were irradiated at about 5:00 p.m. (800 rads) and left overnight. Silica injections were done the next morning between 7:30 and 9:00 a.m. Mice were reconstituted intravenously with $3x10^7$ syngeneic cells and sensitized with $2x10^8$ sheep red blood cells (SRBC). Silica dose was 0.2 mg/gm weight mouse. Both a direct and an indirect assay were done for IgM and IgG on a range of days; 4 to 7 for the direct, 7-11 for the indirect assay respectively.

The assay was based on the Jerne and Nordin (1963) technique, and the modifications of A.K.C. Wong and E. Sabbadini (1974). It involved the layering of plastic Petri dishes (sterile) with 2.5 ml of 0.85% agarose in phosphate buffered saline (PBS) at pH 7.2. These could be stored for several days at four degrees centigrade in air tight plastic boxes which prevent loss of moisture. Immediately before the experiment these dishes were placed at room temperature and allowed to warm up to prevent a too rapid solidification of the top layer to be added later. The top layer consisted of 0.4 ml of double strength Hanks-Hepes, 0.4 ml of 0.85% agarose in double distilled water, 0.1 ml effector cells and 0.1 ml target cells. The target cells were a 25% concentration of SRBC. The antibody forming cells were the spleen cells taken from the sensitized animals, teased, aspirated and made into a single cell suspension at such concentrations as was desired (10^7, 10^6, 10^5, cells/ml).

The Hanks-Hepes and agarose were added in order to a number of tubes (Hanks-Hepes added first and warmed up at 42°C then the hot agarose solution). The effector cells and target cells were then added, usually to two or three tubes at a time, since the water bath in which the tubes containing the Hanks-Hepes and agar must be kept at about 42°C to prevent gelling of the agarose, and the effector cells and target cells might not remain viable at this temperature, if stored for any great length of time. These two or three tubes were then picked up by the tops and swirled around gently in the bath to mix the target cells, effector cells, agarose, and Hanks medium. The contents were then transferred to individual Petri dishes. The dishes were tilted at various angles and contents swirled around to insure the formation of an even layer. The plates were then allowed to cool for ten minutes, (in order for the agarose to solidify) on a pre-levelled solid surface at room temperature. Following this they were placed (inverted) into a moist incubator at 37° and incubated for 45 minutes to one hour. They were then taken from the incubator and to each plate was added 2 ml of Hanks-Hepes containing guinea pig complement, (diluted 1:10 with Hanks-Hepes). After this the plates were again incubated for an additional 30-45 minutes at 37°C in a moist incubator. The complement and medium were then poured out and the plaques counted by the unaided eye, with the aid of a transparent grid placed over the plates which allowed counting of the plates in sections. A microscope at low power was used when in doubt whether a plaque was an air bubble or an actual plaque brought on by an antibody forming cell.

The procedure described served to identify direct or IgM plaques. For indirect, or IgG plaques a developing antiserum (Anti IgG) was added during the second incubation. This serum, a rabbit anti mouse IgG, was produced by Gaylene Pron, a previous MSc. student.

Preliminary experiments were performed to establish the optimal concentration of this antiserum in the plaque assay. The 1:100 dilution (in Hanks-Hepes medium) gave a maximal development of indirect plaques without any significant inhibition of direct (IgM) plaques. Therefore, this dilution was used for all subsequent experiments and the number of indirect plaques was calculated by subtracting the number of plaques obtained without developing antiserum from the number of plaques obtained in the presence of the developing antiserum.

Controls for both the direct and indirect assay consisted of:

i) Irradiated mice reconstituted with syngeneic spleen cells but receiving no SRBC.

ii) Irradiated mice receiving SRBC cells only.

iii) Silica treated irradiated mice reconstituted with syngeneic spleen cells and receiving no SRBC.

iv) Silica treated irradiated mice receiving SRBC cells only.

Statistical Methods:

Statistical methods were used to verify that apparent differences between groups were in fact significant. Standard deviation, analysis of variance, and two way analysis of variance tests were employed.

Standard deviation gives an indication of variability around the mean of a particular group. If within group variability exceeds the differences between groups, it may become difficult to draw any conclusions about the between group differences.

Analysis of variance allows formal comparisons of several groups, comparisons between the variability due to different treatment of groups and the random differences between subjects. The test used is called an "F" test, and requires a comparison of variances (Plutchik 1968). In the present study, for example, it was used in determining whether the difference between controls and silica treated groups in the carbon clearance assay was significant and also whether a significant difference existed in the respective times after treatment that the experiment was run.

Two way analysis of variance is a best designed for use in investigating differences in means obtained from measurements repeated under similar conditions. ie., the same experiment repeated after several days. An example of this is the homing experiments where it was desireable to clarify whether differences existed as the interval between the injection of cells and testing were increased. The same held true for the cell proliferation experiments.

RESULTS:

This section will cover the following areas; (A) Silica dose and mortality, (B) Histology, (C) Carbon clearance, (D) The effect of silica on the homing of lymphocytes, (E) The effect of silica on the capacity of injected cells to survive and proliferate in the recipient's spleen, (F) The effect of silica on cell mediated cytotoxicity and (G) The effect of silica on the humoral response - the plaque forming cell assay.

A. The Silica Dose Mortality Studies:

Increasing doses of silica were injected into B6AF1 mice to determine the appropriate dose to be used in future experiments. The optimal dose range was considered the maximal dose which the animals could tolerate without significant mortality. Any dose above 7 mg. injected intravenously brought about almost immediate convulsions and death, probably due to blockage of the capillaries and venules of the lungs. This held true for non-irradiated and irradiated animals. Non-irradiated animals could withstand a dose of 7 mg for long periods of time (20 weeks) without any noticeable effects and without any deaths (see histology section). Irradiated animals withstood injections of 5-7 mg poorly, usually dying about the third or fourth day after injections. Lower doses usually did not cause mortality within the six days required for most experiments. Irradiated non-reconstituted animals usually died within two weeks, which is normal after a lethal dose of radiation, and indicates that death was probably due to hemopoietic failure. The dose finally chosen for most experiments was 0.2 mg/gm weight of the mouse which for a 20 gm mouse (the average weight of 8-10 week old mice) meant four mg.

While irradiation increased the sensitivity to silica, in terms of mortality, irradiation plus injection of semi syngeneic (parental C57BL/6 cells into B6AF1) cells to silica treated mice proved even more lethal probably due to the graft versus host (GVH) reaction of the injected donor cells. Even a dose of 0.2 mg/gm often proved to be lethal for more than 50% of the mice in this group. In some experiments, however, the effect of silica, radiation and the GVH produced very inconsistent results in terms of mortality ranging from a low of 0% mortality to 100% mortality in different experiments. Deaths usually occurred about four to five days after cell injections. High mortality rates were due in part to the age of animals used; (younger animals appeared more susceptible) and seasonal changes also appeared to affect animal viability, spring and early summer producing higher mortality rates.

More recent results of other investigators in this department indicate that high mortality in irradiated animals may be observed even without any additional treatment and with doses of radiation lower than those expected to produce any mortality. This may be due to latent bacterial and/or viral infections. While this was a serious problem, it did not appear to influence significantly the results obtained in experiments with cell mediated cytotoxicity and with the PFC assay, i.e. identical treatments produced similar results in different experiments irrespective of the high or low mortality observed in a particular experiment. The problem was somewhat alleviated by using extra animals per group.

B. Histology and Alterations in the Spleen Size:

Animals were sacrificed at weekly intervals after the intravenous

injection of 7.1 mg of silica and the lung, liver and the spleens were examined. Intravenous injection of 2.0 mg carbon particles and the subsequent study of the histological localization of the particles in various organs gave an idea of the phagocytic capacity of the reticulo endothelial system and of the localization of active macrophages. The objective of these histological examinations was not to study in detail the effects of the treatment with silica, but rather to obtain a general idea of the magnitude of the alterations produced in different organs, especially in the spleen, in which it was planned to measure different immune responses.

The results demonstrated that the structure and appearance of the organs in question were not altered immediately, but after several weeks time a marked change occurred in the spleen and even more markedly so in the livers.

Infiltrations of mononuclear cells with the appearance of lymphocytes could be seen in the liver (Figure 2&3). At the end of the study these infiltrations were larger and more numerous than initially, but still had the same characteristics as initially. Such infiltrates were prevalently localized around small blood vessels (either in the portal spaces or around the centre lobular venules). However some were seen also within the lobules.

The effect on the spleen was not as striking, although after a week to ten days polynucleated giant cells began appearing in response to the ingested silica (Figure 4&5). These cells were scattered throughout the red pulp, which is the area in which carbon particles were also found. Silica groups also seemed to have a decreased number of lymphocytes in the white pulp (Figure 4). At the close of the study, spleens of silica treated animals also showed the presence of collagen fibers which suggests an increase in fibrogenesis in the spleen.

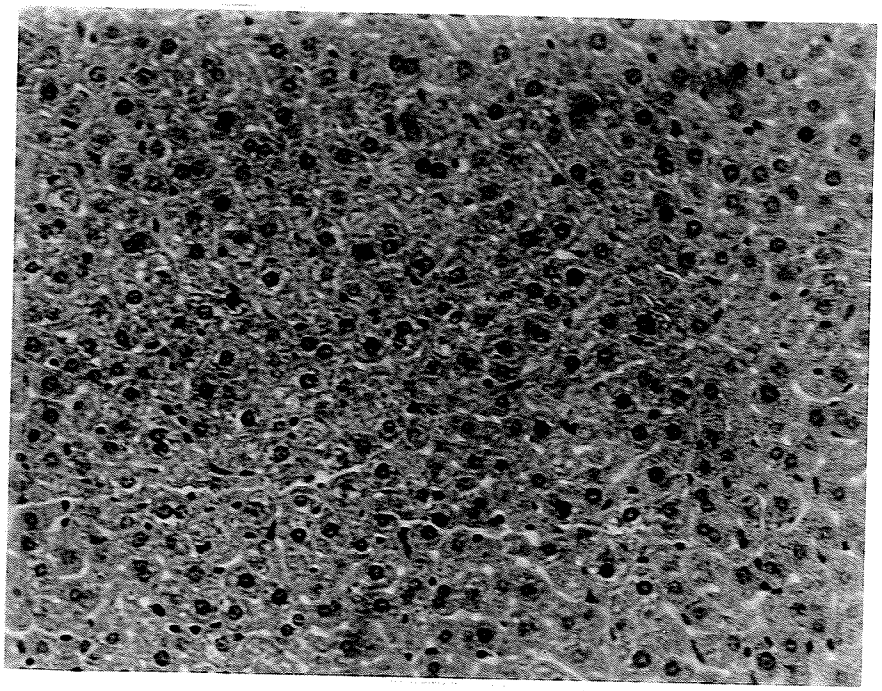
Slides of the lungs did not show any aberrations worthy of note. Carbon localization in the respective organs was not altered appreciably by the silica treatment, although the kinetics of carbon removal from the blood stream was altered as will be shown later.

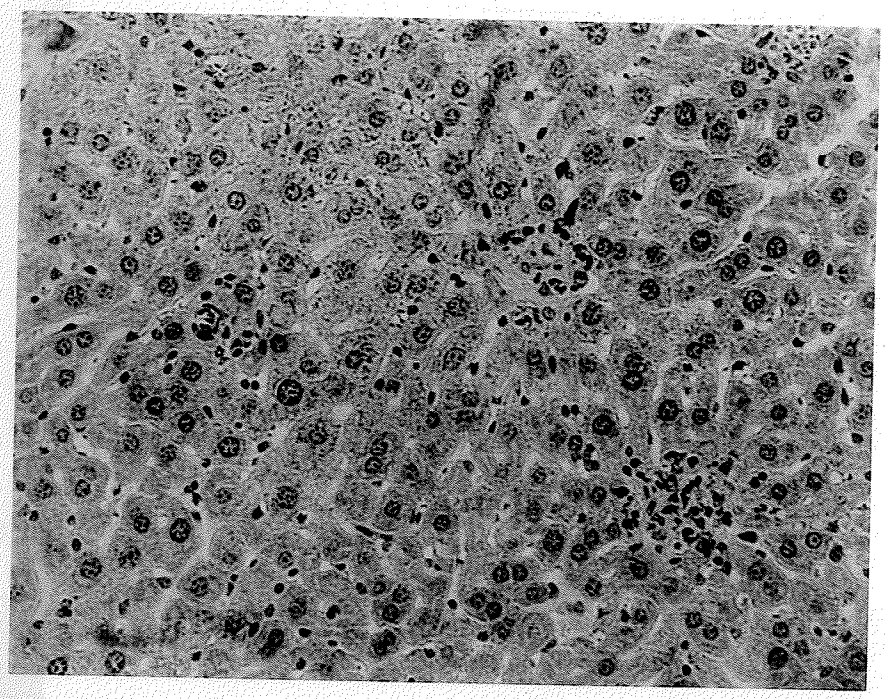
Spleen weights were monitored during the experiment, with the silica group showing an increase of almost 150% above that of the normal animals, (Table V). This increase was not due to the weight of the injected silica alone, as only 7.1 mg were injected per mouse and not nearly all of this is expected to localize in the spleen.

FIGURE 2

TOP: Normal liver. (Magnification 300x)

BOTTOM: Silica treated liver. (Magnification 300x)
Note cellular infiltrates.





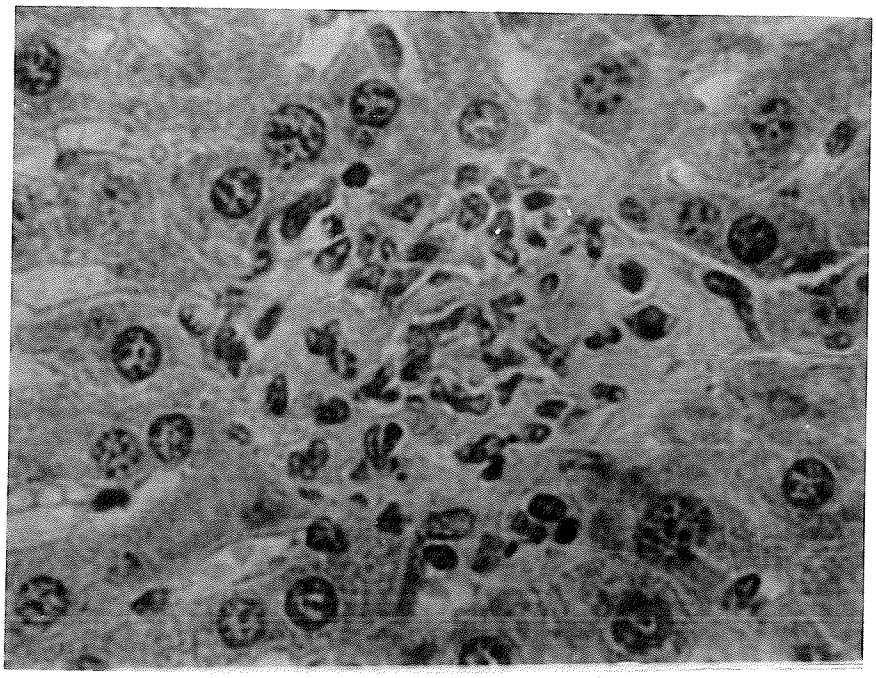


FIGURE 3

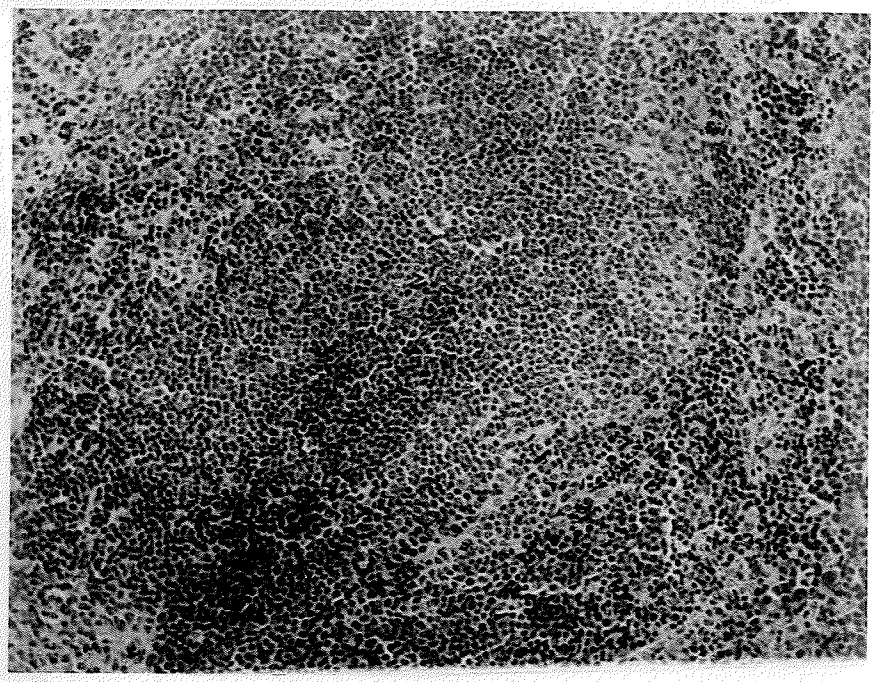
Higher magnification of silica treated liver shown in Figure 2.

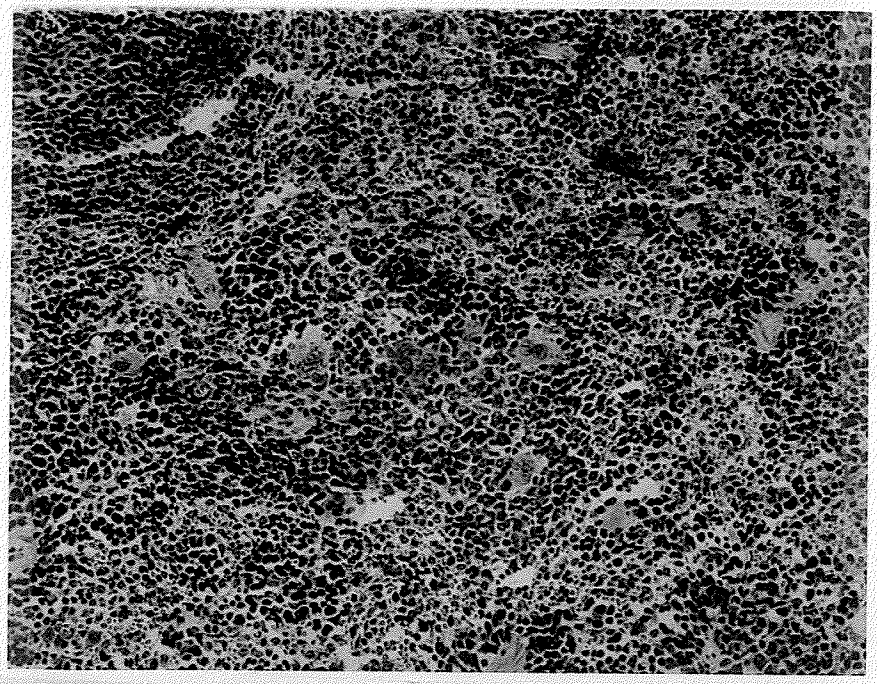
(Magnification 1200x)

FIGURE 4

TOP: Normal spleen. (Magnification 300x)

BOTTOM: Silica treated spleen. Note polynucleated giant cells, and scarcity of lymphocytes. (Magnification 300x).





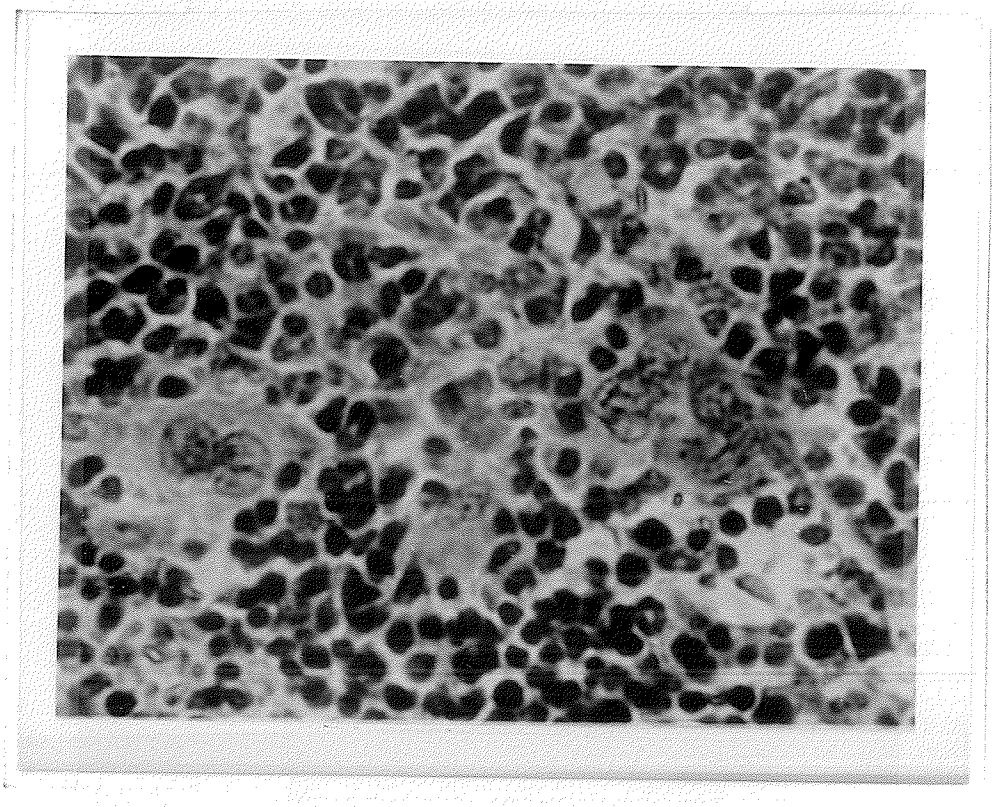


FIGURE 5

Higher magnification of silica treated spleen shown in Figure 4.

(Magnification 1200x)

TABLE V

Increase in Spleen Weight in Silica Treated Mice

	Control	Silica	Carbon Control	Carbon & Silica
Spleen weight in grams	0.1038 ± .02	0.2347 ± .04	0.1260 ± .02	0.3026 ± .07
	mean of 4	mean of 12	mean of 5	mean of 13

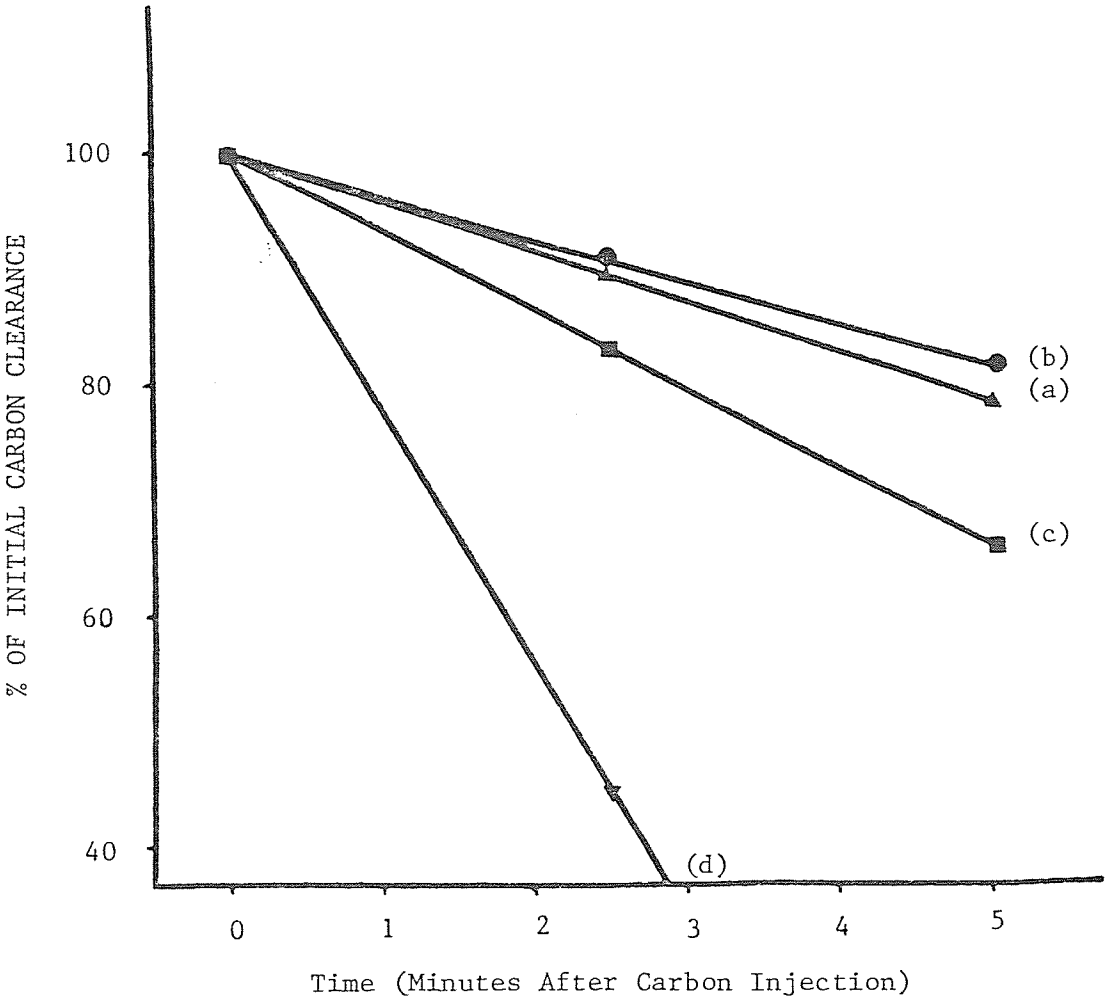
45 Normal B6AF1 male mice were injected intravenously with 7.1 mg of silica and 2-4 sacrificed at six weekly intervals for histological examination. (Controls were not examined every week). The carbon groups received 2.0 mg carbon intravenously 24 hours before being sacrificed.

C. Carbon Clearance:

Any chemical agent which effectively eliminates macrophages should affect the basic macrophage function, phagocytosis. It was thus reasoned that if the macrophage population were depleted to any great extent by silica, this would be evidenced by a lowered phagocytic index. The phagocytic index was measured by injecting carbon particles intravenously and noting the efficiency of its subsequent removal from the blood. The experiments were done with non irradiated animals as it was almost impossible to obtain enough blood from irradiated animals. The animals were bled from the retro orbital plexus to get a zero reading, carbon was then injected into the lateral caudal vein and tail bleedings were taken at one minute intervals to determine the rate of clearance of the injected carbon. Spectrophotometric readings gave the amount of carbon in a particular sample.

Data was treated as described in the methods. This yielded a graph $y = kx + b$ where k is the slope of the line obtained by plotting the amount of carbon per unit of blood taken from the mouse, versus time (one minute intervals). An animal which removed the carbon quickly thus showed a higher k value than an animal which removed carbon more slowly. A graphic illustration of several silica treated groups (figure 6) compared with a control group demonstrates that silica treatment did indeed result in a reduced phagocytic index. Lines a, b and c represent groups that were tested 6 hours, 24 hours and 72 hours respectively after silica treatment. The clearance in the silica treated groups, as indicated by the relative slopes, is significantly less from that of the control group. The reduction in phagocytic index was evident through day 6 but technical problems (animal viability and tail bleeding difficulty) resulted in insufficient data for statistical analysis.

FIGURE 6



Comparison of the mean slopes for the carbon clearance assay. 8-10 week old B6AF1 mice were injected with 0.25 mg/gm silica. At intervals of 6,24 and 72 hours, they were injected intravenously with 0.036 mg/gm carbon particles and the rate of clearance from the blood of this carbon was measured. Mean slopes for the respective groups were (a) 6 hours, -0.033; (b) 24 hours, -0.028; (c) 72 hours, - 0.051 and (d) the control group, -0.165. Values represent the means of 11-40 animals. Control vs experimental differences were significant at a confidence level of 0.01% using analysis of variance.

D. Homing of Donor Spleenocytes:

Since it was planned to investigate the effects of silica on the CMC and PFC responses in the spleen, it was considered necessary to establish if this treatment affects in any way the capacity of injected lymphoid cells to home in this organ. This would allow one to distinguish an increase (or decrease) of the response due to increased (or decreased) tendency of immunocompetent cells to localize in the spleen, from a more direct effect of silica on the immune response. For this reason, spleen cells were labelled with ^{51}Cr and injected intravenously in syngeneic (C57BL/6) or hybrid (B6AF1) recipients which were then sacrificed at various time intervals. The uptake of cells in the different organs, calculated from the radioactivity measured in each organ, was expressed as the percentage of total radioactivity injected. Different experiments included; (a) an exploratory experiment with normal animals receiving syngeneic cells (C57BL/6 cells into C57BL/6), to establish the normal uptake in the liver, spleen, lungs, blood and lymph nodes as well as any fluctuations over a seven day period. (b) the effect of radiation of recipients on the homing of semi syngeneic cells (C57BL/6 cells into B6AF1). (c) the effect of radiation of the donor cells on homing (C57BL/6 cells into B6AF1). (d) the effect of increasing doses of silica on homing of semi syngeneic cells.

(a) Injection of ^{51}Cr labelled syngeneic cells into normal mice (C57BL/6J cells into C57BL/6J) produced the results shown in Table VI and Figure 7. The thymus and the blood retained very little radioactivity and readings were taken only the first 24 hours. (Total blood

counts initially less than one hour were quite high but dropped as the injected cells left the blood stream). The lungs also, after showing high activity initially, dropped rapidly down to less than 1% of the cells injected. This probably was due to initial back-up in the capillaries and venules of the lung. On the other hand, all measurements of radioactivity done after 24 hours, appear to reflect a steady state with a slow progressive reduction of radioactivity probably due to cell death. For this reason, all subsequent experiments involved the measurement of the radioactivity taken up by the spleen and the liver after 24 hours, and in some experiments on subsequent days.

It was concluded from this initial experiment that the injected cells reach the organs of their final destination only after several hours, and that the measurement of radioactivity before 24 hours may not be a reliable index of the ability of cells to home into the spleen.

(b) In an experiment to examine the effect of irradiation on homing of semisyngeneic cells, C57BL/6 cells were injected into irradiated B6AF1 hosts, as well as non irradiated hosts. Irradiated B6AF1 animals received 900 rads whole body radiation from a Cobalt source and 2.7×10^6 ^{51}Cr labelled C57BL/6 cells. The results in the spleen (Figure 8, Table VII) show little difference in chromium uptake in the two groups i.e. irradiated versus non irradiated. In the liver (Figure 9) no significant differences between the two groups was seen either. Thus, it may be concluded that irradiation of the host does not affect the capacity of lymphoid cells to home into the spleen and liver.

(c) C57BL/6 donor cells were given increasing doses of irradiation, up to 1200 rads to see how this affected homing in irradiated B6AF1 hosts. Values for day 1 after cell injection, and day 6 after cell injection are

shown in Table V111 and also graphically in Figures 10 & 11, for the spleen and the liver respectively. Values are given as percent of total radioactivity injected. It may be concluded from these results that the dose of radiation given to the injected cells had very little effect on their homing properties.

(d) The effect of silica on homing, (or trapping) was demonstrated by several dose response experiments. Cells were injected 18 hours after irradiation and 2 hours after silica injections. The results in two different experiments are reported in Table IX and Figure 12, for the first experiment and in Table X and Figure 13 and 14 for the second. In spite of differences, as far as the homing to the spleen is concerned, the two experiments demonstrate the same features. Homing to the liver is negatively affected by silica and even the low dose of 0.05 mg/gm features a marked decrease of the uptake of radioactively labelled cells. After this initial drop, the values remained low throughout the remaining doses tested. In the spleen silica produced an initial increase of the uptake of labelled cells (from 0.05 mg/gm to 0.1 mg/gm in one experiment and from 0.05 to 0.2 in the other, only the latter being significant at $P < 0.05$). Higher doses produced a return to control values or even a reduction below these values in both experiments. There are some differences between these two experiments and between the experiment of Figure 13 and the experiment, examined previously, of Figure 8. On the one hand in the experiment of Figure 13 a higher uptake of radioactive chromium was observed in comparison with both the experiments of Figure 8 and that of Figure 12. This may be due to a better cell viability at the time of cell injection in the experiment of Figure 13 which resulted

in more cells homing to the different organs and fewer being eliminated. On the other hand in the experiment of Figure 13 there was a more marked rise of the uptake of radioactively labelled cells with low doses of silica and a slower drop with higher doses than in the experiment of Figure 12. Since the difference between the two experiments consisted of the different day in which chromium uptake was measured (day 6 in the experiment of Figure 12 and day 1 in the experiment of Figure 13), this may indicate that low silica doses induced a transient increase of homing in the spleen which disappeared by day 6. These data, along with those in cell proliferation and silica dose mortality studies were used in selecting an appropriate silica dose for use in future experiments. The dose selected (0.2 mg/gm) did not significantly reduce the homing of labelled cells to the spleen and actually increased it in one experiment. Since cell proliferation studies tended to support this conclusion, 0.2 mg/gm was considered appropriate.

Table VI

Localization of radioactively labelled spleen cells in different organs of normal syngeneic recipients. (a)

ORGAN	PERCENT OF TOTAL CELLS (b)							
	1 Hour	3 Hours	6 Hours	1 day	4 days	5 days	6 days	7 days
Blood (0.1 ml)	0.25	0.3±.1	.138±.005	.085	--	--	--	--
Liver	13.8	16±2	18±1	17.29	13.3±.5	19.2±.8	15±1	15±1
Lung	17.0	7.09±.07	2.7±.2	.845	--	--	--	--
L.N. (8)	1.83	1.97±.04	2.02±.04	2.27	--	--	--	--
Spleen	24.33	23.4±.3	23±1	25.08	19±2	19±2	16±1	19±2
Thymus	0.11	.10±.02	.098±.007	.133	--	--	--	--

(a) To examine the tendency for cells to home into various organs, 3×10^6 ^{51}Cr labelled C57BL/6 cells were injected into syngeneic hosts. The cells had been incubated at 37° for 45 minutes with 300 microCuries ^{51}Cr for every 5×10^7 cells. Each point represents the mean of two animals.

(b) % of Total injected radioactivity measured in the respective organs at various times.

Figure 7

Homing of spleen cells in different organs of syngeneic recipients.

Activity per organ (a) spleen, (b) liver, (c) lungs, (d)lymph nodes, and (e) blood was measured as percent of total activity injected. The experimental conditions are described in footnote (a) of Table VI.

FIGURE 7

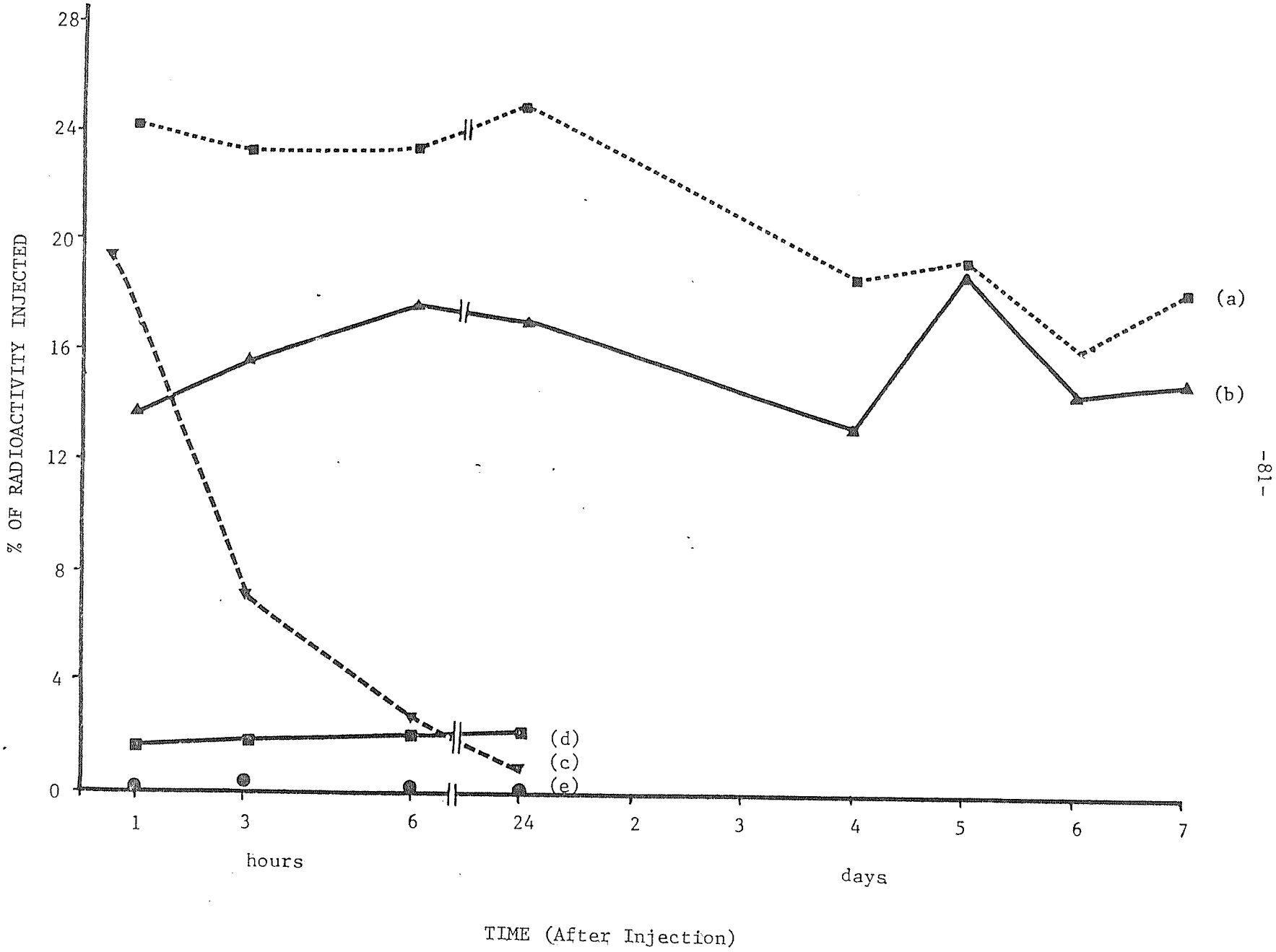

28
24
20
16
12
8
4
0
% OF RADIOACTIVITY INJECTED
(a)
(b)
(c)
(d)
(e)
1
3
6
24
2
3
4
5
6
7
hours
days
TIME (After Injection)

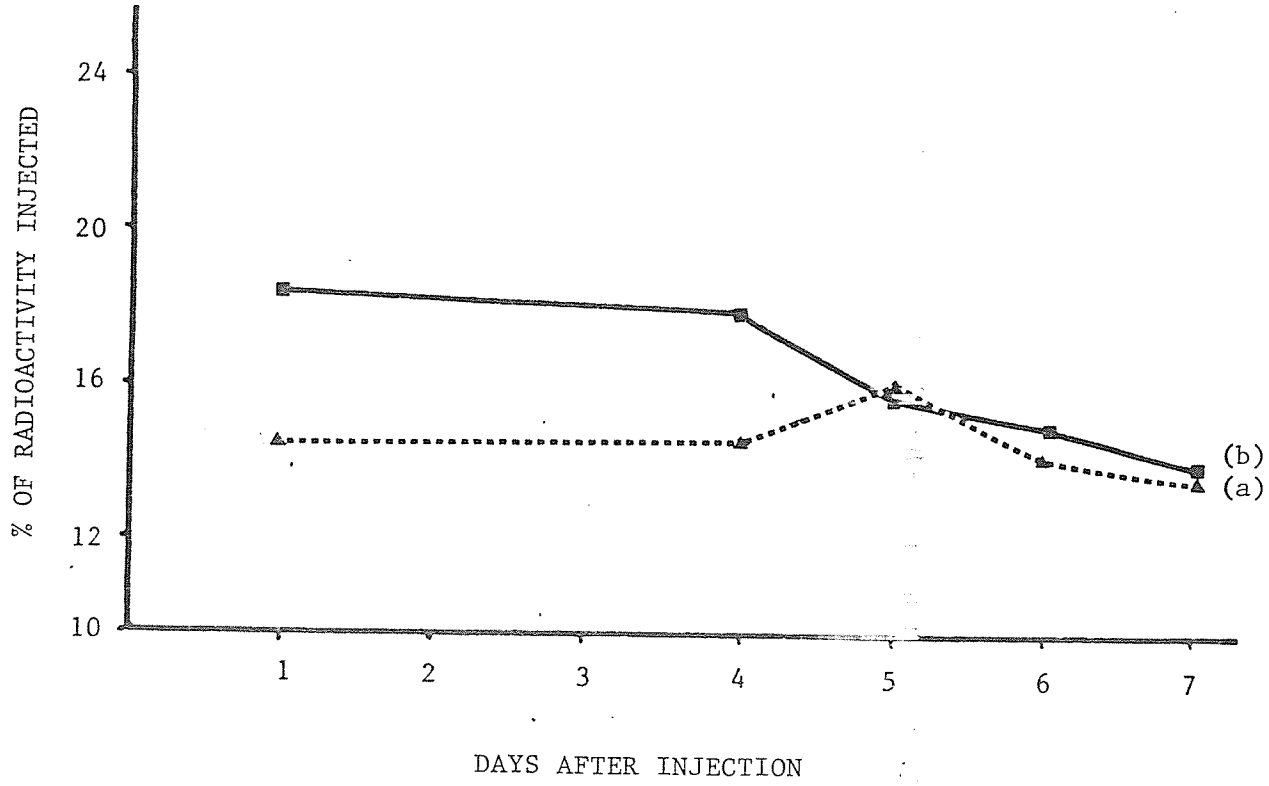
Table VII

Localization of Radioactively Labelled Parental Spleen Cells in the Livers and Spleens of Irradiated and Nonirradiated F1 Hybrid Recipients. [a]

GROUP	TIMES				
Nonirrad.	Day 1	Day 4	Day 5	Day 6	Day 7
Spleen	18.4	18.0	16.0	15.0	14.0
Liver	22.0	21.0	20.0	20.0	18.0
Irradiated					
Spleen	14.0	14.0	16.3	14.2	14.0
Liver	20.0	24.0	22.0	19.0	19.0

a. 9 - 12 week old B6AF1 mice were given 900 rads whole body irradiation. 16 hours later they received 2.7×10^6 ^{51}Cr labelled C57BL/6 spleen cells intravenously. Labelling conditions were: incubation for 45 minutes at 37^o with 300 microcuries ^{51}Cr per 5×10^7 cells. Activity per organ is measured as % of total label injected. Each value represents the mean of two animals.

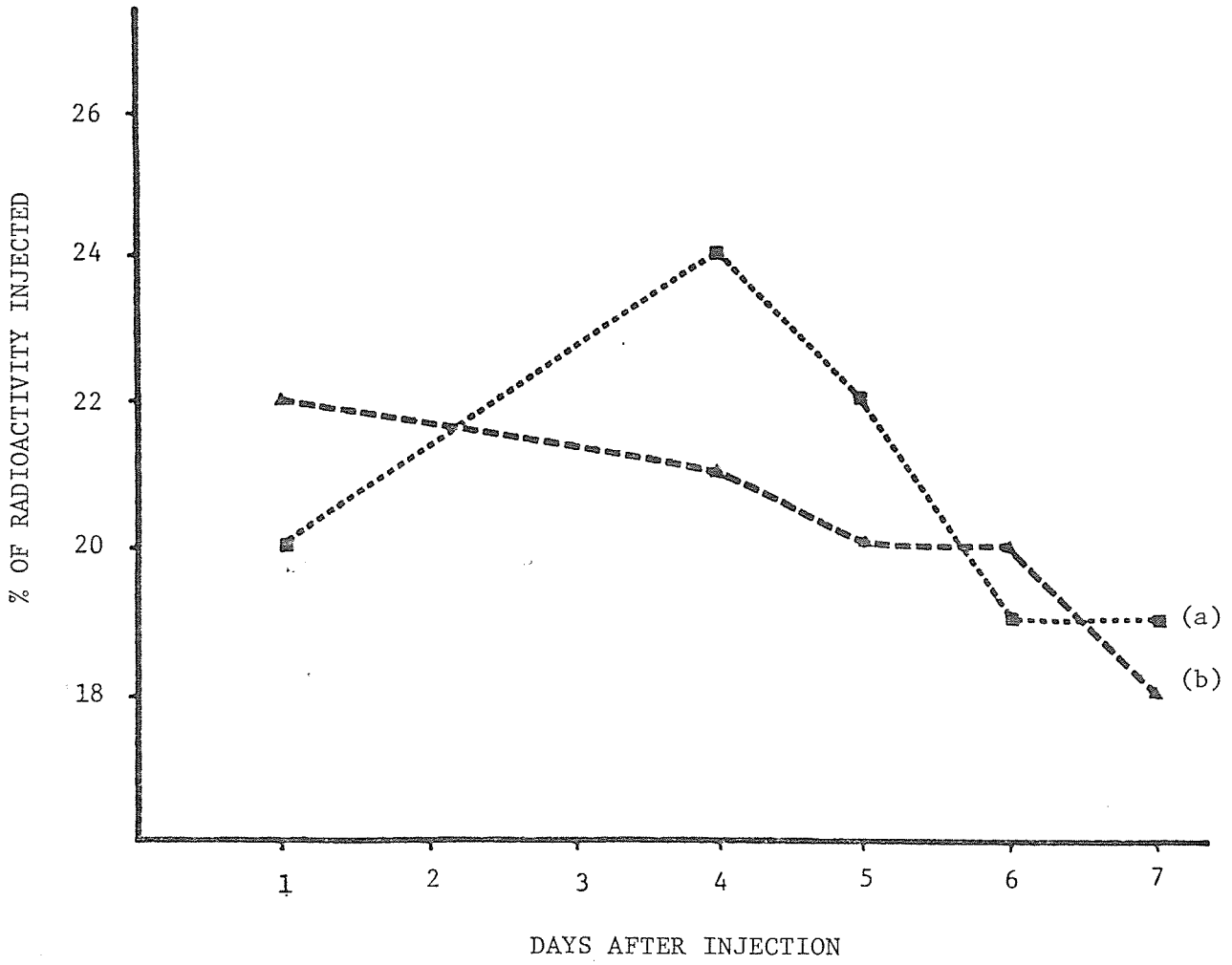
FIGURE 8



Homing of C57BL/6 splenic lymphocytes to the spleen in (a) irradiated and (b) non irradiated B6AF1 recipients.

Experimental conditions were as described in footnote (a) of Table VII. Differences were not statistically significant.

FIGURE 9



Homing of C57BL/6 spleen cells to the liver of (a) irradiated and (b) non irradiated B6AF1 hosts.

Experimental conditions were as described in footnote (a) of Table VII. Differences were not statistically significant.

Table VIII

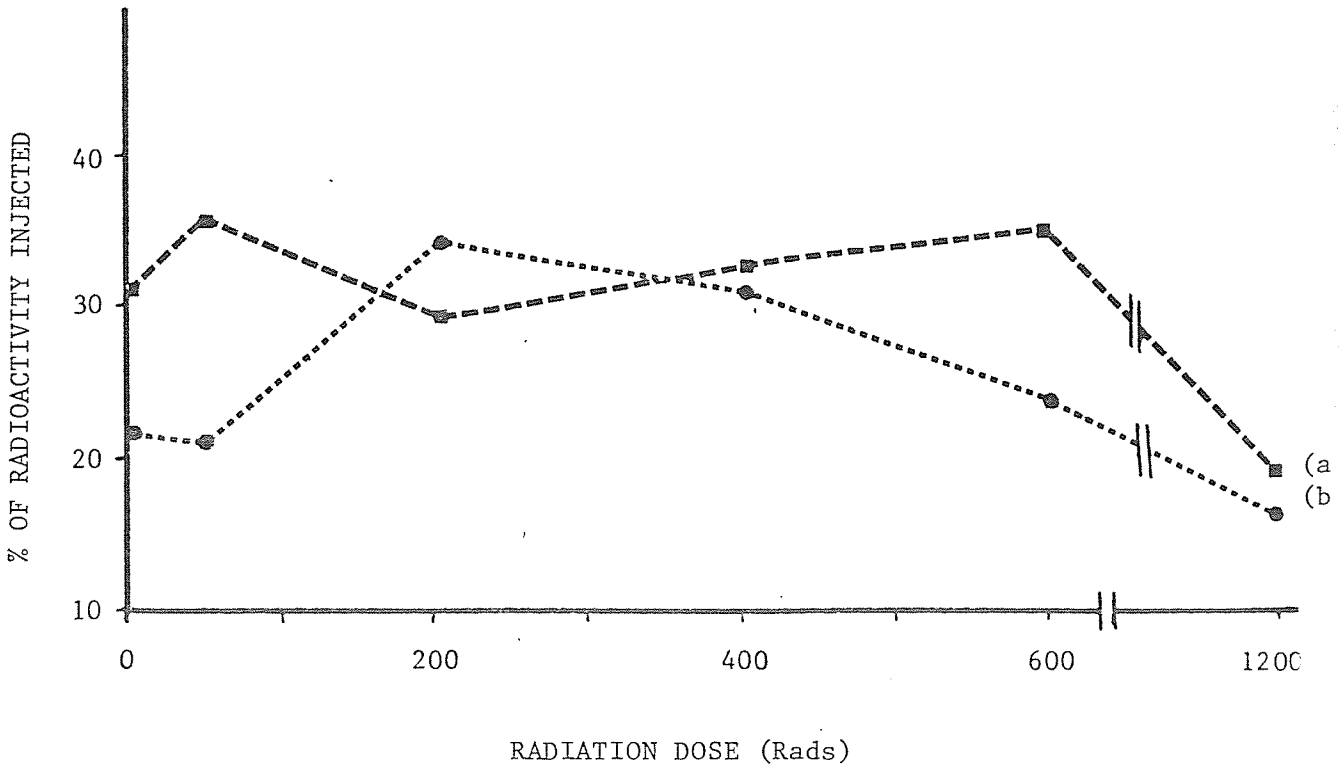
Effect of Irradiating the Donor Spleen Cells (a)

	Rads					
	0	50	200	400	600	1200
Spleen						
% of radioactivity injected.						
Day 1	30.7	35.9	29.3	32.8	35.0	19.2
Day 6	21.7	21.1	31.8	31.2	24.0	16.8
Liver						
Day 1	56.6	40.1	38.6	43.6	42.8	41.6
Day 6	47.2	29.5	32.5	42.0	41.0	39.3

(a) B6AF1 recipients were given 3×10^6 C57BL/6 cells which had been incubated at 37^o for 45 minutes with 300 microCuries ^{51}Cr for every 5×10^7 cells. Recipients were given 900 rads whole body radiation 16 hours before the cells. The differences between irradiated and nonirradiated groups and among the different radiation doses were not statistically significant (using two way analysis of variance).

Values represent the means of two animals. The experiment was repeated with similar results-

FIGURE 10

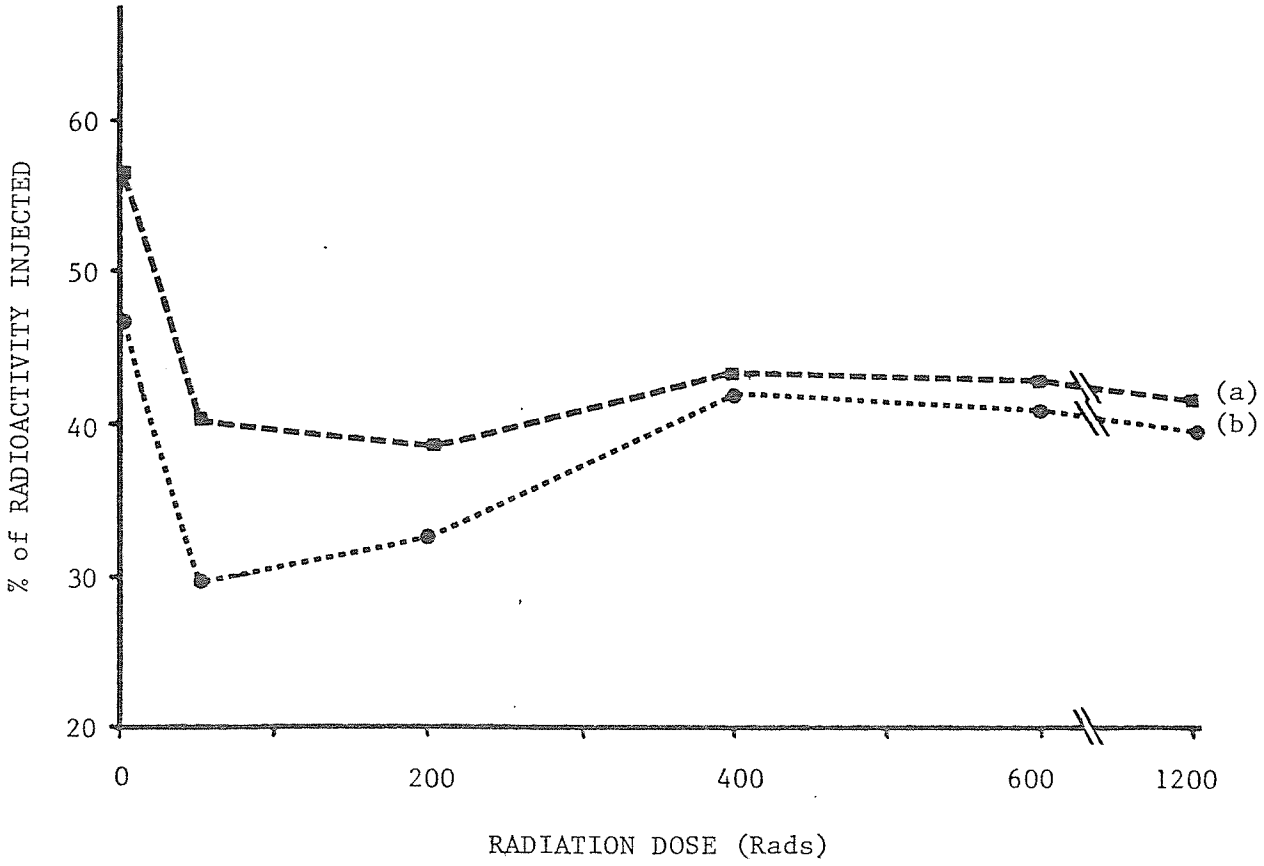


Effect of irradiating the donor cells on homing of C57BL/6 spleen cells to the spleen in B6AF1 mice.

(a) and (b) represent assays taken on day 1 and day 6 respectively after the injection of cells.

Experimental conditions are as described in footnote (a) of Table VIII.

FIGURE 11



Effect of irradiating the donor cells on homing of C57BL/6 spleen cells to the liver in B6AF1 mice.

(a) and (b) represent assays taken on day 1 and day 6 respectively after the injection of cells.

Experimental conditions are as described in footnote (a) of Table VIII.

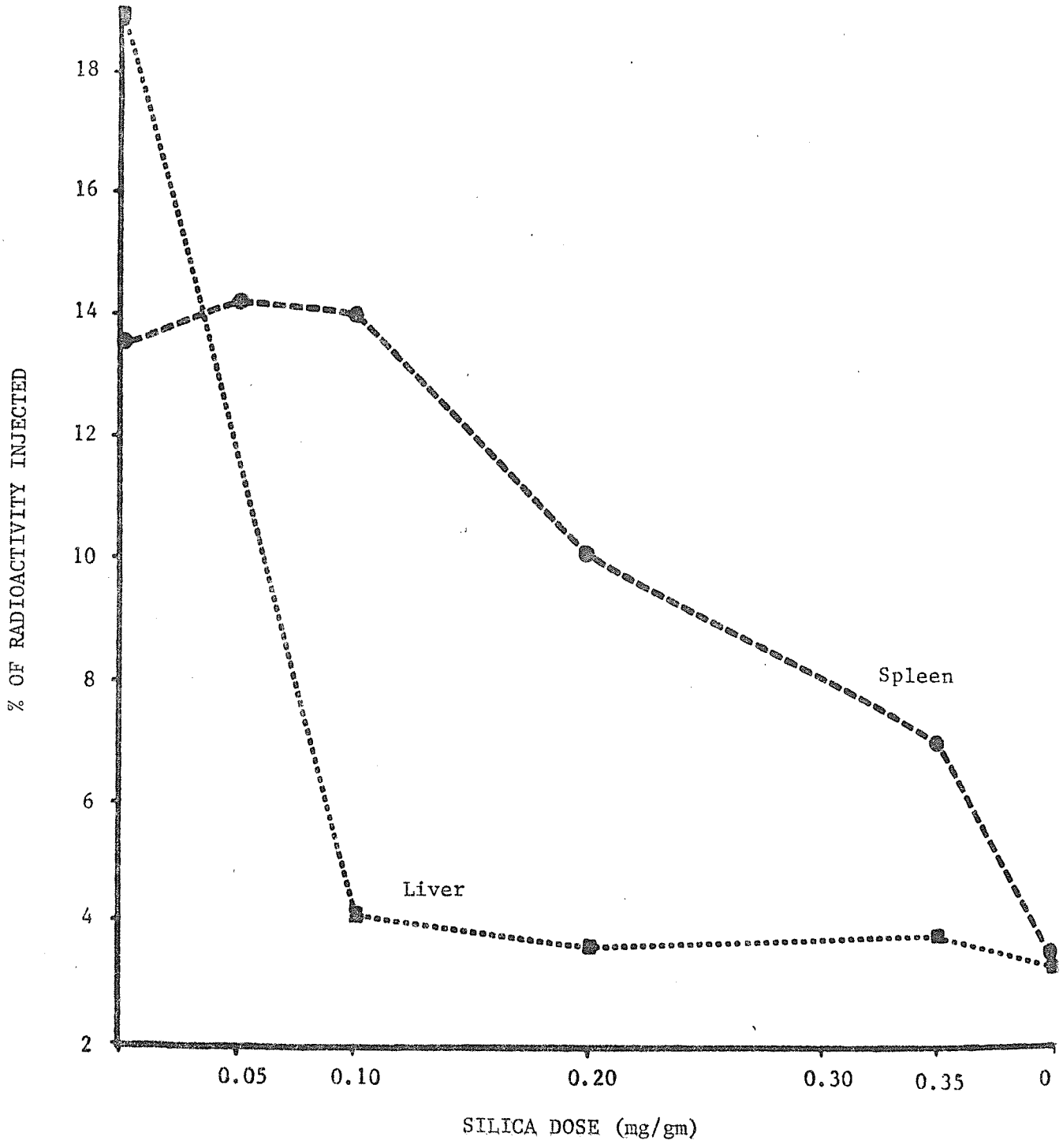
Table IX

Effect of Increasing Silica Doses on Trapping of C57BL/6 Spleen Cells in the Spleen and Liver of B6AF1 Recipients.

	Percent of Radioactivity Injected	
Silica dose (mg/gm)	Spleen	Liver
0	12.49±2	19.03±4
0.05	14.17±.7	8.96±.6
0.10	13.78±1	4.06±.6
0.20	9.87±1	3.64±.2
0.35	7.39±2	3.78±.6
0.40	3.37±1	3.34±.5

9-12 week old B6AF1 mice were given 900 rads whole body radiation, and silica injections 16 hours later. Three hours subsequent to the silica mice were given 3×10^6, ^{51}Cr labelled C57BL/6 cells. Donor cells were incubated at 37^o for 45 minutes with 300 microcuries ^{51}Cr per 5×10^7 cells. Organs were assayed for activity 6 days after injections. Each value represents the mean of at least 3 animals.

FIGURE 12



Effect of increasing silica doses on trapping of C57BL/6 spleen cells in the spleen and liver of B6AF1 recipients.

Experimental conditions were as described in footnote (a) of Table IX.

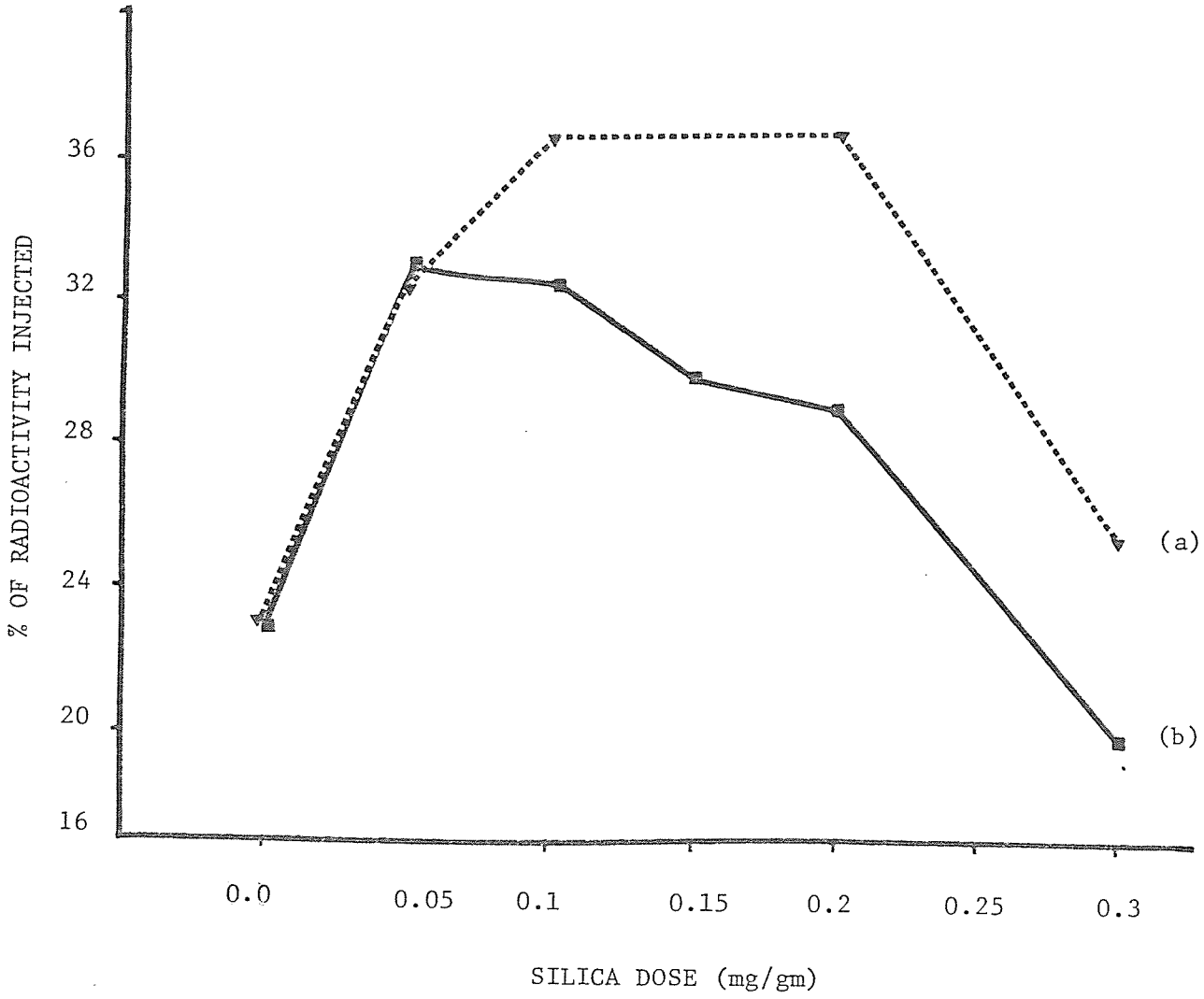
Table X

The Effect of Increasing Silica Doses on Trapping of C57BL/6 Spleen Cells in the Spleen and Liver of B6AF1 Recipients. [a]

Silica Dose	Percent of Radioactivity Injected	
	Spleen	
	A	B
0	19.41	18.95
0.05	28.63	28.91
0.1	32.61	28.47
0.20	32.82	25.07
0.15	-	25.93
0.30	21.38	15.94
	Liver	
0	41.71	36.88
0.05	18.91	13.12
0.1	10.53	8.71
0.15	-	8.53
0.2	11.91	8.19
0.3	10.76	10.01

[a] 9 - 12 week old B6AF1 mice were given 800 rads whole body radiation and silica injections 15 hours later. Three hours subsequent to the silica mice were given (A) 3×10^6 or (B) 5×10^7 ^{51}Cr labelled C57BL/6 cells. Donor cells were incubated at 37^o for 45 minutes with 300 microcuries ^{51}Cr per 5×10^7 cells. Organs were assayed for activity 24 hours after injections. Each value represents the mean of at least 3 animals.

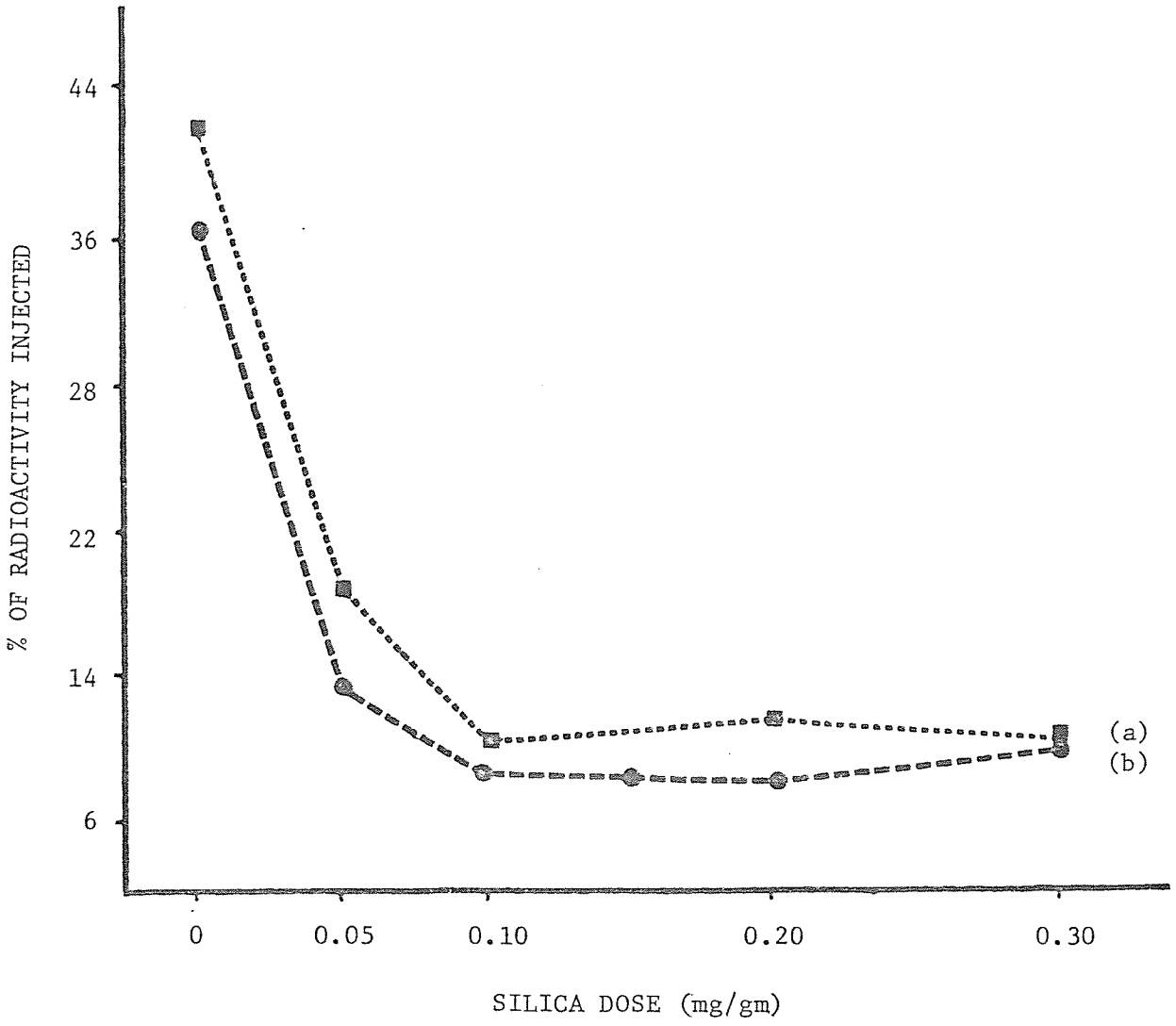
FIGURE 13



Effects of increasing doses of silica on lymphocyte trapping in the spleen.

B6AF1 mice received (a) 3×10^6 or (b) 5×10^7 C57BL/6 spleen cells. Experimental conditions were as described in footnote (a) of Table X.

FIGURE 14



Effects of increasing doses of silica on lymphocyte trapping in the liver.

B6AF1 mice received (a) 3×10^6 pr (b) 5×10^7 spleen cells.

Experimental conditions were as described in footnote (a) of Table X.

E. Cell Proliferation:

The previous experiments with ^{51}Cr labelled cells had indicated that silica doses up to 0.2 mg/gm did not reduce appreciably the accumulation of lymphoid cells in the spleens of irradiated recipients. However, it was necessary to demonstrate that these cells were viable and capable of proliferating. This was demonstrated with the use of ^{125}I-labelled IUdR which is taken up by cells during DNA synthesis. The $^{125}IUdR$ proliferative activity is shown as percent of total radioactivity injected. Briefly, cell proliferation experiments involved determining the kinetics of lymphocyte proliferation in syngeneic and non syngeneic mice, and observing the effect of increasing doses of silica on cell proliferation.

In an experiment on the kinetics of cellular proliferation, B6AF1 and C57BL/6 mice were irradiated and injected with syngeneic or semi-syngeneic cells immediately after. C57BL/6 mice received $5x10^7$ syngeneic spleen cells, and B6AF1 mice received $2.5x10^7$, $5x10^7$ and $7.5x10^7$ C57BL/6 spleen cells intravenously. Mice were sacrificed daily from day 3 to day 6 after the cell injections; 25 hours prior to killing the mice they received 10^{-7} moles FUdR intraperitoneally,* and an hour later received .5 microcuries $^{125}IUdR$. The uptake of radioactivity in the spleen and liver was measured 24 hours later. As seen in figure 15, in the spleen, the proliferative activity of the same cell number was different in the syngeneic hosts as compared to the B6AF1 hybrid ones. In the former, this activity increased almost linearly with time indicating a continuous increase of the proliferating pool of cells. On the other hand, in the hybrid hosts, the activity

* FUdR blocks the endogenous synthesis of thymidine and, therefore, favours the uptake of $^{125}IUdR$ given one hour later.

of parental cells initially increased (from day 3 to day 4) but later levelled off with the exception of the group of mice receiving the highest cell dose (7.5×10^7 cells) which presented a somewhat higher activity on day 6 than on days 4 and 5. The results observed in the liver (Figure 16) and lymph nodes (Figure 17) of the same animals demonstrated that in these organs, syngeneic cells did not produce significant proliferation and ^{125}IUdR uptake was not appreciably different from that in non injected controls. However, in the B6AF1 hybrid hosts proliferation of parental cells was intense both in the liver and the lymph nodes, and peaked on day 4, or 5 depending on the dose of cells injected. This intense proliferation is probably the expression of a GVH reaction.

The effect of various doses of silica on proliferation was next studied. B6AF1 mice received a lethal dose of radiation (850 rads) and were injected with silica, and either syngeneic or semi-syngeneic C57BL/6 spleen cells the next morning. As shown in Figure 18, confirming the results of the previous experiment (Fig. 15), the spleens of mice receiving syngeneic cells showed on the sixth day a higher rate of proliferation, compared to the spleens of mice receiving semi-syngeneic cells. Both these groups showed a modest increase in proliferation corresponding to the increase in silica dose with a levelling off after 0.15 mg/gm. However, these effects were not statistically significant. Liver uptake was significantly higher in the F1 hybrid (Fig. 16). In this group there was a dose-related increase of proliferation with a maximum at the dose of 0.15 mg/gm, which was followed by lower viability which was still considerably higher than that in syngeneic hosts. These data confirmed that the working dose for silica of 0.20 mg/gm indicated as most suitable by the previous experiments on the homing of ^{51}Cr-labelled cells, did not significantly impair the capacity of spleen cells

to proliferate in the spleen . Several experiments employing this dose were then run, comparing silica and non silica groups. The design was identical to the silica dose experiments with B6AF1 mice receiving silica and a lethal dose of radiation and C57BL/6 cells the morning after. Silica and non silica groups were compared between days 3 and 7, after the injection of the cells. In agreement with the previous results (Fig. 18), silica treated groups showed a slightly higher activity than non silica groups in the spleen (Fig. 19) and did not differ appreciably in the liver (Fig.20). Higher activity in the spleen of the silica groups may be related to weakening of the hybrid resistance phenomenon by silica as proposed by Lotzova & Cudkowicz (1974). The implications of this will be discussed later. One can conclude though that the dose of silica used in these experiments does not seem to have a detrimental effect on cell viability and proliferation under the circumstances indicated.

FIGURE 15

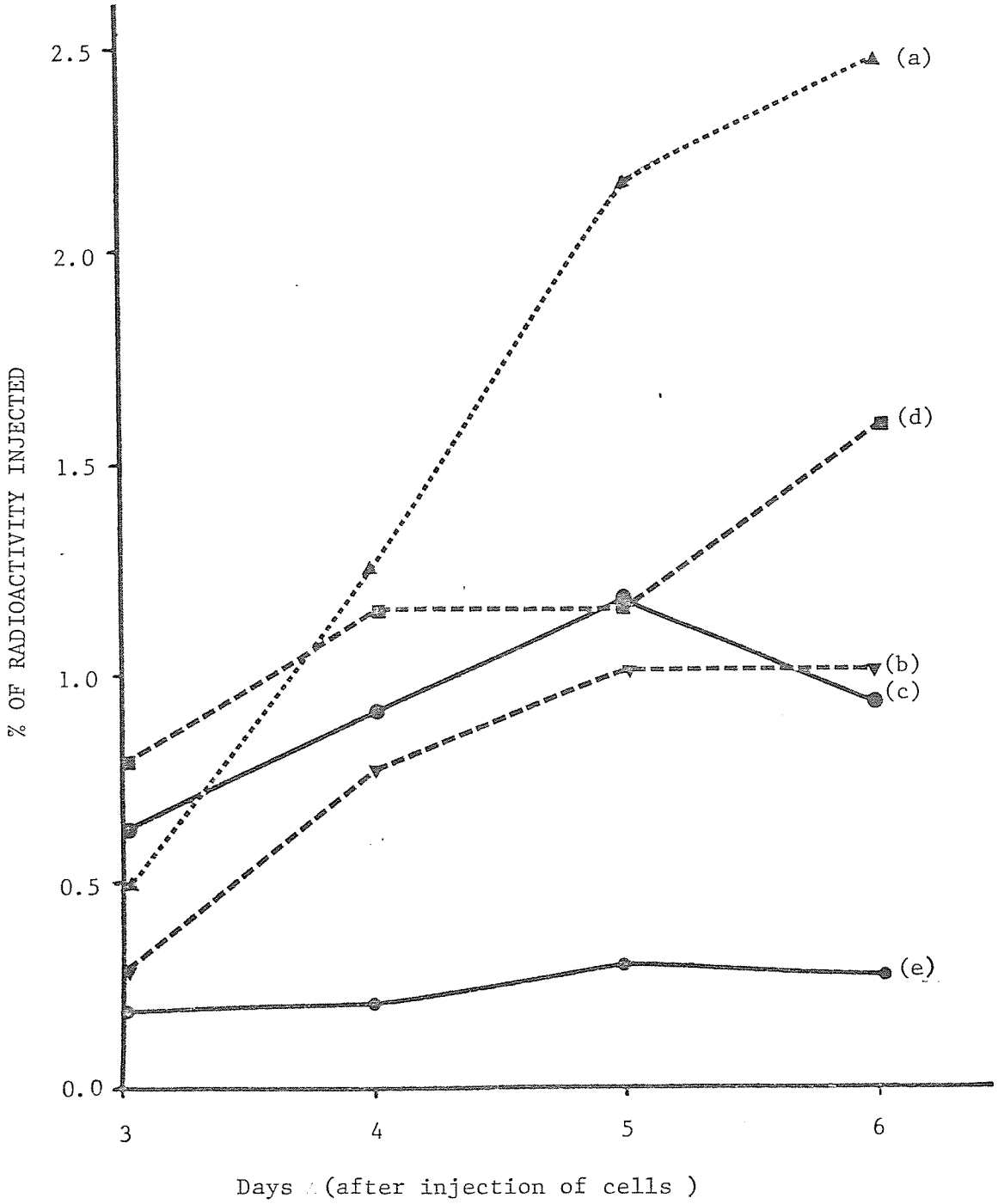
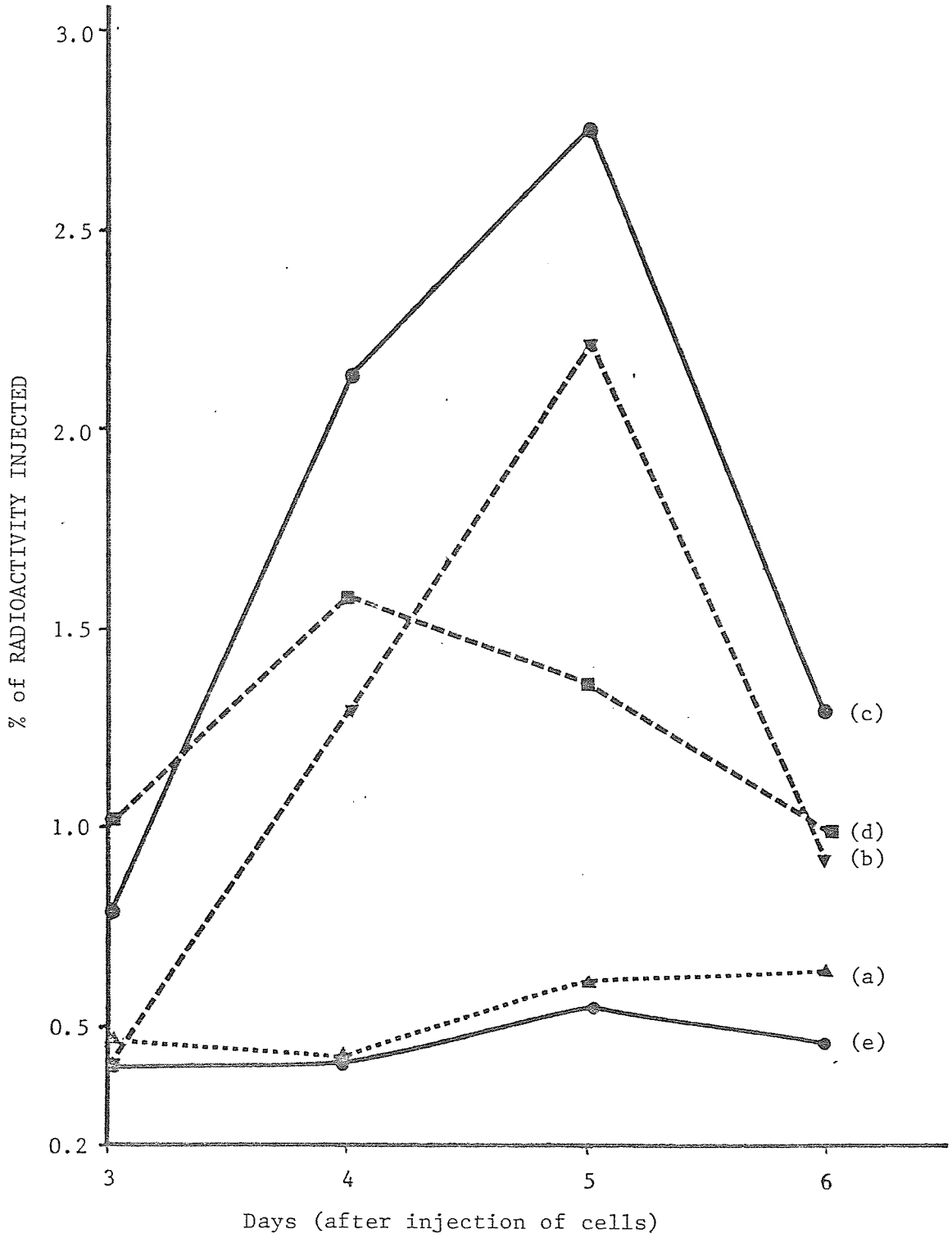

2.5
2.0
1.5
1.0
0.5
0.0
% OF RADIOACTIVITY INJECTED
(a)
(d)
(b)
(c)
(e)
3
4
5
6
Days (after injection of cells)

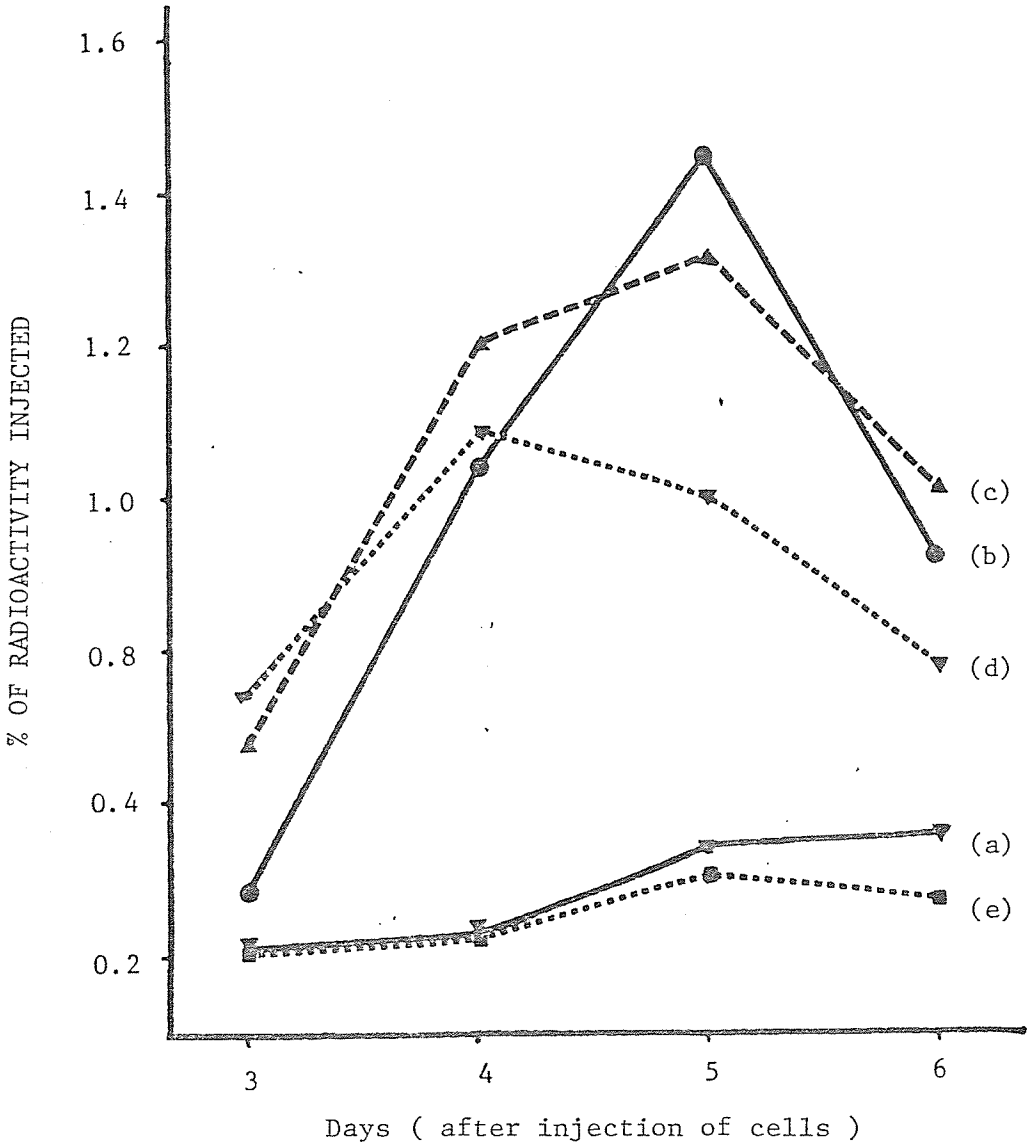
FIGURE 16



The kinetics of syngeneic and semi syngeneic cell proliferation in the livers of irradiated hosts.

Experimental conditions were as described in Figure 15 (a) $5x10^7$ C57BL/6 cells into C57BL/6 mice (b) $2.5x10^7$ C57BL/6 cells into B6AF1 mice (c) $5x10^7$ C57BL/6 cells into B6AF1 mice (d) $7.5x10^7$ C57BL/6 cells into B6AF1 mice (e) no cells (B6AF1).

FIGURE 17



The kinetics of syngeneic and semi syngeneic cell proliferation in the lymph nodes of irradiated hosts.

Experimental conditions were as described in Figure 15.

(a) $5x10^7$ C57bl/6 cells into C57BL/6

(b) $2.5x10^7$ C57BL/6 cells into B6AF1

(c) $5x10^7$ C57BL/6 cells into B6AF1

(d) $7.5x10^7$ C57BL/6 cells into B6AF1

(e) no cells (B6AF1)

FIGURE 18

Effect of increasing silica doses on cell proliferation.

9-12 week old B6AF1 mice were given 850 rads whole body radiation and received silica intravenously 16 hours later. Two hours after silica they received (a) $5x10^7$ B6AF1 cells injected into B6AF1 mice or (b) $5x10^7$ C57BL/6 cells injected into B6AF1 mice.

On the sixth day after injections animals received 0.5 micro Curies ^{125}IUdR. The uptake of radioactivity was measured 24 hours later. Points represent the means of two animals.

FIGURE 18

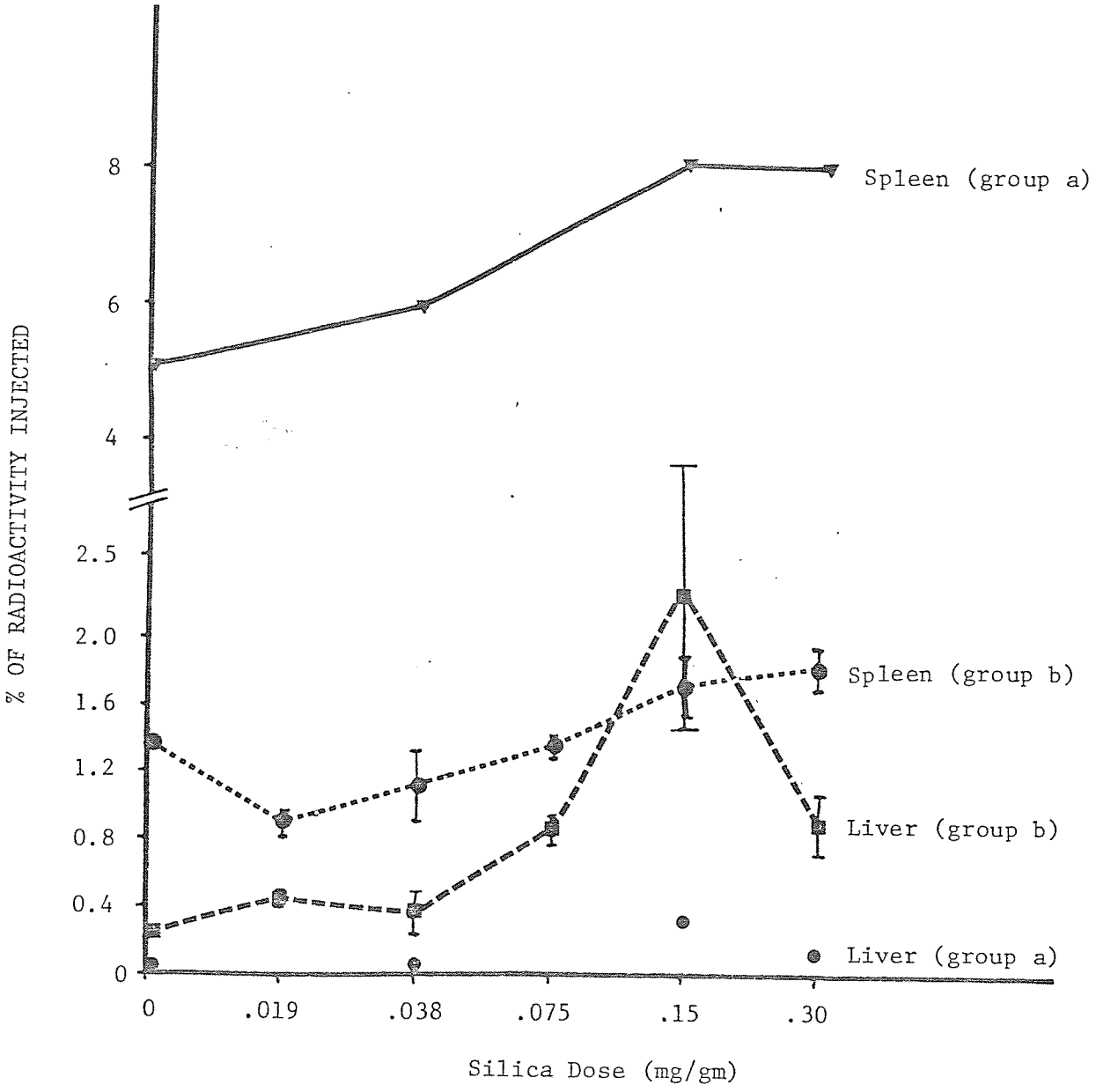

% OF RADIOACTIVITY INJECTED
8
6
4
2.5
2.0
1.6
1.2
0.8
0.4
0
0
.019
.038
.075
.15
.30
Silica Dose (mg/gm)
Spleen (group a)
Spleen (group b)
Liver (group b)
Liver (group a)

FIGURE 19

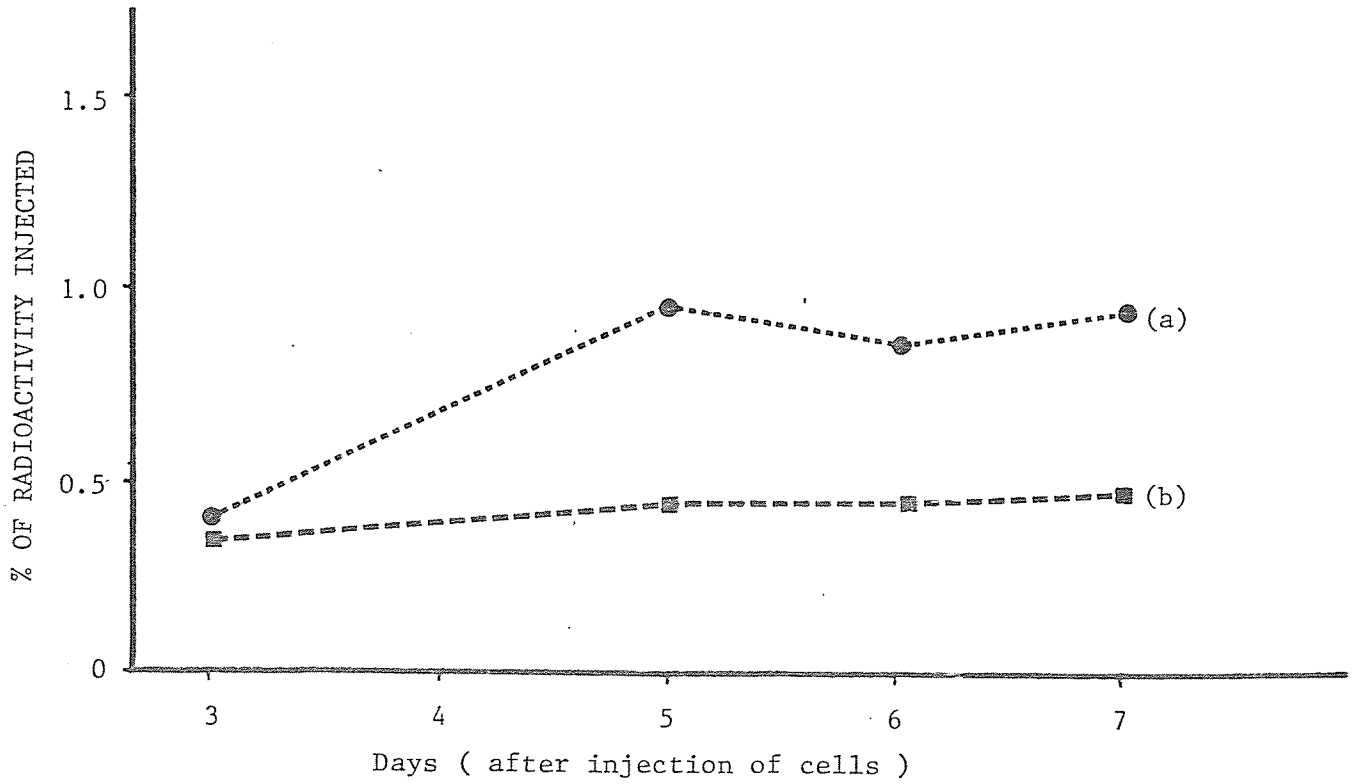
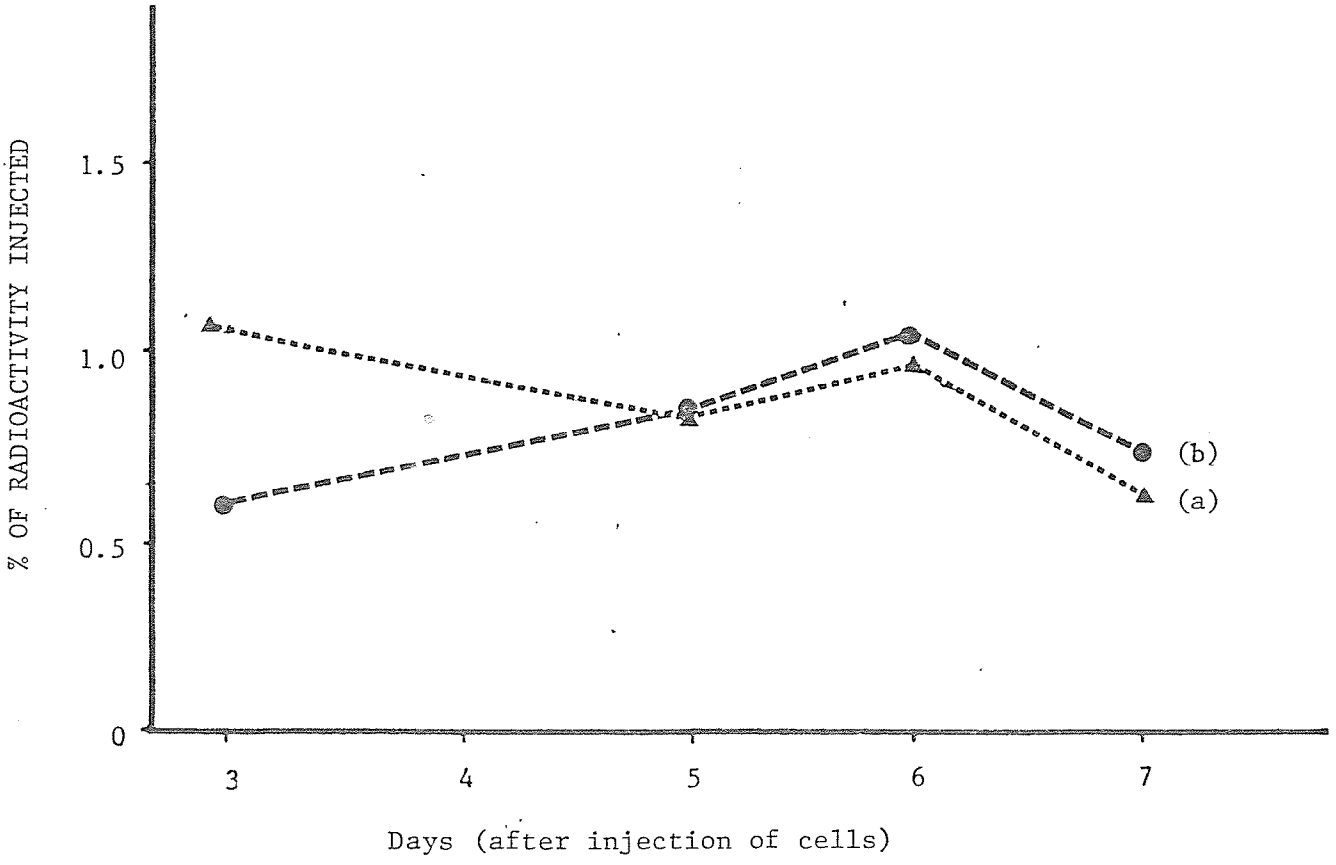

% OF RADIOACTIVITY INJECTED
1.5
1.0
0.5
0
(a)
(b)
3
4
5
6
7
Days (after injection of cells)

FIGURE 20



Effect of silica on cell proliferation in the semi syngeneic liver.

Experimental conditions were as described in Figure 19.

(a) Silica treated group

(b) Received cells only

F. Cell Mediated Cytotoxicity:

As is explained earlier, the assay for cell mediated cytotoxicity involves injecting lethally irradiated B6AF1 mice with parental C57BL/6 spleen cells to induce sensitization of the donor cells to the host allo-antigens. Subsequent measurement of the lysis induced by these cells recovered from the spleen 6 days later, of the mastocytoma tumor cells P815, which share some of their H-2 determinants with the B6AF1 strain, gives an indication of the degree of sensitization of the parental cells. The objective of the present experiments was to measure the effect of silica on this response. The effects of silica on spleen index and on spleen cellularity in the GVH reaction will also be discussed.

B6AF1 female mice were given 800-850 rads whole body irradiation, injected with silica 16 hours later, and with spleen cells 2 hours after the silica. Six days later the spleens of the recipient animals were removed on the sixth day after injections of cells, made up into single cell suspensions and incubated with ^{51}Cr labelled target cells in varying ratios. Cell lysis was determined by chromium release. Four groups were generally used. (a) B6AF1 cells injected into B6AF1; (b) B6AF1 cells injected into silica treated B6AF1; (c) C57BL/6 cells injected into B6AF1; (d) C57BL/6 cells injected into silica treated B6AF1. Groups a and b were the controls, as they received syngeneic cells, and never produced any significant target cell lysis. Therefore, the results are reported only for groups c and d.

As shown in Table XI four different experiments yielded the same results in spite of somewhat different values of lysis in different experiments. Percent lysis in all groups of animals varied with the effector to target cell ratios. From this dose-response effect it was possible to calculate in all

cases the number of cells needed to induce 33% lysis (LD33) and the number of lytic units per spleen. In the F1 hybrid injected with parental cells only LD33 varied from 0.65 to 4.27 and was not significantly different from the LD33 values in the mice receiving parental cells and silica which varied from 0.49 to 2.6 (except for two unrealistically high values). On the other hand the values of lytic units per spleen were significantly lower in the silica treated groups because in these animals an appreciably lower yield of cells was observed. (Table XI).

Lower yields of cells in silica treated mice may have been due to an actual lower number of cells, or to incomplete recovery of these cells because of the increased consistency of the silica treated spleens which were more difficult to tease and to reduce to single cell suspensions. Cell yields in four different experiments (the same experiments as described in Table XI) are reported in Table XII. Cell yields in experiment I are generally low because a problem with cell clumps required resuspension of the cells, resulting in considerable loss of cells. Three out of four experiments however show higher cell yields in silica treated groups (for syngeneic groups) than non silica groups. Semi-syngeneic hosts (F1 hosts receiving parental cells) receiving silica showed lower cell yields than the non silica group in all four experiments listed. Thus the low cell yields in these groups cannot be explained by a low recovery of cells but rather indicate an actual low number of parental cells in the F1 hybrid spleens. This effect may be due to a form of suppression specific to the cell mediated response because as is shown later in the PFC section the humoral response appears to be stimulated by identical treatment with silica.

TABLE XI

THE EFFECTS OF SILICA ON CELL MEDIATED CYTOTOXICITY (a)

Group	Cell Yield ($X 10^5$)	Percent Lysis 30:1	15:1	6:1	3:1	Cells for 33% Lysis = 1 lytic Unit	Lytic Units per Spleen
Experiment I							
C_1	162	68	47		31	0.74	220
C_2	159	55	46		8	1.74	91
C_3	105	79	78		31	0.65	163
$\bar{X}$						1.04	158
D_1	18*	98	92		39	0.50	36
D_2	14*	77	74		38	0.48	30
$\bar{X}$						0.49	33
Experiment II							
C_1	340	80	35	5		2.93	117
C_2	330	85	46	16		2.03	163
C_3	280	78	59	25		1.49	188
C_4	350	69	30	5		3.18	110
C_5	370	87	83	12		1.58	234
$\bar{X}$						2.24	162
D_1	94	104	78	34		1.18	80
D_2	240	77	60	3		1.95	123
D_3	75	85	74	22		1.46	51
D_4	108	27	37	0		N.D.	N.D.
$\bar{X}$						1.53	85

* = mean of 2 animals

TABLE XI -continued

Group	Cell Yield ($\times 10^5$)	Percent Lysis Effector:Target cells		Cells for 33% Lysis ($\times 10^5$)	Lytic Units per Spleen
		50:1	10:1		
Experiment III					
C_1	420	82	25	2.50	168
C_2	855	85	31	2.12	404
C_3	375	61	8	4.27	88
$\overline{X}$				2.96	220
D_1	86	17	0	46.0	2
D_2	155	24	-2	17	9
D_3	45	83	40	1.53	29
$\overline{X}$				21.5	13
Experiment IV					
C_1	190	54	24	3.24	59
C_2	230	69	28	2.43	95
C_3	280	69	28	2.43	115
$\overline{X}$				2.70	90
D_1	43	70	55	3.35	13
D_2	161	44	34	1.70	95
D_3	42	79	24	2.60	16
$\overline{X}$				2.55	41

(a) 9-12 week old B6AF1 female mice were given 800-850 rads whole body radiation injected with silica 16 hours later and with cells (see below) 2 hours subsequent to the silica. The groups were as follows:

(A) B6AF1 cells injected into B6AF1

(B) B6AF1 cells into silica treated B6AF1

(C) C57BL/6 cells into B6AF1

(D) C57BL/6 cells into silica treated B6AF1

In experiments I - II animals received 800 rads and 7.5×10^7 cells and in III - IV they received 850 rads and 5×10^7 cells.

The values given represent individual animals except as stated.

TABLE XII

Cell Recovery From Irradiated Reconstituted Animals.[a]

Group	Experiment #			
	I	II	III	IV
A_1	45	440	181	910
A_2	36	710	870	700
A_3	72	470	--	1360
A_4				420
$\overline{X}$	51	540	526	848
B_1	36	850	740	120
B_2	174	750	2100	1060
B_3	78	--	1930	900
B_4				242
$\overline{X}$	96	800	1590	581
C_1	162	340	268	1060
C_2	30	330	420	1030
C_3	159	280	332	750
C_4	106	350	--	840
C_5	66	370	--	--
$\overline{X}$	104	334	340	920
D_1	12	94	74	148
D_2	24	70	22	168
D_3	15	240	--	320
D_4	12	108	--	286
D_5	--	75	--	--
$\overline{X}$	16	117	22	231

(a) See Table XI for experimental details.

G. Plaque Forming Cell Assay:

In this section the effect of silica on the response to sheep erythrocytes (SRBC) is studied. Adult B6AF1 mice were given a lethal dose of radiation and then injected with silica and syngeneic spleen cells. They also received sensitizing doses of SRBC (0.2 ml of a 2% solution) along with the spleen cells. Preliminary experiments showed that irradiated, reconstituted mice developed a direct (IgM) response not earlier than day four and that this response peaked five to five and a half days after sensitization. Similarily the peak for the indirect (IgG) response was on day 8-9. To cover the expected range of the direct and indirect responses, assays were done on days 4 to 12. Since macrophages are considered necessary for a PFC response (Bice et al 1972a) and since in the present experimental model radioresistant host macrophages may be expected to give a major contribution to the response, the elimination of host macrophages by silica was expected to lead to a reduction of the PFC response. The results in a preliminary assay were contrary to this expectation (Table XIII). The silica treated group, while not differing appreciably on the fifth day after sensitization, showed a much greater response nine days after sensitization. To verify that this was not merely a shift in kinetics for the silica treated group, experiments designed to cover the range of the direct and the indirect response were done. Table XIV shows the direct response on days 5,6 and 8 after sensitization, as well as the indirect response of day 8. As is readily seen, the response in silica treated animals is greater than non silica groups on all days. It was concluded that a shift in kinetics alone would not have caused the increase of direct plaques.

Another experiment was performed to examine the indirect response on

different days. The indirect antibody response is examined on days 7,9 and 11 resulting again with higher responses in silica treated groups (Table XV). Silica treated mice generally had more specific activity (plaques per 10^6 cells) and more activity per spleen (total plaques). Cell yields per spleen were also higher in silica treated groups but varied more than did the specific activity. This may be due to the combination of radiation and silica having a harsher effect on animals in some experiments due possibly to age factors but more likely due to latent bacterial and/or viral infections as discussed earlier. Values as reported represent individual animals. Means are also given.

TABLE XIII

The Effect of Silica on the Antibody Response to SRBC[a]

Group	Cell yield/spleen ($\times 10^6$)		Plaques/10^6 cells		Plaques/spleen	
Cells & SRBC		**DAY 5**				
1	4.8		26		126	
2	15.0	$\overline{X}$=11	30	$\overline{X}$ = 23.7	450	$\overline{X}$ = 258
3	13.2		15		198	
Cells & SRBC & Silica						
1	10.4		34		358	
2	6.6	$\overline{X}$=9.7	11	$\overline{X}$ = 28.7	73	$\overline{X}$ = 306
3	12.0		41		492	
		DAY 9				
Cells & SRBC						
1	21.8		155		3379	
2	4.0		254		1016	
3	0.4	$\overline{X}$ = 10.3	904	$\overline{X}$ = 646	8498	$\overline{X}$ = 5128
4	6.0		1270		7620	
Cells & SRBC & Silica						
1	8.4		1566		13,154	
2	25.6		476		12,186	
		$\overline{X}$= 51		$\overline{X}$ = 1090		$\overline{X}$ = 54985
3	82.0		1672		137,104	
4	89.0		646		57,494	

TABLE XIII - continued.

DAY 12

ells & SRBC Silica	Cell yield/spleen x10^6	Plaques / 10^6 cells	Plaques / spleen
1	32	339	10,848
	$\bar{X}$ = 58	$\bar{X}$ = 203	$\bar{X}$ = 8196
2	84	66	5,544

Control groups receiving either SRBC only, or Donor cells only yielded a negligible number of plaques.

(a) 9-12 week old B6AF1 mice were given 800 rads whole body radiation and injected with silica 16 hours later. Two hours after the silica, animals were reconstituted with $3x10^7$ B6AF1 cells mixed with a sensitizing dose of SRBC cells (.2 ml of a 2% solution). Response to the SRBC was measured on day 5 for the direct, and on days 9 and 12 for the indirect response by a plaque assay. Values represent individual animals.

TABLE XIV

The Effect of Silica on the Antibody Response to SRBC

Group	Cell yield x10^6	Plaques / 10^6 cells	Plaques / Spleen
Day 5			
Cells & SRBC			
1	26	60	1560
2	9 $\overline{X}$ = 23	120 $\overline{X}$ = 99	1080 $\overline{X}$ = 2257
3	35	118	4130
Cells & SRBC & Silica			
1	0.90	420	1890
2	20 $\overline{X}$ = 8.2	305 $\overline{X}$ = 363	6100 $\overline{X}$ = 3995
3	3.6		
Day 6			
Cells & SRBC			
1	145	40	5800
2	81 $\overline{X}$ = 101	47 $\overline{X}$ = 53	3807 $\overline{X}$ = 5048
3	78	71	5538
Cells & SRBC & Silica			
1	5.2	1230	6396
2	81 $\overline{X}$ = 39	248 $\overline{X}$ = 603	20,088 $\overline{X}$=12,248
3	31	331	10,261
Day 8 (direct)			
Cells & SRBC			
1	37	17	629
2	69 $\overline{X}$ = 45	10 $\overline{X}$ = 34	690 $\overline{X}$ = 1545
3	49	40	3120
4	26	67	1742

TABLE XIV cont.

Day 8 (direct)	Cell yield x10^6	Plaques / 10^6 cells	Plaques / Spleen
Cells & SRBC & Silica			
1	19.8	435	8613
2	59 $\overline{X}$ = 46	125 $\overline{X}$ = 271	7375 $\overline{X}$ = 10,389
3	60	253	15,180
Day 8 (Indirect)			
Cells & SRBC			
1	as above	42	1554
2		83 $\overline{X}$ = 14	5727 $\overline{X}$ = 4567
3		117	5733
4		202	5252
Cells & SRBC & Silica			
1	as above	3865	76,527
2		675 $\overline{X}$ = 1976	39,825 $\overline{X}$ = 66,524
3		1387	83,220

Control animals, receiving SRBC, or B6AF1 cells only, showed negligible plaque number. Experimental conditions were as described in footnote (a) of Table XIV. The direct antibody response to SRBC was measured on days 5, 6 and 8, and the indirect measured on day 8 only. Values given represent those of individual animals (means per group are also given).

TABLE XV

The Effect of Silica on the Antibody Response to SRBC

Day 7

Group	Cells / Spleen x10^6	Plaques/10^6 Cells		Plaques / Spleen	
		Direct	Indirect	Direct	Indirect
Cells & SRBC					
1	30	7	23	210	690
2	21 $\overline{X}$ = 31.7	9 $\overline{X}$=7	73 $\overline{X}$=39	189	1533
3	44	5	21	220	924
				$\overline{X}$=206	$\overline{X}$=1049
Cells & SRBC & Silica					
1	54	516	316	27,864	17,064
2	27 $\overline{X}$=52	439	517	11,853	13,959
3	76	248	136	18,848	10,336
		$\overline{X}$ =401	$\overline{X}$ =323	$\overline{X}$=19,522	$\overline{X}$=13,786

Day 9

Cells & SRBC					
1	59	8	18	472	1062
2	87 $\overline{X}$ = 58	8	12	696	1044
3	28.8	32	84	922	2419
		$\overline{X}$ = 16	$\overline{X}$ = 38	$\overline{X}$=697	$\overline{X}$=1508
Cells & SRBC & Silica					
1	165	174	708	28,710	138.060
2	99 $\overline{X}$ = 95	120	669	11,880	58,203
3	20.8	136	945	2829	19,656
		$\overline{X}$=143	$\overline{X}$=774	$\overline{X}$=14,473	$\overline{X}$=71,973

TABLE XV cont.

Day 11	Cells / Spleen x10^6	Plaques/10^6 Cells		Plaques / Spleen	
		Direct	Indirect	Direct	Indirect
Cells & SRBC					
1	105	-	13	-	1365
2	30 $\overline{X}$ = 69	-	47	-	1410
3	73	-	51	-	3723
			$\overline{X}$=37		$\overline{X}$=2166
Cells & SRBC & Silica					
1	162	-	28	-	4536
2	119 $\overline{X}$ = 150	-	84	-	9996
3	173	-	115	-	19,895
4	146	-	295	-	43,070
			$\overline{X}$=131		$\overline{X}$=19,374

Control groups receiving B6AF1 cells, or SRBC only showed a negligible plaque number.

Experimental conditions were as described in footnote (a) of Table XV.

The response to SRBC was measured on days 7, 9 and 11 for the indirect response, and days 7 and 9 for the direct response.

Values given represent individual animals and means per group.

DISCUSSION

Immune reactions are generally thought to involve cooperation of two cells, the T and B lymphocyte and may also require a third cell, an accessary cell. This cell is thought to be a macrophage. Some *in vitro* evidence for macrophage requirement exists, but no conclusive *in vivo* evidence has been documented. In this study an attempt was made to reduce the macrophage population *in vivo* with the use of crystalline silica. Particulate silica is known to be extremely toxic to macrophages. Complete destruction of the total macrophage population was not considered feasible, but a considerable reduction was thought possible. Since intravenous injection of silica is an extremely harsh treatment it was decided to examine silica treated mice histologically to ensure that no serious disruption of organs rich in macrophages (in particular the spleen), was occuring. Significant alterations (necrosis, changed structure etc.) of the spleen could invalidate future immunological studies with silica treated animals. The phenomenon of homing and cell proliferation of donor lymphocytes in silica treated hosts was examined as well. The effects of the silica treatment on the cellular immune response (CMC) and the humoral immune response (PFC) were then studied.

Silica dose mortality studies showed a dose of 7.0 mg. (about 0.35 mg/gm) to be the maximum tolerated dose when injected intravenously. Higher doses resulted in immediate convulsions and death, due probably to embolism and thrombosis in the lungs caused by the mass of silica particles blocking the capillaries and venules in the lungs and causing intravascular coagulation. Animals that survived the initial post-

injection period (about 10 hours) usually tolerated the silica for long periods of time (2 months) without any visible outward effects. Irradiated animals were much more susceptible to silica; even a dose of 0.2 mg/gm at times was 90% lethal by the sixth day after irradiation and silica injection. This was thought to be partially due to opportunistic infection by normal bacterial flora. Latent bacterial or viral infection as discussed earlier may also have been responsible for high and variable animal mortality. The mortality study was not a formal detailed study as only the maximum tolerable dose was of any interest, and even this dose was not reached in future experiments.

Histological studies to ensure that massive disruption of organs rich in macrophages was not occuring followed the mortality experiments. No significant early modification of any of these organs (lung liver spleen) was seen. After several weeks though, alterations in the spleen and liver were noted. Polynucleated giant cells in the red pulp as well as an increased fibrogenesis and a decreased number of lymphocytes in the white pulp were seen in the spleen. Numerous mononuclear cell infiltrations were noted in the liver. These infiltrates may be in response to damaged macrophages and or liver structure, while giant cells are probably an attempt to contain undegradable particles, ie. silica. These alterations did not appear to affect overall animal health, as no mortality was noted and the animals could not be told apart from normal controls. Localization of intravenously injected carbon particles showed that phagocytic capacity was not totally abolished as carbon localization in the spleen and liver did not vary significantly from controls. The presence of carbon particles

inside splenic phagocytes however does not imply that these cells were and would remain functionally intact (Cudkowicz et. al. 1977). Also as is discussed later, the rate of clearance of carbon from the blood is significantly depressed in silica treated groups.

A macroscopic observation of the spleen supporting the earlier findings of Vigliani and Pernis (1959) showed a considerable increase in spleen size and weight. Vigliani and Pernis showed a generalized activation of the reticuloendothelial system by crystalline silica, an observation which is supported by the present increase in spleen size and PFC data (to be discussed later), which shows an increased cell yield and antibody response in silica treated groups. Thus, although carbon particle localization was not significantly altered in silica treated groups and data on hyperplasia of the reticuloendothelial system are not complete, it has been shown that no drastic alterations occur in the spleen and liver in the first ten days after silica injection.

A more direct means of showing the effect of silica on macrophage functions is the phagocytic index. This involves the removal of intravenously injected carbon from the blood stream. Since phagocytosis is the primary function of macrophages, the effect of silica if any, should be readily demonstrable. Initial experiments with irradiated animals had to be abandoned as the dehydrated state of these groups (after irradiation) made it impossible to take tail bleeds as was required. Non irradiated animals were subsequently used and since a significant depression in phagocytosis was noted it was reasoned that such a depression would have been more marked in irradiated mice

from which radiosensitive macrophage precursors had been eliminated. Sporadic results from irradiated animals indicate that this was the case. Since results from unirradiated groups showed a significant depression in clearance of carbon, it was concluded that silica was at least partly effective in depressing the macrophage population and its function of phagocytosis.

Donor lymphocytes when injected into new hosts, redistribute in distinct patterns to the host lymphoid organs. This tendency is known as homing; cells are known as "spleen seeking, lymph node seeking" etc. Since both the CMC and the PFC (to be described later) involved the use of spleen cells that had been injected into irradiated hosts and were later recovered from the spleen, it became necessary to show that the treatments used such as irradiation and silica did not adversely affect homing. Initial experiments with chromium labelled cells injected into syngeneic hosts in the absence of silica showed that a 24 hour equilibration period is required for cells to "find" their respective organs. This is largely due to congestion in the capillaries and venules of the lung as the amount of label found there, while being quite high initially falls to near zero and remains there. The spleen and liver contain the bulk of the label injected and remained steady over the seven day period tested.

The effect of radiation of either the host or the donor cells on homing was next studied. No appreciable effect was found, even when donor cells were given up to 1200 rads. Homing thus appears to be relatively insensitive to the doses of radiation used in the present study.

While no direct comparison was made, in a single experiment there also do not appear to be any major differences in homing of cells in either syngeneic or semisyngeneic hosts. The one difference is a shift in the relative degree of label found in the liver. In syngeneic hosts this falls below the spleen while in semisyngeneic hosts the liver values are higher. This is likely to be due to more phagocytosis as a result of the GVH reaction in semisyngeneic hosts, or may be due to active migration of immunocompetent cells capable of developing a GVH reaction.

The effect of silica on the homing of cells in silica treated animals was examined and while some differences were seen when groups tested six days after injections were compared to those tested 24 hours after injection, these were minor. Homing experiments done at 24 hours or six days showed an initial increase in homing to the spleen with low silica doses but only the 24 hour experiments showed a significant rise. At high silica doses the homing dropped off sharply in both cases. High doses of silica seem to make the spleen inhospitable for lymphoid cells, while a possible chemotactic effect at low silica doses may account for the increase in homing to the spleen at these doses. The liver shows much more clear and consistent results, with a marked drop even at very low silica doses. This low level is then maintained at all other doses. Since most of the authors who have examined this problem (Lance, Bainbridge and Gowland) have attributed localization of chromium-labelled cells in the liver to phagocytosis of poorly viable cells, it is likely that these low values for the liver may be due to a decrease in phagocytosis due to macrophage inactivation by silica.

In conclusion, the results of these homing studies show that moderate doses (0.1 to 0.2 milligrams per gram weight of mouse)

of silica do not have a negative effect on homing of cells to the spleen, a range which included the dose used in lowering the phagocytic index.

A series of experiments designed to complement the homing experiments above involved cell proliferation. Cell proliferation as shown by uptake of a radioactive DNA precursor indicated cell viability and not just accumulation of label carried by dead cells as may be the case in the homing experiments. An initial study examined proliferation of syngeneic and parental spleen cells in F1 hosts. A burst of cell division was seen an days 3-5 in the spleens of F1 hosts receiving parental or syngeneic donor cells. Hosts receiving syngeneic cells however greatly surpassed those receiving parental cells even when receiving only two thirds as many cells. Proliferation also dropped off by day 6 in hosts receiving parental cells, while in those receiving syngeneic cells no decline was seen.

Cell division in the liver and lymph nodes showed the opposite trend. Animals receiving syngeneic cells did not differ significantly from animals receiving no cells at all while F1 hosts receiving parental cells showed a rapid rise in uptake of label at days 3-5 followed by a rapid decline thereafter. This likely represents GVH activity which is known to be strong in the liver and lymph nodes.

As was seen in the homing experiments, silica did not negatively affect cell proliferation in the spleen in F1 hosts receiving syngeneic or parental cells. Groups receiving parental cells actually showed an increase

in proliferation . Even at high silica doses the level of proliferation did not fall but merely tended to level off, which was not seen in the homing experiments. Proliferation in the liver was very low in syngeneic hosts, while groups receiving parental cells were considerably higher. Increased cell proliferation at high silica doses in F1 hosts receiving parental cells may be explained by abrogation of hybrid resistance by silica as proposed by Lotzova and Cudkowicz (1974). This resistance is the natural resistance of hybrid mice to donor cells that differ at specified H-2 loci. Hybrid resistance is thought to be largely due to macrophages so an anti-macrophage agent like silica should in effect increase the proliferation of the parental donor cells. Abrogation of hybrid resistance may also be effected by overloading the host with donor cells, a phenomenon which was also seen in the spleens of F1 hosts receiving high numbers of parental spleen cells.

While hybrid resistance may not be the sole reason for the proliferation patterns as described, these results do show a dose of 0.2 mg/mg not to have a negative effect on proliferation. These results as well as those from the homing studies and the silica dose mortality experiments were evaluated and the dose to be used in future experiments was set at 0.2 mg/gm. Studies on the effect of silica on the immune response were now carried out at the above dose.

The effect of silica on the cellular immune response, the CMC, was first studied. This involved sensitization of parental cells to F1 host cell alloantigens, and an attempt to block this sensitization by treating the hosts with silica. Measurement of the degree of sensitization was

determined by lysis of cells of the tumor line P815 which shares allo-antigens with the F1 hosts. Lysis of 33% of a given number of the target cells (described as one lytic unit, or LD33) as well as total lytic units per spleen were calculated. Lytic units per spleen was thought to be a more valid means of determining immunological activity, as this more closely measures the total activity generated by the animal in question. Silica treated animals while requiring fewer cells for one lytic unit, had significantly fewer units per spleen in all cases. Silica thus appeared to cause a lowered cellular immune response. Low lytic unit values per spleen in silica treated groups may have been due to the gritty texture of the spleens of silica treated groups resulting in a poor recovery of spleen cells. However, in three out of four experiments where cell yields were monitored, silica treated syngeneic hosts showed higher cell yields than non silica groups. In all four of these same experiments, F1 hosts receiving parental cells and silica had lower cell yields than non silica groups. These results are not easy to explain in view of the slightly enhanced proliferative activity of parental cells in F1 hosts. However, they demonstrate that poor recovery of cells from silica treated spleens cannot be the sole explanation for the low numbers of LU per spleen. This is further supported by the results from the PFC experiments discussed below where silica is shown to behave more like an adjuvant.

The effect of silica on the humoral immune response, showed results quite contrary to those in the CMC. An increase in spleen cell yield, plaques per 10^6 spleen cells, and consequently also in plaques per spleen were noted. These results were obtained in the direct, and indirect antibody response to SRBC bringing to mind the adjuvant properties

discussed by Pernis and Paronetto (1962). They reported antibody titers in silica treated groups that significantly surpassed the control groups and the spleens and lymph nodes of the animals used (rabbits) were enlarged up to 10 times the normal size. This adjuvant effect increased as the length of time between silica injection and antigen increased. Hyperplasia of lymphoid cells and the reticuloendothelial system was not seen to the same extent in the present study, however considering the animals used were irradiated, it was significant. Increased spleen size was however also seen in mice used in histology experiments. These mice were not irradiated and were left for about 12 weeks after silica injections.

Adjuvants have been suggested to operate via one or several of the following mechanisms (Allison 1973). (a) Slowing the release of antigen e.g. antigen injected in water and emulsions, (b) antigen denaturation, where antigen is denatured or made particulate by adsorption e.g. alum or bentonite, (c) recruitment of reactive cells, e.g. granuloma formation with cell infiltration in Freund's adjuvant, (d) stimulation of the proliferation and differentiation of immunocompetent cells, e.g. lipopolysaccharide, a B cell mitogen, (e) trapping of immunocompetent cells in lymphoid organs, and (f) increasing the number of effector cells in antibody dependent cell mediated immunity e.g. Freund's complete adjuvant. An additional mechanism is thought to involve labilization of lysosomal membranes (Spitznagel & Allison 1970a). Adjuvants such as retinol (vitamin A alcohol and and E coli lipopolysaccharide labilize lysosomal membranes and are potent adjuvants. These adjuvants have been shown not to cause enhancement of immune responses when incubated with lymphocytes alone, whereas macrophages treated with adjuvants stimulated not only antibody producing cells,

but also a generalized hyperplasia of the reticuloendothelial system (Spitznagel & Allison 1970b).

Silica, the compound used in the present study is also known to be a lysosomal labilizer and has been shown to stimulate hyperplasia (Pernis & Paronetto 1962) of the reticuloendothelial system and the proliferation of lymphoid cells. Silica, and other adjuvants that weaken and break lysosomal membranes may thus cause the release of factors which then bring on the adjuvant effects.

The suggestion has also been made (White 1973) that adjuvants debilitate or destroy a specific macrophage subpopulation whose function was the regulation of the immune response. Silica may interfere with the normal function of removal of excess antigen by macrophages. This would disrupt a feedback mechanism by free antibody, causing sustained antibody synthesis. Regulation of antibody synthesis is an area that at present has more questions than answers. The compound used in the present study however has been shown to cause an increased antibody synthesis, while at the same time it is a known labilizer of lysosomal membranes. Given the latter, the adjuvant effect which was quite unexpected, may be partially explained. The exact mechanism, however is not known at present.

SUMMARY

Properties and effects of crystalline silica were studied in the murine immune system. The depression of the phagocytic index as shown by other investigators was duplicated. Studies on the effects of silica on homing showed no negative effects except at high doses. Studies on the effects of silica on cell proliferation also showed no negative effects at moderate doses rather an adjuvant like increase was seen. The effect on cellular immunity was a depression in treated mice while the humoral response to SRBC was considerably enhanced. These results may shed light on some of the regulatory functions of macrophages, but at the least appear to indicate that the CMC and PFC operate via somewhat different mechanisms.

LITERATURE CITED

Allison, A.C., Lysosomes and toxicity of particulate pollutants. Arch. Intern. Med. 128 131, 1971

Allison, A.C., 1973. Effects of adjuvants on different cell types and their interactions in immune responses in immuno potentiation. (Ciba Fdtn. Series 18) 73-99. Assoc. Scientific Pub. N.Y.

Allison, A.C., Harrington, J.S., Birbeck, M. An examination of the cytotoxic effects of silica on macrophages. J. Exp. Med. 124, 141, 1966.

Allison, A.C., D'Arcy Hart, P. Potentiation by silica of the growth of mycobacterium tuberculosis in macrophage cultures. British Journal of Exp. Path. 465-477 49, 1968.

Andersson, B., Blomgren, H. Evidence for a small pool of immunocompetent cells in the mouse thymus. Its role in the humoral antibody response against sheep erythrocytes, bovine serum albumin, ovalbumin and the NIP determinant. Cell. Immunol. 1 362, 1970.

Andre'Schwartz, J. The morphologic responses of the lymphoid system to homografts. III Electron microscopy study. Blood 24 113, 1964.

Ascheim, L., Raffel, S. The immunodepressant effect of carrageenin. J. Reticuloendothel. Soc. 11 253, 1972.

Babu, U.M., Sabbadini, E. 1977. Regulation of cell-mediated cytotoxicity. III Synergism of cortico-resistant with anti-thymocyte serum-resistant splenic T cells in the generation of cytotoxic lymphocytes in graft-vs-host reactions. J. Immunol., 119 781.

Balner, H. 1963. Identification of peritoneal macrophages in mouse radiation chimeras. Transplantation 1 217.

Bartfeld, H., Kelly, R., 1968. Mediation of delayed hypersensitivity by peripheral blood lymphocytes *in vitro* and by their products *in vivo* and *in vitro*, morphology *in vitro* lymphocyte-macrophage interaction. J. Immunol., 100 1000.

Barton, M.A., Diener, E. 1975. A new perspective on B cell triggering. Control of the immune response by organizational changes in the lipid bilayer. Transp. Rev. 23 5.

Bast, R.C., Manseau, E.J., Dvorak, H.F. 1971. Heterogeneity of the cellular immune response. I Kinetics of lymphocyte stimulation during sensitization and recovery from tolerance. J. Exp. Med., 133 187.

Benezra, D., Gery, I., Davies, A.M. 1971. Peripheral lymphocyte populations. Changes in antigen requirements for stimulation *in vitro*. Int. Arch. Allergy Appl. Immunol. 41 381.

Bennet, et al. 1968. Hemopoietic progenitor cells of W anemic mice studied in vivo and in vitro. J. Cell. Physiol., 71 211.

Bice, D.E., Gruwell, D.G., Salvaggio, J. 1972a. Suppression of anti-SRBC antibody fromation by carrageenan - a macrophage toxic substance. Fed. Proc., 31 929.

Bice, D.E., Gruwell, D.G., Salvaggio, J.E., Hoffman, E.O. 1972b. Immunol. Commun. 1 615.

Billingham, R.E., 1968. The biology of graft-vs-host reactions. Harvey Lec. 62 21.

Biozzi, G., Benacerraf, B., Halpern, B.N., 1953. Quantitative study of the granulopectic activity of the reticulo-endothelial system. II A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. Brit. J. Exp. Path. 34 441.

Blomgren, H., Andersson, B., 1971. Reappearance and relative importance of immunocompetent cells in the thymus, spleen and lymph nodes following lethal x-irradiation and bone marrow reconstitution in mice. J. Immunol. 106 831.

Boyle, W. 1968. An extension of the ^{51}Cr release assay for the estimation of mouse cytotoxins. Transplantation 6 761.

Bretscher, P., 1975. The two signal model for B cell induction. Transpl. Rev., 23 37.

Bretscher, P., Cohn, M., 1970. A theory of self-nonself discrimination. Science 169 1042.

Buurman, W.A., VanBruggen I., 1976. Weakening of genetic resistance. II The effect of injection of the macrophage toxic agents, silica and carrageenan and the cytostatic drugs cyclophosphamide busulphan and vinblastin. Exp. Hemat. 4 382.

Calderon, J., Unanue, E.R., 1974. The release of antigen molecules from macrophages: Characterization of the phenomena. J. Immunol., 112 1804.

Cantor, H., 1972. The effects of anti-theta antiserum upon graft-vs-host activity of spleen and lymph node cells. Cell. Immunol. 3 461.

Cantor, H., Asofsky, R., 1970. Synergy among lymphoid cells mediating the graft-vs-host response. II Synergy in graft-vs-host reactions produced by BALB/C lymphoid cells of differing anatomic origin. J. Exp. Med. 131, 235.

Cantor, H., Asofsky, R., 1972. Synergy among lymphoid cells mediating the GVH response. III Evidence for interaction between two types of thymus derived cells. J. Exp. Med., 135, 764.

Cantor, H., Asofsky, R., Talal, 1970a. Synergy among lymphoid cells mediating the graft-vs-host response. I Synergy in graft-vs-host reactions produced by cells from NZB/B1 mice. J. Exp. Med. 131 223.

Cantor, H., Asofsky, R., Talal, 1970b. Synergy among lymphoid cells mediating the graft-vs-host response. II Synergy in GVH reactions produced by BALB/C lymphoid cells of differing anatomic origin. J. Exp. Med. 131 235.

Cantor, H., Boyse, E.A., 1975a. Functional subclasses of T lymphocytes bearing different Ly antigens. II Cooperation between subclasses of of Ly + cells in the generation of killer activity. J. Exp. Med. 141 1390.

Cantor, H., Boyse, E.A., 1975b. Functional subclasses of T lymphocytes bearing different Ly antigens. I The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J. Exp. Med., 1397.

Cantor, H., Simpson, E., Soto, V.L., Furthman, C.G., Herzenberg, L.A., 1975. Characteristics of subpopulation of T lymphocytes. Separation and functional studies of peripheral T cells binding different amounts of flourescent Anti-Thy 1.2 (theta) antibody using ACS. Cell. Immunol. 15 180.

Cantor, H., Shen, F.W., Boyse, E.A. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. II Activation by antigen: after immunization, antigen-specific suppressor and helper activities are mediated by distinct T cell subclasses. J. Exp. Med. 143 1391.

Chen,D., Hirsh, J.G., 1972. The effects of mercaptoethanol and of peritoneal macrophages on the antibody forming capacity of nonadherent mouse spleen cells in vitro. J. Exp. Med. 136 604.

Cline, M.J., Swett, V.C., 1968. The interaction of human monocytes and lymphocytes. J. Exp. Med., 128 1309.

Cohen, B.E., Rosenthal, A.S., Paul, W.E., 1973a. Antigen-macrophage interaction. I Hapten-specific inhibition of antigen interaction with macrophages from immune animals. J. Immunol., 111 811.

Cohen, B.E., Rosenthal, A.S., Paul, W.E., 1973b. Antigen-macrophage interaction. II Relative roles of cytophilic antibody and other membrane sites. J. Immunol. 111 820.

Cohen, B.E., Paul, W.E., 1974. Macrophage control of time-dependent changes in antigen sensitivity of immune T lymphocyte populations. J. Immunol. 112 359.

Coutinho, A. 1975. The theory of the One-Nonspecific signal model for B cell activation. Transpl. Rev. 23 49.

Cudkowicz, G., Yung, Y.P. Abrogation of resistance to foreign bone marrow grafts by carrageenans. I Studies with the anti-macrophage agent, Seakem Carrageenan. J. Immun. Vol. 119 #2, p. 483, 1977.

Davie, D.M., Paul, W.E., 1973. Immunological maturation. Preferential proliferation of high affinity precursor cells. J. Exp. Med. 137 200.

Davie, D.M., Paul, W.E., 1972. Receptors on immunocompetent cells: V Cellular correlates of the "maturation" of the immune response. J. Exp. Med. 135 660.

Davies, A.J.S., Leuchars, E., Wallis, V., Doenhoff, M., 1971. A system for lymphocytes in the mouse. Proc. Royal Soc. Ser. B. 179 369.

Dixon, W.J., Massey, F.J. Jr., 1969. Introduction to statistical analysis 3rd Ed. McGraw Hill Book Co. N.Y.

Dorsch, S., Roser, B. 1977. Recirculating suppressor T cells in Transplantation Tolerance. J. Exp. Med. 145 1144.

Eisen, H.N., Siskind, G.W., 1964. Variations in affinities of antibodies during the immune response. Biochemistry 3 996.

Ellner, J.J., Lipsky, P.E., Rosenthal, A.S., 1977. Antigen handling by guinea pig macrophages: Further evidence for the sequestration of antigen relevant for activation of primed T lymphocytes. J. Immunol. 118 2053.

Erb, P., Feldmann, M., 1975a. Role of macrophages in in vitro induction of T helper cells. Nature 254 352.

Erb, P., Feldmann, M., 1975b. The role of macrophages in the generation of T helper cells. II The genetic control of the macrophage T cell interaction for helper cell induction with soluble antigens. J. Exp. Med. 142 460.

Erb,P., Feldmann, M., 1975c. The role of macrophages in the generation of T helper cells. I The requirement of macrophages in helper cell induction and characteristics of the macrophage T cell interaction. Cell. Immunol. 19 356.

Erb, P., Meir, B., Feldmann, M., 1976. Two gene control of T helper cell induction. Nature 363 601.

Erb, P., Vogt, P., Meier, B., Feldmann, M., 1977. The role of macrophages in the generation of T helper cells. V Evidence for differential activation of short lived T_1 and long lived T_2 lymphocytes by the macrophage factors GRF and NMF. J. Immun. 119 206.

Feldmann, M., 1972. Cell interactions in the immune response in vitro. II The requirements for macrophages in lymphoid cell collaboration. J. Exp. Med. 135 1049.

Feldmann, M., Erb, P., 1977. Requirement for interaction between two subpopulations of T cells for helper cell induction in vitro. Z. Immunitaetsforsch. Immunobiol. 153 217.

Forbes, I.J., Mackaness, G.B., 1963. Mitosisin Macrophages. Lancet, 2 1203.

Ford, C.E., Micklem, H.S., Evans, E.P., Gray, J.G., Ogden, D.A., 1966. The inflow of bone marrow cells to the thymus: Studies with part-body irradiated mice injected with chromosome-marked bone marrow and subjected to antigenic stimulation. Ann. N.Y. Acad. Sci. 129 283.

Frost, P., Lance, E.M., 1974. The cellular origin of the lymphocyte trap. Immunology, 26 175.

Fung, K.Y., Sabbadini E., 1976. Cytotoxicity in graft-vs-host reaction. II Lysis of target cells of parental genotype by F1 hybrid macrophages. Transplantation 22 449, 1976.

Gillette, R.W., Boone, C.W., 1974. Changes in the homing properties of labelled lymphoid cells caused by solid tumor growth. Cell. Immunol. 12 363.

Gillman, T., Wright, L.J., 1966. Probable in vivo origin of multinucleated giant cells from circulating mononuclears. Nature (Lond.) 209 263.

Hanifin, J.M., Cline, M.J., 1970. Human monocytes and macrophages: Interaction with antigen and lymphocytes. J. Cell Biol. 46 97.

Hanks, J.H., Wallace, R.E., 1949. Relation of oxygen and temperature in preservation of tissues by refrigeration. Proc. Soc. Expt. Biol. Med. 71 196.

Hirsch, Gary, Murphy. In vitro and in vivo properties of anti macrophage sera. J. Immunology Vol. 102 #3, 1969 pp. 656-661

Huber, B., Cantor, H., Shen, F.W., Boyse, E.A., 1976. Independent differentiative pathways of Ly_1 and $Ly_{2,3}$ subclasses of T cell. Experimental production of mice deprived of selected T cell subclasses. J. Exp. Med. 144 1128.

Hughes, W.L., 1964. Deoxyribunocleic acid metabolism in vivo: I. Cell proliferation and death as measured by incorporation and elimination of iododeoxyuridine. Fed. Proc. 23 640.

Ishizaka, S., Otani, S., Morisawa, S., Effects of carrageenan on immune responses. I Studies on the macrophage dependency of various antigens after treatment with carrageenan. J. Immunology Vol. 118 #4 p. 1213 1977.

Jerne, N.K., Nordin, A.A., 1963. Plaque formation in agar by single antibody producing cells. Science 140 405.

Kaw, J.L., Zard, S.H. In vitro studies on the cytotoxic action of different varieties of asbestos dust on macrophages. Acta pharmacol. et toxicol. 36 225-235 1975

Kierszenbaum, F., Knecht, E., Budzko, D.B., Pizziment, M.C. 1974. Phagocytosis: A defense mechanism against infection with trypanosoma Creizi. J. Immunol. 112 1839.

Leckband, E., 1970. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 29 621.

Levy, M.H., Wheelock, E.F., 1975. Effects of intravenous silica on immune and non immune functions of the immune host. J. Immunol. 115, 41.

Lipscomb, M.F., Ben-Sasson, S.Z., Uhr, J.W., 1977. Specific binding of T lymphocytes to macrophages. I Kinetics of binding. J. Immunol. 118 1748.

Lipsky, P.E., Rosenthal, A.S., 1973. Macrophage lymphocyte interaction. I Characteristics of the antigen independent binding of guinea pig thymocytes and lymphocytes to syngeneic macrophages. J. Exp. Med. 138 900.

Lipsky, P.E., Rosenthal, A.S., 1975a. Macrophage-lymphocyte interaction: II Antigen-mediated physical interactions between immune guine pig lymphnode lymphocytes and syngeneic macrophages. J. Exp. Med. 141 138.

Lipsky, P.E., Rosenthal, A.S., 1975b. Macrophage-lymphocyte interaction: Antigen independent binding of guinea pig lymphnode lymphocytes by macrophages. J. Immunology 115, 440.

Loegering, D.J., Carr, F.K., 1977. Humoral factor activity and carbon clearance rate during the early stages of hemorrhagic shock. J. Res. 21 263.

Loewi, Temple, Nind, Axelrod. A study of the effects of antimacrophage sera. Immunology 1969. 16, 99.

Lopez, L.R., Vatter, A.E., Talmage, D.W., 1977. The requirement of viable thymocytes for species specific attachment to and release from macrophages. J. Immunol. 119 1668.

Lotzova, Cudkowicz. Abrogation of resistance to bone marrow grafts by silica particles. J. Immunology Vol. 113 #3, 1974.

Loveren, H.V., Snock, M., Otter, W.D., 1977. Effects of silica on macrophages and lymphocytes. J. Reticuloendo. Soc. 22 523.

McFarland, W., Heilman, D.H., Moorhead, J.F., 1966. Functional anatomy of the lymphocyte in immunological reactions in vitro. J. Exp. Med. 124 851.

Miller, H.R.P., Avrameas, S., 1971. Association between macrophages and specific antibody producing cells. Nat. New Biol 229 184.

Miller, J.F.A.P., Mitchell, G.F., 1969. Thymus and antigen reactive cells Transpl. Rev. 1 3.

Miller, S.D., Zarkower, A., 1974. Alterations of immune immunologic responses after silica dust inhalation. J. of Immun. 113 1533.

Miller, S.D., Zarkower, A., 1976. Silica induced alterations of murine lymphocyte immunocompetence and suppression of B lymphocyte immunocompetence: A possible mechanism. J. Ret. End. Soc. 19, 47.

Mitchison, A., 1971. Cell cooperation in the immune response: The hypothesis of an antigen presentation mechanism. Immuno pathology. 6 52.

Möller, E., Bullock, W., Makela, E., 1973. Affinity of T and B lymphocyte receptors for hapten determinants. Eur. J. Immunol. 3 172.

Möller, G., 1975. One nonspecific signal triggers B lymphocytes. Transplant. Rev. 23 126.

Möller, G., 1970. Immunocyte triggering. Cell Immun. 1 573.

Moore, M.A., Owen, J.J.T., 1967. Experimental studies on the development of the thymus. J. Exp. Med. 197 715.

Mosier, D.E., 1969. Cell interactions in the primary immune response in vitro: a requirement for specific cell clusters. J. Exp. Med. 129 351.

Mosier, D.E., Pierce, C.W., 1972. Functional maturation of thymic lymphocyte populations in vitro. J. Exp. Med. 136 1484.

Osmond, D.G., Nossal, E.J.V., 1974. Differentiation of lymphocytes in mouse bone marrow. II Kinetics of maturation and renewal of antiglobulin binding cells studied by double labelling. Cell. Immunol. 13 132.

Owen, J.J.T., Raff, M.C., 1970. Studies on the differentiation of thymus derived lymphocytes. J. Exp. Med. 132 1216.

Owen, J.J.T., Ritter, M.A., 1969. Tissue interaction in the development of thymus lymphocytes. J. Exp. Med. 129 431.

Pearsall, N.N., Weiser, R.S.; The macrophage in allograft immunity I Effects of silica as a specific macrophage toxin. J. Ret. End. Soc. 5, 107-121, 1968.

Pernis, B., Paronetto, F., 1962. Adjuvant effect of silica (tridymite) on antibody production. Proc. Soc. Exper. Biol. & Med. 110 390.

Plutchik, R., 1968. Foundations of experimental research. P. Ziegler, ed. Harper Row, New York.

Raff, M.; Evidence for subpopulation of mature lymphocytes within mouse thymus. Nature, (New Biol.) 229 182, 1971a.

Raff, M.C., 1970. Role of thymus derived lymphocytes in the secondary humoral response in mice. Nature (Lond.) 226 1257.

Raff, M.C., Cantor, H., 1971. Subpopulations of thymus cells and thymus derived lymphocytes. Prog. In Immunol. 1 83.

Robock, K., 1973. Standard quartz DQ12 less than 5 microns in size, for experimental pneumoconiosis research projects in the federal republic of Germany. Ann. Occup. Myg. 16 63.

Sabbadini, E., 1974. Regulation of cell mediated cytotoxicity. I Augmentation of cell mediated cytotoxicity induced by radiation. J. Exp. Med. 140 470.

Sabbadini, E., 1975. Regulation of cell mediated cytotoxicity., II Synergism of two types of thymus dependent cells in vivo. Cell. Immunol. 18 76.

Sabbadini, E., Neri,A., Sehon, A.H., 1974. Localization of non-immune radioactively labelled cells in the lesions of contact sensitivity in mice. J. Immunolog. Methods. 5 9.

Sabet, Tawf, K.Y., 1972. The mechanism of immunosuppression by RES blockade. J. Ret. End. Soc. 11 503.

Schechter, G.P., MacFarland, W.J., 1970. Interaction of lymphocytes and a radioresistant cell in PPD-stimulated leukocyte cultures. Immunol. 105 661.

Schlesinger, M.; Immune lysis of thymus and spleen cells of embryonic and neonatal mice. J. Immunol. 94 358, 1965.

Schmidke, J.R., Unanue, E.R., 1971 Interaction of macrophages and lymphocytes with surface immunoglobulin. Nature (New Biol) 233, 84.

Shipman, C.,Jr. 1969. Evaluation of 4- (2-hydroxyethyl) -1- piperazine-ethane sulfonic acid (HEPES) as a tissue culture buffer. Prof. Soc. Exp. Biol. & Med. 130 305.

Siegel, I., 1970. Natural and antibody induced adherence of guinea pig phagocyte cells to autologous and heterologous thymocytes. J. Immunol. 105 879.

Simonsen, M., 1962. Graft-vs-host reactions: Their natural history and applicability as tools of research. Prog. Allergy 6 349.

Siskind, G.W. Benacerraf, B., 1969. Cell selection by antigen in the immune response. Adv. Immunol. 10 1.

Snell, G.D.; T cells, T cell recognition structures, and the major histocompatibility complex. Transpl. Rev. 38 3, 1978.

Spitznagel, J.K., Allison A.C., 1970a. Mode of action of adjuvants: Tetinol and other lysosome labelizing agents as adjuvants. J. of Immunol. 104 119.

Spitznagel, S.K., Allison A.C., 1970b. Mode of action of adjuvants: Effects on antibody responses to macrophage associated bovine serum albumin. J. Immunol. 104 128.

Strauss, R.R., Patel, N., Patel, C., 1977. Suppression of the immune response in Balb/C mice by trypan blue. J. Ret. End. Soc. 22 533.

Strober, S., 1975. Immune function, cell surface characteristics, and maturation of B cell subpopulations. Transpl. Rev. 24 84.

Sulitzeanu, D., Kleinman, R., Benezra, D., Gery, I., 1971. Cellular interactions and secondary response in vitro.

Tigelar, R.E., Asofsky, R., 1973. Synergy among lymphoid cells mediating the graft-vs-host response. V Derivation by migration in lethally irradiated recipients of two interacting subpopulations of thymus derived cells from normal spleen. J. Exp. Med. 137 239.

Turner, E.N., Higginbotham, R.D., 1977. Carrageenan induced alterations of antigen distribution and immune responses in mice. J. Ret. End. Soc. 22 545.

Unanue, E.R., 1968. Properties and some uses of anti macrophage antibodies. Nature, 218 36.

Unanue, E.R., 1972. The regulatory role of macrophages in antigenic stimulation. Adv. Immunol. 15 95.

Unanue, E.R., Askonas, B.A., Allison, A.C., 1969. A role of macrophages in the stimulation of immune responses by adjuvants. J. Immunol. 103 71.

Unanue, E.R., Cerrotini, J.C., 1970. The immunogenicity of antigen bound to the plasma membrane of macrophages. J. Exp. Med. 131 711.

Unanue, E.R., Cerrotini, J.C., Bedford, M., 1969. Persistance of antigen on the surface of macrophages. Nature (Lond) 222 1193.

Van Furth, R., Ed. 1970. Mononuclear Phagocytes. F.A. Davis Co. Philadelphia, Pa.

Van Furth, R., Diesselhoff-Den Dulk, M.M.C., 1970. The kinetics of promonocytes in the bone marrow. J. Exp. Med. 132.

Vigliani, E.C., Pernis, B., 1959. An immunological approach to silicosis. Journal of Occ. Med. 319 1959.

Waldron, J.A., Horn, R.G., Rosenthal, A.S., 1973. Antigen induced proliferation guinea pig lymphocytes in vitro: Obligatory role of macrophages in the recognition of antigen by immuen T lymphocytes. J. Immunol. III 58.

Waldron, J.A., Horn, R.G., Rosenthal, A.S., 1974. Antigen induced proliferation of guinea pig lymphocytes in vitro: Functional aspects of antigen handling by macrophages. J. Immunol. 112 746.

Waters, M.D., Gardner, D.E., Aranyi, C., Coffin D.L.; Metal Toxicity for rabbit alveolar macrophages in vitro. Environmental Res. 9 32-47, 1975.

White, R.G. 1973. Immunopotentiation by mycobacteria in complete Freund-type adjuvent as the failure of normal immunological homeostasis. In Immunopotentiation (CIBA 47-72 foundation symposium 18). Associated Scientific Pub. N.Y.

Wilkinson, P.C., White, R.G., 1966. The role of mycobacteria and silica in the immunological response of the guinea pig. Immunol. 11 229.

Wong, A.K.C., Sabbadini, E., 1974. Plaque assay for the detection of allo-antibody producing cells in mice. J. Immunol. 112 2251.

Yamashita, V., Shevach, E.M., 1977. The expression of Ia antigens on immunocompetent cells in the guinea pig. II Ia antigens on macrophages. J. Immunol. 119 1584.

Zatz, M.M., 1976b. Effects of BCG on lymphocyte trapping. J. Immunol. 116 1587.

Zatz, M.M., 1976a. Lymphocyte trapping. Differential effects of ATS and irradiation on trapping in lymphnodes and spleen. Immunol. 30 749.

Zatz, M.M., Gershon, R.K., 1975. Regulation of lymphocyte trapping: The negative trap. 115 450.

Zatz, M.M., Lance, E.M., 1970. The distribution of chromium 51-labelled lymphoid cells in the mouse: A survey of anatomical compartments. Cell Immunol. 1 3.

Zatz, M.M., Lance, E.M., 1971. The distribution of ^{51}Cr labelled lymphocytes into antigen stimulated mice. J. Exp. Med. 134 224.

Zatz, M.M, Gershon, R.K., 1974. Thymus dependence of lymphocyte trapping. J. Immunol. 112 101.

Zembala, M., Agherson, G.L., 1973. Depression of the T cell phenomenon of contact sensitivity by T cells from unresponsive mice. Nature (Lond) 244 227.