The University of Manitoba

The Influence of Host Mediated Natural Resistance on the Tumor Progression of the SL2-5 Murine Lymphoma

by

Garth W.A. Brown, B.Sc.

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science

Department of Immunology

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DEDICATION

This thesis is dedicated to my wife, Helen. The completion of this work is a tribute to her enduring patience, support and love.

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LIST OF ABBREVIATIONS

ATxBM	adult thymectomized, irradiated and bone marrow reconstituted
BM	bone marrow
dddH2O	double distilled and deionized water
E:T	effector lymphocytes to tumor target cell ratio
F	Fischer's medium
FBS	fetal bovine serum
10% FFBS	Fischer's medium supplemented with 10% fetal bovine serum
HBSS	Hank's balanced salt solution
INVITRO cells	SL2-5 cells grown exclusively in tissue culture
INVITRO LONG	INVITRO cells subcloned after 3 months in tissue culture
INVITRO SHORT	INVITRO cells subcloned after 3 days in tissue culture
IP	intraperitoneal
Lps	lipopolysaccharide
NAb	natural antibody
NK	natural killer
NR	natural resistance
P	probability
poly I:poly C	polyinosinic-polycytidylic acid
SC	subcutaneous
S.D.	standard deviation
S.E.	standard error

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ABSTRACT

The ability of natural resistance (NR) and the putative effectors of host mediated anti-tumor mechanisms, natural killer (NK) cells and natural antibody (NAb), to influence the development of the NK-sensitive and NAb-sensitive SL2-5 murine lymphoma was examined using an *in vivo* model of tumor progression. Reductions in sensitivity to host mediated anti-tumor defences and an increased heterogeneity for susceptibility to syngeneic NR and serum NAb were consistent with a variant generation and selection mechanism as a basis for the progression of this tumor. The further characterization of the model revealed that the decreased tumor susceptibility to NR exhibited by the SL2-5 cells grown subcutaneously in syngeneic mice was related to an increased tumor frequency and that the observed alterations in the NR-sensitive phenotype were dependent upon the anatomical site of *in vivo* growth. The fact that tumors passaged within the peritoneum for comparable periods of time actually exhibited increases in sensitivity to host mediated anti-tumor mechanisms suggested that under these conditions the SL2-5 lymphoma may have progressed towards a decreased malignancy. In addition, the thymus independent nature of the reduction in sensitivity to NR in conjunction with decreases in sensitivity to NK cell and NAb activity for SC grown tumors supported a role for NK cells and NAb as effectors of a host mediated anti-tumor mechanism against the SL2-5 lymphoma. It appeared that a decreased osmotic fragility and in the case of NK cytolysis, a decreased expression of tumor target structure,

likely contributed to tumor resistance to lysis. Finally, the fact that the susceptibility of the SL2-5 cells to hypotonic shock was dependent upon temperature, energy metabolism, protein synthesis and microtubule polymerization suggested that a counterlytic mechanism was an integral determinant of tumor sensitivity to osmotic lysis. Based upon these observations, it was postulated that the capacity of the SL2-5 lymphoma to actively resist and repair lytic damage may have contributed to the reduced susceptibility of these cells to host mediated NR mechanisms and thus was an important aspect of tumor progression in vivo.

CHAPTER I

INTRODUCTION

INTRODUCTION

(A) TUMOR PROGRESSION

(1) A Historical Perspective

Foulds (1954) proposed the term "tumor progression" to describe the appearance of irreversible, qualitative alterations in one or more of the characteristics that collectively contribute to the behaviour of a tumor throughout its development. He distinguished this type of change from that of tumor modulation, the environmentally induced reversible variation of tumor phenotype (Foulds, 1954). The concept was based upon many observations derived from the clinical histories of human and animal neoplasia (Foulds, 1954). Specifically, the earlier work on experimentally induced rabbit, skin (Rous and Beard, 1935; Rous and Kidd, 1939) and mammary tumors (Greene, 1940) contributed to the genesis of Foulds' thesis. These investigators emphasized the fact that the evolution of a tumor appeared to occur in a graded or stepwise manner. Additional information was obtained by Foulds (1949a, 1949b, 1954, 1969), while he studied the development of hormone independence in murine mammary tumors. He delineated the complex phenomenon of tumor progression in a series of generalizations (Foulds, 1954).

(2) The Principles of Tumor Progression

Foulds originally formulated these principles prima-

rily based on his own research. However, his contemporaries determined that most of these rules also applied to chemically induced murine skin tumors (Shubik et al., 1953). Thus, the focus of the concept was broadened in an attempt to encompass the progression of tumors in general. The first generalization stated that the progression of any particular tumor should be independent of other tumors. He suggested that it was the innate properties of a neoplasm rather than the environment in which it developed, that ultimately determined the progression of a tumor. This idea was typified by the observation that given the conditions where multiple human or animal benign tumors existed, rarely did more than one tumor become malignant (Dukes, 1952; Shubik et al., 1953).

Secondly, the individual characteristics of a tumor may be capable of independent progression. The behaviour and structure of a tumor can be subdivided into a diversified variety of "unit characters"; gross tumor traits observable at the cellular level, that help to define and contribute to the malignant nature of a particular tumor (Foulds, 1954). Examples of unit characters include responsiveness to hormones, sensitivity to various drugs, metastatic and invasive potentials, and the ability to grow in ascites form. Typically, tumors may be cytologically malignant without being invasive (Hamperl, 1967) or locally invasive, but do not disseminate (Foulds, 1954). The display of a random assortment of these characteristics during progression suggested that each tumor underwent a unique evolution (Medina, 1975).

However, Foulds (1954) realized that some characteristics may

have been dependent upon others, such as the morphology of a tumor which may have depended on its responsiveness to a hormone. In addition, it was noted that unit characters were not indivisible entities, for example, hormone responsiveness involved the uptake, translocation and fixation of the hormone (O'Malley and Means, 1974). Progression could take place at any or all of these levels, the net result of which would be to alter the unit character of hormone responsiveness.

The third principle has been refined to state that tumor progression can occur in the absence of the original inciting stimulus (Medina, 1975). This generalization was based on the initiation and promotion sequence of carcinogen induced skin cancers (Rous and Beard, 1935) and the observation that regressed mammary tumors reappeared as hormone independent tumors without the hormonal fluxes of pregnancy (Foulds, 1954). Foulds originally interpreted these data to mean that progression was independent of cell growth, though the mitotic activity of the tumors was never established. Medina added that the progression of a tumor was probably enhanced under the influence of the inciting stimulus, as more intense and prolonged stimuli were associated with a greater capacity for progression.

Foulds' fourth principle was concerned with the continuity and terminus of tumor progression in the primary host. He suggested that tumors generally developed along different paths towards one of alternative end points (Foulds, 1954). The development of tumors towards "dissimilar" end points followed from the independent progression of unit characters and the demonstration

that a range of different tumors could evolve by the "divergent differentiation" of identical stem cells (Foulds, 1940). Rous and Kidd (1941) remarked that it was only the "successful" tumors that were clinically detected. This statement emphasized the point that the growth of a tumor did not necessarily have to culminate in the death of the host (Foulds, 1954). Foulds concluded that a tumor could potentially progress to any point between and including the extremes of host extinction and the complete regression of the tumor.

Conversly, Foulds (1954) noted that other tumors developed directly or indirectly towards "similar" end points. Other workers observed that human intestinal carcinoma arose as a carcinoma or evolved from an originally benign papillary adenoma (Sunderland et al., 1948). Foulds added that the development of "direct" tumors may involve the progressive expression of innate tumor characteristics. He also observed that this developmental pathway could skip intermediate steps detectable in other tumors (Foulds, 1954). In contrast, the evolution of "indirect" tumors often involved the abrupt and unpredictable appearance of new tumor phenotypes as opposed to the modification of previously established characteristics (Rous and Kidd, 1939). The sudden progression of murine mammary tumors from hormone responsiveness to unresponsiveness was an example of the latter process (Foulds, 1954).

Finally, the fifth generalization stated that tumor progression essentially had no fixed end point. The method of serial transplantation of tumors clearly demonstrated the extent and potential infiniteness of this phenomenon (Foulds, 1950). The

limits of this process were likely determined by the age and species of the host (Medina, 1975).

(3) The Progression of Specific Tumor Characteristics

A more in depth review of a number of experimental systems involving the progression of various unit characters provides the necessary background information for a discussion of the possible mechanisms of tumor progression.

(a) Hormone Responsiveness

Studies have shown that prolonged, continuous alterations of normal hormonal levels may evoke tumors of various endocrine and reproductive organs in rodents (Furth, 1953; Furth et al., 1960). Upon studying the F1 hybrids of certain inbred mouse strains, prone to multiple mammary tumors, Foulds (1949a,b) observed two basic patterns of tumor development. Most tumors were "responsive" to the changing hormonal environment of pregnancy as they grew during this period, but regressed in whole or in part after parturition. The proliferation and even survival of the tumors were conditional on the presence of hormone. The majority of these tumors did not progress past hormone induced hyperplasia or a benign adenoma during the life of the primary host (Foulds, 1954). However, Foulds (1954) observed that some of these tumors abruptly changed from a hormone responsive to an unresponsive state. This conversion was random and permanent. It was determined that pregnancy was not an essential impetus of this

process as the alteration in hormone responsiveness could occur in its absence. His experiments demonstrated the inevitability of this conversion. It was shown that if the life of a hormone responsive tumor was indefinitely extended by serial transplantation, it would eventually progress to the unresponsive state (Foulds, 1949a).

In contrast, the second pattern of tumor development was associated with hormone unresponsive carcinomas. These tumors were malignant upon first appearance, grew continuously regardless of the reproductive state of the animal and culminated in the death of the primary host. Foulds (1954) concluded that this phenomenon was due to the sequential acquisition of new tumor properties and/or the modification of existant characteristics. Other investigators have since expanded on this interpretation. They maintained that these data were consistent with the selective survival of cells that were no longer responsive to hormone from an original population which was heterogenous for this parameter (Klein, 1959; Medina, 1975). More recently, based upon the fact that some neoplastic cells can both synthesize and respond in vitro to potent factors capable of influencing cell growth and proliferation, it was postulated that the autonomous behaviour of a tumor may, at least in part, be related to "autocrine" secretion (Sporn and Todaro, 1980). Though the effect of these "transforming growth factors" in vivo is presently unclear, the ability of a tumor to progress to a hormone unresponsive state may be related to this phenomenon (Todaro et al., 1982).

(b) Ascites Conversion

G. and E. Klein (1955, 1956) showed that the conversion of a solid tumor, to one capable of growing intraperitonealy in ascites form, resulted from the serial passage of the peritoneal exudate. Once the change occurred, it was stable and irreversible. They suggested that the process, associated with a decreased cellular adhesiveness (Klein, 1955) and an increased surface charge (Purdon et al., 1957) and invasive potential (Ringertz et al., 1957), was the result of a sequential progression of events. In addition, the Kleins (1956) demonstrated that the conversion of cells to the ascites form was directly related to the size of the inoculated test populations. It was postulated that the change was due to the selection of a small fraction of preexistant variant cells in the original population (Klein and Klein, 1956). The subpopulation was thought to have been endowed with an enhanced ability to become established in the ascites form. This idea was supported by the fact that the percentage of variants in the original unconverted population was inversely proportional to the time required for ascities conversion (Klein and Klein, 1956).

(c) Drug Susceptibility

Despite an often promising initial response to hormone or chemotherapy, anti-neoplastic treatment was frequently unsuccessful (Foulds, 1954). Foulds (1954) attributed this failure to the progression of the tumor to an unresponsive or resis-

tant condition. It was determined that tumor progression towards drug resistance occurred in a distinct stepwise fashion and that once attained was permanent even in the absence of the chemical agent (Law, 1958; Klein, 1963). The random appearance and clonal growth of tumors resistant to a variety of drugs suggested to Klein (1963) that variant subpopulations arose as a result of the instability of this phenotype. The drug susceptability phenotype was unstable in the sense that with time, tumors that were initially sensitive to a particular chemical agent often became resistant to the same drug. As in the ascites conversion model, Klein (1963) postulated the preexistence of cells resistant to the drug in the original population. It was suggested that the effect of a drug was to selectively eliminate the most sensitive variants rather than to influence variant generation (Klein, 1963).

(d) Isoantigenic Variation

Foulds (1954) often noted that during the first few serial passages of a transplanted tumor, a permanent decrease in tumor rejections occurred. The success of a tumor transplant was determined to be proportional to the exactness of the match between the membrane histocompatability protein determinants of the tumor cell and the recipient animal (Medina, 1975). Universal tumors, such as the Ehrlich ascites tumor, were exempt from this restriction as they had lost their H-2 alleles (Medina, 1975). Similarly, tumors which progressively acquired the ability to grow in a histoincompatible host always exhibited a deletion of isoantigens resulting in a decreased specificity (Klein, 1963). Tumors

which arose in hybrid mice exhibited isoantigenic variants (loss of one parental H-2 allele) and as a result were able to grow in the H-2 compatible parental strain. Klein (1963) postulated that the acquisition of isoantigenic variants that were less immunogenic and thus more likely to survive upon transplantation was a result of the selection of a preexistent subpopulation of cells within the original tumor.

(e) Karyotypic Alterations

Hsu (1961) and Hauschka (1961) have demonstrated that the karyotype of some transplanted tumors progressed towards and often attained aneuploid patterns. The observed changes were random, stable and occurred in a continuous fashion associated with tumor proliferation (Hsu, 1961). Klein (1963) described such tumors as "... complex mosaics from the chromosomal point of view, containing one or several predominant stem cell lines with numerically and often structurally distinct chromosome sets". In contrast, Rowley's (1975) work documented the emergence of a predominant subpopulation associated with one or more characteristic cytogentic change including the appearance of the Philadelphia chromosome during the progression of human chronic granulocytic leukemia.

Studies suggested that the development of a neoplasm with characteristic alterations in chromosome number or structure was a result of the selection of cells with that particular karyotype, from a population of cells with an entire spectrum of such changes (Makino, 1957; Hsu, 1961). Koller (1972) predicted that the

resultant variants had some survival advantage over the bulk of the original population. However, he was unable to consistently associate the cell karyotype with a specific alteration in a biological phenotype such as drug susceptibility, antigenicity or hormone responsiveness.

However, more recently karyotypic alterations have consistently been detected in association with some forms of human tumors including site specific chromosomal translocations and deletions in Burkitt's lymphoma and retinoblastoma, respectively (Yunis, 1983). This investigation concluded that such kayrotypic abnormalities likely represented a mechanism of altered oncogene expression as the basis for certain human neoplasms. In addition, the presence of unstable or stable amplified genes found on small acentric chromosomes called double minutes or within homogeneously staining regions, respectively, have been linked with mammalian cell resistance to the drug methotrexate (Snapka and Varshavska, 1983). Based upon this evidence, it is likely that the progression of other tumor phenotypes, when examined in more detail, will be associated with a particular karyotypic modification.

(4) Postulated Mechanisms of Tumor Progression

In addition to the frequently cited variant generation and selection mechanism as a basis for tumor progression, several other rationales have been considered. Any one or all of these mechanisms may be responsible for the evolution of tumor phenotype because they are not necessarily mutually exclusive.

(a) Spontaneous Hybridization

Evidence in the literature supported the hypothesis that an alteration in tumor phenotype, as observed during tumor progression, may have been due to the spontaneous fusion of tumor-tumor and/or tumor-host cells. The spontaneous fusion of transplanted tumor and host cells has been reported for cells grown in vivo (Wiener et al., 1972; Lala et al., 1980) and in vitro (Marshall et al., 1982). The hybridization process may produce a new tumor with properties distinctly different from either contributing cell. For example, Hart (1984) described that the fusion of neoplastic cells with different metastatic capacities generally resulted in a more metastatic tumor, while the hybridization of normal and metastatic tumor cells appeared to reduce the metastatic potential of the hybrid compared with the parental population. Kerbel and associates (1984) postulated that spontaneous in vivo tumor cell fusion and the subsequent chromosomal segregation may result in the emergence of a range of variants with respect to malignant potential. They suggested that the selective survival of the hybrid cells best suited to growth in the host environment could account for an alteration in tumor phenotype observed during tumor progression.

(b) Host-Induced Adaptation

Barrett and associates (1953) observed that a transplanted murine mammary carcinoma of the inbred C3H strain, passaged once through a sensitive F1 hybrid, grew better in the

backcross resistant host than tumors not so passaged. The change was permanent and not enhanced with repeated passage through the hybrids. It was determined that the phenomenon was independent of cellular contact with the F1 hybrid and that small and large inocula produced the same effect (Barrett et al., 1953; Klein, 1963). Klein (1963) showed that the increased transplantability of these tumors in the F1 hybrid was probably due to a decreased quantitative or qualitative expression of H-2 genes. These results suggested that this particular alteration in tumor histocompatability phenotype was due to the adaptation of the original tumor population to a new environment. However, the relative heterogeneity of their original tumor population was not determined and as a result the outgrowth of a high frequency variant, even at the lower doses tested, could potentially have been selected by some host mediated mechanism, as conceded by Barrett and coworkers (1953). In support of this point, Gronberg et al. (1981) observed that a tumor selected repeatedly in vitro and in vivo for a decreased sensitivity to NK cell lysis was associated with a reduced expression of NK target structure and an increased tumorigenicity (Klein et al., 1982).

In addition to the studies of Barrett and associates, other investigators observed an enhanced ability to transplant genetically foreign tumors to normal, mature mice following the passage of the tumor in irradiated adult mice (Krebs et al., 1942) or murine embryos still within the mother (Koprowski et al., 1956). These studies were designed to preclude the ability of the host to respond to the tumor with an efficient homograft reaction.

Similarly Klein (1963) suggested that the experimental manipulations of Barrett and Deringer may have induced a state of tolerance in the sensitive F1 hybrids, with respect to the tumor. He noticed that a compromised host response to tumors was a recurrent theme in these experiments but that there was insufficient evidence to determine the precise relationship between the immune status of the host and this alteration in tumor transplantability.

(c) Endogenous Tumor Growth Factors

It was suggested that a tumor, initially dependent on an exogenus hormone for growth, could become hormone independent by virtue of an increasing proportion of the tumor cells which synthesize and secrete endogenous hormone (Klein and Klein, 1957; Revesz, 1958). Tumor cells have been shown to generate factors capable of stimulating tumor cell proliferation (Puck and Marcus, 1955; Revesz, 1958; Todaro et al., 1982). Though the contribution of endogenous tumor growth factors to tumor progression has not been conclusively demonstrated, Todaro and coworkers (1982) suggested that the expansive proliferation of a heterogeneous tumor population may be dependent upon the autocrinous secretion of a number of essential growth factors from a variety of variants within the tumor mass. Based upon this model, they contended that the variants capable of colonizing distant sites would likely be able to directly supplement the hormonal growth requirements previously supplied by the tumor mass through an alteration in the qualitative or quantitative autocrinous production of crucial tumor growth factors.

(d) Variant Generation and Selection

Klein (1959) was an early proponent of a variant generation and selection mechanism to account for the behaviour of tumors undergoing progression to ascites growth, hormone unresponsiveness, drug resistance and karyotypic variation. He postulated that new variant cells appeared in a random manner in connection with cell division, independent of any specific inducing action of the environment. These variants were thought to have differed from the original tumor with regard to one or several unit characters capable of providing the cell with some survival advantage. Any endogenus (host mediated) or exogenous (therapeutic) growth regulationg mechanisms would provide potential selection pressures upon the expanding tumor mass (Klein, 1959). This process would eliminate the more susceptible variants, the remainder of which may become the precursors of a new dominant subpopulation, capable of continued variation.

In addition, Nowell (1976) focused on the origin of phenotypic variation during neoplastic progression. He reviewed the evidence obtained from the studies of abnormal tumor karyotypes, isoenzymes from the tumors of heterozygous women and the immunoglobulins produced by plasmacytomas (Nowell, 1957; Linder and Gartler, 1965; Milstein et al., 1967). Based upon this body of evidence, Nowell (1976) sugested that the tumors likely developed as a clone from a single cell. These results were consistent with the observation that the tumors which developed clinically, usually represented the progeny of a single cell or at most a few

cells. The development of a tumor from a common progenitor cell was an important idea. It appeared that the generation of tumor cell variants was due to progressive phenotypic alteration in the progeny of generally a single stem cell as opposed to the existance of a tumor derived from many stem cells. Currently, many researchers believe that most neoplasms have a unicellular origin. However, it was noted that carcinogens can simultaneously effect many cells in vivo (Nowell, 1976; Fialkow, 1979) and that tumors of viral etiology or those cancers associated with a familial gene defect were multiclonal (Linder and Gartler, 1965; Friedman and Fialkow, 1976).

Finally, Nowell (1976) suggested that the observed rate of progression depended upon the frequency of variant generation and the intensity of the selection pressures. It was thought that many variants were selectively eliminated due to metabolic disadvantage, immune destruction or other forms of host mediated growth restraint (Nowell, 1976). He contended that the environmental influences generated by the deteriorating health of the host, associated with a malignant growth and therapy, may in fact foster the appearance of new variants. It was concluded that the capacity for variant generation and selection which initally permitted the development of the tumor was likely responsible for endowing the tumor with the ability to rapidly adjust to a range of host environments to the detriment of the host (Nowell, 1976).

(5) A Genetic Versus an Epigenetic Basis of Tumor Progression

The appearance of new tumor phenotypes was thought to have either a genetic or an epigenetic basis, associated with some structural alteration of the genome or the changed expression of preexistent genes, respectively (Foulds, 1954; Klein, 1959; Medina, 1975). Arguments advanced in support of an epigenetic basis for tumor progression included the absence of detectable new gene products in many tumor cell systems, the instability or reversibility of some tumor phenotypes (Gelbion, 1967) and the lack of an effect of ploidy on the rate of variant formation (Harris, 1973, 1974). In addition, Harris (1973, 1974) maintained that the frequency of the appearance of altered tumor characteristics exceeded the known rate of somatic mutation for normal cells.

In contrast, Nowell (1976) contended that the phenomenon of tumor progression involved the sequential selection of variant subpopulations which arose as a result of an acquired genetic instability. He countered that a change in the expression of existent genes may have in fact resulted from mutations in regulator genes or the integration of a virus into the host's genome. He added that the investigators that cited a disparity between the rate of somatic mutation and alteration of tumor phenotype as evidence for an epigenetic basis, apparently ignored the recognized increase in the genetic liability of neoplastic cells. Koller (1972) noted that this genetic liability may become more evident as the neoplasm evolves. It was suggested that this alteration

was due to a higher frequency of mitotic errors and other genetic changes associated with an increased proliferative activity (Hellstrom et al., 1963). The basis of this instability may have been associated with inherited defects of DNA repair (German, 1972), the continued presence of carcinogen (Cole and Nowell, 1965), a local nutritional deficiency (Freed and Schatz, 1969), or as mentioned, host genome rearrangements due to an integrated oncogenic virus (Handen, 1974). Finally, it was emphasized that "major genetic errors do occur in tumor cell populations with sufficient frequency to permit the sequential selection of mutant subpopulations' (Nowell, 1976).

Collectively, the results of these studies did not conclusively substantiate or negate either mechanism. Foulds (1954) noted a parallelism between the sequential acquisition and/or modification of tumor characteristics observed during tumor progression and the similar scenerio of embryonic epigenetic development. Though most of the evidence suggested that the effects of tumor progression were the result of a genetic mechanism, he thought it inadvisable to completely exclude epigenetic processes. Similarly, Harris (1974) contended that genetic and epigenetic mechanisms were not mutually exclusive, as tumor cell variants could conceivably arise as a result of a stable alteration in gene expression and/or the structural mutation of genetic components.

(B) IMMUNE SURVEILLANCE

Any factor capable of influencing the development of an

incipiant neoplasm *in vivo* could potentially have an important bearing on the progression of the tumor and ultimately on the outcome of host-tumor interactions. Accordingly, the ability of the host to respond immunologically to a tumor has been the focus of much investigation, speculation and controversy.

(1) *Host Mediated Control of Neoplasia*

Some researchers proclaimed that the development of a neoplastic growth must of necessity indicate the failure of host mediated tumor defense mechanisms (Currie, 1976). The fact that the deleterious effect of neoplasia on the host is generally much more evident than the ability of the host to eliminate cancerous cells or influence the malignant process once initiated, has only served to foster such rationales (Woodruff, 1982). Woodruff (1982) reviewed the evidence that supported the existence of a host mechanism capable of exerting some form of restraint on neoplastic growth. A report by Eversen and Cole (1966) documented the spontaneous regression of human tumors and the phenomenon of tumor dormancy, an extended latent period observed between apparent tumor irradication and reappearance. The study of tumor cell population kinetics also supported this view (Woodruff, 1980). It was demonstrated that the slow growth rate of some tumors, in spite of a high proliferation rate, was due to an increased frequency of cell death. Ehrlich (1909) contended that in the absence of any regulating process, the incidence of cancer would be predictably higher. Woodruff (1980) reiterated this view based upon the relative ease with which cells could be transformed *in*

vitro and the high estimated frequency of mutation during an average human lifetime.

Several postulated anti-tumor mechanisms have been examined (Woodruff, 1980), including the extinction of a tumor due to a lethal mutation, correction of a malignant mutation via DNA repair, selective shedding of deviant cells from the epithelium of the skin or gastroinstinal tract and the destruction of tumors by host immune processes. Somewhat prior to the formulation of the immune surveillance theory, Ehrlich (1909) postulated the existence of some form of "natural immunity" that was capable of controlling neoplasia. Interest in the possible role of host immunity in carcinogenesis was prompted by the realization that the rejection of a transplanted tumor from a genetically distinct animal, was an immune phenomenon. The fact that a small but significant increase in the incidence of spontaneous tumors was observed in patients maintained on extended immunosuppression after a renal transplant, provided additional impetus for this work (Burnet, 1971).

(2) The Immune Surveillance Theory

The idea of immune surveillance was revitalized as a justification for the existence of cell mediated immunity. Thomas (1959) thought that it was unlikely that transplantation immunity evolved as a defense against surgical homografts. He suggested that as a result of the "univeral requirement of multicellular organisms to preserve uniformity of cell type ... homograft rejec-

tion will turn out to represent a primary mechanism for the natural defense against neoplasia". Burnet was inspired by this rationale and postulated two basic requirements for the existence of such a system. He thought that incipient tumors must be antigenically distinct from normal host cells and such differences could, under the appropriate conditions, stimulate a thymus dependent immune response. This process was thought to culminate in the elimination of the tumor, equivalent to a homograft rejection (Burnet, 1971). The purported role of immune surveillance was then, not to mediate the regression of established tumors, but rather to seek and destroy clinically unrecognizable in situ tumors. The evolutionary significance of immune surveillance was thought to be the prevention of the emergence of aberrant cells in order to maintain the cellular integrity of the body (Burnet, 1964).

The possibility of an immune response to spontaneous tumors was generally discounted until various researchers demonstrated that many types of experimental cancers carried distinct antigenic determinants. Furthermore, it was shown that these determinants were capable of stimulating such a reaction in their isogenic host, given the appropriate experimental conditions (Foley, 1953; Prehn and Main, 1957; Habel, 1961; Sjogren et al., 1961; Old and Boyse, 1965). The concept of immune surveillance was initially accepted uncritically as it explained the phenomenon of foreign graft rejection and provided a normal biological role for T lymphocytes (Moller and Moller, 1976).

(3) Criticisms of the Theory

Various clinical and experimental predictions were made on the basis of the immune surveillance theory (Burnet, 1970). At first glance, most of these expectations appeared to have been fulfilled, yet Burnet (1971) conceded that some findings were rare and that others relied upon the subjective interpretation of selected results. Critical analysis of the evidence has since weakened the interpretation of certain aspects of the theory. Woodruff (1982) reviewed the general objections to the immune surveillance hypothesis including the fact that many tumors are not contained before killing the host and that the results of immune therapy have generally been disappointing. Furthermore, many cancers appear to be restricted to age groups that are not reproductively active, thus questioning the evolutionary significance of such a mechanism. Finally, many human and animal tumors appeared to be poorly antigenic. Hewitt and coworkers (1976) examined a wide variety of spontaneous murine neoplasms during repeated isotransplantations in an attempt to determine the immunogenicity of these neoplasms. They concluded that there was no evidence to suggest that any of the tumors studied were immunogenic as a result of the apparent inability of the host to reject a tumor inoculum even with the coinjection of specifically sensitized lymphocytes from regional lymph nodes of tumor bearing mice.

Most of these criticisms have been reasonably explained within the context of the theory. For instance, though some tumors may have escaped host defences, many more tumors may have

been destroyed, and just because immune therapy has not worked to date does not preclude any future advances in treatment. In addition, even if cancer was confined to age groups which contribute a negligible fraction to the birth rate, this fact would not exclude the possibility that many tumors were aborted early in life (Woodruff, 1982). Furthermore, most of the evidence suggested that human tumors were in fact antigenic (Currie, 1975). The difficulty in detecting their antigenicity may have been related to the inability to perform the same syngeneic transplantation tests as used to define these antigens in inbred animals (Kripke, 1981). With regard to the studies of Hewitt and associates (1976), had they searched for evidence of a thymus independent response against the tumors tested, they may have found that those neoplasms were indeed antigenic.

Despite these considerations, numerous other observations have made it clear that the immune surveillance concept as stated was improbable, especially the intregal role given to thymus dependent mechanisms. It has been determined that a main function of T lymphocytes is to defend the host against otherwise lethal viral and parasitic infections (Moller and Moller, 1976). It has furthermore been suggested that the microbial milieu and not spontaneous tumor generation was the selection pressure that maintained T cell mediated immunity during evolution (Rygaard and Povlsen, 1976). More specifically, Moller and Moller (1976, 1979) have compiled an extensive summary of the most incriminating evidence including the fact that the monoclonal origin of most tumors contradicted the prediction that multiple tumors should occur in

immunosuppressed individuals. They also refer to the lack of an enhanced tumor frequency associated with spontaneous or chemically induced tumors in nude mice, the restricted range of neoplasms in immunodeficient patients and the absence of an increased tumor incidence in immunoprivileged sites. Woodruff (1982) pointed out that the observed increase in the rate of viral oncogenesis in nude and immunosuppressed mice was consistent with the proposition that T-cell mediated immune surveillance played an important role in promoting the rejection of cells transformed by ubiquitous oncogenic viruses. However, this may only have been a gratis consequence of virus irradication. The generality and efficiency of the surveillance system was also questioned by other workers (Prehn, 1976, 1977; Kripke and Borsos, 1974). They contended that a thymus dependent immune response to tumors would not likely act early enough during tumor growth so as to eliminate the tumor before it had a chance to escape host defenses. Furthermore, they suggested that such a response may indirectly stimulate tumor development due to tumor induced immunosuppression.

(4) A Revised Immune Surveillance Theory

Stutman (1975) stressed that if immune surveillance against incipient tumors existed, it must be mediated by a different mechanism from the "post factum" immune response against an established tumor, to which T-cells may contribute. Currently, a modified thymus independent form of the surveillance hypothesis has generally been accepted as a likely primary defense against

neoplasia (Allison, 1977; Ioachin, 1976). Furthermore, the close physical association of lymphoid and neoplastic cells within the complex "ecosystem" of a tumor mass (Woodruff, 1982), and the fact that malignant cells can be destroyed *in vitro* by various cells of immune origin (Klein et al., 1960; Hibbs et al., 1972; Herberman et al., 1975; Keissling et al., 1975) emphasized the potential role of certain lymphoid cells in host mediated tumor resistance and immune surveillance. Based on the revised criteria for potential effectors of an immune surveillance system, this type of mechanism must be capable of distinguishing between "self" and the tumor and be equipped with the capacity to kill malignant cells without the complex time consuming intermediate steps characteristic of a classic T-cell mediated immune response (Currie, 1976).

(C) HOST MEDIATED ANTI-TUMOR NATURAL RESISTANCE

Once the attention to elucidate potential mediators of immune surveillance shifted from T-cells, it initially focused on "natural" immunity, as this system basically satisfied the requirements for a surveillance mechanism. Interest in this field stemed from the study of F1 hybrid resistance to transplanted tumors (Snell, 1958; Hellstrom, 1963; Kiessling et al., 1975) and the important observation that lymphocytes from normal rats, mice and humans were cytotoxic for tumor cells, especially of lymphoid origin (Takasugi et al., 1973; Greenberg and Playfair, 1974; Keissling et al., 1975a,b). Presently, the abilities of natural killer (NK) cells, natural antibodies (NAb) and macrophages to

influence tumor growth and collectively mediate host anti-tumor "natural resistance" (NR) are being extensively studied.

(1) The Characterization of NK Cell Activity

NK cell activity is thought to be mediated by a heterogenous subpopulation of non-phagocytic lymphoid cells (Minato et al., 1981). These cells, without previous sensitization, have been shown to lyse a variety of tumor, virus infected, embryonal or normal cells (Herberman and Holden, 1978; Hallen et al., 1977). The development of NK cell activity does not depend on a functional spleen or thymus (Haller et al., 1978; Herberman et al., 1975a). NK cells likely originated from cells of a pre-T cell lineage (Herberman et al., 1979) residing in bone marrow (Haller et al., 1977; Keissling and Wigzell, 1979). NK cell activity has been detected in the peripheral blood, peritoneum and most lymphoid organs, excluding the thymus, of a variety of mammalian and avian species (Herberman and Holden, 1978; Welsh, 1978). In addition, the NK cell has been extensively characterized for the expression of a variety of cell surface markers including asialo GM1 and GM2, LY-5, Mph-1, NK-1, Qa-2, Qa-5, Thy-1 and Thy-200 (Glimcher et al., 1977; Dennert et al., 1981; Minato et al., 1981; Kasai et al., 1981). Receptors for the Fc portion of immunoglobulins were detected (Herberman et al., 1977), but surface immunoglobulins were absent (Welsh, 1978). The NK cell reactivity of both human and murine peripheral blood was essentially consistent throughout the majority of adult life (Lanza and Dejeu, 1982). In

contrast, murine splenic NK activity peaked between 4-8 weeks of age, but was undetectable past 12 weeks. Other data sugested that NK cell activity was under polygenic control. For the inbred strains of mice tested, it was shown that the dominant inheritance of high NK cell reactivity was dependent upon both MHC-linked and unlinked genes including pigment mutations (Petranyi et al., 1975; Klein et al., 1978; Kiessling and Welsh, 1980; Orn et al., 1982).

(a) A Role for NK Cells in Tumor Resistance

Numerous studies have provided evidence for the participation of NK cells in in vivo tumor resistance. In vivo resistance to tumor growth for many tumor-strain combinations was positively correlated with levels of NK cell activity assessed in vitro (Kiessling et al., 1975; Sendo et al., 1975; Harmon et al., 1977). More direct evidence was obtained from the work of Hanna et al. (1982) demonstrating that a decreased frequency of metastases was correlated with increased NK levels in nude mice. Other researchers found that NK cell deficient mutant beige/beige homozygous mice were more susceptible to some, but not all, syngeneic NK cell sensitive tumors, compared with their normal beige/+ heterozygous littermates (Talmadge et al., 1980; Karre et al., 1980). Kasai (1979) demonstrated that tumor growth was significantly reduced if the tumor cells were co-inoculated with NK cells that were cytotoxic for the tumor in vitro. Similarly, tumors sensitive to NK cell cytolysis were eliminated faster from various organs when injected intravenously with NK cells of a high rather than a low reactivity (Riccardi et al., 1980). Kasai et al.,

(1981) found that an almost complete abolition of NK cell activity after injecting the mice with anti-asialo GM1, correlated with increased tumor incidence and growth.

The bulk of the evidence strongly supported a role for NK cells in host mediated resistance to tumors. However, many researchers have noted an inconsistency between the survival of some tumors and host levels of NK cell activity or tumor sensitivity to NK cell cytolysis (Karre et al., 1980; Riccardi et al., 1980; Collins et al., 1981.). For example, it was determined that in vivo variation in susceptibility to NR correlated well with in vitro sensitivity to NK cells for NK-sensitive but not NK-resistant tumors (Gorelik and Herberman, 1981; Chow et al., 1981). These data suggested that other mechanisms, in addition to NK cells, were operative in host mediated anti-tumor natural resistance. As a result of this evidence, the participation of anti-tumor NAb and macrophages in this phenomenon has increasingly been examined.

(2) The Characterization of NAb Activity

Antibodies that are observed in the sera of normal non-intentionally immunized individuals have been termed "natural antibodies". NAb activity has been detected in a wide range of species, including humans and mice (Martin and Martin, 1975; Aoki, 1976; Gronberg et al., 1980), directed against a diverse list of antigens such as synthetic haptens (Jormalainen and Makela, 1971), thymocytes (Shira and Mellors, 1972), murine and primate oncogenic

viruses (Aoki et al., 1966; Nowinski and Kaehler, 1974; Kende et al., 1981) and numerous tumor cells (Herberman and Aoki, 1972; Menard et al., 1977; Wolosin and Greenberg, 1979; Chow et al., 1981). The presence of NAb *in vitro* was assessed through the inhibition of immunoglobulin binding in a radioimmuno assay and in a complement mediated cytotoxicity assay (Wolosin and Greenberg, 1979; Chow et al., 1981). Wolosin and Greenberg (1981) determined that NAb activity was a function of a thymus independent immune system. Furthermore, murine anti-tumor NAb activity was found to be age dependent and predominantly associated with IgM and to a lesser extent, IgG immunoglobulin fractions (Herberman and Aoki, 1972; Pierotti and Colnoghi, 1976; Menard et al., 1977; Chow et al., 1981). Work with monoclonal NAb prompted the suggestion that an anti-tumor NAb response was the result of the synergistic action of a broad range of NAb's with different specificities (Colnaghi et al., 1982).

(a) A Role for NAb in Tumor Resistance

The hypotheses that NAb is an effector of host mediated anti-tumor NR was supported by the observations that the level of host NAb activity and the sensitivity of a tumor to NAb lysis assessed *in vitro*, correlated with tumor growth *in vivo* (Menard et al., 1977; Chow et al., 1980). Furthermore, it was found that the ontogeny of host resistance also correlated with that of NAb production (Menard et al., 1977; Chow et al., 1981). Wolosin and Greenberg (1979) demonstrated that tumors rapidly acquired NAb after *in vivo* implantation, while Chow et al. (1981)

showed that pretreatment of tumor cells with NAb in vitro reduced their tumorigenicity in vivo. The fixing of complement in vivo appeared not to be involved in the anti-tumor action of NAb (Carlson and Terres, 1976; Scornik and Klein, 1978). Additional evidence in support of the hypothesis that NAb was an effector of NR was derived from the fact that modulated levels of serum NAb through adjuvant stimulation and silica suppression, correlated with host mediated resistance to tumors (Greenberg et al., 1980; Chow et al., 1981). Chow (1983) also showed that resultant cells from an initially heterogeneous tumor population, selected in vitro with syngeneic NAb plus complement, were reduced in their sensitivity to syngeneic NAb plus complement and to syngeneic NR measured in an in vivo ^{131}IUdR labeled tumor elimination assay.

(3) The Characterization of Macrophage Activity

Macrophages or mononuclear phagocytes actively participate in host resistance to infection and function as accessory and regulatory cells in humoral and cellular immunity (Benacerraf and Unanue, 1979; Nelson, 1981). Differentiated precursors from the bone marrow enter the circulation and tissues, become widely distributed throughout the body and can be recruited from distant sites to an inflammatory foci where further differentiation and proliferation may occur (Allison, 1978; Keller, 1980). The diversity of macrophage function may be associated with different subpopulations of cells (Keller, 1980; Lee, 1980; Pelus et al., 1981). Macrophages have been associated with a diverse list of

secretory products and can express cytostatic or cytolytic activity *in vitro* against tumor cells. They function spontaneously or at an enhanced level of activity, subsequent to specific or non-specific stimulation (Ogmundsdottir and Weir, 1980; Keller, 1980). Herberman (1980) showed that silica inhibited macrophage mediated cytotoxicity *in vitro*, without affecting NK cell activity.

(a) A Role for Macrophages in Tumor Resistance

The observations that macrophages were physically associated with many tumors *in vivo* and cytotoxic for tumor cells *in vitro* support the hypothesis that macrophages participate in host mediated tumor resistance (Hibbs, 1972, 1976; Keller, 1973, 1976; Piessens et al., 1975; Haskill et al., 1975; Currie and Basham, 1975; Evans, 1980). Eccles and Alexander (1974) found that the macrophage content of tumors was inversely related to the incidence of tumor metastases, suggesting a role for macrophages in the control of tumor dissemination. Other investigators showed that the suppression or stimulation of macrophage function resulted in a decrease or increase in host resistance to tumor growth, respectively (Keller, 1976a; Chow et al., 1979). More recent studies were unable to detect a correlation between the extent of macrophage infiltration and the metastatic behaviour of a variety of different rodent neoplasms (Fidler and Poste, 1982; Talmadge et al., in press). Fidler and Poste (1982) concluded that the role of macrophages within a tumor mass based upon these conflicting results is presently controversial and likely dependent upon the unique properties of individual tumors.

Collectively, the evidence indicated that NK cells, NAb and macrophages were able to influence tumor growth. The specificity, rapidity and thymus independent nature of these responses implicate them all as effectors of NR and likely participants in host mediated anti-tumor surveillance.

(D) CURRENT MODELS OF THE VARIANT GENERATION AND SELECTION MECHANISM OF TUMOR PROGRESSION

(1) Metastases

It was generally conceded that the most devastating aspect of cancer was the formation of metastases (Nicolson, 1979). The development of metastases is thought to be a complex phenomenon, dependent on an interplay between host defence mechanisms and the intrinsic properties of the tumor cells (Fidler and Hart, 1982). To establish metastases the tumor cell must successfully traverse all of the steps of the process (Fidler et al., 1978). The acquisition of new phenotypes including the synthesis and release of degradative enzymes, resistance to host immunity, decreased cellular adhesiveness, and increased cell motility are all thought to contribute to the formation of metastases (Nicolson, 1979).

Most malignant cells released into the circulation are rapidly destroyed suggesting that the few remaining cells were endowed with an enhanced ability to survive (Poste and Fidler, 1980). Fidler and Kripke (1971) postulated that the surviving

cells may have originated from a population of cells, that as a whole, had adapted to an altered host environment and as such were no different from the cells that perished. The other possibility that they considered was that the surviving cells were unique. They suggested that these cells may have been the progeny of one or more preexistant subpopulations within the primary tumor, that possessed some growth advantage over the remainder of the population.

The relative contribution of these factors to the successful outcome of blood-borne metastasis was examined with an in vivo model of metastatic cancer. It was designed to mimic the spread of neoplastic disease by injecting murine B16 melanoma cells directly into the circulation of the host (Fidler et al., 1978). Fluctuation analysis of the B16 melanoma and an ultra violet irradiation induced fibrosarcoma revealed that a series of subpopulations gave rise to a significantly heterogeneous frequency of lung colonies compared to the uncloned parental lines (Fidler and Kripke, 1971). This observation suggested that the original unselected lines were heterogeneous, with high and low metastatic variants preexisting in the population. By the repeated process of intravenous injection of melanoma cells, lung colonization, tissue culture and harvesting for reinjection into mice for ten cycles, Fidler and Nicolson (1976) selected a variant melanoma line (B16-F10). The B16-F10 cells formed significantly more tumor colonies in the lung and exhibited a much more uniform metastatic potential than the parental B16 cells, under the experimental conditions employed (Talmadge and Fidler, 1982).

Further evidence in support of the existance of predetermined variants as a basis for the progression of the metastatic phenotype was sought by assessing the factors that were thought to influence the pattern of metastatic distribution. A population of cells, that predominantly colonized a specific organ, was injected into the host in such a manner as to trap the cells at a different site. The results indicated that the cells could eventually detach from the original arresting location and recirculate until they reached the preferred colonization environment (Nicolson, 1979). Analysis of the B16 cells was also performed in vitro. Variants were selected in vitro for the expression of properties considered important in one of the steps in the metabolic process, including resistance to T-lymphocytes (Fidler et al., 1976), NK cells (Hanna and Fidler, 1981) and complement dependent antibody cytoxicity (Frost and Kerbel, 1981), decreased adhesiveness (Liotta et al., 1978) and increased invasive capacity (Poste et al., 1980). The selected cells were assayed in vivo in order to assess the metastatic potential of the resultant population. It was determined that the cells selected with respect to these parameters, exhibited an increased metastatic potential.

Together, these data suggested that the malignant tumors were not homogeneous, but rather were composed of different subpopulations with a range of characteristics related to the metastatic potential. Other studies demonstrated that the evaluation of the metastatic phenotype of a cloned cell population, after experimental conditions in which the effect of selection was thought to be limited, indicated the emergence of a heterogeneous population of

cells with respect to this parameter (Talmadge et al., 1979). In addition it appeared that the generation of metastatic heterogeneity was a random bidirectional process. Cloned cells from the parental population were obtained which expressed a higher (Fisher and Cifone, 1981) similar or lower (Kerbel, 1979; Dennis and Kerbel, 1981) metastatic phenotype compared with the original tumor. The concensus was that metastases likely occurred due to the selective survival and proliferation of a specialized subpopulation of cells within the primary tumor at the metastatic site (Fidler et al., 1978; Nicolson, 1979; Poste and Fidler, 1980).

Nowell (1976) proposed that during tumor progression, variant cells would likely arise as a result of an acquired genetic liability, which if subjected to host selective pressures, would favor the survival of variants with an increased malignant capacity. Cifone and Fidler (1981) demonstrated that an increased metastatic capacity was frequently associated with an increased rate of spontaneous mutation to various chemical agents. Thus, the evidence obtained using the metastatic model supported a variant generation and selection mechanism as the basis for the progression of the metastatic phenotype.

(2) Tumor Nascence

Evidence for the existence of an immune surveillance system was sought by examining the fate of a small tumor inoculum which was intended to simulate the growth of an incipient neoplasm (Greenberg and Greene, 1976). This model was subsequently used to

determine the stability of a cloned murine lymphoma during growth in a syngeneic host in an attempt to test the hypothesis that tumor progression proceeds through the generation and selection of variants (Chow and Greenberg, 1980). Using the NK-resistant and NAb-sensitive L5178Y-F9 clone, small, 25 cell inocula were injected into syngeneic mice subcutaneously in their lower middle backs. The cells grew at this site for 3 1/2 weeks before they were returned to tissue culture. Subclones from the *in vivo* passaged cells exhibited an increased heterogeneity in sensitivity to complement mediated lysis by normal allogeneic serum (Chow and Greenberg, 1980). Chow and Greenberg (1980) contended that the extensive phenotypic heterogeneity was not due to structural alterations in the tumor cell genome because the observation was inconsistent with the reported frequency of mutation for mammalian cells.

In addition, the progression of tumors was assessed in terms of changes in tumor sensitivity to NR. The elimination rate of IP inocula of ^{131}IUdR labeled tumor cells was determined as a measure of NR against the L5178Y-F9 cells grown *in vivo* or maintained only *in vitro* (Chow et al., 1982). The *in vivo* grown tumor cells were eliminated significantly slower than the cells grown only in tissue culture (Chow et al., 1983). These results suggested that the *in vivo* grown cells from an originally homogeneous population were selected for a reduced sensitivity to NR during growth in syngeneic mice. Chow and co-workers (1983) also demonstrated that the subclones from the cells grown *in vivo* exhibited an increased heterogeneity in sensitivity to NR. Based upon these observations,

it was postulated that an initially homogeneous clone of cells had generated variants upon proliferation in vivo, including some which expressed an altered sensitivity to host mediated anti-tumor NR. Furthermore, these investigators contended that NR mechanisms may have imposed a selection pressure upon the expanding tumor population eliminating the variants most sensitive to NR. As a result, the remaining cells were less sensitive to NR and thus able to escape host defenses, and progress to a detectable neoplasm. This explanation accounted for the observed decrease in tumor susceptability to NR and increase in heterogeneity for sensitivity to syngeneic NR and allogeneic serum NAb, following the in vivo passage of the tumor.

Chow and co-workers (1983) concluded that the extent of heterogeneity argued against an adaptation mechansim as a basis for this phenomenon, while the results of a karyotypic analysis made it unlikely that a new tumor was formed via hybridization. These data supported a variant generation and selection mechanism as a basis for the evolution of this tumor for sensitivity to NR.

CHAPTER II

OBJECTIVES

OBJECTIVES

The first goal of this study was to assess the ubiquity of tumor variant generation and selection as a mode for the progression of the SL2-5 lymphoma. Secondly, in order to determine the ability of NR mechanisms to influence the progression of the SL2-5 tumor, the model was further characterized with respect to: (1) tumorigenicity, (2) the site dependent kinetics of *in vivo* tumor growth, (3) thymus independence, (4) the role of NK cell and NAb activity, and (5) the effect of tumor progression at the molecular level of the tumor cell.

CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

(A) ANIMALS

Male inbred DBA/2 and CBA strain mice were obtained from Jackson Laboratories (Bar Harbor, Maine) or the University of Manitoba Vivarium (Gunton, Manitoba) and used for experiments between the ages of six to ten weeks, unless otherwise stated. The mice were housed seven to a cage, held in laminar flow containment hoods and allowed food and water *ad libitum*.

(B) TUMOR CELLS

The SL2-5 tumor is a murine T cell lymphoma. It is a twice cloned subline of the 3-methylcholanthrene induced SL2 tumor (Wolosin and Greenberg, 1979) and is relatively sensitive to syngeneic and allogeneic NK cell and NAb cytolysis (Chow et al., 1981).

(1) *In Vitro* Cells

A sample of SL2-5 tumor cells was maintained exclusively in tissue culture (INVITRO cells). All of the *in vivo* grown cell populations were derived by passaging these INVITRO cells in syngeneic mice for various periods of time.

(2) In Vivo Cells

Various tumor cell populations were produced by injecting INVITRO cells (1×10^3 - 5×10^4) into syngeneic DBA/2 mice. Generally, these cells were passaged once in vivo at the subcutaneous (SC) site of the lower middle back or the intraperitoneal (IP) site, for specified lengths of time before returning the cells to tissue culture. Certain cell populations were subjected to a maximum of two consecutive in vivo passages.

(C) TUMOR CELL MAINTENANCE

(1) Tissue Culture

SL2-5 lymphoma cells were grown as suspension cultures in sterily filtered Fischer's medium (F), supplemented with 10% fetal bovine serum (FBS) and 5.0 mls of a penicillin (10,000 U/ml), streptomycin (10,000 mcg/ml) solution per 1.0 L of medium (Gibco Laboratories, Grand Island, N.Y.). The FBS was incubated for one-half hour in a 56°C water bath prior to its use, in order to heat inactivate complement. An aliquot of 8×10^5 cells was aseptically transfered to 35 mls of 10% FFBS (Fischer's medium supplemented with 10% fetal bovine serum) every two days, so as to maintain the cells in exponential growth between 3×10^4 to 6×10^5 cells per ml. The cells were cultured in parafilm sealed 100 ml glass bottles and incubated at 37°C in a 3% CO2, humidified atmosphere provided by a Napco Controlled Environment Incubator (Portland, Oregon).

(2) Long Term Cell Storage

As soon as a sufficient number of cells were grown in tissue culture, aliquots of 3-5 x 10^6 cells of all tumor populations were frozen in 10% FFBS containing 10% dimethyl sulfoxide. In order to control the rate of freezing, the cells were frozen in an insulated container at -70°C in an Ultra Low Freezer (Revco Inc., West Columbia, S.C.). After 24 hours, the frozen cells were transfered to uninsulated cardboard boxes within the freezer. The frozen cells served as a stock of fresh cells, and every 2 to 3 months all cell populations were recultured from frozen stock. Frozen cells were rapidly thawed in a 37°C water bath and immediately washed once with 25 mls of warm (37°C) 10% FFBS. The cells were resuspended at a concentration of 2 x 10^5 cells per ml in 10% FFBS, in order to initiate growth in tissue culture.

(D) IN VIVO TUMOR CELL PASSAGE

Tumor cells from tissue culture were washed three times with 25 mls of cold Hanks Balanced Salt Solution (HBSS), (Gibco Laboratories, Grand Island, N.Y.), and centrifuged at 210 g for 10 minutes in a refrigerated (4°C) centrifuge. The cell pellets were resuspended in cold HBSS and set on ice. Aliquots of 0.10 mls or 0.20 mls (1 x 10^3 - 5 x 10^4 cells) were injected into mice at the SC or the IP site, respectively. The cells were grown in vivo for predetermined periods of time (see Results Section A1, B2a and b) and then sterily removed in a Laminar Flow Biological Safety

Cabinet (Nu Aire Inc., Minneapolis, Minn.). Palpable SC masses at the injection site were removed and teased into a single cell suspension in cold HBSS. The cells passaged at the IP site were retrieved by two 10 ml HBSS lavages of the peritoneum with an 18 gauge needle and 10 ml syringe. All cell lines were washed twice more before they were resuspended in tissue culture medium and grown *in vitro*.

(E) TUMOR CELL CLONING

Tumor cell clones were obtained using a modification (Chow and Greenberg, 1980) of the Chu and Fisher (1968) method. Agar Noble (80 mg) (Difco Laboratories, Detroit, Mich.) was combined with 5 mls of ddd H20 (double distilled and deionized water), autoclaved and diluted to 50 mls with warm (44°C) 15% FFBS. This agar solution was placed in a 44°C water bath until required. The tumor cells to be cloned were diluted with 15% FFBS to a concentration of 10 cells per ml of medium. Three mls of the agar solution were added to each of ten sterile tubes and allowed to cool slightly. Once 2 mls of the cells were added to all of the tubes, the cloning cultures were placed in a 4°C fridge for 5 minutes in order to allow the agar to gel. The mixing of the agar solution with the cells was performed in a reverse order to that described by Chow and Greenberg (1980). These cells were incubated for 10-14 days in the 37°C, C02 incubator. Ten small, medium and large sized, well separated colonies were chosen near the agar surface and extracted with a Pasteur pipette. Only experiments

that resulted in a cloning efficiency of greater than 90% were harvested. Individual colonies were released into 20 ml glass culture tubes and maintained in 10% FFBS at a concentration of 2 x 10^5 cells per ml. Once these cells had doubled they were transfered to the 100 ml glass bottles and cultured as previously described (Methods Section B1).

(F) PREPARATIONS OF MURINE SERA

(1) Normal Versus Adjuvant Stimulated Serum

Normal serum was obtained by bleeding male or female, 10-20 week old mice per axilla into Pasteur pipettes. The blood was transfered to test tubes, allowed to clot on ice and centrifuged at 890 g for 10 minutes. The serum was removed from pelleted cells, passed through a 0.45 uM millipore filter (Millipore Corp., Bedford, Mass.) and used immediately or stored at -20°C. In order to produce adjuvant stimulated serum, a 0.20 ml aliquot of trichloracetic acid extracted, E. coli lipopolysaccharide (Lps), (Serotype No. 0127.B8) (Sigma Chemical Co., St. Louis, Mo.) containing 100 ug of Lps dissolved in HBSS, was injected into each mouse at the IP site. The stimulated mice were bled as described, 3 days after Lps injection. Normal or Lps stimulated DBA/2 (H2d haplotype) mice were bled to obtain normal or non-specifically stimulated syngeneic serum, respectively. Normal serum that was allogeneic to the DBA/2 mouse strain was acquired from CBA (H2k haplotype) mice.

(2) Anti-H2d Serum

Anti-H2d (B10.Br anti-B10.D2) antiserum was obtained following six injections of B10.D2 splenocytes (one spleen per three recipients) biweekly at the IP site into B10.Br mice. The mice were bled 8 days after the final injection.

(3) Anti-Thy 1.2 Serum

This monoclonal antibody preparation was obtained from Dr. P. Lake, Dept. of Zoology, University College, London, England. The antibody was originally dissolved in distilled water and further diluted with HBSS to obtain a stock dilution of 1:20. Aliquots of this preparation were stored frozen and diluted with 10% FFBS according to individual experimental requirements.

(G) SERUM ABSORPTION WITH TUMOR CELLS

According to an established procedure (Chow et al., 1981), tumor cells were washed twice in 25 mls of cold HBSS. During the second wash the cells were centrifuged for an additional 2 minutes at 890 g to firmly pack the cells. The supernate was completely removed by suction. A volume of 0.20 mls of serum per 1×10^7 tumor cells was incubated on ice for one hour with gentle mixing every 15 minutes. After this incubation the mixture was centrifuged at 890 g for 10 minutes and the serum was carefully removed with a Pasteur pipette. The absorbed serum was stored overnight

at 4°C and used the next day.

(H) PREPARATION OF COMPLEMENT

Lyophilized rabbit serum as a source of complement (The Buxted Rabbit Co. Ltd., Sussex, England) was used for all antibody plus complement cytolysis assays. The serum was reconstituted with ddd H20. SL2-5 tumor cells were used to absorb any non-specific toxicity from the preparation in a 1:2 volume ratio of tumor cells to rabbit serum. The absorbed complement was passed through a 0.45 uM millipore filter and stored at -70°C until required.

(I) THYMECTOMY AND BONE MARROW RECONSTITUTION

A suction technique (Chow et al., 1979) was used to thymectomize 4 week old male DBA/2 mice. Average sized mice were anesthetized with a 0.20 ml IP injection of Phenobarbitol Nembutal Sodium (Abbott, Montreal), diluted with HBSS to a final concentration of 5 mg/ml. Smaller mice were given slightly less (0.15-0.19 mls) anesthetic. Four weeks later the mice were lethally irradiated (950 R) with a Theratron F Cobalt Unit and 24 hours after that were reconstituted with 1.5×10^7 bone marrow (BM) cells from age and sex matched syngeneic DBA/2 mice. The BM cells were removed from the femur of the rear limbs, diluted in cold HBSS, and injected intravenously. These adult thymectomized, irradiated, BM reconstituted (ATxBM) mice were used approximately 4

weeks after reconstitution. The ATxBM mice were checked for the presence of residual thymic tissue at the conclusion of the experiment and any mice with thymic tissue were excluded from the study.

(J) DETECTION OF NATURAL KILLER CELL ACTIVITY

The method of Greenberg et al. (1975) was used for this assay. Tumor cells were pelleted (210 g for 10 minutes) and resuspended in ^{51}Cr(Na2CrO4) (New England Nuclear, Boston, Mass.), diluted in HBSS or normal saline (100 uCi/10^7 cells). The cells were incubated with the radiolabel for 45 minutes in a 37°C water bath and washed twice in cold HBSS. The concentration of the cells was adjusted to 1 x 10^5 cells per ml in 10% FFBS. Spleen effector cells were harvested from mice 20 hours after the IP injection of polyinosinic-polycytidilic acid (poly I:poly C) (Sigma Chemical Co., St. Louis, Mo.), dissolved in HBSS (100 ug/0.1 mls/mouse).

The spleen cells from 3 to 4 mice were pooled, teased into a single cell suspension in cold HBSS and washed once. The pellet of effector cells was resuspended in 5-10 mls of a 0.16 M ammonium chloride solution. This incubation continued for 4 minutes at room temperature in order to lyse the red blood cells. The spleen cells were washed 3 more times and resuspended in cold 10% FFBS. If unlabeled tumor target cell inhibition of NK cell cytolysis was to be determined, unlabeled tumor cells were added to the microtiter plate at a ratio of unlabeled to labeled tumor cells of 10:1,

5:1 or 2.5:1. All NK cell assays were performed at a ratio of effecter lymphocytes to target tumor cells (E:T) of 150:1, in a final 0.20 ml volume of 10% FFBS. The microtiter plate was centrifuged for 2 minutes at 50 g to bring the effecter and the target cells into close proximity. The cells were incubated for 5 hours in the 37°C, CO2 incubator. The assay was carried out in triplicate. The plates were centrifuged for 10 minutes at 210 g and the supernatants from each well were sampled (0.10 mls) for gamma counting in a Packard 5230 Auto Scintillation Spectrometer. The percentage of ^{51}Cr released in the presence of effector cells was determined using the following formula:

$$\frac{\%\ ^{51}Cr\ \text{release (experimental)} - \%\ ^{51}Cr\ \text{release (control)}}{100 - \%\ ^{51}Cr\ \text{release (control)}} \times 100$$

The spontaneous release of ^{51}Cr from the control wells was usually between 5 and 20 percent.

(K) DETECTION OF NATURAL ANTIBODY ACTIVITY

This two step assay was employed by Wolosin and Greenberg (1979). Tumor cells from tissue culture were pelleted and labeled with ^{51}Cr as described for the NK cell assay. After radiolabeling, the cells were washed twice in HBSS and a pellet of 5×10^5 cells was incubated with 0.20 mls of test serum or 40% FFBS as a control, for 45 minutes in the 37°C incubator. The cells were washed once and resuspended in 0.30 mls of 10% FFBS. The assay

was carried out in quadruplicate in a microtiter plate. An aliquot of 0.20 mls of cells was incubated for one hour at 37°C with or without complement (diluted to 1/6 with 10% FFBS). A 0.160 ml aliquot of cold 10% FFBS was added to each well and the plate was centrifuged at 210 g for 10 minutes. Aliquots of 0.10 mls were removed from the supernatant for quantitation of ^{51}Cr release. Specific complement mediated NAb cytotoxicity was calculated as follows:

$$\frac{\%\ ^{51}Cr\ release_{(Ab+C)} - \%\ ^{51}Cr\ release_{(Ab)} - \%\ ^{51}Cr\ release_{(C)}}{100 - \%\ ^{51}Cr\ release_{(Ab)} - \%\ ^{51}Cr\ release_{(C)}} \times 100$$

(Ab = antibody, C = complement)

The percentage of ^{51}Cr released in the presence of test antibody and 10% FFBS was usually in the order of 5-20%, which was similar to the spontaneous release with 40% FFBS. Complement toxicity usually ranged from 5-10%.

(L) ASSESSMENT OF A GENERAL SUSCEPTIBILITY TO LYSIS

(1) Tumor Susceptibility to Hypotonic Lysis

This assay was a modification of a hypotonic shock technique described by Russell (1980). Tumor cells from tissue culture were pelleted, incubated with ^{51}Cr radiolabeled for 30 minutes as described for the NK assay and washed three times with cold HBSS. The cells were resuspended in cold 10% FFBS and ali-

quots of 2 x 10^4 cells in 0.10 mls of media were added to each well of a microtiter plate on ice. The experiment was performed in triplicate. Various percentages of isotonic media (5-50%) were added to the cells, to a total volume per well of 0.20 mls. These hypotonic media were made by diluting 10% FFBS with the appropriate volume of ddd H20 supplemented with 10% FBS. A 100% isotonic medium (undiluted 10% FFBS) was used as a control. Separate plates were incubated for 1.5 to 4.5 hours on ice or in the 37°C C02 incubator. At the end of the incubation period, the microtiter plate was centrifuged at 210 g for 10 minutes and a 0.10 ml sample of each well was removed for gamma counting.

(2) The Effect of Metabolic Inhibitors on Tumor Susceptibility to Hypotonic Lysis

The cells were incubated with ^{51}Cr radiolabel for 30 minutes and washed once with cold HBSS. Each pellet was resuspended in either 3.0 mls of warm (37°C) 10% FFBS or a solution of cycloheximide, colchicine, or sodium azide dissolved in warm (37°C) 10% FFBS (for specific concentrations see Results Section 5cii). The cells were incubated for 30 minutes at 37°C, immediately followed by another 30 minute incubation on ice. The cells that were preincubated with 10% FFBS or poison were washed once in cold, 10% FFBS or a 1 mM solution of the appropriate poison, respectively. They were resuspended in 10% FFBS or a metabolic inhibitor solution and aliquoted (2 x 10^4 cells/0.01 mls) into the plates on ice. The plates were set up as described in the Methods

Section L, except that the remainder of the assay was carried out in the presence of the poisons. The various inhibitors (0.05 mls) were added to the 100% isotonic medium, dissolved in warm (37°C) 10% FFBS and to the hypotonic media dissolved in warm (37°C) 10% FBSddd H20, so as not to alter the ionic strength in the wells. The cells were incubated for 1.5 to 4.5 hours in the 37°C, C02 incubator. At the end of each incubation the plates were centrifuged and sampled as described in the Methods Section J. The percentage specific cytotoxicities for both assays (Methods Section L1,2) were determined using the following formula:

$$\frac{\%\ ^{51}Cr\ release_{(hypotonic)} - \%\ ^{51}Cr\ release_{(isotonic)}}{100 - \%\ ^{51}Cr\ release_{(isotonic)}} \times 100$$

The spontaneous release of ^{51}Cr from the cells incubated in 100% isotonic medium in the presence or absence of poison was usually between 1 and 10 percent.

(M) DETECTION OF NATURAL RESISTANCE IN VIVO

(1) ^{131}IUdR Tumor Elimination Assay

The susceptibility of SL2-5 tumor cell populations to host mediated NR was determined using a modification of the method published by Carlson et al. (1980). The rate of tumor elimination observed in this assay was considered to be a measure of tumor sensitivity to thymus independent NR since tumor cells were

eliminated at comparable rates from normal and thymus deficient mice (Carlson et al., 1980). Tumor cells, grown to a cell density of 3-5 x 10^5 cells per ml, were radiolabeled in vitro by adding approximately 1 uCi of ^{131}I-deoxyuridine (^{131}IUdR) (Edmonton Radiopharmaceutical Laboratories, Edmonton, Alberta) per 10^6 tumor cells directly to the tissue culture medium. These cells were grown for 3 to 4 hours in the 37°C, C02 incubator, then washed twice with cold HBSS. Once resuspended in HBSS, inocula of each tumor cell population (5 x 10^5 - 1 x 10^7) cells were injected into a group of 5 DBA/2 mice. The cells were injected at the IP site or the SC site in 0.20 ml or 0.10 ml volumes, respectively. The mice were given drinking water supplemented with 0.1% KI during the experiment to minimize thyroid uptake of iodine released from dead tumor cells (Hofer et al., 1969). Immediately after injection, at time zero, and at four consecutive intervals of one day thereafter, the mice were whole body gamma counted with an Ortec Modular Gamma Counting System. The percentage of ^{131}I retained on each day of the assay was determined using the following formula:

$$\frac{(^{131}\text{I}_{(\text{day } x)} - \text{background}_{(\text{day } x)})\ R}{^{131}\text{I}_{(\text{day o})} - \text{background}_{(\text{day o})}} \times 100$$

x = day 1, 2, 3 or 4 of the assay.

R was a correction factor for the decay of the radioisotope. R = standard (day o)/standard (day x). This calculation was carried out for each of the five mice in a group. The mean of these

values was then determined with the standard deviation (SD) and the standard error (SE). The percentage ^{131}I retained for each tumor population corresponded to the surviving fraction of the original radiolabeled tumor inoculum as the radiolabel was released from dead tumor cells and excreted (Hofer et al., 1969).

(2) Small Tumor Inocula

Host mediated NR was also assessed by examining the fate of a threshold SC tumor inocula producing tumor frequencies of less than 100 percent (Greenberg and Greene, 1976). The tumor frequency for a given cell population was determined by injecting pre-washed tumor cells ($1 \times 10^3 - 5 \times 10^4$ cells/0.10 mls of HBSS), into a group of DBA/2 mice at the SC or the IP site. The proportion of mice that developed a tumor at the injection site, eventually culminating in the death of the animal, was used to determine the tumor frequency.

(N) STATISTICS

(1) Student's T-Test

The student's T-test was used to determine the significance of differences in: (a) the mean percentage of ^{131}I retained in mice following the IP injection of ^{131}IUdR labeled tumor cells and (b) the mean percentage of specific cytotoxicities of cell populations assayed for sensitivity to NK cell, NAb or

hypotonic cytolysis.

(2) The "F" Statistic

The F statistic (F = S.D.^{2} population A/S.D.^{2} population B) was used to compare the variance (standard deviation)^2 of: (a) the percentage ^{131}I retained on each day of the tumor elimination assay, or (b) the percentage specific cytotoxicity of cells assayed for sensitivity to NAb plus complement, for groups of subclones derived from tumors passaged *in vivo* or grown only in tissue culture.

(3) Fischer's Exact Test

The significance of differences in the tumorigenicity of various tumor cell populations, assayed in small groups of mice, was determined using the Fischer's exact test.

(4) Linear Regression Analysis

Linear regression analysis of the percentage ^{131}I retained in mice on days one through four of the tumor elimination assay and: (a) the length of time that individual tumor cell lines were passaged *in vivo* or, (b) the percentage specific cytoxicities for the same series of cells as assessed in NK cell, NAb or hypotonic cytolysis assays were used to detect correlations with tumor sensitivity to NR.

Probability (P) values of less than or equal to five percent were considered significant for all of the above tests.

CHAPTER IV

RESULTS

RESULTS

(A) The Tumor Progression of the SL2-5 Lymphoma in Syngeneic Mice

Previously Chow and co-workers demonstrated that a reduction in tumor susceptibility to syngeneic NR, observed after the *in vivo* passage of a small inoculum of cells from the L5178Y-F9 clone, was associated with an increased heterogeneity for sensitivity to syngeneic NR and allogeneic CBA serum (Chow and Greenberg, 1980; Chow et al., 1983; Chow, 1984b). They concluded that the generation of a range of L5178Y-F9 tumor variants and the selective elimination of those cells most sensitive to NR could best account for this alteration in tumor phenotype. Corroboration of the L5178Y-F9 murine model of tumor progression was sought by examining the stability of the *in vivo* grown SL2-5 cells, in order to determine the ubiquity of tumor variant generation and selection as a mode of tumor progression.

(1) An Alteration in Tumor Susceptibility to Natural Resistance

According to the experimental design of Greenberg and Greene (1976), a small inoculum of 1 x 10^3 clone SL2-5 cells grown exclusively *in vitro* was injected into the SC site of syngeneic mice. This protocol was intended to simulate the development of an incipient neoplasm since there was evidence to suggest that

many tumors developed through the progression of a single or a few transformed cells (Nowell, 1976) and that the effect of immune surveillance would likely be optimal before a tumor reached a critical mass (Greenberg and Greene, 1976). These cells were grown in vivo for three weeks before a tumor was removed from the injection site and reestablished in tissue culture (3WKSC cells). The effect of in vivo growth on the cloned SL2-5 tumor was assessed by comparing the susceptibility to syngeneic NR of the in vivo grown cells with that of the INVITRO cells maintained in tissue culture for a comparable period of time.

Inocula of 2.5×10^6 ^{131}IUdR tumor cells were injected into the IP or the SC site of syngeneic mice (Figure 1). The 3WKSC cells were eliminated significantly slower than the INVITRO cells at both the IP and the SC site on all but the first day of the assay. The decreased tumor elimination rate of the 3WKSC cells suggested that a reduction in tumor cell susceptibility to NR was an effect of the three week in vivo passage at the SC site.

In addition, it appeared that the rejection of the SL2-5 tumor populations at the SC site was generally slower than from the peritoneum. It was possible that the retarded SC elimination of the tumor cells was due to a site dependent difference in the clearance of dead cells, as opposed to an increased tumor survival. A sample of INVITRO tumor was radiolabeled with ^{131}IUdR and then incubated for twenty minutes in a 75°C water bath in order to heat kill the cells. The clearance of the dead INVITRO cells from the SC or the IP site was determined using a tumor elimination assay (Figure 2). Overall, the heat killed tumors

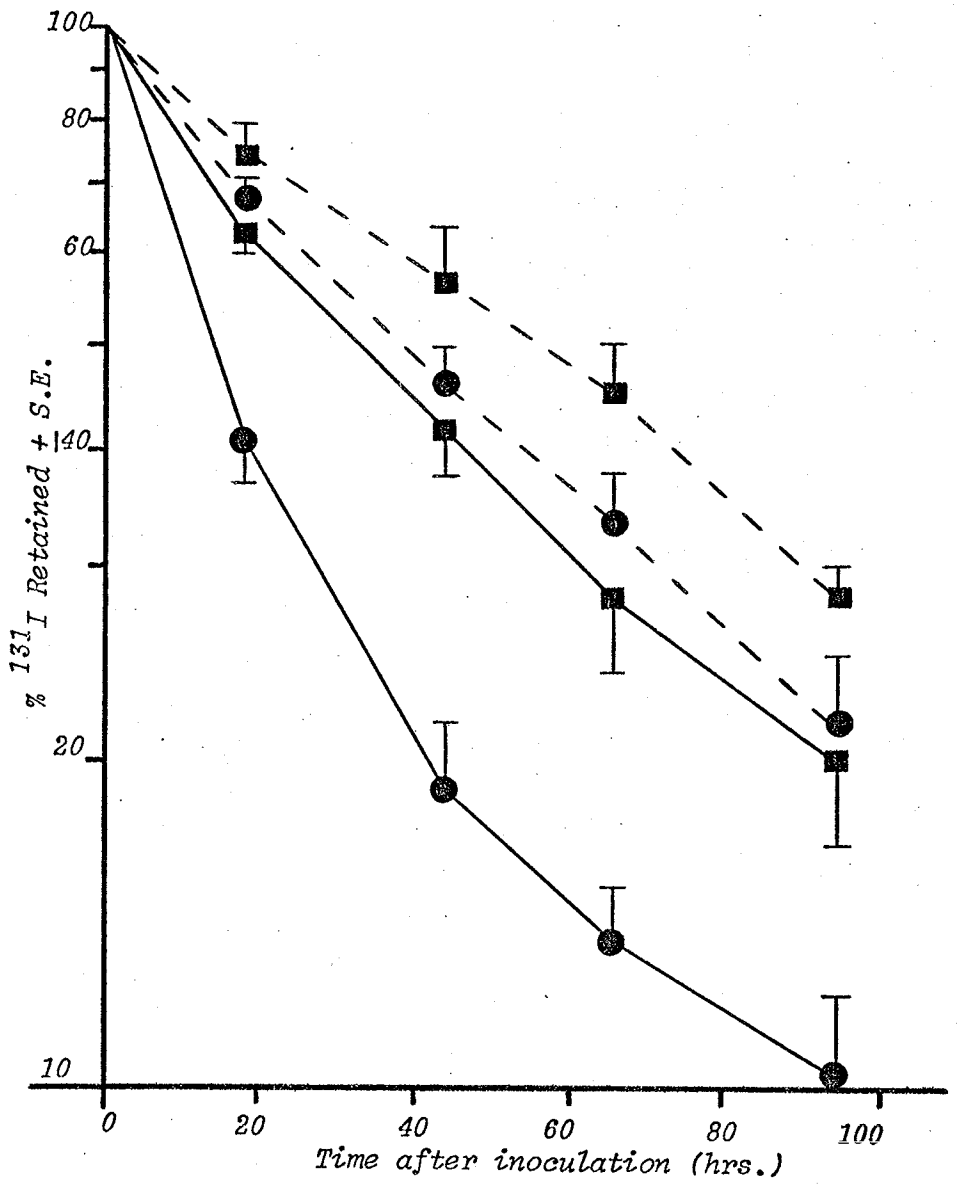

Figure 1. The susceptibility to NR of tumor cells assessed at the IP and the SC sites. Aliquots of $2.5x10^6$ ^{131}IUdR labeled INVITRO (●) or 3WKSC (■) cells were injected into groups of 5 DBA/2 mice at the IP (——) or the SC (- -) site. The results represent the combined data from two experiments. The mean percentages of ^{131}I retained for the two tumors were significantly different (P<0.05) on the last 3 days of the assay at both the IP and the SC sites.

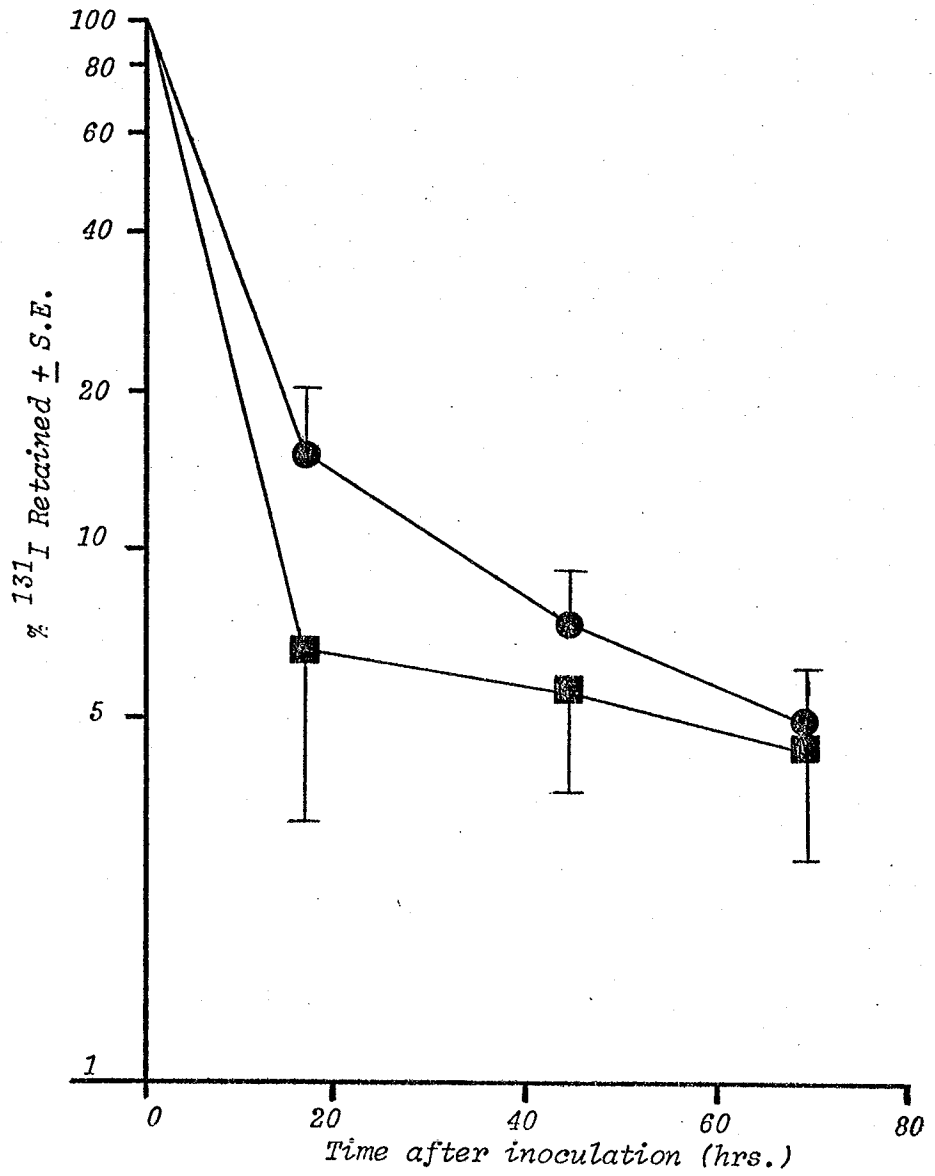

Figure 2. The elimination of dead SL2-5 cells from the SC or the IP site. Aliquots of $1x10^6$ ^{131}IUdR labeled, heat killed INVITRO cells were injected into groups of 5 DBA/2 mice (5 wks. old) at the SC (●) or the IP (■) site. The results represent the data from a single experiment.

were cleared very rapidly from both sites, however, the cells appeared to be eliminated slightly faster from the peritoneum. These differences, though more marked on day one of the assay, were not significant. However, early site dependent differences in the clearance of dead tumor cells would likely affect the percentage of ^{131}I retained for live tumor inocula during the latter period of the assay. These results suggested that the ability of the IP site to reject live tumor cells faster than the SC site may have been at least partially due to an enhanced clearance of dead tumor cells from the peritoneum. Furthermore, it appeared that the ability of the host to distinguish between live *in vitro* and *in vivo* grown cells was slightly greater at the IP site (Figure 1).

In addition, the ability of syngeneic mice to reject different doses of labeled INVITRO cells from both sites was examined in an attempt to determine if the elimination of labeled tumor cells from the IP site was a more sensitive assay for measuring tumor cell susceptibility to NR (Figure 3). The inoculum of 1×10^6 INVITRO cells was eliminated from the peritoneum significantly faster than that of the 1×10^7 cell IP dose on all three days of the assay, while the 5×10^6 IP tumor inoculum was eliminated significantly different from that of the 1×10^6 and 1×10^7 cells injected at the IP site on days 1 and 3 and days 2 and 3, respectively. In contrast to these observations, there were no significant differences in the elimination rates of the INVITRO cells injected at the SC site. It appeared that the IP site was more effective in distinguishing between different doses of INVITRO

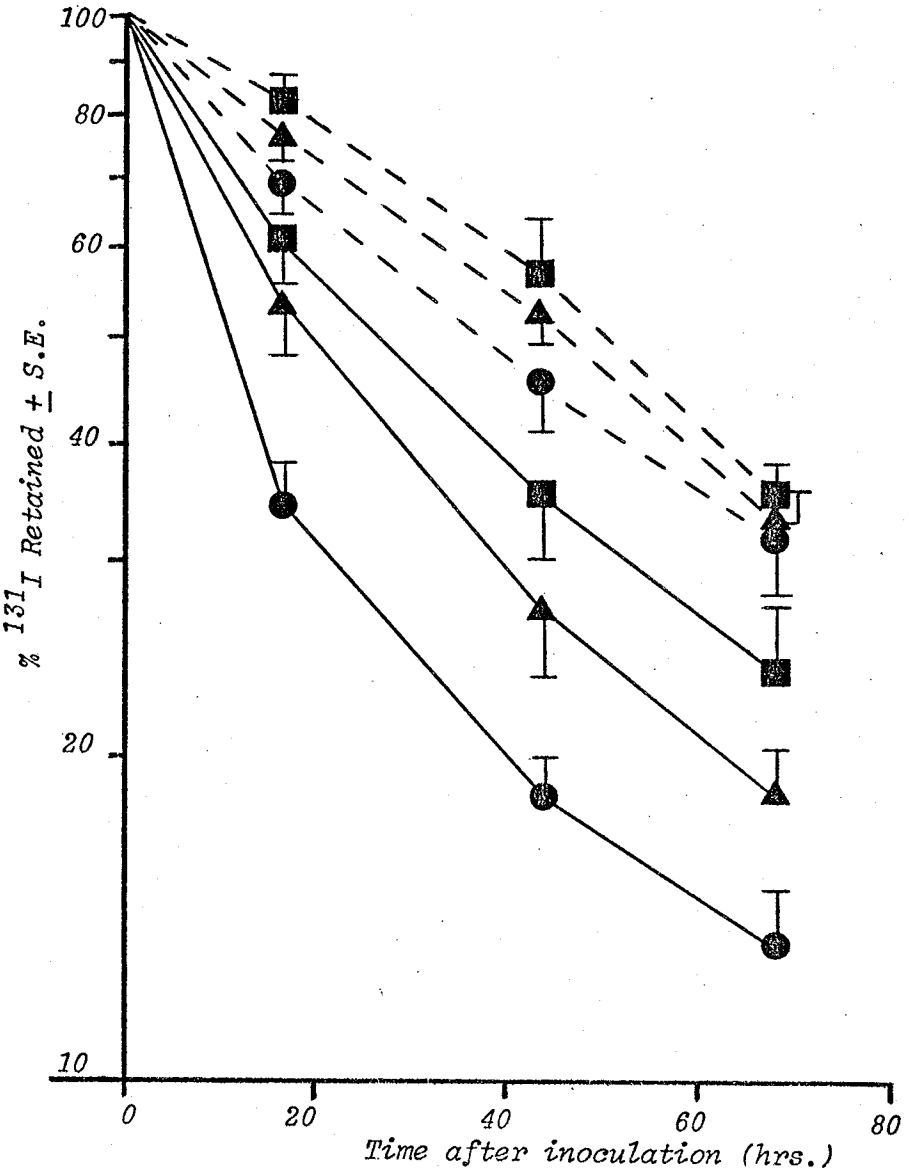

Figure 3. A tumor elimination dose response plot for cells injected at the SC or the IP site. Aliquots of $1x10^7$ (■), $5x10^6$ (▲) or $1x10^6$ (●) ^{131}IUdR labeled INVITRO cells were injected into groups of 5 DBA/2 mice at the SC (- -) or the IP (——) site. The results represent the data from a single experiment. The mean percentages of ^{131}I retained for the $1x10^6$ IP cell inoculum were significantly different ($P<0.03$) from that of the $1x10^7$ IP tumor dose on all 3 days of the assay. In addition the mean percentages of ^{131}I retained for the $5x10^6$ IP cell inoculum were significantly different ($P<0.05$) from that of the $1x10^6$ IP tumor dose on days 1 and 3 and from that of the $1x10^7$ IP cell inoculum on days 2 and 3. The various doses of INVITRO cells injected at the SC site were not eliminated significantly differently from one another.

cells than the SC site. These results suggested that for the doses tested, tumor elimination from the peritoneum may have been a more sensitive assay in which to examine the NR susceptibility of different SL2-5 tumor populations. Thus, the IP site was chosen to conduct all subsequent ^{131}IUdR tumor elimination experiments.

In order to confirm the initial observation that the effect of growing the SL2-5 tumor at the SC site was to produce a population of cells with a decreased susceptibility to NR, a second independent in vivo passage of the INVITRO tumor was performed. In order to examine this possibility, aliquots of 1 x 10^4 INVITRO cells were injected into DBA/2 mice at the SC site and passaged for six weeks (6WKSC cells). The susceptibility of the 6WKSC cells to syngeneic NR was assessed using the tumor elimination assay (Figure 4). Inocula of 5 x 10^6 radiolabeled 6WKSC, 3WKSC or INVITRO cells were injected at the IP site into separate groups of syngeneic mice. The INVITRO cells were eliminated significantly faster than both the 3WKSC and the 6WKSC cells, while the 6WKSC cells were rejected significantly slower than the 3WKSC population.

A final demonstration of the effect of in vivo growth upon tumor susceptibility to NR was attempted by repassaging an aliquot of 1 x 10^4 6WKSC cells at the SC site for an additional five weeks (5WK+6WKSC cells). The resultant tumors were more necrotic and appeared to kill the mice faster than the tumors produced by the SC injection of a comparable number of INVITRO cells. The relative sensitivity of these cells to host mediated anti-tumor mecha-

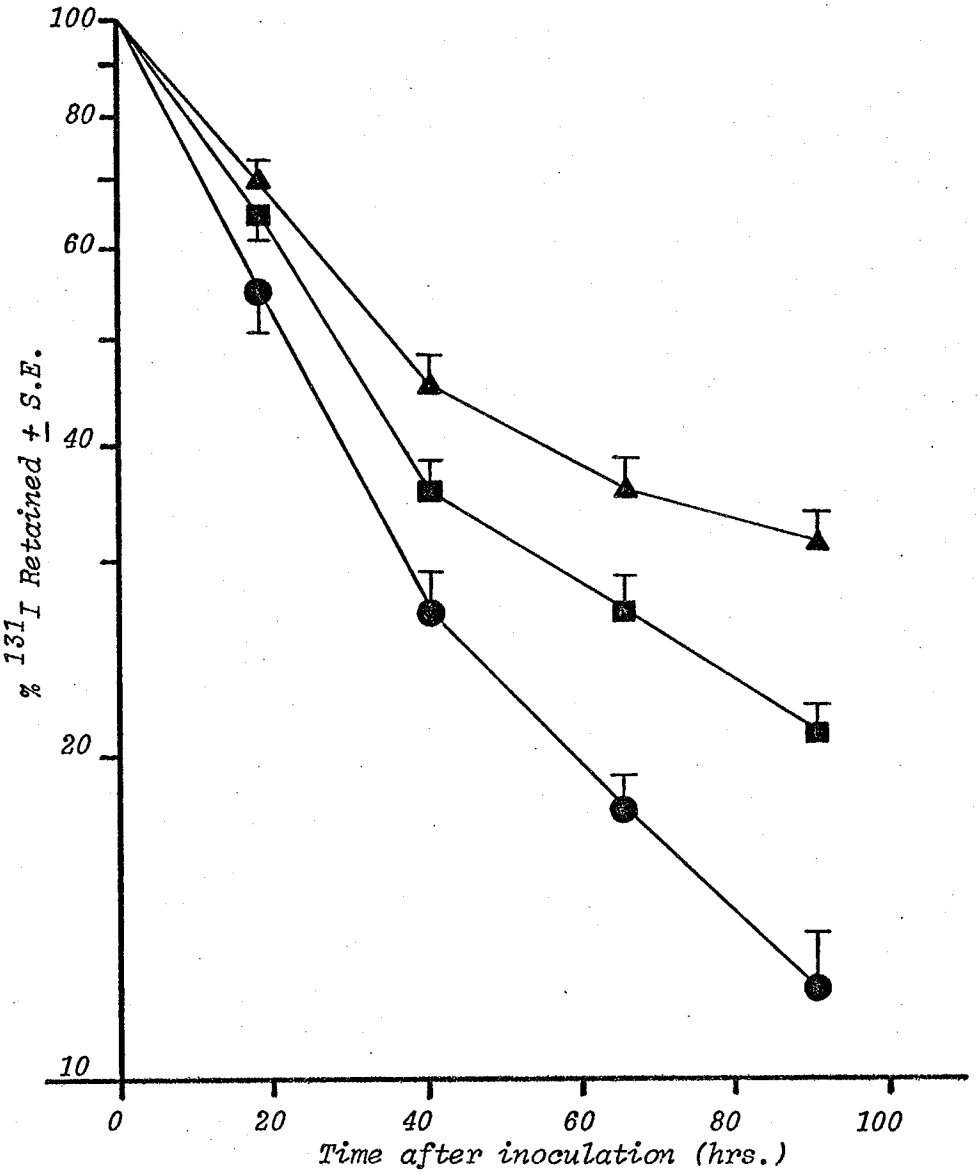

Figure 4. The susceptibility to NR of tumor cells grown at the SC site for a single passage. Aliquots of $5x10^6$ ^{131}IUdR labeled INVITRO (●), 3WKSC (■) or 6WKSC (▲) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from two experiments. The mean percentages of ^{131}I retained on the last 3 days of the assay for the 3WKSC and the 6WKSC cells were significantly different from that of the INVITRO cells ($P<0.01$) and from each other ($P<0.01$).

nisms was examined by injecting DBA/2 mice with 5 x 10^6 radiolabeled 5WK+6WKSC, 6WKSC or INVITRO cells (Figure 5). The 5WK+6WKSC cels were eliminated significantly slower then the 6WKSC cells which were eliminated significantly slower than the INVITRO cells. The differences in sensitivity to NR were repeatedly observed during a six week period of tissue culture maintenance and were not related to detectable differences in tissue culture growth rates.

In addition the sensitivity to NR of the INVITRO cells was assayed periodically during six weeks of tissue culture maintenance in order to determine the stability of this population. The elimination of a sample of radiolabeled INVITRO cells grown in tissue culture for ten days, did not differ by more than 8 percent on days one and two of the assay and by 4 percent on the remaining days as compared with that for the cells cultured in vitro for longer periods of time up to 42 days (Figure 6). These differences were not significant and within the experiment to experiment variation of the assay. This observation suggested that the NR-sensitive phenotype was stable during tissue culture maintenance for at least six weeks. In summary, the results suggested that the observed decrease in tumor susceptibility to host mediated anti-tumor NR was a consequence of the growth of SL2-5 cells at the SC site.

(2) The Detection of New Tumor Variants

An assessment of the heterogeneity of the SL2-5 tumor was performed in an attempt to determine if the alteration in

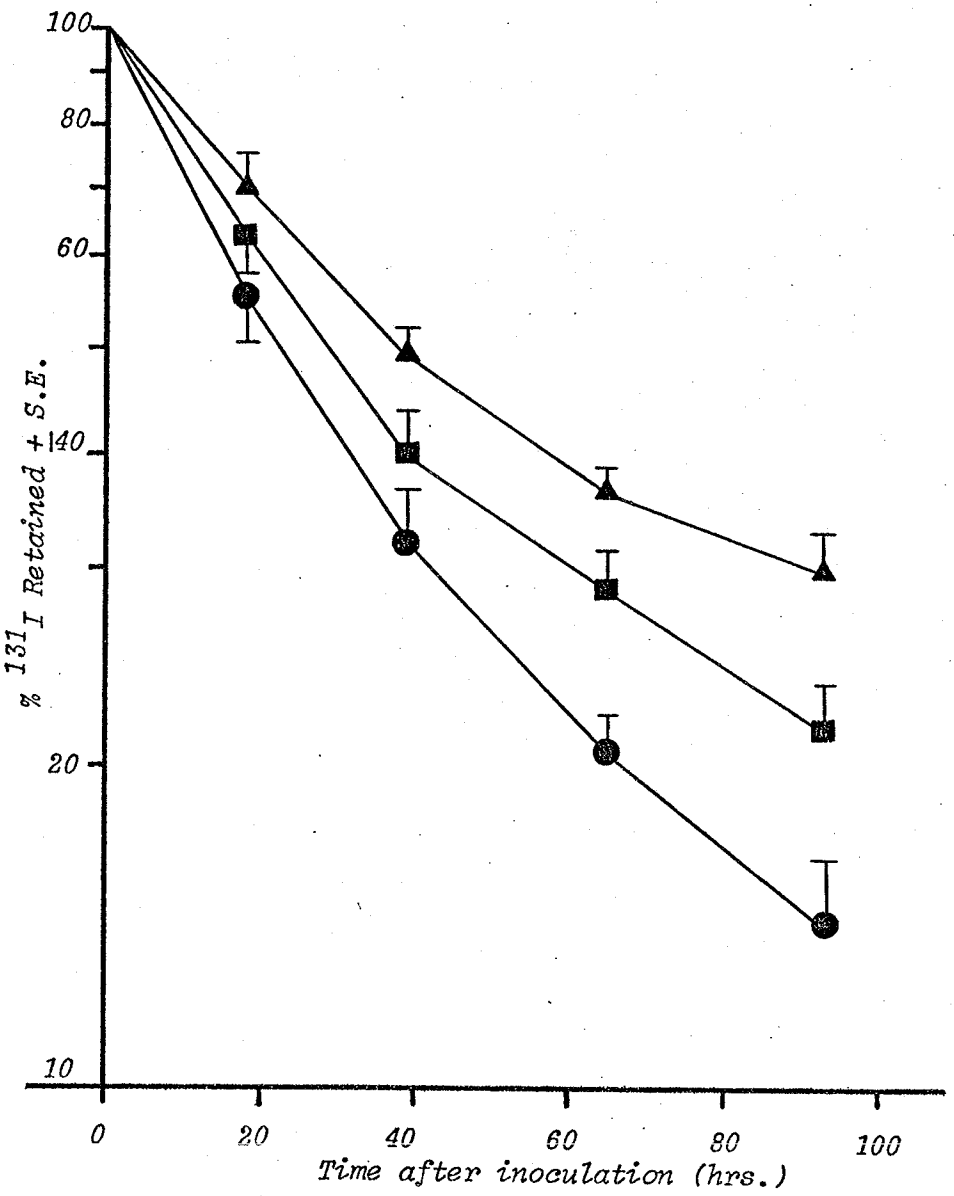

Figure 5. The susceptibility to NR of tumor cells grown at the SC site for one or two passages. Aliquots of $5x10^6$ ^{131}IUdR labeled INVITRO (●), 6WKSC (■) or 5WK+6WKSC (▲) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from two experiments. The mean percentages of ^{131}I retained on days 2, 3 and 4 of the assay for the 6WKSC and the 5WK+6WKSC cells were significantly different from that of the INVITRO cells ($P<0.01$) and from each other ($P<0.01$).

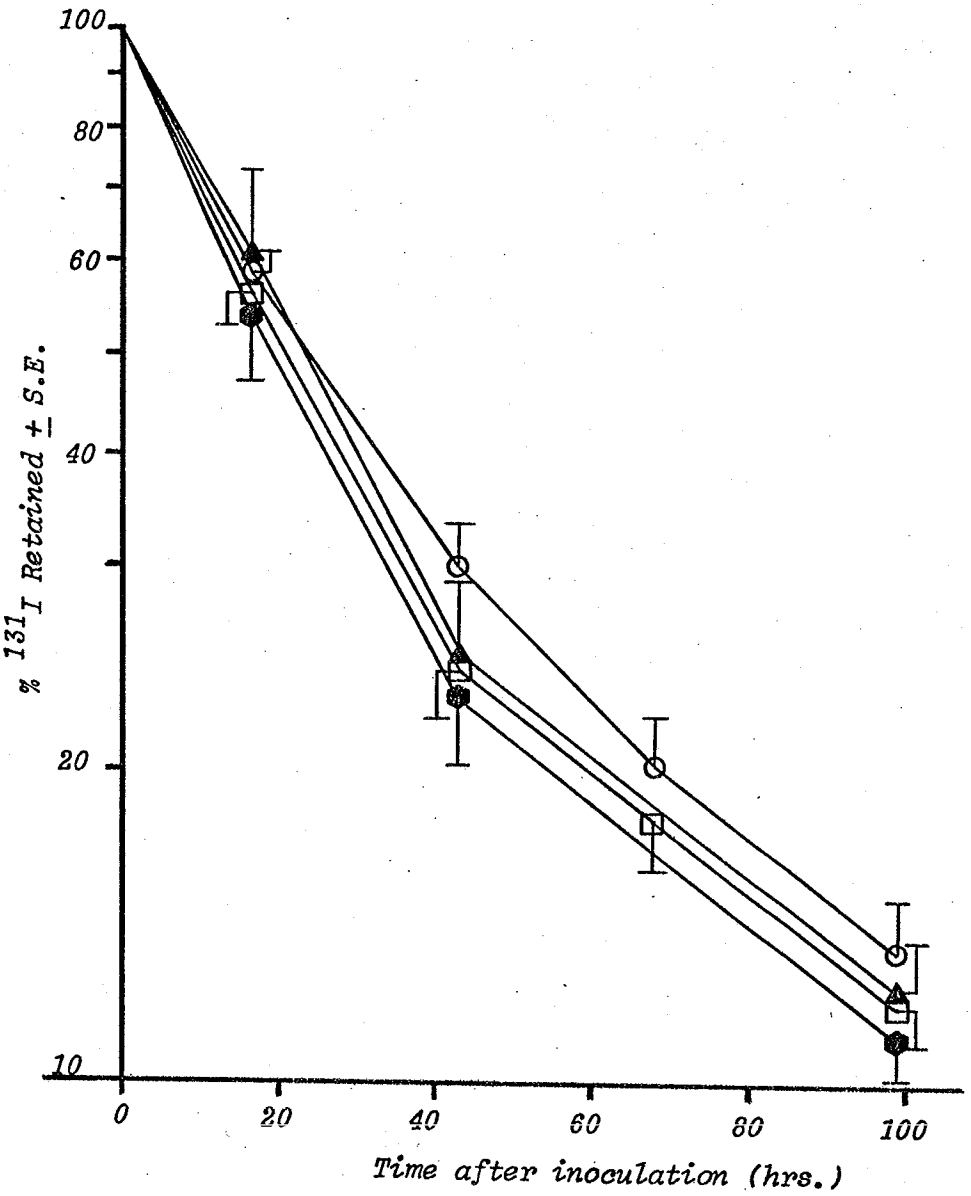

Figure 6. The sensitivity of the INVITRO cells to syngeneic NR assessed periodically during growth in tissue culture. Aliquots of $5x10^6$ ^{131}IUdR labeled INVITRO cells were injected into groups of 5 DBA/2 mice at the IP site, after 10 (□), 12 (○), 17 (●) or 42 (▲) days of tissue culture maintenance. For each sample of INVITRO cells the results represent the data from a single experiment carried out on different days with different lots of age matched mice. There were no significant differences in the mean percentages of ^{131}I retained for the various samples of INVITRO cells.

tumor susceptibility to NR following *in vivo* passage was associated with the presence of tumor variants.

(a) *Tumor sensitivity to natural antibody plus complement as an in vitro marker of tumor heterogeneity*

(i) *In vitro* subclones

The relative homogeneity of the cloned SL2-5 tumor population maintained exclusively in tissue culture was determined as a basis from which to compare the stability of the SL2-5 cells grown *in vivo*. An aliquot of INVITRO cells was started from frozen stock and grown in tissue culture for only three days before it was subcloned (INVITRO SHORT subclones). The relative heterogeneity of ten INVITRO SHORT subclones was examined using Lps stimulated syngeneic serum. The mean percentage cytolysis for the group of ten subclones and the standard deviation from this mean was determined. The standard deviation value has previously been used as a measure of the extent of phenotypic heterogeneity (Chow and Greenberg, 1980; Chow et al., 1983; Chow, 1984a).

The stability of the SL2-5 tumor cells grown only in tissue culture was determined by comparing the extent of phenotypic heterogeneity in sensitivity to syngeneic serum for the subclones from a sample of SL2-5 cells grown for three months in tissue culture (INVITRO LONG) with that of the INVITRO SHORT subclones

TABLE I.

The sensitivity to syngeneic serum of the INVITRO SHORT and the INVITRO LONG subclones.

SUBCLONE NUMBER	MEAN % SPECIFIC CYTOTOXICITY ± S.D.[a]	
	INVITRO SHORT	INVITRO LONG
1	69.2 ± 3.6	59.8 ± 1.5
2	71.8 ± 9.1	66.1 ± 5.2
3	71.4 ±12.5	62.8 ± 3.3
4	66.5 ± 4.8	68.2 ± 2.0
5	74.0 ± 1.4	61.6 ± 3.0
6	62.1 ± 8.8	57.7 ± 3.7
7	76.9 ± 2.5	59.5 ± 2.7
8	71.2 ± 4.6	70.8 ± 5.8
9	74.1 ± 4.2	64.9 ± 5.4
10	71.2 ± 5.4	64.4 ± 1.7
$\bar{x}$ ± S.D.[b]	70.8 ± 4.2	63.6 ± 4.1

a. Lps stimulated syngeneic serum, diluted to 1/2 with 10% FFBS, was used in these experiments.

b. The results represent the combined data from 3 experiments for each subclone set. The phenotypic heterogeneity for sensitivity to syngeneic serum of the INVITRO LONG subclones was not significantly different from that of the INVITRO SHORT subclones.

(Table I). It appeared that both subclone sets were relatively homogeneous. Furthermore, the phenotypic heterogeneity for sensitivity to stimulated syngeneic serum of the INVITRO LONG subclones was not significantly different from that of the INVITRO SHORT subclones as assessed with the "F" statistic. These data suggested that the sensitivity to syngeneic serum of the SL2-5 cells maintained for at least three months in tissue culture was a stable phenotype.

(ii) In vivo subclones

Based upon the hypothesis that the generation and selection of tumor variants may have been a basis for the progression of the SL2-5 lymphoma, the detected alterations in the NR-sensitive phenotype suggested that variants had previously been generated. Thus, the criteria for analyzing the heterogeneity of a particular in vivo grown tumor population was initially the ability to exhibit a reduction in sensitivity to syngeneic NR.

Based upon the successful demonstration of heterogeneity for the L5178Y-F9 model, the sensitivity of SL2-5 subclones to allogeneic CBA murine serum was used as an in vitro marker of tumor heterogeneity. Allogeneic serum was chosen for this analysis based upon the probability that the tumor populations grown in syngeneic mice would not have been selected by CBA NAb and therefore should express a more complete range of tumor variants. The sensitivity to CBA serum of the INVITRO SHORT subclones was determined (Table II) as a reference point from which to compare the extent of phenotypic heterogeneity with that of ten subclones derived from the

TABLE II.

The sensitivity to allogeneic CBA serum of the INVITRO SHORT subclones.

SUBCLONE NUMBER	MEAN % SPECIFIC CYTOTOXICITY ± S.D.[a]
1	41.2 ± 15.8
2	40.3 ± 15.5
3	53.6 ± 17.6
4	39.4 ± 14.7
5	38.5 ± 14.2
6	39.0 ± 13.8
7	40.3 ± 12.2
8	53.5 ± 12.9
9	31.6 ± 14.2
10	40.2 ± 15.5
$\bar{x}$ ± S.D.[b]	40.2 ± 6.8

a. Normal allogeneic CBA serum (whole) was used for these experiments.

b. These results represent the combined data from 3 experiments.

TABLE III.

The sensitivity to allogeneic CBA serum of subclones from a variety of in vivo passaged SL2-5 cells.

SUBCLONE NUMBER	MEAN % SPECIFIC CYTOTOXICITY ± S.D.[a]			
	3WKSC	5WK+6WKSC	6WKIP	5WK+6WKIP
1	52.1 ± 0.8	45.2	29.5 ± 7.5	29.0 ± 6.2
2	59.0 ± 2.3	61.4	21.6 ± 5.6	25.7 ±12.0
3	55.4 ± 4.9	56.5	30.5 ± 8.1	23.4 ±10.4
4	59.4 ± 4.2	56.1	13.2 ± 6.8	28.6 ± 7.7
5	64.0 ±14.8	58.1	37.2 ± 4.0	23.0 ± 7.2
6	61.3 ± 4.9	45.9	19.7 ± 1.8	31.7 ± 4.1
7	55.2 ± 4.4	49.8	23.6 ±10.5	23.1 ± 5.2
8	54.4 ± 6.4	61.2	29.8 ± 5.5	21.1 ± 7.4
9	53.5 ± 8.2	53.1	31.4 ±12.6	28.4 ±13.0
10	54.4 ±20.2	58.9	24.8 ± 7.7	21.5 ±10.7
$\bar{x}$ ± S.D. (b)	56.9 ± 3.8 (2)	54.6 ± 5.9 (1)	26.1 ± 6.9 (3)	25.6 ± 3.7 (3)

a. Normal allogeneic CBA serum (whole) was used for all assays.

b. The number of experiments from which the data was combined for each set of subclones is indicated in the brackets.

3WKSC population (Table III). After the analysis of the data from duplicate experiments, it appeared that the tumor heterogeneity of the 3WKSC subclones was certainly no greater than that of the INVITRO SHORT subclones.

However, it was possible that the conditions which were suitable to observe heterogeneity for the L5178Y-F9 tumor may have been inappropriate for the SL2-5 lymphoma. Thus, in order to determine if a longer in vivo growth period at the SC site was necessary to demonstrate heterogeneity, the 5WK+6WKSC cells were also cloned. The sensitivity to CBA serum of ten 5WK+6WKSC subclones was assessed (Table III). The extent of tumor heterogeneity for the 5WK+6WKSC cells was not markedly increased and thus the subclones were not further examined. These results suggested that either the in vivo grown cell populations were not more heterogeneous than cells maintained in vitro or that the experimental conditions to detect such changes were still not optimized.

Various observations including the fact that the IP site required a lower tumor inoculum to produce a viable neoplasm and the IP passaged cells did not exhibit a reduction in sensitivity to NR (Results Section B.2.b and c), suggested that the peritoneum may provide a more optimal environment for tumor survival. It was possible that in vivo growth under these conditions could have potentiated the survival of a wider range of variants that may otherwise have been eliminated during SC tumor passage. Aliquots of 1×10^4 INVITRO cells were injected at the IP site of syngeneic mice and grown for six weeks (6WKIP cells). The 6WKIP cells were cloned and ten subclones were assayed for their sensitivity to CBA

serum NAb (Table III). The extent of phenotypic heterogeneity of the 6WKIP subclones was not significantly different from that of the INVITRO SHORT subclones. However, it was still possible that the IP growth period was too short to allow for the generation of an appreciable level of heterogeneity. Thus, an aliquot of 1 x 10^4 6WKIP cells was repassaged at the IP site of syngeneic mice for an additional five weeks (5WK+6WKIP cells). This twice in vivo passaged cell population was cloned and ten subclones were assayed for their sensitivity to CBA serum. The extent of tumor heterogeneity for the 5WK+6WKIP subclones was not significantly greater than that of the INVITRO SHORT subclones.

In an attempt to approach this problem from a slightly different perspective, the sensitivity of the 5WK+6WKIP subclones to syngeneic and allogeneic NK cell cytolysis was also examined. It was determined that there was no significant reduction in the sensitivity of the uncloned 5WK+6WKIP cell to syngeneic NK cells (Results Section B.4.a). Thus, if alterations in tumor phenotype were due to the generation and selection of tumor variants and the ability to generate variants was comparable at both sites, then growth at the IP site could have allowed for the survival of more NK cell sensitive variants. The extent of phenotypic heterogeneity of the 5WK+6WKIP subclones was so small for sensitivity to both syngeneic and allogeneic NK cells (Table IV) that a comparison with INVITRO subclones was not considered useful.

Concurrent studies examining the titration profile of Lps stimulated syngeneic serum against the INVITRO and the 5WK+6WKSC uncloned cell populations (Results Section B.4.b) indicated that

TABLE IV.

The sensitivity to syngeneic and allogeneic NK cells of the 5WK+6WKSC subclones.

SUBCLONE NUMBER	MEAN % SPECIFIC CYTOTOXICITY ± S.D.[a]	
	CBA (normal)	DBA/2 (poly I: poly C)
1	44.9 ± 3.2	14.8 ± 4.2
2	51.0 ± 9.1	20.8 ± 0.8
3	47.4 ± 6.3	19.3 ± 0.7
4	41.3 ± 6.0	12.9 ± 4.3
5	40.3 ± 2.9	13.6 ± 0.6
6	45.2 ± 3.1	12.6 ± 2.9
7	48.4 ± 3.5	18.1 ± 3.3
8	50.3 ± 2.9	15.4 ± 2.2
9	44.2 ± 8.1	23.0 ± 2.6
10	41.7 ± 3.5	15.5 ± 1.9
$\bar{x}$ ± S.D.	45.5 ± 3.8	16.6 ± 3.5

a. The results represent the combined data from 3 experiments for both the CBA and DBA/2 splenic NK cells.

dilution of whole serum may accentuate differences in tumor sensitivity to syngeneic serum cytolysis. Thus, the sensitivity of the 5WK+6WKIP subclones to a one-half dilution of stimulated syngeneic serum was assessed. The extent of the 5WK+6WKIP subclone heterogeneity was significantly greater than that of the INVITRO LONG subclones assayed under the same experimental conditions (Table V). In fact, the sensitivity to syngeneic serum of the 5WK+6WKIP subclone 9 was significantly different ($P<0.05$) from that of subclones 5 and 7. A similar comparison of the 5WK+6WKIP phenotypic heterogeneity with that of the INVITRO SHORT subclones, for data previously presented, was also significant ($F = 6.68$, $P<0.005$). The observed heterogeneity was not due to random variation as repeated testing, using different pools of sera with the same complement, resulted in a reproducible ranking of the clones. However, the demonstrated increase in heterogeneity for the 5WK+6WKIP subclones as compared to the INVITRO subclones may have been due to differences in the sensitivity of the NAb assay at different mean percentage cytotoxicity values.

In order to examine this possibility, the sensitivity to syngeneic serum of the INVITRO LONG subclones was determined at a mean level of cytolysis comparable to that used to assay the 5WK+6WKIP subclones. A reduction in the net level of tumor kill for the INVITRO LONG cells was accomplished by diluting the stimulated serum to 1/8 rather than 1/2 (Table VI). It appeared that the extent of phenotypic heterogeneity for the INVITRO LONG subclones had significantly increased as compared with the sensitivities of the INVITRO LONG cells assessed at the lower serum dilu-

TABLE V.

The sensitivity to syngeneic serum of the INVITRO LONG and the 5WK+6WKIP subclones.

SUBCLONE NUMBER	MEAN % SPECIFIC CYTOTOXCITY ± S.D.[a]	
	INVITRO LONG	5WK+6WKIP
1	59.8 ± 1.5	37.0 ± 8.4
2	66.1 ± 5.2	46.8 ± 3.0
3	62.8 ± 3.3	37.3 ± 4.8
4	68.2 ± 2.0	43.2 ± 5.0
5	61.6 ± 3.0	28.1 ± 3.9
6	57.7 ± 3.7	29.0 ± 9.7
7	59.5 ± 2.7	28.2 ±11.3
8	70.8 ± 5.8	23.3 ± 6.8
9	64.9 ± 5.4	54.9 ± 7.4
10	64.4 ± 1.7	49.0 ±12.0
$\bar{x}$ ± S.D.[b]	63.6 ± 4.1	37.7 ±10.6

a. Lps stimulated syngeneic serum, diluted to 1/2 with 10% FFBS, was used in these experiments.

b. The results represent the combined data from 3 experiments for each subclone set. The standard deviation from the mean percentages of specific cytotoxicity for the 5WK+6WKIP subclones was significantly different (F=6.37, P=0.005) from that of the INVITRO LONG subclones.

TABLE VI.

The sensitivity to syngeneic serum of the INVITRO LONG subclones at a reduced mean level of cytotoxicity.

SUBCLONE NUMBER	MEAN % SPECIFIC CYTOTOXICITY ± S.D.[a]
1	27.8 ± 11.9
2	49.2 ± 6.4
3	34.8 ± 10.3
4	44.5 ± 12.5
5	24.1 ± 9.3
6	28.7 ± 8.4
7	49.6 ± 13.2
8	44.5 ± 14.9
9	40.8 ± 11.1
10	19.7 ± 3.6
$\bar{x}$ ± S.D.[b]	37.7 ± 10.6

a. Lps stimulated syngeneic serum, diluted to 1/8 with 10% FFBS, was used for these experiments.

b. The results represent the combined data from 3 experiments.

tion. Consequently, the level of heterogeneity for the 5WK+6WKIP and the INVITRO LONG subclone sets, assayed at similar mean percentage cytotoxicity values, was no longer significantly different.

However, Chow (unpublished observations) has subsequently determined that the dilution of stimulated syngeneic serum with another syngeneic serum of lower activity rather than 10% FFBS, substantially reduced the extent of tumor heterogeneity observed for the SL2-5 INVITRO LONG subclones. Though this evidence was only preliminary, it suggested that the analysis of the INVITRO LONG subclones with a serum diluted to 1/8 with 10% FFBS did not provide an accurate assessment of tumor heterogeneity for sensitivity to syngeneic serum. As a result of these observations, it appeared that under conditions of similar serum concentration and mean percentage of cytolysis, the 5WK+6WKIP subclones did exhibit a greater degree of heterogeneity than the INVITRO LONG subclones.

(b) Tumor Susceptibility to NR as an In Vivo Marker of Tumor Heterogeneity

Based upon the previous observations, it appeared that the sensitivities of the 5WK+6WKIP subclones to syngeneic serum plus complement were more heterogenous as compared with that of the INVITRO subclones. In order to confirm these results with a more biologically relevant parameter for determining tumor heterogeneity, the susceptibility to syngeneic NR of the 5WK+6WKIP and the INVITRO SHORT subclones was determined.

Initially, aliquots of 1 x 10^7 radiolabeled 5WK+6WKIP or INVITRO SHORT subclones were injected into the IP site of syngeneic mice. The standard deviation from the mean percentage of ^{131}I retained on each day of the assay has been considered to reflect the relative extent of clonal tumor heterogeneity for susceptibility to NR (Chow et al., 1983). After a single experiment with both sets of subclones, the phenotypic variation in sensitivity to NR for the 5WK+6WKIP cells was not significantly different from that of the INVITRO SHORT subclones. However, the overall clearance of tumor from the syngeneic mice was slightly faster for the 5WK+6WKIP subclone set (data not shown). This difference was particularly evident on the first day of the assay. The fact that the 5WK+6WKIP cells were initially eliminated more rapidly than the INVITRO SHORT cells may have reduced the sensitivity of the assay to detect a difference in tumor heterogeneity during the latter portion of the experiment.

In order to equalize the overall rate of tumor clearance for the two subclone sets, an attempt was made to retard the clearance of the 5WK+6WKIP cells by increasing the tumor inoculum to 3 x 10^7 radiolabeled cells. This modification slightly decreased the mean elimination rate of the 5WK+6WKIP subclones, especially on day one of the assay, and thus made possible a more reasonable comparison of the relative levels of heterogeneity for the _in vitro_ and the _in vivo_ grown cells. The determinations of subclone sensitivity to host mediated natural anti-tumor mechanisms was repeated for the INVITRO SHORT and the 5WK+6WKIP cells at tumor inocula of 1 x 10^7 and 3 x 10^7 cells, respectively. The combined data from two

replicate experiments indicated that the 5WK+6WKIP subclones were significantly more heterogeneous for susceptibility to NR than the INVITRO SHORT subclones on days three and four of the assay (Table VII). In fact, the sensitivity to NR of the 5WK+6WKIP subclones 2 and 9 were significantly different ($P<0.05$) from that for subclones 6, 8 and 10 on the last three days of the assay.

Throughout the course of this study, it was noted that an increased cell inoculum generally enhanced differences in the elimination rate of tumors with varied sensitivities to NR. Thus, in order to determine if the observed increase in heterogeneity for the 5WK+6WKIP subclones was only a consequence of the use of different doses of cells for the two subclone sets, the susceptibility of the INVITRO SHORT subclones to NR was also examined at a 3×10^7 ^{131}IUdR tumor inoculum. The larger cell dose appeared to reduce the mean elimination rates of the subclones on day one, however, the clearance of tumor during the remainder of the assay was actually slightly more rapid than that at the lower inoculum. The experiment was repeated and similar results were obtained (data not shown). These observations were in contrast to all previous SL2-5 tumor elimination dose response data, where increasing the number of injected tumor cells resulted in a reduced rate of clearance.

Based upon this evidence, it appeared that the rejection of the INVITRO SHORT subclones at the 3×10^7 dose was mediated by a mechanism unlike that previously encountered for the SL2-5 cells. This phenomenon may have involved a tumor induced augmentation of NR similar to the transient increases in splenic NK cell cytolysis

TABLE VII

The sensitivity to syngeneic NR of the INVITRO SHORT and the 5WK+6WKIP subclones.

DAY OF TUMOR ELIMINATION	MEAN % ^{131}I RETAINED ± S.D.[a]	
	INVITRO SHORT	5WK+6WKIP
1	51.0 ± 5.2	61.2 ± 5.6
2	24.9 ± 3.8	28.3 ± 5.9
3	15.0 ± 2.5	18.2 ± 4.7
4	9.9 ± 1.9	13.8 ± 4.3

a. *Aliquots of either $1x10^7$ INVITRO SHORT or $3x10^7$ 5WK+6WKIP ^{131}IUdR labeled subclones were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from two experiments for each subclone set. The standard deviations from the mean percentages of ^{131}I retained for the two populations were significantly different from each other on day 3 (F=3.53, P=0.037) and day 4 (F=5.12, P=0.012) of the assay.*

observed, following the IP or the SC inoculation of some tumors (Herberman et al., 1977; Karre et al., 1980). Due to the apparent difference in the basis for the elimination of the INVITRO SHORT and the 5WK+6WKIP subclones at the 3×10^7 tumor inoculum, any comparison of these experiments, in order to assess levels of tumor heterogeneity, was considered inappropriate. Despite this fact, it appeared that the difference between the standard deviation from the mean percentage of ^{131}I retained, for the 5WK+6WKIP and the INVITRO SHORT subclones, both assessed at the 3×10^7 cell dose, was nearly significant by day four of the assay ($F = 2.74$, $P = 0.075$) with the 5WK+6WKIP cells exhibiting a larger standard deviation. The results represented the combined data from two experiments. Collectively, the evidence indicated that the extent of tumor heterogeneity for susceptibility to syngeneic NR and NAb was greater for the 5WK+6WKIP tumor compared with that of the SL2-5 cells maintained exclusively in tissue culture. These observations suggested that the cells within the twice IP passaged tumor population were more heterogeneous for sensitivity to host mediated anti-tumor NR mechanisms and, therefore, supported the hypothesies that tumor variants were generated during growth in vivo.

Finally, a comparison between clonal sensitivity to syngeneic NR and to stimulated DBA/2 serum was performed in order to determine if there was a relationship between these two parameters of cytolysis (Figure 7). Linear regression analysis of the phenotypic heterogeneity for susceptibility to NR and to stimulated serum for both the INVITRO SHORT and the 5WK+6WKIP subclones

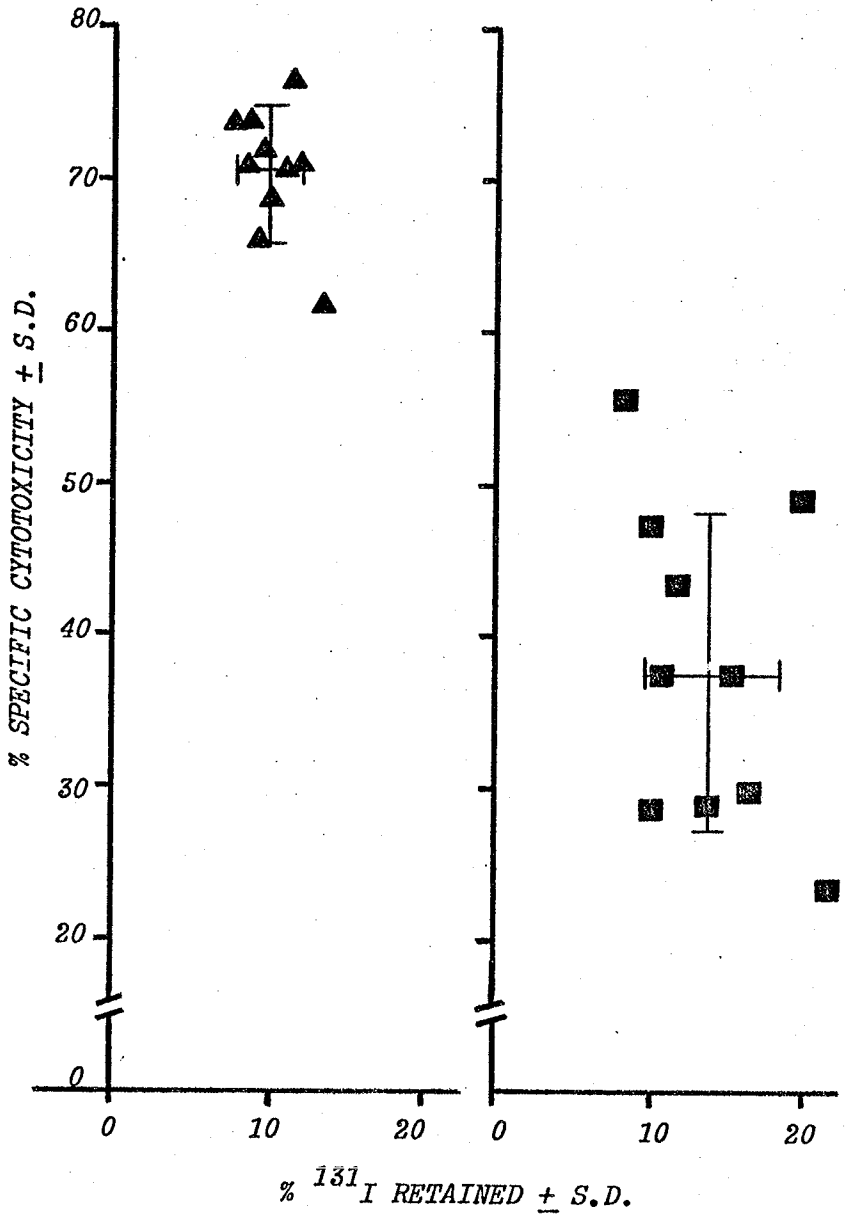

Figure 7. A comparison of clonal sensitivity to syngeneic serum NAb and NR. Each point represents the mean susceptibility of the INVITRO SHORT (▲) or the 5WK+6WKIP (■) subclones to Lps stimulated serum (3 experiments) and to NR (2 experiments). The serum used was diluted to 1/2 with 10% FFBS. Aliquots of either $1x10^7$ INVITRO SHORT or $3x10^7$ 5WK+6WKIP subclones were injected into groups of 5 DBA/2 mice at the IP site. The mean percentages of ^{131}I retained on day 4 of the assay are illustrated. No correlation was observed for either population of subclones.

failed to demonstrate a significant correlation. These observations were consistent with the independent progression of tumor phenotypes and provided supportive evidence for the presence of a variety of tumor variants with different characteristics in the 5WK+6WKIP population.

(B) The Further Characterization of the Progression of the SL2-5 Tumor

(1) Tumorigenicity - an In Vivo Measure of Malignancy

It was predicted that the in vivo grown tumors which exhibited a reduction in sensitivity to NR would have been in effect, selected for an increased ability to survive. Thus, the fate of threshold tumor inocula which produced tumor frequencies of less than one hundred percent were examined in order to assess the ability of a variety of SL2-5 derived populations to produce palpable tumors in opposition to host mediated natural defense mechanisms. Aliquots of 5×10^4 INVITRO, 3WKSC, 6WKSC or 5WK+6WKSC cells were injected subcutaneously into the lower middle back of syngeneic mice and the initial appearance of tumors was recorded (Figure 8). There were no tumor regressions detected during this experiment. It appeared that the tumor incidence of the 5WK+6WKSC cells was markedly and significantly greater than that of the INVITRO population. Though the ability of the 3WKSC and the 6WKSC cells to form tumors was greater than that of the INVITRO cells, these differences were not significant.

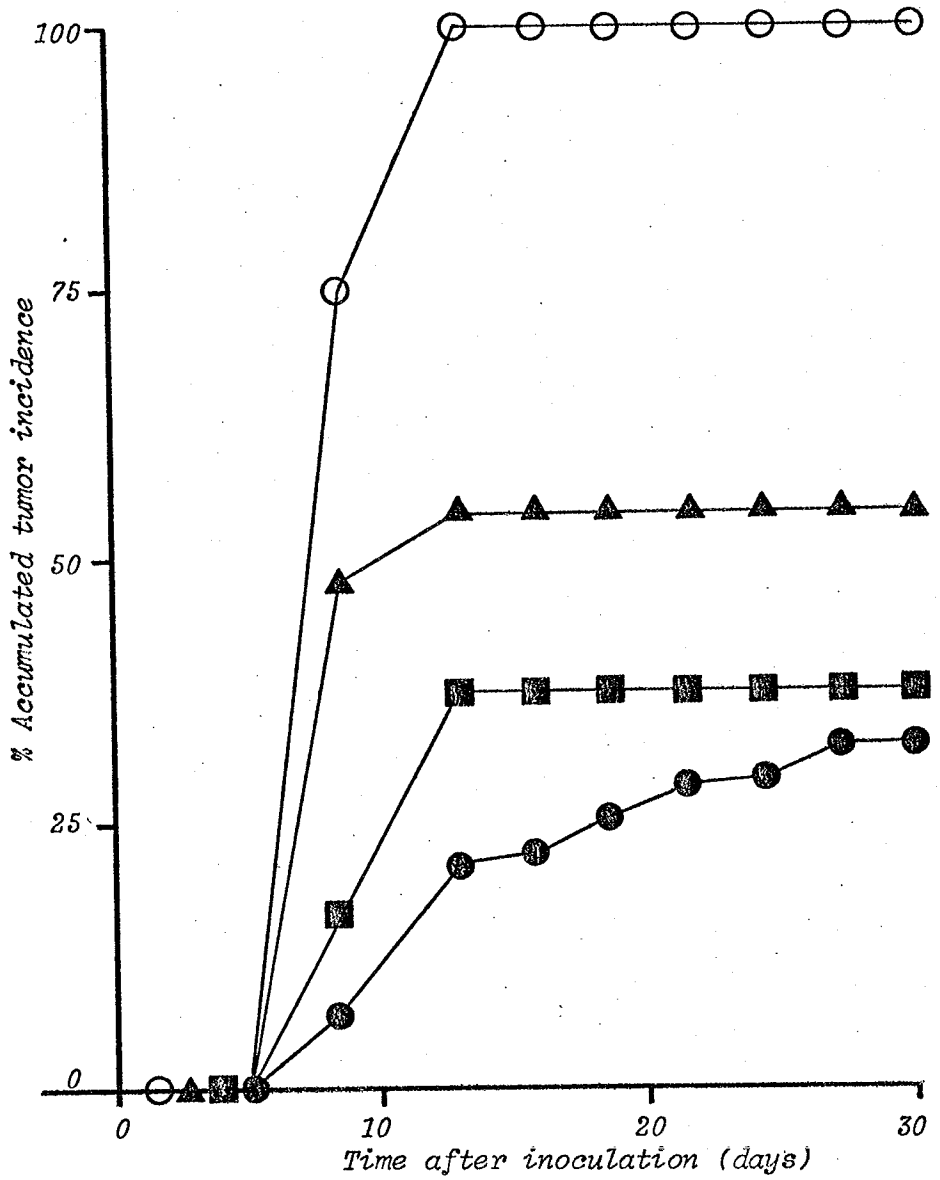

Figure 8. An assessment of the tumorigenicity of various SL2-5 tumor populations. Aliquots of $5x10^4$ INVITRO (●), 3WKSC (■), 6WKSC (▲) or 5WK+6WKSC (○) cells were injected at the SC site of groups of 41, 10, 7 or 8 DBA/2 mice, respectively. The results represent the data from a single experiment. Only the percentage accumulated tumor incidence of the 5WK+6WKSC cells was significantly different from that of the INVITRO cells ($P<0.001$).

In addition, the latent time between the injection of tumor cells and the appearance of a neoplasm was inversely related to the tumorigenicity of the population. For example, the 5WK+6WKSC cells, which exhibited the greatest tumor frequency, were associated with the shortest latent period. Furthermore, a comparison of the ability of the INVITRO, 3WKSC, 6WKSC and the 5WK+6WKSC populations to produce a tumor with their susceptibility to syngeneic NR (Table VIII) revealed a clear trend between increasing malignant potential and reductions in sensitivity to host mediated natural anti-tumor mechanisms. As previously discussed, the differences in the sensitivity to NR of these cells were significant on days two, three and four of the tumor elimination assay. These observations suggested that an increased tumorigenicity was also a consequence of tumor growth at the SC site and that tumor susceptibility to NR was inversely related to malignant potential.

(2) The Site Dependence of Tumor Progression

(a) Kinetics of the subcutaneous site

It was previously established that the effect of tumor growth at the SC site was to produce a tumor population with a decreased sensitivity to host mediated NR. The following experiment was performed in order to determine if there was a relationship between the length of time that the tumor cells were grown at the SC site and the extent to which they had undergone tumor progression towards an altered sensitivity to NR. This inoculum

TABLE VIII.

The tumorigenicity and the sensitivity to syngeneic NR of a variety of SL2-5 cells.

TUMOR CELLS	% ^{131}I RETAINED ± S.E.[a]	% ACCUMULATED TUMOR INCIDENCE[b]
INVITRO	12.4 ± 0.8	34.1
3WKSC	21.1 ± 1.0	40.0
6WKSC	30.7 ± 1.4	57.1
INVITRO	14.6 ± 1.2	34.1
6WKSC	22.3 ± 1.8	57.1
5WK+6WKSC	29.9 ± 1.8	100.0

a. The sensitivity to NR of the tumors included in the first block were assessed independently from those cells in the second block and thus the data were not grouped together. The percentage of ^{131}I retained on day four of the tumor elimination assay were summarized from data previously presented in figures 4 and 5, respectively.

b. These results were obtained from the experiment depicted in figure 8.

resulted in a tumor frequency of thirty-two percent (16/50). An aliquot of 5 x 10^4 INVITRO cells was injected at the SC site of DBA/2 mice and grown for 11, 25, 32 or 39 days (11DAYSC, 25DAYSC, 32DAYSC and 39DAYSC cells) before returning the cells to tissue culture. The elimination of 5 x 10^6 radiolabeled tumor cells from the IP site was assessed as a measure of the susceptibility of the INVITRO and the various *in vivo* grown tumor populations to syngeneic NR (Figure 9). The cells that were passaged at the SC site for thirty-nine days were eliminated significantly slower than the INVITRO cells. Linear regression analysis of the mean percentage of ^{131}I retained and the duration of tumor growth at the SC site revealed a significant direct correlation for all four days of the tumor elimination assay (Figure 10). It appeared that the longer the cells were passaged at the SC site, the more resistant they became to host natural anti-tumor defences. These results suggested that the effect of SC growth upon the alterations of tumor phenotype towards a decreased susceptibility to host mediated NR occurred in a time dependent manner.

(b) Kinetics of the Peritoneal Site

In order to determine whether the effect of tumor progression for an altered susceptibility to NR was dependent upon the site of *in vivo* growth, a range of tumor populations was produced by injecting 1 x 10^4 INVITRO cells at the IP site of syngeneic mice. This inoculum resulted in a tumor frequency of 67 percent (10/15). These cells were grown *in vivo* for two, four or

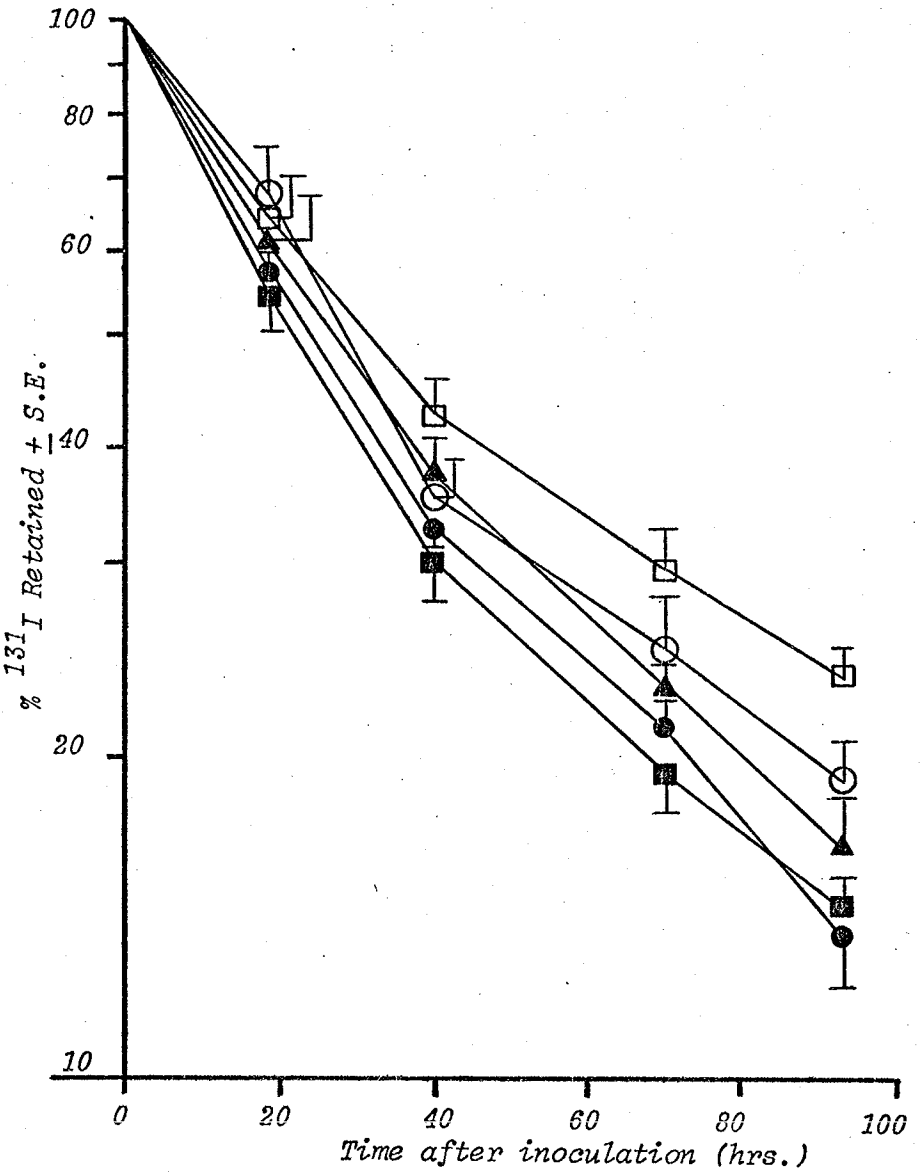

Figure 9. The effect of an increased SC growth period on tumor susceptibility to NR. Aliquots of $5x10^6$ ^{131}IUdR labeled INVITRO (●), 11DAYSC (■), 25DAYSC (▲), 32DAYSC (○) or 39DAYSC (□) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from 3 experiments. Only the mean percentages of ^{131}I retained for the 39DAYSC cells were significantly different from that of the INVITRO cells ($P<0.05$) on the last day of the assay.

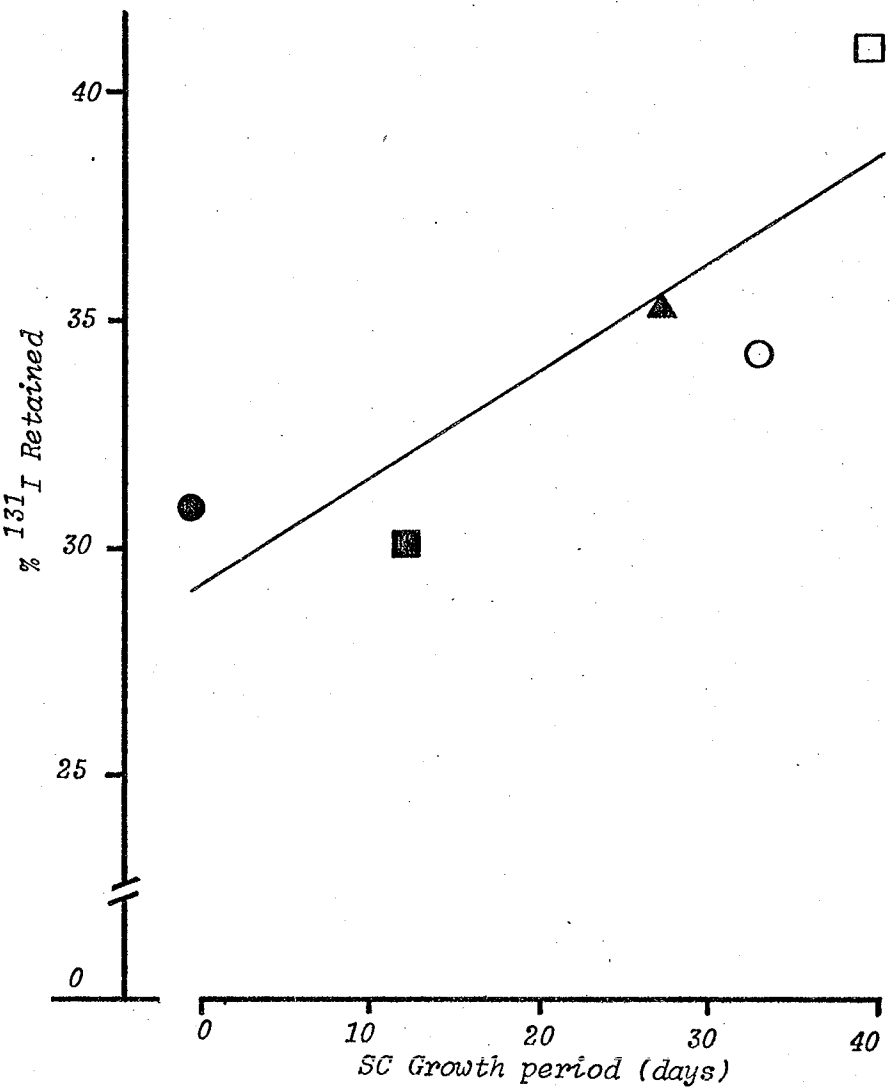

Figure 10. A correlation between tumor susceptibility to NR and the length of time that the cells were grown at the SC site. Linear regression analysis of ^{131}I retained for the INVITRO (●), 11DAYSC (■), 25DAYSC (▲), 32DAYSC (○) and 39DAYSC (□) cells and the number of days that the cells were passaged in vivo revealed a significant, direct correlation (LR=0.897, P=0.039). The percentage ^{131}I retained was the mean percentage of ^{131}I remaining in mice on day 2 of the assay, for 3 replicate experiments.

six weeks (2WKIP, 4WKIP and 6WKIP cells) before returning them to tissue culture. A series of three assays was performed in which aliquots of 6×10^6 radiolabeled tumor cells were injected into syngeneic mice for an assessment of their susceptibility to NR (Figure 11). When the data from the three experiments were pooled, there was no significant decrease in sensitivity to NR for any of the IP passaged cells compared with the INVITRO cells. This observation was in contrast to reductions in sensitivity to NR for SC grown SL2-5 cells. There was, however, some indication that the peritoneal tumor populations were actually eliminated slightly faster than the INVITRO cells. Linear regression analysis revealed a significant inverse correlation between the percentage of ^{131}I retained on day three of the tumor elimination assay and the lengths of the IP growth period (Figure 12). This information suggested that the longer the cells were passaged at the IP site, the more susceptible they became to host mediated NR.

In an attempt to determine if a longer in vivo growth period would accentuate the detectable increase in NR susceptibility, an aliquot of 1×10^4 INVITRO cells was injected into the IP site of DBA/2 mice and grown for eight weeks (8WKIP cells). The sensitivity of this tumor population was compared with that of the INVITRO cells in a tumor elimination assay (Figure 13). The 8WKIP cells were eliminated significantly faster than the INVITRO cells on all three days of the assay. This observation was consistent with the correlation between increased tumor susceptibility to NR and longer periods of growth at the IP site. This alteration in tumor phenotype was stable during at least six weeks of tissue culture

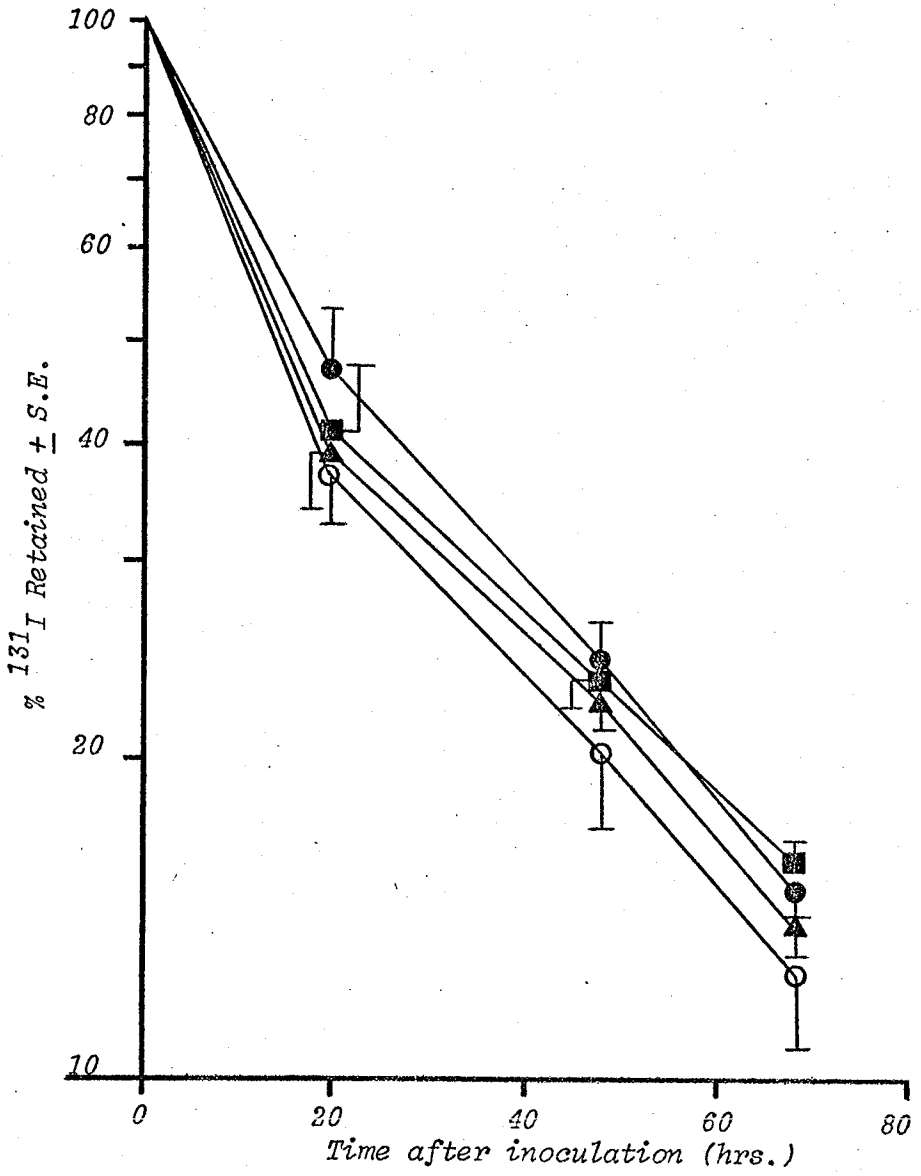

Figure 11. The susceptibility to NR of the cells grown for different lengths of time at the IP site. Aliquots of 6x10^6 ^{131}IUdR labeled INVITRO (●), 2WKIP (■), 4WKIP (▲) or 6WKIP (○) cells were injected at the IP site of groups of 5 DBA/2 mice. The results represent the combined data from 3 experiments. The mean percentages of ^{131}I retained for the IP passaged populations were not significantly different from that of the INVITRO cells.

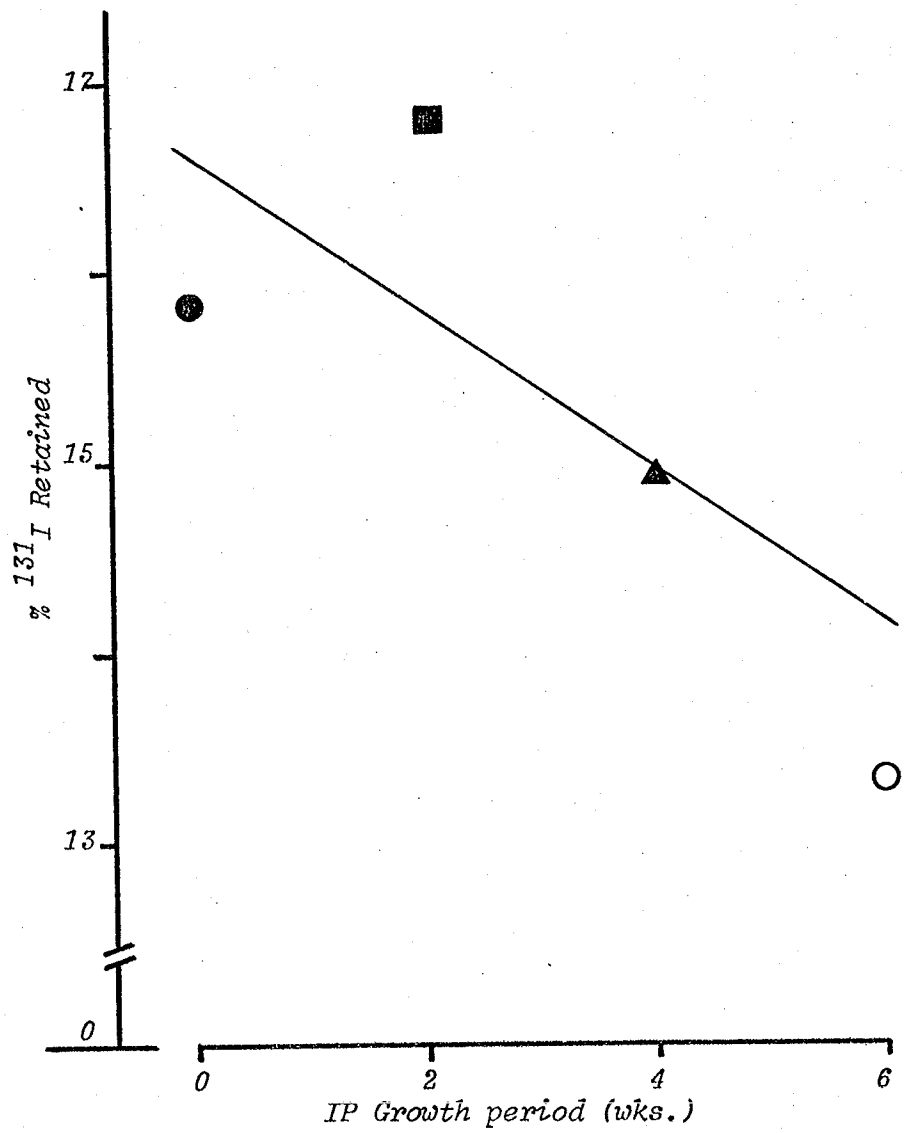

Figure 12. An inverse correlation between tumor susceptibility to NR and the length of time that the cells were grown at the IP site. Linear regression analysis of ^{131}I retained for the INVITRO (●), 2WKIP (■), 4WKIP (▲) and 6WKIP (○) cells and the number of weeks that the cells were passaged in vivo revealed a significant, inverse correlation (LR=-0.835, P=0.05). The percentage of ^{131}I retained was the mean percentage of ^{131}I remaining in mice on day 3 of the assay, for 3 replicate experiments.

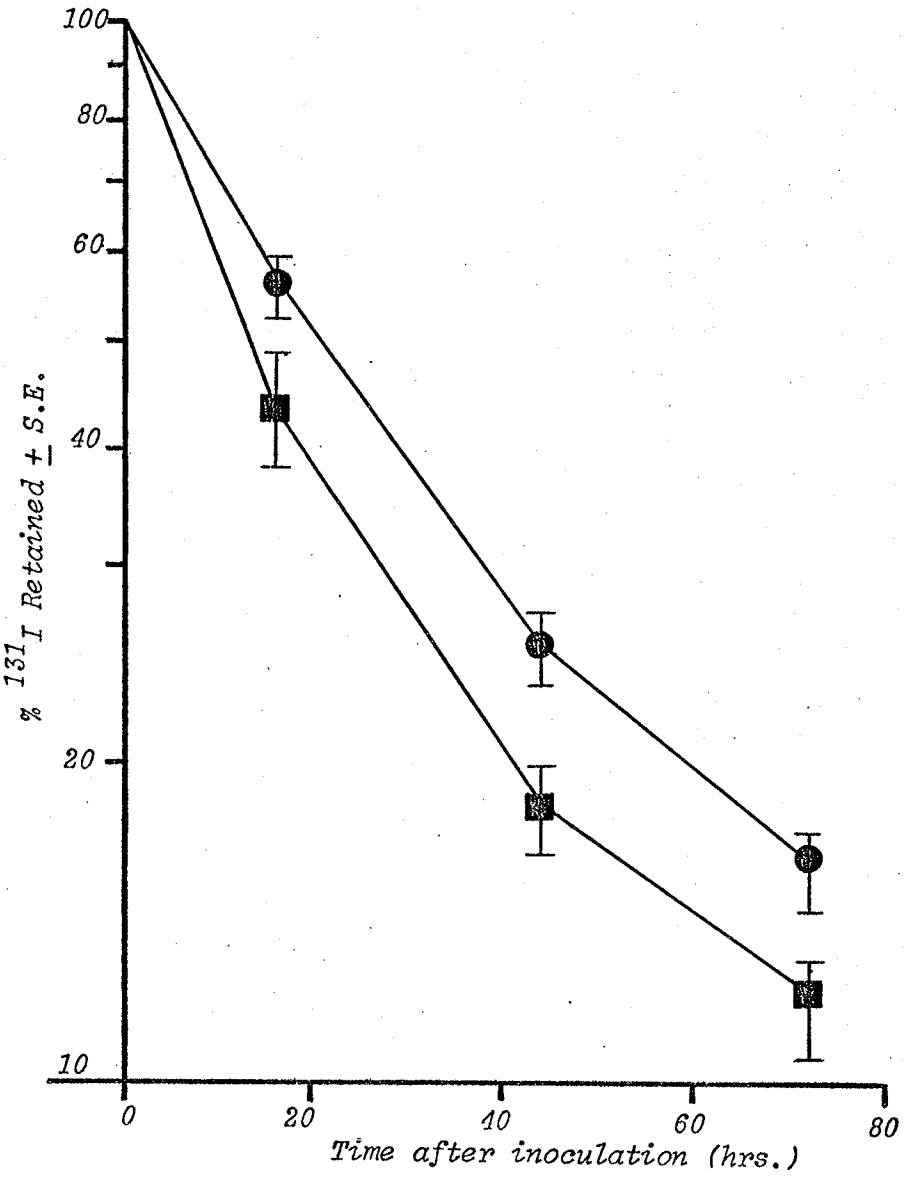

Figure 13. The susceptibility to NR of the INVITRO and the 8WKIP cells. Aliquots of $7.5x10^6$ ^{131}IUdR labeled INVITRO (●) or 8WKIP (■) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from two experiments. The mean percentages of ^{131}I retained for the 8WKIP cells were significant from that of the INVITRO cells on days 1 ($P=0.05$), 2 ($P=0.01$) and 3 ($P<0.02$) of the assay.

maintenance.

Since the repassage of the 6WKSC population at the SC site further reduced the tumor sensitivity to NR, the susceptibility of the 5WK+6WKIP cells was assessed in order to determine if a similar but opposite effect was obtained for the cells grown in the peritoneum for an extended period of time. The sensitivity of the 5WK+6WKIP cells to host mediated NR was compared with that of the INVITRO, 6WKIP and 5WK+6WKSC tumor cell populations (Figure 14). Upon pooling the data from two separate experiments, a significant difference was observed between the elimination rates of the INVITRO and the 5WK+6WKSC cells, included in this experiment as a positive control. The IP grown tumors did appear to be slightly more sensitive to NR than the INVITRO cells, but these differences were not significant. The elimination rates of the IP cells and the INVITRO population were quite rapid and this may have reduced the sensitivity of the assay to distinguish between tumor cell populations with slightly different susceptibilities to NR. So the tumor elimination assay was repeated at a tumor inoculum of 2 x 10^7 cells in order to retard the clearance of these tumors. The overall elimination rates of the cells tested were somewhat decreased by the larger tumor dose, but the elimination rate of the 5WK+6WKIP cells was not significantly different from that of the 6WKIP cells or the INVITRO cells (data not shown). In summary, the evidence suggested that an increase in tumor susceptibility to NR was a consequence of growth at the IP site. However, this alteration in tumor phenotype was not as marked as the reductions in sensitivity to NR, observed for cells grown at the SC

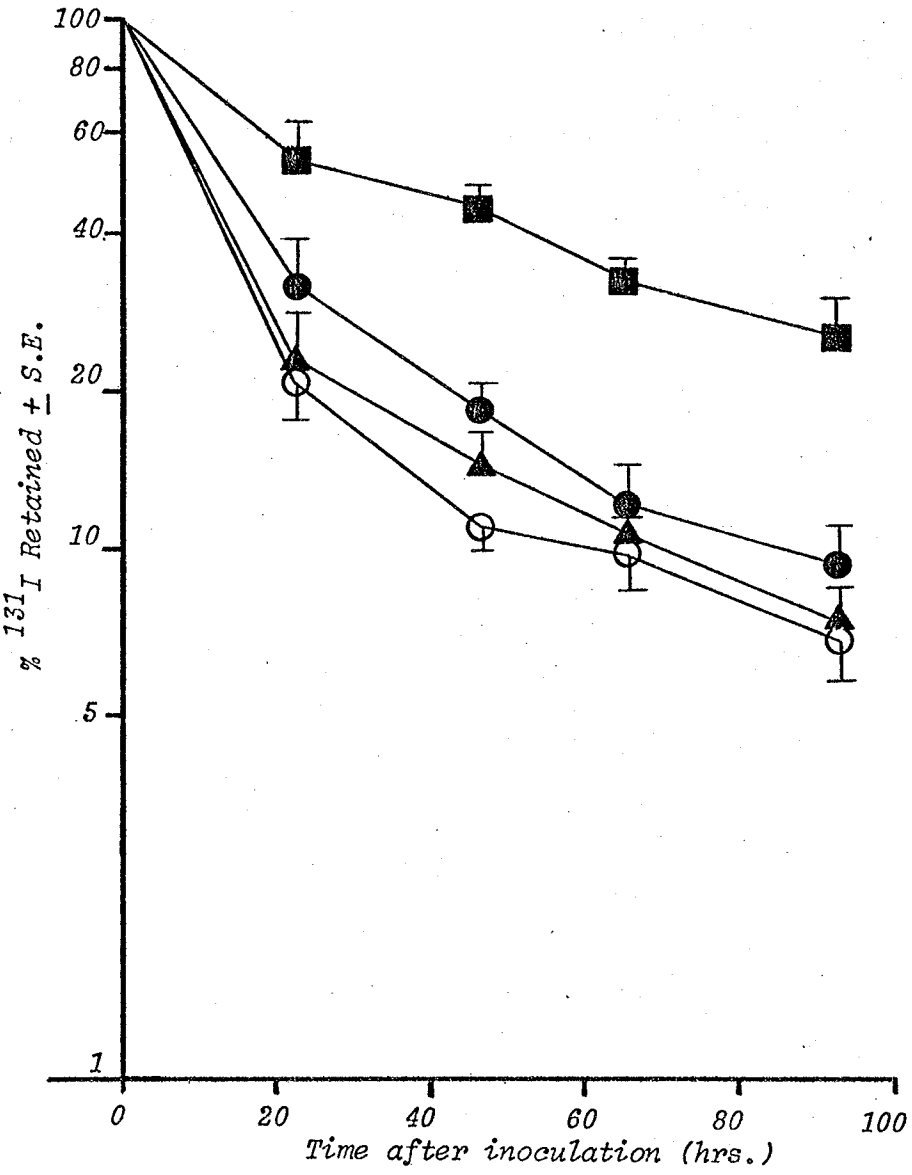

Figure 14. The susceptibility to NR of tumor cells grown at the IP site for one or two passages. Aliquots of 7.5x10^6 ^{131}IUdR labeled INVITRO (●), 6WKIP (○), 5WK+6WKIP (▲) or 5WK+6WKSC (■) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from two experiments. The mean percentages of ^{131}I retained on all of the days for the 5WK+6WKSC cells were significantly different from that of the INVITRO cells (P<0.001). However the IP passaged populations were not eliminated significantly different from the INVITRO cells.

site.

In addition, it has been suggested that host mediated NR would likely act early during tumor development, while the tumor mass was still small, in order to provide the host with an effective tumor defence mechanism (Greenberg and Greene, 1976; Wolosin and Greenberg, 1979; Chow et al., 1983). Thus, a reduction in the susceptibility to NR for tumors grown at the IP site may initially have occurred, but was subsequently masked by continued tumor proliferation. In order to pursue this possibility, 1 x 10^4 INVITRO cells were injected into the IP site of syngeneic mice and grown for twenty-four or forty-eight hours (24HRIP and 48HRIP cells). The susceptibility of these tumor populations to NR was assessed in a tumor elimination assay (Figure 15). The elimination rates of the cells passaged at the IP site for twenty-four and forty-eight hours were slightly, but not significantly, greater than that of the INVITRO cells. Though the reductions in susceptibility to NR of these cells were slight, the fact that the alterations in tumor phenotype were not directed towards an increasing sensitivity, suggested that host mediated anti-tumor mechanisms may exert a limited influence early during IP tumor growth.

(c) Tumorigenicity

Based upon the previous results, it appeared that there was a difference in the ability of the SC and the IP site to influence tumor phenotype with respect to susceptibility to NR. In another attempt to determine if the ability of the host

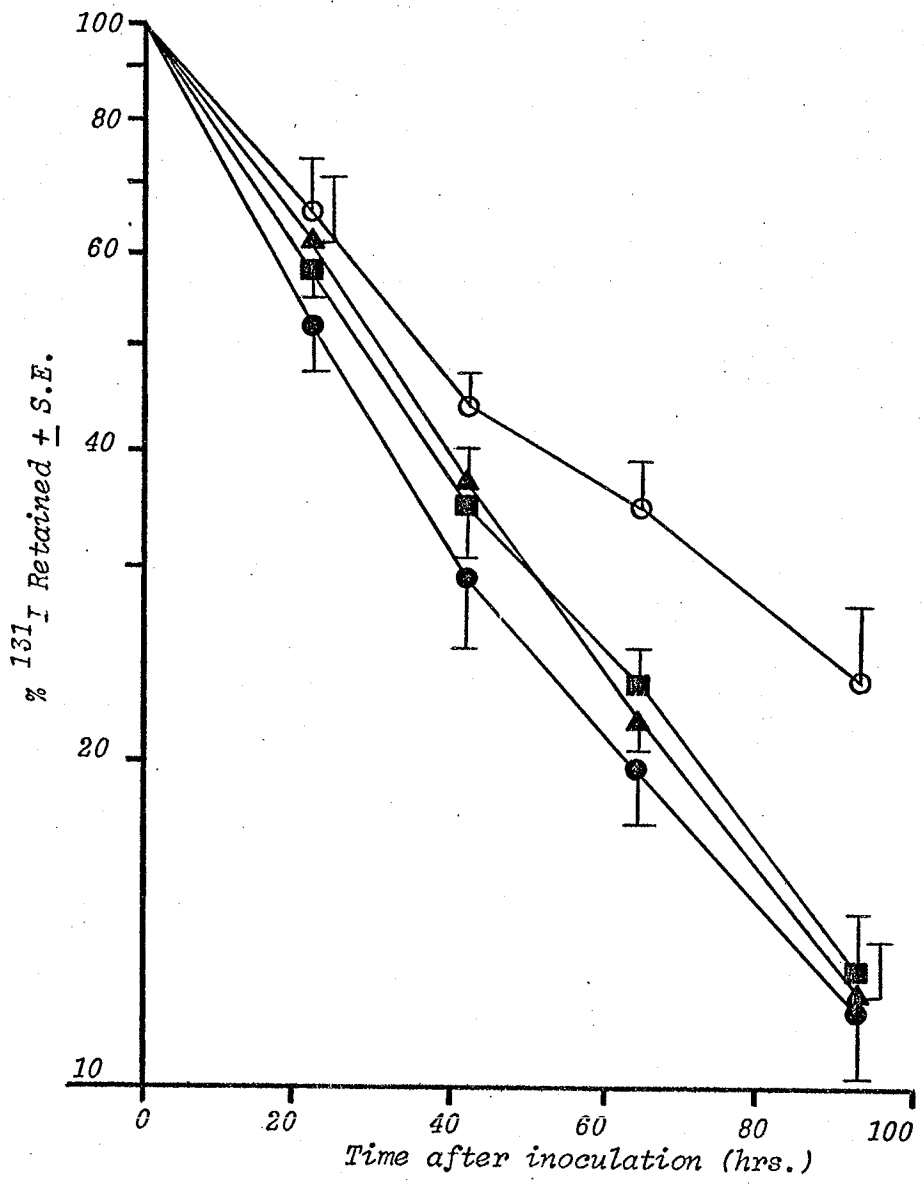

Figure 15. The susceptibility to NR of tumors grown at the IP site for 24 or 48 hours. Aliquots of $5x10^6$ ^{131}IUdR labeled INVITRO (●), 24HRIP (▲), 48HRIP (■) or 5WK+6WKSC (○) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the data from a single experiment. Only the mean percentages of ^{131}I retained for the 5WK+6WKSC cells on days 1 through 4 were significantly different from that of the INVITRO cells (P<0.01).

to affect tumor growth was site dependent, 5×10^4 INVITRO cells were injected into groups of syngeneic mice at the SC or the IP site. The accumulated tumor incidence for the cells injected at the SC site (18/52) was significantly less ($P = 0.0067$) than that of the IP inoculated cells (8/10). These results represented the data from a single experiment at the IP site and the combination of two experiments at the SC site, one of which was carried out in parallel with the peritoneal trial. These observations confirmed the previous results of Greenberg and Greene (1976) and suggested that the SC site was better able to reject a small tumor inoculum as compared with the IP site.

(3) The Thymus Independence of Tumor Progression

The ability of SL2-5 cells to survive growth in ATxBM mice was examined in order to test the hypothesis that the progression of the SL2-5 tumor towards a decreased susceptibility to host mediated NR was a thymus independent phenomenon. Aliquots of 5×10^4 INVITRO cells were injected into the SC site of a group of ATxBM or normal age and sex matched syngeneic mice. The net tumor frequencies for the normal and the ATxBM mice were comparable, though the tumors initially appeared at a slower rate in the ATxBM mice (Figure 16). After five weeks of growth at the SC site, a tumor was removed from the injection site of a normal (5WKSC-N) and an ATxBM (5WKSC-ATxBM) mouse. It was predicted that if the mechanism responsible for the reduction in sensitivity to NR for SL2-5 cells grown at the SC site of normal mice was thymus inde-

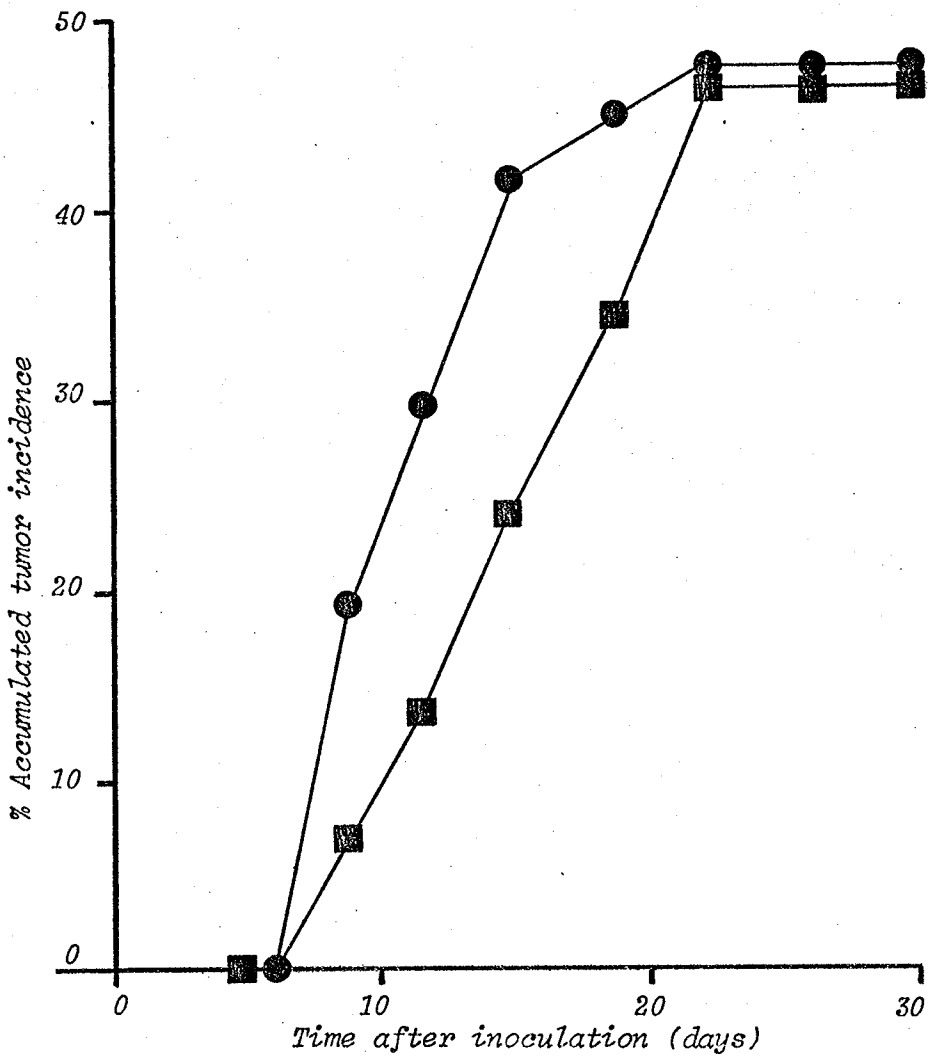

Figure 16. The percentage accumulated tumor incidence after the injection of INVITRO cells into normal or ATxBM mice. Aliquots of $5x10^4$ INVITRO cells were injected at the SC site of 33 syngeneic normal (●) or ATxBM (■) mice. The results represent the data from a single experiment. The percentage accumulated tumor incidence after 30 days of growth at the SC site in the ATxBM mice was not significantly different from that in the normal mice.

pendent, then tumors grown at the same site in ATxBM mice should exhibit a similar decrease in sensitivity to NR. The susceptibility to NR of the 5WKSC-N, 5WKSC-ATxBM and INVITRO cell populations were assessed in a tumor elimination assay (Figure 17). The elimination rates for the tumor cells passaged in vivo in normal or ATxBM mice could not be distinguished statistically, while both populations were rejected significantly slower than the INVITRO cells. These results suggested that the effect of in vivo growth towards a reduced susceptibility to NR was a thymus independent phenomenon.

(4) The Role of Natural Killer Cell and Natural Antibody Activity in Tumor Progression

Based upon the thymus independent SL2-5 reduction in sensitivity to NR, it was postulated that similar decreases in susceptibility to the putative effectors of NR would be observed if NK cells and NAb were involved in this process and contributed to the increased resistance to host mediated anti-tumor mechanisms. Evidence of a correlation was sought between the changes in susceptibility to NR exhibited following the in vivo growth of various tumor populations and alterations in sensitivity to NAb and NK cell activity.

(a) The Sensitivity of In Vivo Grown Tumor Cells to Natural Killer Cell Cytolysis

The sensitivity of a variety of in vivo grown

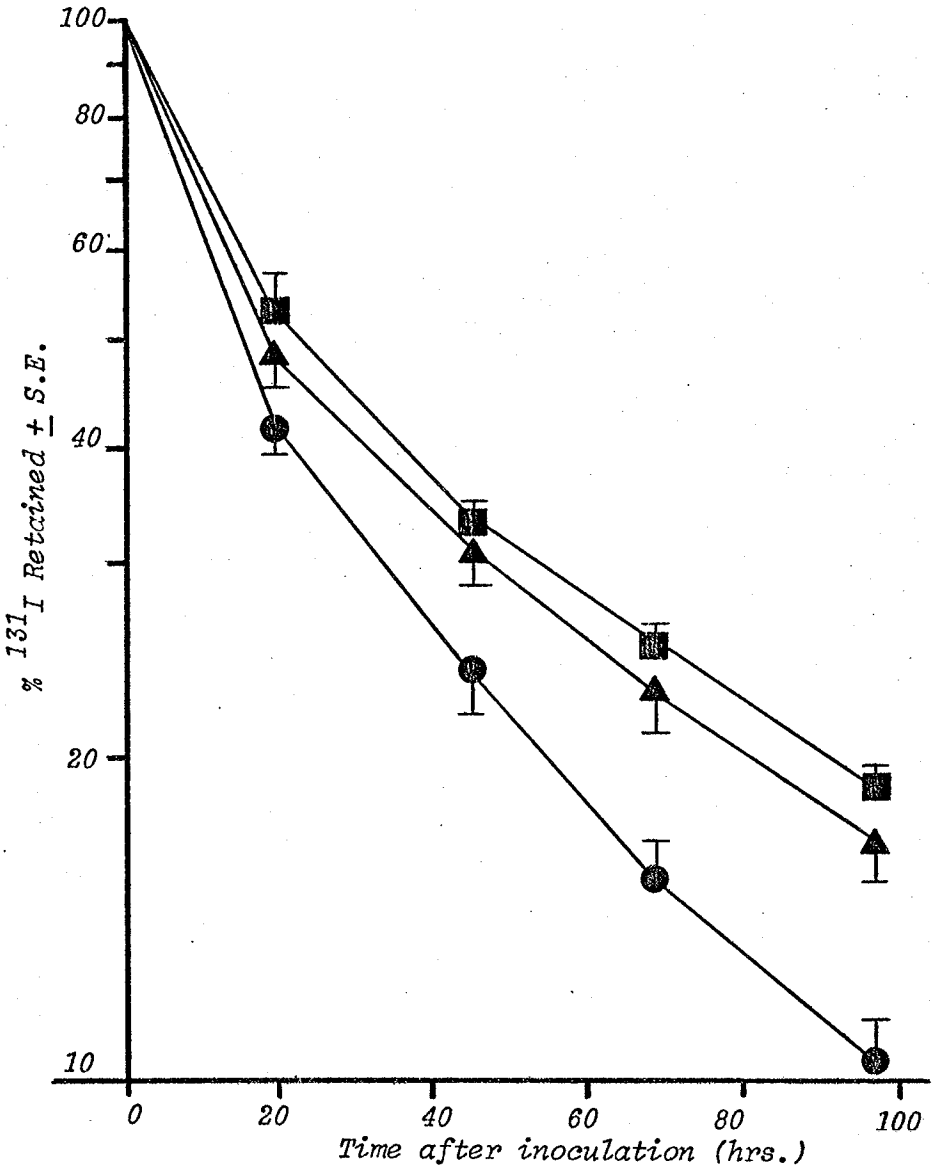

Figure 17. The susceptibility to NR of SL2-5 cells passaged in normal or ATxBM mice. Aliquots of 7x10^6 ^{131}IUdR labeled INVITRO (●), 5WKSC-ATxBM (▲) or 5WKSC-N (■) cells were injected into groups of 5 DBA/2 mice at the IP site. The results represent the combined data from three experiments. The mean percentages of ^{131}I retained for the 5WKSC-ATxBM and the 5WKSC-N cells were significantly different from that of the INVITRO cells (P<0.05 and 0.04, respectively), but not from each other on days 2, 3 and 4 of the assay.

SL2-5 populations to syngeneic splenic NK cells was determined using an *in vitro* ^{51}Cr release assay. It appeared that the 3WKSC and 5WK+6WKSC tumors were significantly less sensitive to stimulated DBA/2 NK cell cytolysis than the INVITRO cells (Table IX). In addition, the 5WK+6WKSC tumor was significantly more resistant to syngeneic NK cell cytolysis than the 3WKSC population. The detectable decreases in sensitivity to NK cells that were observed *in vitro* for these tumors corresponded to a marked reduction in their susceptibility to NR, as demonstrated previously. Linear regression analysis of the mean percentage ^{131}I retained for the INVITRO, 3WKSC and 5WK+6WKSC cells on day four of the tumor elimination assay and the percentage specific NK cell cytotoxicity revealed a significant inverse correlation (Figure 18). It appeared that a decrease in susceptibility to host mediated NR following growth at the SC site was associated with a concomitant decrease in sensitivity to NK cell lysis.

In contrast to these results, the sensitivity of the 5WK+6WKIP cells to stimulated syngeneic NK cell cytolysis was not significantly different and was essentially unaltered from that of the INVITRO cells. However, both of these populations were significantly more sensitive than the 5WK+6WKSC cells (Table X). Based on these observations, it appeared that the effect of *in vivo* growth upon tumor sensitivity to NK cell cytolysis was site dependent. In addition, it appeared that both the susceptibility of 5WK+6WKIP cells to syngeneic NR and to NK cell cytolysis were not significantly different and certainly not decreased from that of the INVITRO cells. This observation was consistent with the

TABLE IX.

The sensitivity to syngeneic NK cells for the SL2-5 cells passaged at the SC site.

TUMOR CELLS	% SPECIFIC CYTOTOXICITY ± S.E.[a]
INVITRO	33.7 ± 4.8
3WKSC	26.4 ± 2.5
5WK+6WKSC	20.5 ± 2.7

a. *The results represent the combined data from 8 NK assays using poly I:poly C stimulated, syngeneic spleen cells. The percentage specific cytotoxicities of the 5WK+6WKSC and the 3WKSC cells were significantly different from each other (P<0.001) and from that of the INVITRO cells (P<0.04).*

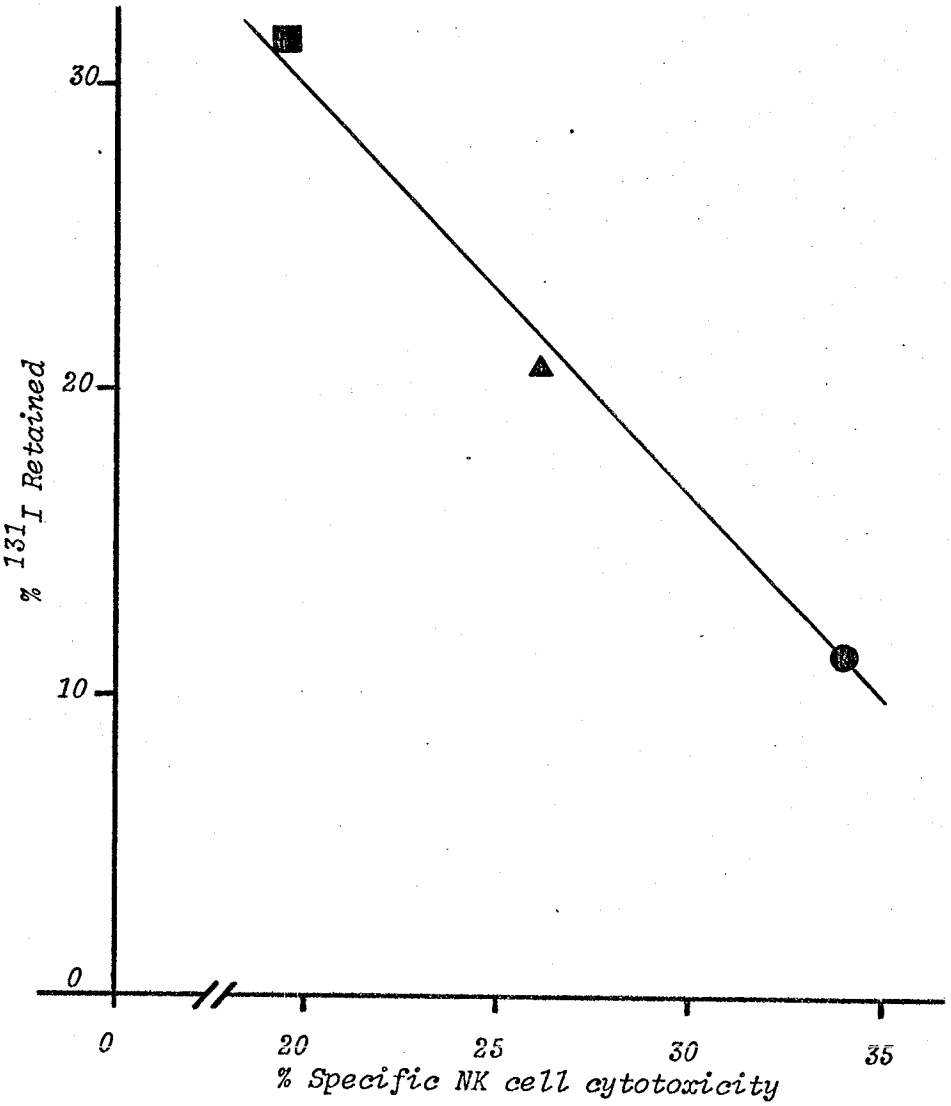

Figure 18. A correlation between tumor susceptibility to NR and sensitivity to NK cell cytolysis. Linear regression analysis of ^{131}I retained and the percentage specific NK cytotoxicity for the INVITRO (●), 3WKSC (▲) and 5WK+6WKSC (■) cells revealed a significant, inverse correlation (LR=-0.987, P<0.02). The percentage ^{131}I retained was the mean percentage of ^{131}I remaining in mice on day 4 of the tumor elimination assay, for three replicate experiments. The NK assays (8) were performed with poly I: poly C stimulated spleen cells.

TABLE X.

The sensitivity to syngeneic NK cells of the SL2-5 cells passaged at the SC or the IP site.

TUMOR CELLS	% SPECIFIC CYTOTOXICITY ± S.E.[a]
INVITRO	35.3 ± 3.0
5WK+6WKIP	34.7 ± 3.3
5WK+6WKSC	26.8 ± 3.7

a. *The results represent the combined data from 10 NK assays using poly I:poly C stimulated syngeneic spleen cells. Only the percentage specific cytotoxicity of the 5WK+6WKSC cells was significantly different from that of the INVITRO cells (P<0.002).*

significant correlation between tumor sensitivity to NR and NK cells for tumors grown at the SC site, which supported the hypothesis that NK cells were mediators of NR and contributed to alterations in tumor susceptibility to NR.

(b) The Sensitivity of In Vivo Grown Tumor Cells to Natural Antibody Cytolysis

In an attempt to determine whether the alterations in tumor susceptibility to syngeneic NR observed following periods of *in vivo* growth correlated with changes in the sensitivity of the SL2-5 populations to NAb activity, tumor cells passaged at the SC or the IP site were assessed for their susceptibility to syngeneic serum NAb cytotoxicity in the presence of rabbit complement. Initially, the sensitivity of the 3WKSC cell population to both syngeneic normal and Lps stimulated serum was compared with that of the INVITRO cells. It appeared that the susceptibility of the 3WKSC cells to both sera was significantly reduced from that of the INVITRO cell population (Table XI). Mosmann and Longnecker (1982) determined that the specificity of normal murine NAb against chicken red blood cells was very similar to that of a panel of Lps induced monoclonal antibodies derived from the fusion of spleen cells from unimmunized mice. These observations suggested that either normal or stimulated DBA/2 serum could be used to detect differences in tumor cell sensitivity to NAb cytolysis. The remainder of experiments were conducted with stimulated serum only.

TABLE XI.

The sensitivity to syngeneic normal or Lps stimulated serum of the INVITRO and the 3WKSC cells.

TUMOR CELLS	% SPECIFIC CYTOTOXICITY ± S.E.[a]	
	NORMAL	Lps STIMULATED
INVITRO	21.0 ± 1.8	41.3 ± 4.5
3WKSC	13.1 ± 1.4	35.2 ± 5.2

a. *Normal or Lps stimulated (diluted to 1/2 with 10% FFBS) syngeneic serum was used in these experiments. The results represent the combined data from 12 and 7 experiments, respectively. The percentage specific cytotoxicity of the 3WKSC cells was significantly different from that of the INVITRO cells assessed with normal ($P<0.01$) and stimulated ($P=0.05$) serum.*

A more extensive analysis of the alterations in tumor sensitivity to stimulated syngeneic serum was performed on various SL2-5 populations grown at the SC or the IP site. The purpose of these experiments was to determine if changes in sensitivity to syngeneic NR and NAb paralleled the site correlation between tumor susceptibility to host mediated natural anti-tumor mechanisms and the cytolytic action of NK cells. It appeared that the sensitivity to syngeneic serum of the 5WK+6WKSC, 6WKIP and the 5WK+6WKIP populations were all significantly reduced from that of the INVITRO cells (Table XII). In addition, the susceptibility of the 5WK+6WKIP cells was significantly less than that of the 6WKIP tumor. The increase in resistance to stimulated serum of IP passaged tumors was in sharp contrast to the lack of change in sensitivity to splenic NK cells for the 5WK+6WKIP cells.

In fact, the results indicated that repeated passage was associated with a greater reduction in sensitivity to syngeneic serum. Linear regression analysis of the sensitivity to this serum for the INVITRO, 6WKIP and the 5WK+6WKIP cells and the total length of time that the cells were passaged at the IP site revealed a significant inverse correlation (Figure 19). This result suggested that a reduction in sensitivity to syngeneic serum following tumor growth was dependent upon the number of passages <u>in vivo</u>. The sensitivity to serial dilutions of stimulated syngeneic serum of the 5WK+6WKSC and the INVITRO cells was examined in order to determine if the observed decrease in tumor sensitivity to NAb was dependent upon the concentration of serum (Figure 20). It appeared that the overall sensitivity of the

TABLE XII.

The sensitivity of SL2-5 cells grown at the SC or the IP site to syngeneic stimulated serum.

TUMOR CELLS	% SPECIFIC CYTOTOXICITY ± S.E.[a]
INVITRO	65.0 ± 2.4
6WKIP	38.6 ± 4.2
5WK+6WKIP	10.7 ± 1.8
5WK+6WKSC	38.6 ± 2.3

a. *Lps stimulated syngeneic serum, diluted to 1/8 with 10% FFBS, was used in this experiment. The results represent the combined data from 6 experiments. The percentage specific cytotoxicities of the 6WKIP, 5WK+6WKIP and 5WK+6WLSC cells were significantly different from that of the INVITRO cells (P<0.001). In addition, the percentage specific cytoxicities of the 6WKIP and the 5WK+6WKSC cells were significantly different from that of the 5WK+6WKIP cells (P<0.01).*

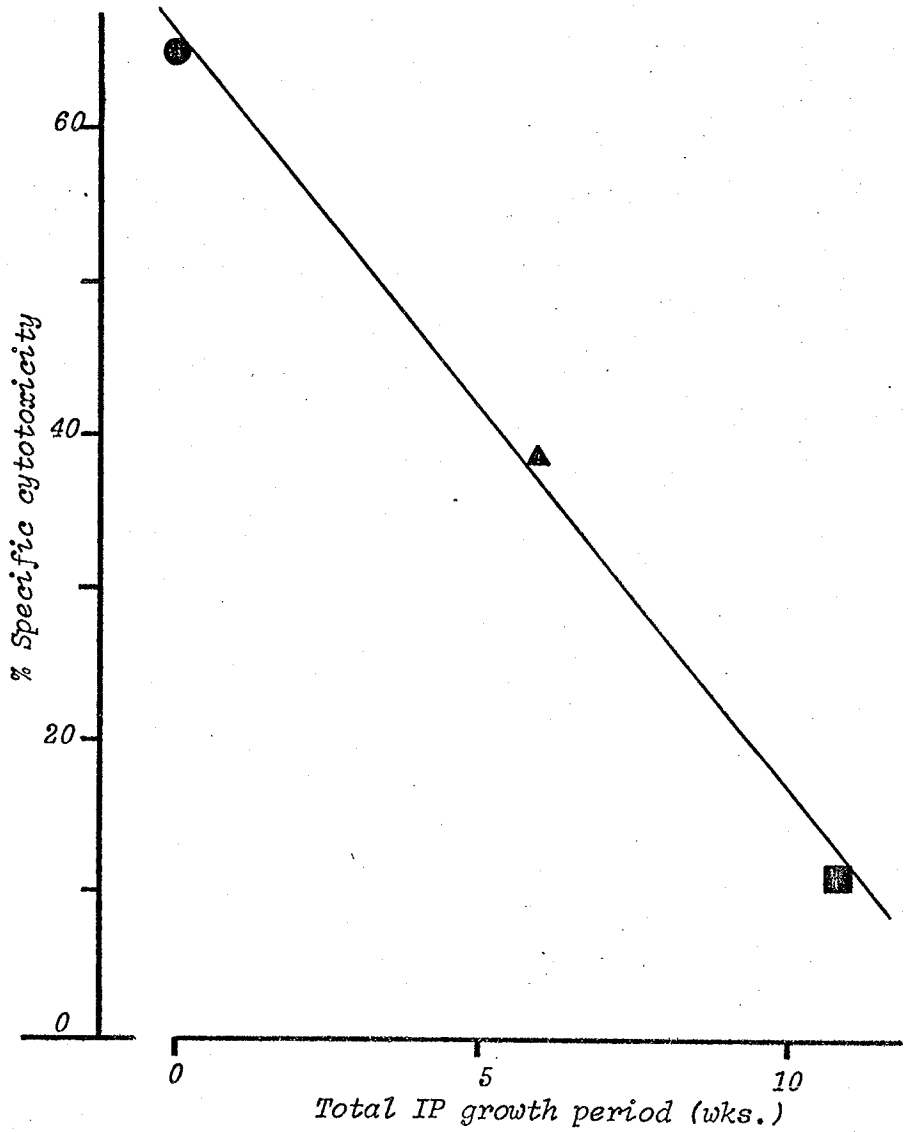

Figure 19. An inverse correlation between tumor sensitivity to syngeneic serum NAb and the total length of the IP growth period. Linear regression analysis of the percentage specific NAb cytotoxicity for the INVITRO (●), 6WKIP (▲) and 5WK+6WKIP (■) cells and the total length of time that the cells were passaged at the IP site revealed a significant, inverse correlation (LR=-0.998, P=0.04). The results represent the combined data from 6 NAb cytotoxicity experiments, using Lps stimulated syngeneic serum diluted to 1/8 with 10% FFBS.

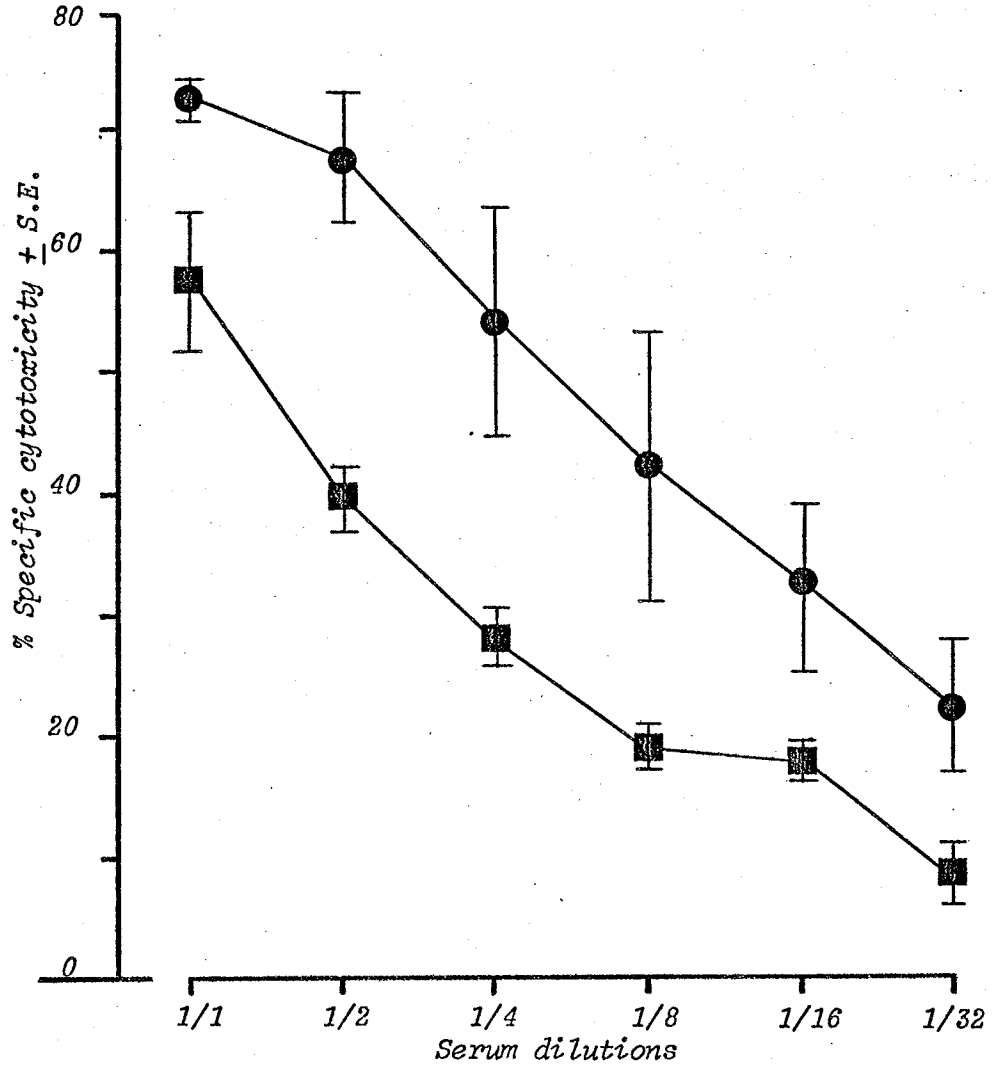

Figure 20. The sensitivity of the INVITRO and the 5WK+6WKSC cells to serial dilutions of stimulated syngeneic serum. The Lps stimulated serum was diluted with 10% FFBS. The results represent the combined data from two experiments. The percentage specific cytotoxicities of the 5WK+6WKSC (■) cells over the range of dilutions were significantly different from that of the INVITRO (●) cells ($P<0.004$).

5WK+6WKSC cells to the titration of serum activity was significantly less than that of the INVITRO cells and that these differences were more pronounced at the intermediate dilutions.

Collectively, the results suggested that in vivo passage reduced tumor sensitivity to syngeneic serum independent of the site of tumor growth. Though decreases in susceptibility to syngeneic NR correlated with reductions in sensitivity to NAb for the tumors grown at the SC site, there did not appear to be a similar relationship for the IP passaged cells.

(5) The Effect of Tumor Progression at the Molecular Level of the Tumor Cell

The purpose of this aspect of the study was to examine a variety of factors which might account for the observed reductions in tumor cell sensitivity to syngeneic NR, NK cells and NAb.

(a) The Expression of Tumor Target Structure Recognized by Natural Killer Cells and Natural Antibody

It was postulated that the decreased expression of tumor target structure recognized by NK cells and NAb contributed to the ability of the 5WK+6WKSC cells to exhibit an increased resistance to these effector mechanisms. The expression of tumor target structure recognized by syngeneic NK cells was indirectly assessed for the INVITRO and the 5WK+6WKSC populations by inhibit-

ing NK cytotoxicity with the addition of unlabeled tumor target cells. The inhibition of cytolysis with unlabeled tumor cells was thought to reflect tumor binding to NK cells and thus the expression of tumor target structure recognized by these effector lymphocytes (Becker et al., 1978; Roder et al., 1979; Brooks et al., 1981; Chow et al., 1981).

Accordingly, the abilities of unlabeled 5WK+6WKSC or INVITRO cells to inhibit the NK cytolysis of either labeled target cell was determined. The sensitivity of the 5WK+6WKSC cells to stimulated syngeneic NK cytolysis was significantly less than that of the INVITRO cells, as previously demonstrated. The ability of the unlabeled 5WK+6WKSC cells to inhibit NK cytolysis directed against the INVITRO or the 5WK+6WKSC ^{51}Cr labeled targets was assessed at ratios of unlabeled to labeled tumor targets of 10:1, 5:1 and 2.5:1. The number of unlabeled cells required to inhibit fifty percent of NK cytolysis (I_{50}) was determined as a means of standardizing the inhibition of kill for each of 8 replicate experiments (Table XIII). The I_{50} values were obtained directly or extrapolated from a linear regression plot of the percentage inhibition of lysis and the ratio of unlabeled to labeled targets. The mean I_{50} was determined for both unlabeled tumor lines in the presence of either labeled target. The I_{50} of the unlabeled 5WK+6WKSC cells was significantly greater than that of the unlabeled INVITRO tumor only when assayed with the INVITRO cells as the labeled targets ($P=0.049$). The fact that this trend was detectable but not significant for the experiments performed with labeled 5WK+6WKSC targets, may have been a result of the decreased

TABLE XIII.

The inhibition of NK cell cytolysis with unlabeled tumor target cells.

TUMOR CELLS	% SPECIFIC CYTOTOXICITY ± S.E.[a]
INVITRO	36.5 ± 2.4
5WK+6WKSC	28.7 ± 3.6

RATIO OF UNLABELED TO LABELED TUMOR TARGETS	LABELED TUMOR TARGETS	% INHIBITION OF CYTOLYSIS ± S.E. UNLABELED TUMOR TARGETS: INVITRO	UNLABELED TUMOR TARGETS: 5WK+6WKSC
10 : 1	INVITRO	59.3 ± 5.3	48.9 ± 8.6
	5WK+6WKSC	73.7 ± 4.3	74.4 ± 5.8
5 : 1	INVITRO	41.4 ± 5.6	35.4 ± 5.2
	5WK+6WKSC	57.3 ± 3.4	46.1 ± 5.2
2.5 : 1	INVITRO	26.5 ± 5.6	24.4 ± 4.8
	5WK+6WKSC	33.9 ± 1.8	31.9 ± 3.6

LABELED TUMOR TARGETS	I_{50} ± S.E.[b] UNLABELED TUMOR TARGETS: INVITRO	UNLABELED TUMOR TARGETS: 5WK+6WKSC
INVITRO	8.0 ± 1.1	14.3 ± 3.6
5WK+6WKSC	5.1 ± 0.5	6.1 ± 0.8

a. The results represent the combined data from 8 NK assays using poly I: poly C stimulated syngeneic spleen cells. The percentage specific cytotoxicity of the 5WK+6WKSC cells was significantly different from that of the INVITRO cells (P=0.004).

b. The I_{50} value refers to the number of unlabeled cells required to inhibit 50% of the NK cytolysis. The I_{50} of the unlabeled 5WK+6WKSC cells was significantly different (P=0.049) from that of the unlabeled INVITRO cells only when labeled INVITRO cells were used as the tumor targets.

sensitivity of this determination due to the reduced susceptibility to NK cells of the 5WK+6WKSC tumor compared with the INVITRO population. These results suggested that a reduction in sensitivity to NK cells, following the SC growth of SL2-5 cells, was associated with a decreased expression of tumor target structure recognized by NK cells.

Similarly, the association of the reduced sensitivity to syngeneic serum following the SC growth of SL2-5 cells with a decreased expression of tumor antigen recognized by syngeneic NAb was examined. The relative ability of an aliquot of tumor cells to absorb the cytotoxic activity from the serum has been assessed as a measure of the expression of tumor antigen recognized by NAb (Chow et al., 1981). Initially, a range of tumor cells (5×10^6 - 2×10^7) was used to determine the number of cells required to absorb approximately fifty percent of the anti-tumor cytotoxic activity from the stimulated syngeneic serum (data not shown). Finally, a standard aliquot of 1×10^7 INVITRO or 5WK+6WKSC cells was used to absorb 0.20 mls of serum. The sensitivity of the INVITRO and the 5WK+6WKSC cells to the absorbed and unabsorbed serum was then assessed in a NAb plus complement assay (Table XIV). The susceptibility of the 5WK+6WKSC cells to unabsorbed serum was significantly less than that of the INVITRO cells. However, there was no significant difference between the abilities of the 5WK+6WKSC and the INVITRO cells to inhibit the cytolysis of either target population as a result of their previous absorption of the serum. This observation suggested that the reduction in sensitivity to syngeneic serum following SC growth was not associ-

TABLE XIV.

The sensitivity of the INVITRO and the 5WK+6WKSC cells to tumor cell absorbed syngeneic serum.

TUMOR CELL TARGETS	% SPECIFIC CYTOTOXICITY ± S.E.[b]	% INHIBITION OF CYTOLYSIS ± S.E.[a]		
		ABSORBING TUMOR CELLS		
		INVITRO		5WK+6WKSC
INVITRO	71.6 ± 3.2	44.3 ± 2.8		43.9 ± 2.5
5WK+6WKSC	61.2 ± 2.4	43.2 ± 4.2		45.6 ± 2.6

a. *Tumor-absorbed or unabsorbed, Lps stimulated syngeneic serum, previously diluted to 1/2 with 10% FFBS, was used in these experiments. The results represent the combined data from 3 experiments. There were no significant differences in the percentage inhibition of cytolysis for any of the absorbing tumor-target cell combinations.*

b. *The percentage specific cytotoxicity of the 5WK+6WKSC cells was significantly different from that of the INVITRO cells using unabsorbed serum ($P<0.008$).*

ated with concomitant decreases in the expression of tumor target structure recognized by NAb.

(b) A General Alteration in Tumor Susceptibility to Lysis

It was postulated that similar reductions in sensitivity to other antibodies thought to be unrelated to the syngeneic selection process would be observed if the reductions in susceptibility to NAb and NK cells were due to a general increase in tumor resistance to lysis. In order to examine this possibility, the sensitivities of the 3WKSC and the INVITRO cells to various dilutions of normal allogeneic CBA, anti-thy 1.2 and anti-H2d sera were assessed (Figure 21). It appeared that the 3WKSC cells were slightly, but consistently, less sensitive to the titrated activities of all three sera, as compared with that of the INVITRO population. This information suggested that either these antisera exhibited some activity in common with that present in the syngeneic growth environment, or that a general decrease in susceptibility to lysis was an underlying characteristic effect of tumor growth at the SC site.

The latter possibility was further examined by assessing the susceptibility to non-specific hypotonic lysis of the 3WKSC and the INVITRO cells. It appeared that the 3WKSC cell population was significantly more resistant to hypotonic lysis at the isotonic media concentrations tested than the INVITRO cells (Table XV). In order to confirm this observation, the sensitivity to hypotonic

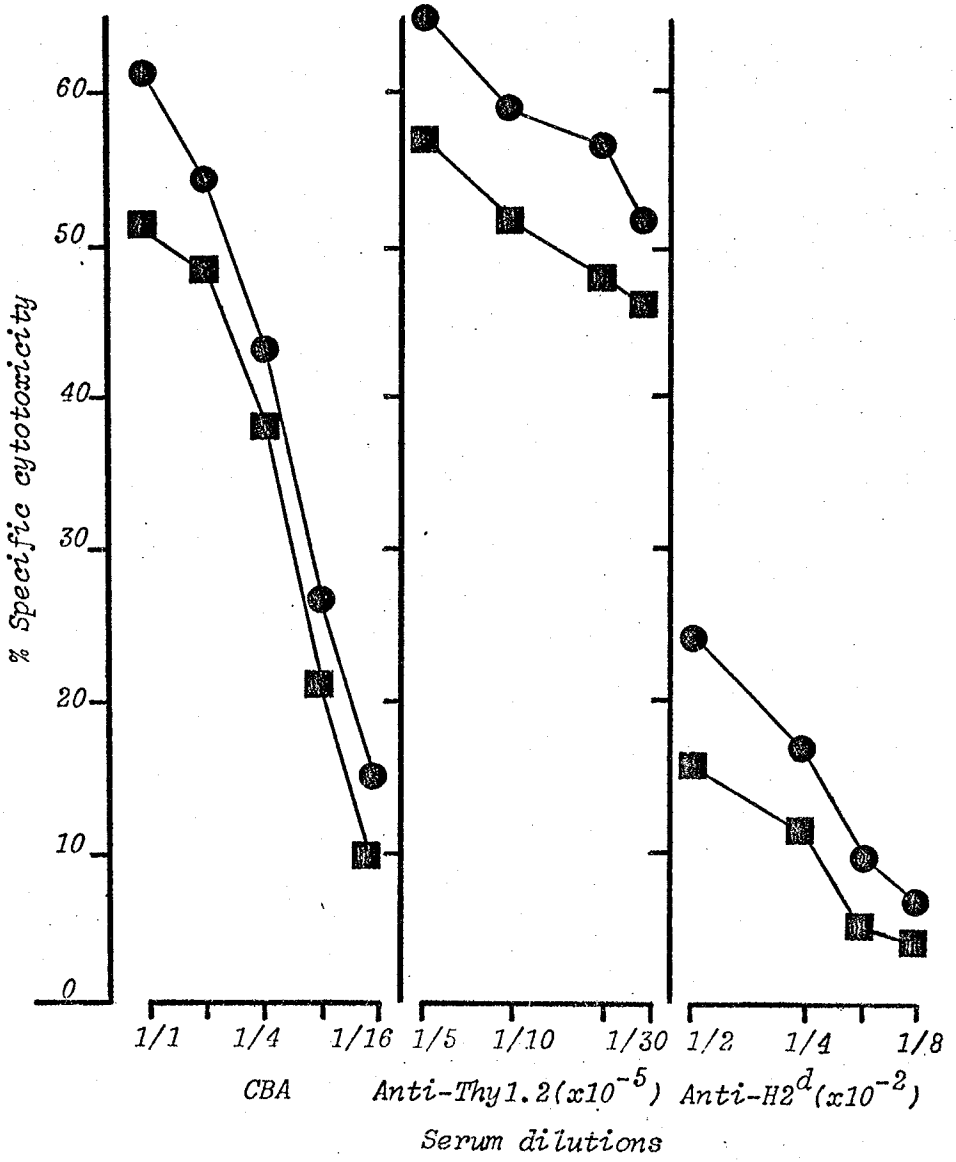

Figure 21. The sensitivity of the INVITRO and the 3WKSC cells to dilutions of various anti-sera. The susceptibility of the INVITRO (●) and 3WKSC (■) cells to the titrated activities of CBA, anti-Thy1.2 and anti-H2^{d} sera was assessed in the presence of rabbit serum as a source of complement. The results represent the data from a single experiment, conducted independently, for each serum tested.

TABLE XV.

The susceptibility of the INVITRO and the 3WKSC cells to hypotonic lysis.

TUMOR CELLS	% HYPOTONIC LYSIS ± S.E.[a]	
	ISOTONIC MEDIUM 50%	25%
INVITRO	14.9 ± 3.2	31.7 ± 6.1
3WKSC	9.0 ± 1.9	22.3 ± 4.9

a. *Tumor cells were incubated for 2 hours at 37 °C in 50% or 25% isotonic media. The results represent the combined data from 4 experiments. The percentage hypotonic lysis of the 3WKSC cells incubated in 50% and 25% isotonic medium was significantly different from that of the INVITRO cells with P values of 0.05 and 0.02 respectively.*

shock of other in vivo passaged tumor cell populations was determined using conditions of greater hypotonic stress at one hour intervals over a four and one-half hour period. Generally, the differences in the sensitivity of SL2-5 populations to hypotonic lysis were more pronounced at lower percentages of isotonic medium (5-10%). The overall susceptibility of the 5WK+6WKSC cells to hypotonic lysis was significantly less than that of the INVITRO cells. In addition, the sensitivity of the 5WK+6WKIP to hypotonic shock was significantly greater than that of the 6WKIP cells, which were both significantly more susceptible than the INVITRO tumor (Figure 22). These results indicated that reductions in osmotic fragility were associated with the SC passaged cells, while those tumors grown in the peritoneum exhibited an increased osmotic fragility.

In fact, linear regression analysis of the percentage of ^{131}I retained on day four of the tumor elimination NR assay for the INVITRO, 6WKIP, 5WK+6WKIP and 5WK+6WKSC cells, and the percentage hypotonic lysis at 2.5 hours (data not shown) and 4.5 hours (Figure 23) of incubation revealed significant inverse correlations. This relationship was nearly significant ($P<0.07$) for the other time points. These observations suggested that alterations in tumor susceptibility to NR following growth in vivo were directly related to the osmotic fragility of the SL2-5 populations and that an effect of in vivo growth upon these cells was to alter their general susceptibility to lysis in a site dependent manner.

The observed changes in tumor resistance to hypotonic shock, NK cell and NAb cytolysis were stable characteristics during main-

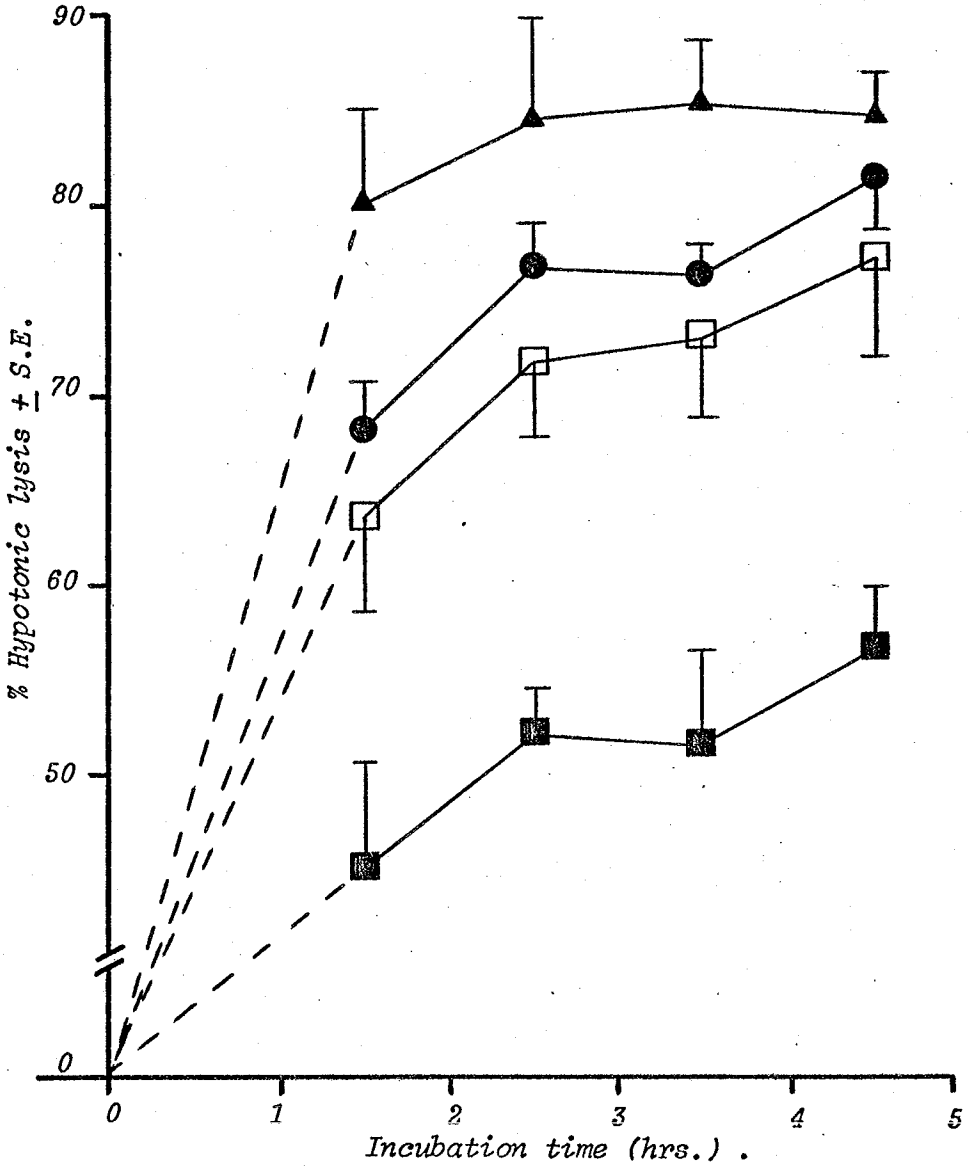

Figure 22. The susceptibility to hypotonic lysis of a variety of SL2-5 tumor populations. The INVITRO (□), 6WKIP (●), 5WK+6WKIP (▲) or 5WK+6WKSC (■) cells were incubated in 10% isotonic medium at 37°C. The results represent the combined data from three experiments. The percentages of hypotonic lysis of the 6WKIP, 5WK+6WKIP and 5WK+6WKSC cells were significantly different over the entire period from that of the INVITRO cells ($P<0.05$, $P<0.01$, $P=0.001$, respectively). In addition the sensitivity to hypotonic shock of the 6WKIP cells was significantly different from that of the 5WK+6WKIP cells ($P<0.03$).

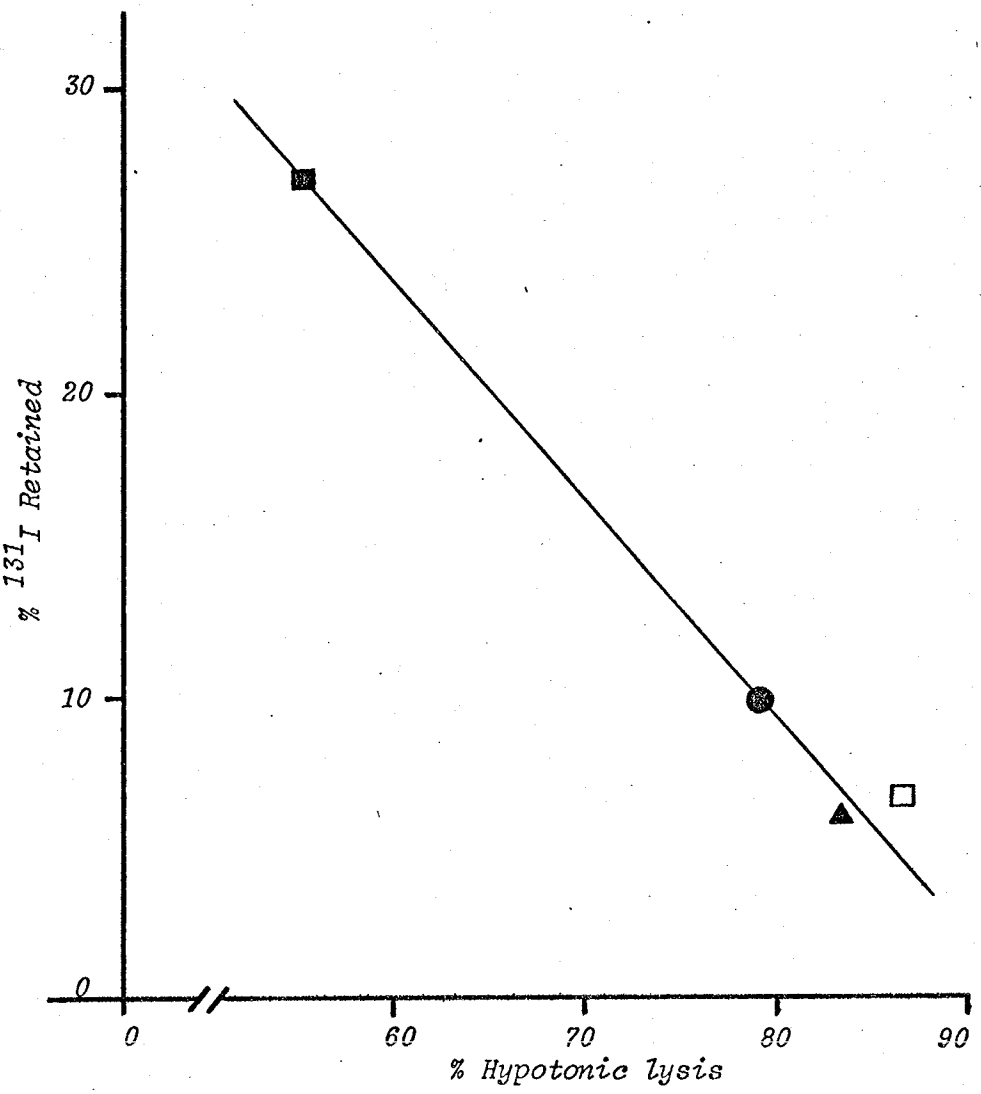

Figure 23. A correlation between tumor sensitivity to hypotonic lysis and susceptibility to NR. The INVITRO (●), 6WKIP (▲), 5WK+6WKIP (□) and 5WK+6WKSC (□) cells were incubated for 4.5 hours in 10% isotonic medium, at 37°C. There was a significant correlation between the percentage of ^{131}I retained on day 4 of the tumor elimination assay and the percentage hypotonic lysis (LR=-0.986, P<0.02).

tenance in tissue culture for at least a six week period. This fact suggested that these alterations in tumor phenotype were relatively irreversible and not simply due to temporary alterations in response to a changed growth environment.

(c) The Observation of Tumor Metabolic Processes with Resistance to Lysis

It has been suggested that the observed resistance of many tumors to lysis mediated by antibody in the presence of complement (Schlager et al., 1978; Ohanian et al., 1983), NK cells (Kunkel and Welsh, 1981; Collins et al., 1981), hypotonic shock and activated macrophages (Brooks et al., 1981), was under the metabolic control of the tumor cell. These researchers postulated that alterations in tumor sensitivity to lysis may for some tumors be reflected in the ability of the cells to actively repair the damage caused by the above mentioned lytic processes. Similarly, evidence of a counterlytic mechanism was sought in order to determine if the ability of the SL2-5 tumor to resist or actively repair damage due to hypotonic shock contributed to the reduced osmotic fragility of the 5WK+6WKSC cells.

(i) Temperature dependence

If the osmotic fragility of the 5WK+6WKSC was dependent, to a greater extent, than the INVITRO cells upon an active cellular metabolism and/or the physical integrity of the tumor membrane and cytoskeleton, then a marked reduction in temp-

erature would be expected to preferentially increase the sensitivity of the 5WK+6WKSC population to hypotonic lysis. Based upon this hypothesis, it was predicted that a decrease in the hypotonic incubation temperature to 4°C would reduce the difference in the susceptibilities of the 5WK+6WKSC and the INVITRO populations previously observed at 37°C. Thus, the sensitivity of these two tumors to hypotonic shock was assessed after a 3.5 hour incubation in 5% isotonic medium at 37°C or 4°C. It appeared that the susceptibility of the 5WK+6WKSC cells to hypotonic lysis was significantly less than that of the INVITRO cells when assayed at 37°C. But there was essentially no difference in the osmotic fragility of the 5WK+6WKSC and the INVITRO cells incubated at the lower temperature (Figure 24). These results indicated that the enhanced ability of the 5WK+6WKSC cells to resist hypotonic shock was temperature dependent, which suggested that tumor metabolism and/or structural components contributed to this phenomenon.

(ii) Metabolic poisons

Numerous investigators have recognized the important role of an active counterlytic mechanism in the sensitivity of a variety of tumors to antibody plus complement, NK cell and hypotonic cytolysis (Boyle et al., 1975; Schlager and Ohanian, 1980a,b; Brooks et al., 1981; Collins et al., 1981; Kunkel and Welsh, 1981). In order to determine which aspects of tumor metabolism were integral to the lytic repair process, these researchers examined the effect of various poisons, including sodium azide and cycloheximide as inhibitors of energy metabolism and protein

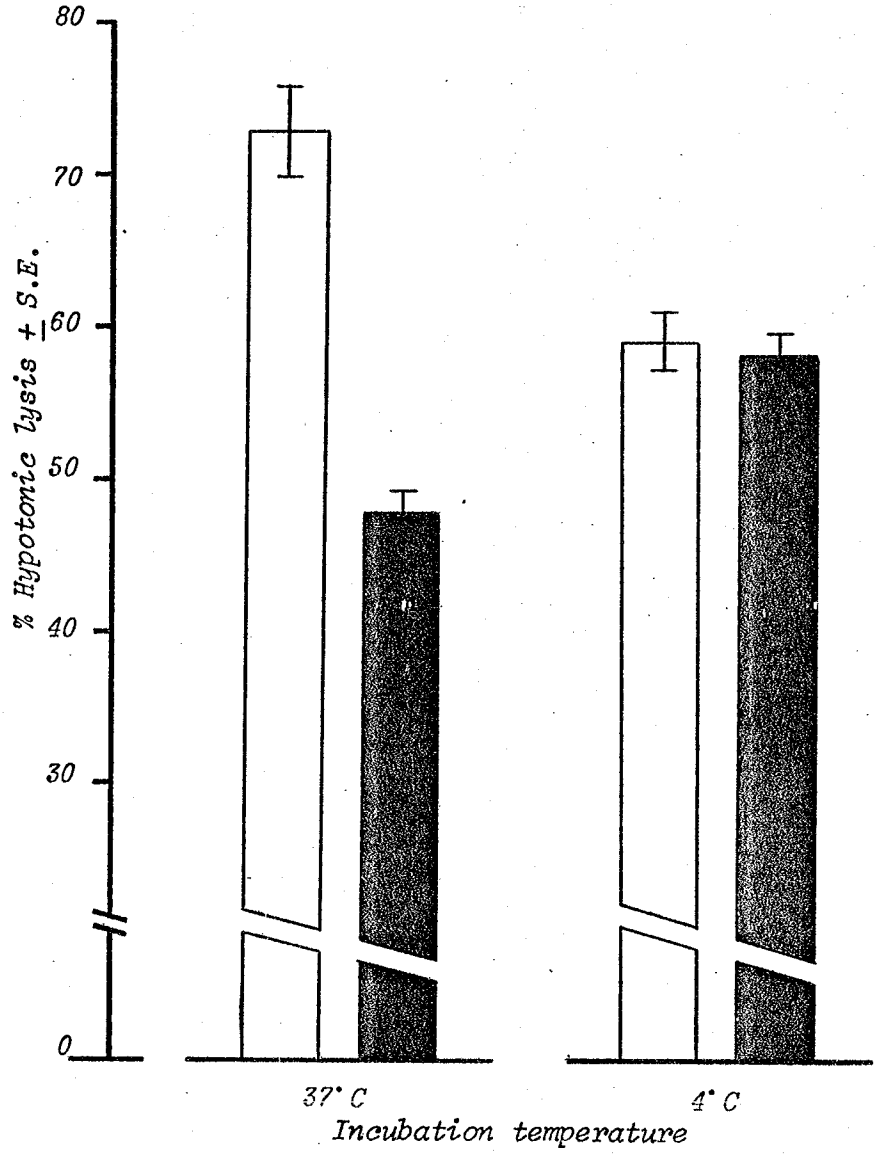

Figure 24. The effect of temperature on tumor cell susceptibility to hypotonic lysis. The INVITRO (□) or the 5WK+6WKSC (■) cells were incubated in 5% isotonic medium for 3.5 hours, at 37°C or 4°C. The results represent the combined data from three experiments. The sensitivity of the 5WK+6WKSC cells to hypotonic shock was significantly different from that of the INVITRO cells ($P<0.01$) when assayed at 37°C, but not at 4°C.

synthesis, respectively.

In addition, Schlager and Ohanian (1980a,b) stated that factors affecting the fluidity or permeability of the tumor cell plasma membrane may also influence the ability of some tumor cells to resist lysis. In fact, both the structural integrity of tumor cells and the organization and the mobility of molecules within the plasma membrane have been associated with cytoskeletal components, including microtubules (Dyson, 1977; Sundqvist and Ehrnst, 1976; Poste et al., 1975). However, the complex movement of intracellular constituents pertaining to, for example, protein synthesis and membrane recycling, was thought not to be a random event, but an active process specifically directed by the contractile elements of the nuclear and cytoskeletal lattice (Barrack and Coffey, 1982). Thus, a dynamic cytostructure could also potentially be involved in a counterlytic mechanism. This information suggested that a tumor's ability to structurally resist and/or actively repair lytic damage may be affected by microtubule formation. Colchicine has typically been used to disrupt microtubules, their associated structures and functions (Dennis et al., 1981; Katz et al., 1982).

Thus, the ability of sodium azide, cycloheximide and colchicine to inhibit SL2-5 cell processes potentially involved in tumor resistance to osmotic shock was examined. It was predicted that if the decreased osmotic fragility of the 5WK+6WKSC cells was due to a process affected by one of the poisons, then the presence of the inhibitor should increase the susceptibility of this tumor population to hypotonic lysis. The 5WK+6WKSC and the INVITRO

cells were subjected to hypotonic shock at 37°C in the presence or absence of each poison in order to determine if a lytic repair pathway and/or the structural integrity of the tumor cell contributed to the reduced sensitivity of the 5WK+6WKSC cells to hypotonic cytolysis. The concentrations used for each of the inhibitors corresponded to the doses cited as effective in other murine or guinea pig tumor cell systems (Boyle et al., 1975; Collins et al., 1981; Katz et al., 1982). The concentration of sodium azide and colchicine used was 10 mM. Solutions of these poisons greater than 10 mM were cytotoxic to the control cells incubated in 100 percent isotonic medium, while those less than this value appeared to have little effect upon the tumor cell susceptibility to hypotonic lysis (data not shown). There was a small range of concentrations of cycloheximide from 5 mM to 10 mM, which was effective in altering tumor cell sensitivity to hypotonic lysis and the data from these experiments were pooled.

The sensitivities of the tumor populations to hypotonic lysis in the presence and absence of each poison was assessed independently for sodium azide, colchicine and cycloheximide. Though the net level of percentage hypotonic lysis for the control trials varied somewhat from experiment to experiment, the differences in the susceptibility of the tumors to hypotonic shock in the absence of inhibitors were consistent and significant. The osmotic fragility of the 5WK+6WKSC cells was significantly greater than that of the INVITRO cells throughout the control experiments (Figure 25A,B,C).

In contrast to these results, the sensitivity of the

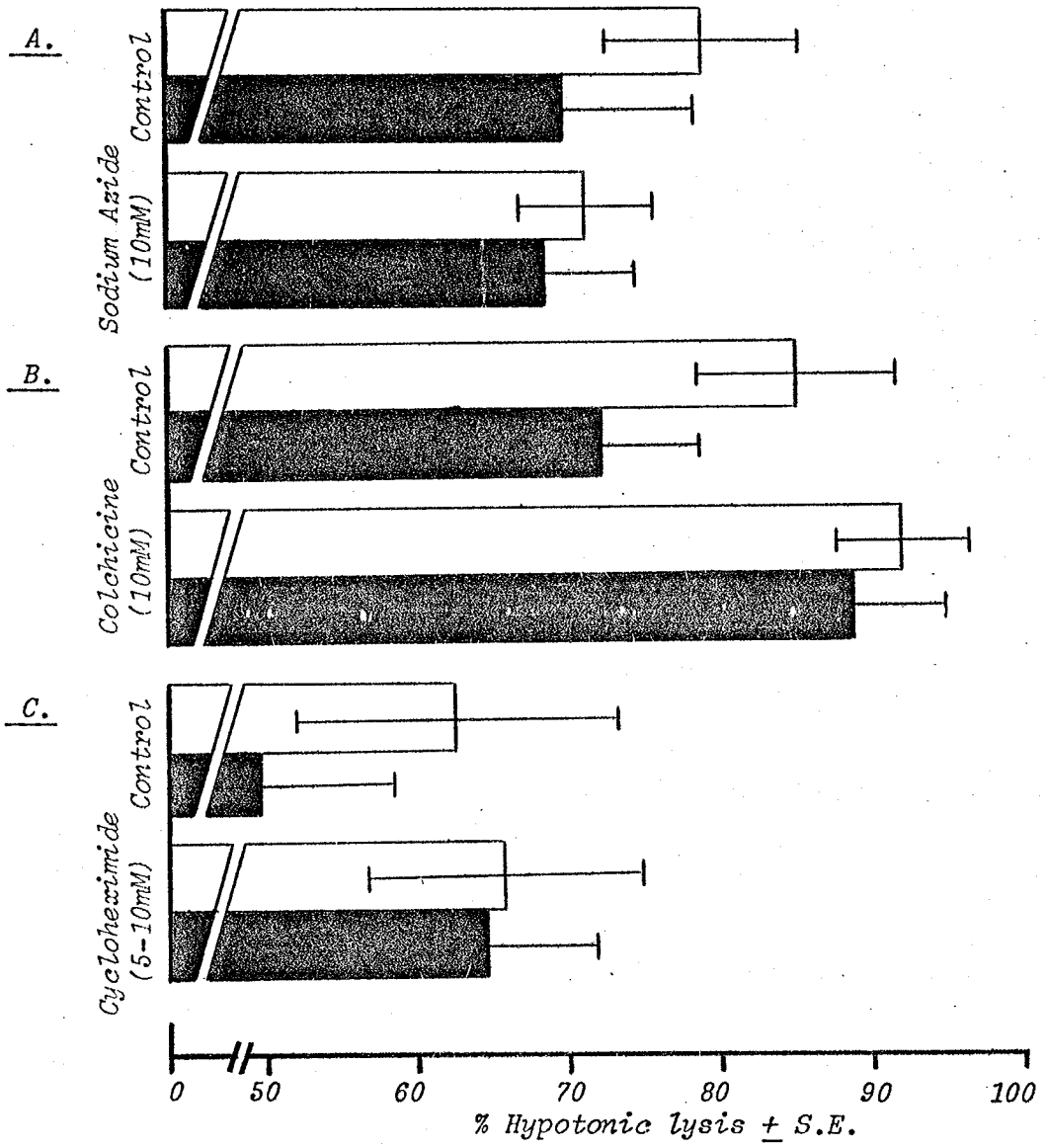

Figure 25. The effect of sodium azide, colchicine or cycloheximide on tumor susceptibility to hypotonic lysis. The INVITRO (□) or the 5WK+6WKSC (■) cells were incubated with or without (control) poison for 4.5 hours in 10% isotonic medium, at 37°C. The results represent the combined data from 4, 3 and 5 experiments for A., B. and C., respectively. Only in the absence of poison was the percentage hypotonic lysis of the 5WK+6WKSC cells significantly different from that of the INVITRO cells, A.($P<0.02$), B.($P=0.002$) and C.($P=0.006$).

5WK+6WKSC cells to hypotonic lysis was not significantly different from that of the INVITRO cells when both cell lines were incubated in the presence of sodium azide (Figure 25A). This observation suggested that the difference in the osmotic fragility of the two populations was dependent upon energy metabolism. Similarly, there was no difference in the osmotic fragility of the 5WK+6WKSC cells as compared with the INVITRO tumor in the presence of colchicine (Figure 25B). This result indicated that the increased ability of the 5WK+6WKSC cells to resist hypotonic shock was associated with an intact microtubule network.

Finally, for the experiments performed in the presence of cycloheximide, the susceptibility of the 5WK+6WKSC cells to hypotonic lysis was not significantly different from that of the INVITRO population (Figure 25C). This observation suggested that the reduced susceptibility to hypotonic lysis of the 5WK+6WKSC cells was also dependent upon protein synthesis. Collectively, it appeared that the presence of each of the poisons tested had a marked effect upon the osmotic fragility of the SL2-5 tumor, which suggested that some form of counterlytic mechanism contributed to the reduced sensitivity of the 5WK+6WKSC cells to hypotonic shock.

CHAPTER V

DISCUSSION

DISCUSSION

The stability of the cloned SL2-5 lymphoma for sensitivity to host mediated anti-tumor NR was examined following tumor growth *in vivo*. Reductions in susceptibility to syngeneic NR were observed for the tumor cells retrieved from the SC site of DBA/2 mice and reestablished in tissue culture. Compared with the original neoplastic cells, the SC passaged populations also exhibited an increased tumor frequency for small SC inocula suggesting that the effect of the alteration in the NR-sensitive phenotype was to increase the ability of the tumor to survive in opposition to host anti-tumor mechanisms. On the other hand, both the susceptibility to NR of the SL2-5 cells that were grown exclusively in tissue culture and its ability to form a tumor from a small inocula of cells remained unchanged. The evidence, therefore, suggested that the decreased sensitivity to host mediated anti-tumor defences was not simply a consequence of tumor proliferation but rather was dependent upon exposure to the *in vivo* milieu. These results could be explained on the basis of several underlying mechanisms including the generation of a range of tumor variants followed by the selective elimination of those cells most sensitive to NR.

During an initial attempt to confirm the presence of tumor variants, it appeared that the extent of phenotypic heterogeneity for sensitivity to allogeneic NAb was no greater for subclones derived from the *in vivo* SC passaged SL2-5 populations than that of the subclones obtained from cells grown exclusively *in vitro*. However, the slight to moderate increases in sensitivity to NR

detected for the cells grown IP for two to eight weeks suggested that the peritoneal tumors had not developed in a selective environment and as a result, could potentially contain a more complete range of variants. This interpretation was based upon the prediction that the phenotypic heterogeneity of a tumor would likely be more extensive in the absence of host mediated selection (Chow et al., 1983). In fact, the subclones derived from the twice IP passaged tumor were more heterogeneous than the subclones obtained from the SL2-5 cells grown only in tissue culture for susceptibility to syngeneic NR and nonspecifically stimulated serum. In addition, the lack of a correlation for subclone sensitivity to NR and to stimulated NAb suggested the presence of a range of different cells with independently altered phenotypes. This evidence was indicative of the ability of the SL2-5 cells to generate tumor variants in vivo.

The stability of the altered phenotypes during at least a six week period of tissue culture maintenance argued against the temporary modulation of tumor characteristics in response to an altered growth environment as an underlying basis for this phenomenon. In addition to the evidence in support of a range of tumor variants, the graded effect on the alterations in the NR-sensitive phenotype associated with increasing the duration of the in vivo growth period or repeating the tumor passage in vivo was not characteristic of "tumor adaptation" as described by Barett and Deringer (1953). In contrast to the SL2-5 results, these investigators observed that once an increase in transplantibility was attained, it was not further affected by additional exposure to

the in vivo growth environment. Klein (1963) also demonstrated that these adaptive changes were independent of inoculum size and thus not likely associated with the presence of tumor variants. Therefore, the marked reduction in tumor susceptibility to NR following growth in vivo in conjunction with the demonstrated ability of the SL2-5 lymphoma to generate a range of variants at the IP site, supported the hypothesis that variant generation and selection formed the basis for the tumor progression of these cells in vivo.

Alterations in tumor phenotype apparently due to the selection of tumor variants have been studied extensively by other investigators. G. and E. Klein (1956) postulated that the conversion of a solid tumor to one capable of growing in ascites form was due to the selective survival of a small fraction of preexistant variant cells in the original population, which were endowed with an enhanced ability to become established in ascites form. In addition, increases in the malignant potential of an uncloned, UV-induced regressor fibrosarcoma (Urban et al., 1982) or in the tumorigenicity and NK cell resistance of a cloned, NK-sensitive regressor fibrosarcoma (Collins et al., 1981) were observed after tumor passage in immune deficient hosts followed by growth in normal mice. Their results were consistent with the hypothesis that the absence of selection in immune deficient animals permitted the survival of an extended range of tumor variants and that the most sensitive variants were subsequently eliminated upon exposure to the selective environment of the normal host.

Based solely upon this evidence, it was not possible to

determine if the alterations in tumor phenotype observed by these investigators were due to the random generation of variants, the expansive proliferation of a single variant or the formation of a new tumor. However, in a previous study from this laboratory, the chromosomal analysis of clone L5178Y-F9 cells grown in vivo or maintained only in vitro revealed that the karyotypic constitution of these populations were comparable. This observation suggested that the L5178Y-F9 tumor obtained following growth in vivo was unlikely a new tumor as a result of a fusion event or viral infection (Chow et al., 1983). Chow and co-workers (1983) contended that the extent of the phenotypic variation in susceptibility to syngeneic NR and allogeneic NAb argued against the possibility that tumor adaptation had occurred in vivo. They concluded that the generation and selection of tumor variants as a basis for the tumor progression of the L5178Y-F9 lymphoma provided the most reasonable explanation of their results.

Finally, the consensus based upon the study of the metastatic model of tumor progression, using the uncloned murine B16 melanoma, was that metastases likely occurred due to the proliferation and selective survival of subpopulations of preexistant variants endowed with the ability to colonize specific metastatic sites (Fidler et al., 1978; Nicolson, 1979; Poste and Fidler, 1980). In addition, other investigators demonstrated the generation of tumor heterogeneity for metastatic potential following the in vivo growth of a cloned tumor (Talmadge et al., 1979, 1981; Cifone and Fidler, 1981). Furthermore, Ling and co-workers (1984) concluded that the increased metastatic potential observed subsequent to the

repeated selection for metastasis in vivo was directly proportional to the ability of the tumor to generate variants rather than an increased metastatic phenotype. Collectively, the evidence supported the generation and selection of tumor variants as a basis for the progression of these tumors towards an increased metastatic potential.

Based upon the distinct differences in the alteration of the SL2-5 NR-sensitive phenotype, depending upon the anatomical site of tumor growth, it was apparent that the progression of this lymphoma was more complex than expected. The rapid clearance of radiolabeled SL2-5 cells from the IP site during the four day period immediately following tumor injection suggested that NR was active early during tumor growth at this site. The fact that the cells passaged intraperitoneally for forty-eight hours or less were slightly but consistently less sensitive to host mediated anti-tumor defences, was consistent with this interpretation. Chow (1984b) similarly detected a reduction in sensitivity to NR following the growth of a larger inoculum of L5178Y-F9 cells at the IP site for a comparable period of time. These observations suggested that the peritoneum was capable of selecting for a reduction in susceptibility to NR early during tumor growth. However, the fact that the cells passaged twice at the IP site for a total of eleven weeks exhibited a slight increase in susceptibility to host mediated anti-tumor mechanisms and that this change was associated with an increased phenotypic heterogeneity for the same parameter suggested that NR selection pressures were unable to appreciably influence the growth of these cells in the perito-

neum over the long term.

Other investigators have similarly detected an inability of the IP site to select for an increase in NK cell resistance of a rat adenocarcinoma (Brooks et al., 1981) or an increase in the metastatic potential of a murine UV-induced fibrosarcoma (Cifone and Fidler, 1981) and a transformed rat hepatoma (Talmadge et al., 1979). In fact, Talmadge and associates (1979, 1981) and Cifone and Fidler (1981) observed that subclones from the tumors passaged in the peritoneum exhibited a range of metastatic potential, both higher and lower than the original tumor. They contended that random variant generation and thus an increase in tumor heterogeneity had occurred in the absence of selection. The relatively high tumorigenicity of the SL2-5 cells injected at the IP site compared with the SC site suggested that under the conditions of a limiting tumor dose, the NR mechanisms within the peritoneum were less able to reject the tumor burden.

Consequently, it was postulated that due to a less growth restrictive environment, possibly as a result of a decreased ability to sustain a natural anti-tumor response in the presence of a growing tumor, the early selective effects of host mediated NR within the peritoneum were soon masked by the generation of new variants in the rapidly expanding neoplastic population. The fact that many tumors achieved most of their mass within a short period of time after their inception (Laird, 1965), and that natural immune surveillance mechanisms appeared to be effective only against a small number of tumor cells (Greenberg and Greene, 1976), supported this interpretation. Under the conditions of

small tumor inocula, the random generation of tumor variants would have been expected to produce sensitive and resistant variants at equal rates and thus no net shift in NR susceptibility would have been predicted. However, the SL2-5 populations grown at the IP site for increasing numbers of weeks exhibited corresponding increases in sensitivity to host mediated anti-tumor mechanisms and to hyptonic lysis. In fact, based upon the direct correlation between tumor sensitivity to NR and hypotonic shock, the determination of the osmotic fragility of the in vivo passaged SL2-5 cells appeared to provide a direct and sensitive assessment of alterations in lytic sensitivity to NR, independent of effector-tumor interactions. These observations suggested that the sensitive variants might have been generated or expressed to a greater extent in the population. Alternatively, a reduction in the appearance of resistant tumor variants could account for these results.

In summary, this evidence supported the contention that the net change in the SL2-5 NR-sensitive phenotype was dependent upon a balance between the presence of sensitive and resistant variants in combination with the ability of the host to impose selective pressures at the in vivo site of tumor growth. Furthermore, the fact that SL2-5 cells passaged within the peritoneum exhibited an increased sensitivity to NR provided interesting speculation as to the therapeutic benefits that could be gained as a result of the progression of a tumor towards a decreased malignancy.

This murine model of tumor progression was further analyzed in order to determine more precisely the ability of particular NR

effectors to direct or influence the development of the SL2-5 lymphoma. Urban and co-workers (1982) predicted that the selective elements active in vivo would have a characteristic effect upon a population of tumor cells and that the subsequent examination of the "fingerprints" remaining after selection should reveal the nature and hierarchy of the natural anti-tumor mechanisms involved in this process. The fact that the SC tumor frequencies of small SL2-5 inocula were no greater in syngeneic ATxBM mice than in normal animals and that tumors removed from a normal and an ATxBM mouse exhibited similar reductions in sensitivity to NR, indicated that the host mediated anti-tumor mechanism capable of influencing the development of this tumor functioned in a thymus independent manner. These results were consistent with the previous observations of similar tumor frequencies in ATxBM and normal mice using the murine 1509a fibrosarcoma, the L5178Y lymphoma and the P815-X2 mastocytoma (Greenberg and Green, 1976; Chow et al., 1979). The fact that NK cell (Holler et al., 1978; Herberman et al., 1975a), NAb (Wolosin and Greenberg, 1981), and macrophage (Chow et al., 1979) activities were shown to be independent of a functional thymus suggested a role for these natural effector mechanisms against incipient neoplasia.

Reduction in SL2-5 sensitivity to syngeneic NK cytolysis correlated with decreases in tumor susceptibility to NR regardless of the anatomical site of tumor growth. These data were consistent with the hypothesis that NK cells contributed to their alteration in NR-sensitive phenotype. Chow and colleagues (1981) also observed a relationship between tumor sensitivity to syngeneic NR

and NK cell activity for a pair of SL2 lymphoma clones chosen on the basis of disparate susceptibilities to NK cells. In addition, numerous other reports have implicated NK cells as a putative effector of NR (Kiessling et al., 1975; Hanna et al., 1982; Karre et al., 1980; Kasai, 1979; Ricarde et al., 1980). Beyond supporting a role for NK cells in vivo, the results of the present study suggested that these natural effector lymphocytes may have directed the progression of the in vivo passaged SL2-5 cells.

Similarly, reductions in SL2-5 sensitivity to syngeneic serum NAb, for cells passaged at the SC site, correlated with changes in tumor susceptibility to NR. This evidence was consistent with the relationship previously observed between a reduced susceptibility to serum NAb and a decreased sensitivity to NR for a pair of L5178Y-F9 clones chosen on the basis of disparate susceptibilities to NAb (Chow et al., 1981). Chow (1984b) also observed reductions in sensitivity to both syngeneic NR and NAb cytolysis for tumors obtained following the SC, IP or intravenous growth of L5178Y-F9 cells. However, this correlation was not evident for the SL2-5 cells grown for longer periods at the IP site, as slight increases in susceptibility to NR were observed in association with marked decreases in sensitivity to syngeneic serum NAb. The observed discrepancy between the susceptibility of the IP passaged tumors to the NR and NAb could be explained on the basis that either NAb may not have been a primary mediator of NR against the SL2-5 lymphoma or that due to some peculiar aspect of SL2-5 growth within the peritoneum, the previously observed correlation between these parameters was not readily detectable. The first explanation

implied that the decreased sensitivity to serum NAb of the SC passaged SL2-5 cells may not have been causally related to the corresponding reductions in sensitivity to NR but due only to the parallel decreases in tumor susceptibility to NK cells.

In support of a role for NAb in host mediated anti-tumor mechanisms, the slight but consistent decreases in sensitivity to NR exhibited by the SL2-5 cells grown at the IP site for 48 hours may have been based upon the selective action of NAb. However, the subsequent random generation of variants during further tumor growth, very likely in the absence of NR selection, may have allowed alterations in the numerous parameters, which constitute tumor susceptibility to NR without necessarily changing sensitivity to NAb, a much less complex phenomenon. Alternatively, it was possible that selection at the IP site had occurred based on some effector which had an inverse effect on tumor sensitivity to NAb, but bore no direct relation to a host mediated NR mechanism. This interpretation could account for these results without compromising the hypothesis that NAb acts as an effector NR. The fact that Urban and associates (1982) observed that a progressor fibrosarcoma variant, selected in vivo on the basis of a reduction in sensitivity to tumor-specific T cell-mediated immunity, actually exhibited an increased susceptibility to NK cells compared with the unselected UV-induced 1591 regressor fibrosarcoma, gave some support to the latter hypothesis.

The examination of SL2-5 sensitivity to the putative mediators of NR revealed that the reductions in susceptibility to NAb were most pronounced for cells grown within the peritoneum, while

decreases in sensitivity to NK cells were the greatest for tumor populations passaged at the SC site. In addition, Chow and co-workers (unpublished observations) demonstrated that syngeneic tumor susceptibility to NR correlated with sensitivity to NK cells or NAb for tumors eliminated from the SC or the IP site, respectively. Collectively, these results suggested that, although NK cells and NAb would likely have been active during tumor growth at both anatomical sites, the predominant effector of host mediated anti-tumor NR was NK cells at the SC site and NAb within the peritoneum.

When considering the ability of a neoplasm to resist or "escape" an elaborate system of host anti-tumor defences, a variety of mechanisms including the altered expression of tumor antigen or more recently, a counterlytic capacity, have been cited as being of crucial importance (Klein, 1966; Nicolson, 1976; Fidler, 1978; Schlager et al., 1978; Gatenby et al., 1981; Woodruff, 1982). Numerous investigators have previously assessed the contribution of an altered expression of tumor antigen to the NK cell sensitivity of a variety of human and animal tumors (Roder et al., 1979; Brooks et al., 1981; Chow et al., 1981; Kunkel and Welsh, 1981). The general consensus from these researchers was that the ability of a tumor to inhibit NK cell lysis reflected the expression or accessibility of tumor antigens recognized by these natural effector lymphocytes. However, Trinchieri and associates (1981) suggested that cold target experiments might not only assess competitive inhibition, but also the inactivation of the NK cells by the tumor targets. This contention was based upon dis-

crepancies between the results of competitive inhibition experiments and the data from: (1) the absorption fo NK cell activity on fibroblast monolayers, and (2) the agarose single cell binding assay, for the NK-sensitive K562 human tumor.

In the present study the association of a decreased sensitivity to NK cells for a SC grown SL2-5 population with a reduced ability to cold target inhibit NK cell cytolysis suggested that the decreased expression of tumor antigen may have contributed to the resistance of these cells to natural effector lymphocytes. Previous studies have similarly shown a direct correlation between the sensitivity to allogeneic NK cells and the ability of a tumor to inhibit NK cell cytolysis for the NK-sensitive SL2-5 clone and the NK-resistant SL2-9 clone (Chow et al., 1981). However, a more direct assessment of NK cell-tumor target binding should be determined in order to confirm the apparent reduced expression of tumor antigen for the in vivo grown SL2-5 cells and to examine the capacity of the SL2-5 lymphoma to inactivate NK cells.

In addition, Trinchieri and colleagues (1981) suggested that the differences in the ability of some tumors to resist NK cell cytolysis may have been due to the induction of interferon production which was shown to decrease tumor susceptibility to NK cells. These observations were consistent with the fact that exogeneous interferon treatment of the SL2-5 cells decreased both tumor sensitivity to NR and NK cells (Greenberg et al., 1984). In this regard a temporary reduction in sensitivity to NK cells observed following in vivo growth of a rat adenocarcinoma (Brooks et al., 1981) may only have been due to a prolonged effect of endogenous

interferon. However, the fact that reductions in sensitivity to syngeneic NK cells of the SL2-5 tumor were stable in tissue culture for an extended period of time, made the latter possibility unlikely in our case.

In contrast, the decrease in tumor sensitivity to stimulated syngeneic NAb in the presence of complement for the same SC passaged SL2-5 population was not associated with a decreased ability to absorb the cytotoxic activity from this serum. This result suggested that the increased resistance of these cells to syngeneic NAb was not associated with a reduction in the expression of tumor antigen recognized by NAb. Other investigators have also reported that the variation in sensitivity to antibody plus complement observed for certain tumor cell lines was not due to differences in tumor antigen expression (Ohanian et al., 1983; Celis and Celis, 1983). The ability of tumor cells to actively resist and repair membrane damage caused by antibody and complement was implicated in their studies.

Similiar counterlytic mechanisms have been associated with the resistance of tumor and normal cells to NK cytolysis, with or without concomitant reductions in the expression of NK cell target antigens (Hansson et al., 1979; Brooks et al., 1981; Collins et al., 1981; Kunkel and Welsh, 1981). Brooks and co-workers (1981) determined that normal fibroblasts and many NK-resistant tumors bound to but were not lysed by NK cells and that the NK-resistant targets were significantly less sensitive to hypotonic lysis than NK-sensitive targets. Lachmann (1983) recently reviewed the evidence which suggested that the cytotoxicity of a subpopulation of

NK cells may also be mediated by analogues of the complement components and ultimately dependent upon osmotic lysis.

Based upon this evidence, it was postulated that the counterlytic ability of the SL2-5 cells was an underlying feature in tumor resistance to lysis in general, since there was a direct relationship between susceptibility to hypotonic shock for tumors which had apparently undergone selection for reduced sensitivity to syngeneic NR and the putative mediators of NR, NK cells and NAb. The fact that the susceptibility to hypotonic shock at 4°C of the twice SC passaged tumor preferentially increased over that of the SL2-5 cells maintained only in vitro indicated that the reduced sensitivity to osmotic lysis of the in vivo grown cells observed at 37°C was temperature dependent. Since a marked reduction in incubation temperature would have been expected to induce microtubule depolymerization (Davis et al., 1980) and to generally inhibit tumor cell metabolism, the previous observations suggested that either the structural integrity of the tumor cell or an active repair mechanism, or both, contributed to the ability of the in vivo passaged cells to resist hypotonic shock. This evidence supported the existance of an SL2-5 counterlytic process as a basis for this phenomenon.

Furthermore, in the presence of sodium azide, the osmotic fragility of the in vivo passaged tumors was no longer less than that of the cells maintained exclusively in vitro which suggested that the difference in the osmotic fragility of these populations in the absence of poison was energy dependent. However, it appeared that this difference in sensitivity to hypotonic shock

may have been eliminated as a result of a decreased susceptibility to osmotic lysis on the part of the tumor grown only in tissue culture, rather than an increase in the sensitivity of the SL2-5 cells passaged at the SC site. This observation suggested that the in vitro grown SL2-5 cells may have actively contributed to their own demise under conditions which constituted passive lysis. Wyllie and associates (1980) contended that every cell likely had the capacity to destroy itself and that such autolytic events were essential to the normal differentiation of a multicellular organism. Thus, it appeared that a difference in the structural integrity of the tumor cells may not account for the reduced sensitivity of the in vivo grown SL2-5 lymphoma to hypotonic lysis. Alternatively, it was also possible that in the presence of sodium azide, the sensitivity to hypotonic shock of both populations decreased due to the disruption of an autolytic mechanism, but that the susceptibility to osmotic lysis of the in vivo grown cells simultaneously increased to a level comparable with that of the in vitro grown cells, due to the inhibition of an energy dependent counterlytic mechanism. In accordance with this rationale, an enhanced metabolic repair process may have contributed to the decreased osmotic fragility of the SC passaged tumor in keeping with the interpretation of the temperature dependence of tumor sensitivity to hypotonic lysis discussed previously.

In addition to the observed effect of temperature and sodium azide upon tumor cell sensitivity to hypotonic shock, it appeared that cycloheximide and colchicine selectively increased the susceptibility of the in vivo grown tumor to osmotic lysis. This

evidence suggested that the enhanced capacity of the SC passaged cells to resist hypotonic shock was also dependent upon protein synthesis and an intact microtubule cytostructure, respectively. In addition, the fact that the sensitivity of the cells maintained only in vitro was slightly increased in the presence of colchicine suggested that this population also possessed some degree of counterlytic capability. On the basis of all the evidence which supported the existance of a counterlytic mechanism for the in vivo grown SL2-5 cells, it was likely that this process was involved in some crucial aspect of plasma membrane repair as various investigators contended that it was at this site that the cell must ultimately defend itself against lysis (Schalager et al., 1978; Ohanian et al., 1978; Kunkel and Welsh, 1981). In fact, alterations in plasma membrane lipid (Schlager et al., 1978, 1979; Schlager and Ohanian, 1980a,b) and protein (Celis and Celis, 1983) composition have been associated with tumor cell resistance to humoral immune lysis. Other investigators have previously shown an effect of the inhibition of protein synthesis upon an increased tumor susceptibility to NK cell lysis which was associated with an underlying sensitivity to hypotonic lysis (Brooks et al., 1981; Collins et al., 1981; Kunkel and Welsh, 1981).

In contrast, Schlager and co-workers (1977) reported that the inhibition of protein synthesis or energy metabolism did not affect the resistance of an in vivo passaged guinea pig hepotoma to antibody plus complement. Instead they determined that the modification of cellular lipid and fatty acid composition had marked effects on membrane fluidity, permeability or thickness,

which were related to tumor cell susceptibility to lysis (Schlager and Ohanian, 1980a,b). This discrepancy likely reflected the different tumors analyzed since Russel and Debos (1980) contended that tumor cytolysis mediated by antibody plus complement and hypotonic shock were similar, as in both cases "cell death is presumed to be the result of increasingly large lesions in the target cell membrane sustained by the influx of extracellular water in an attempt to balance the intracellular osmotic pressure from cytoplasmic proteins". In addition, the modification of cytoskeletal elements, specifically microtubules and microfilaments, have also been shown to influence tumor growth and tumorigenicity (Dennis et al., 1981).

Collectively the evidence from these investigators suggested that osmotic lysis may have been the common terminal event in tumor killing mediated by hypotonic shock, antibody plus complement and at least a subpopulation of NK cells. Thus, a counterlytic mechanism could potentially contribute to the susceptibility of a tumor to any cytotoxic effector that was ultimately dependent upon osmotic lysis in order to destroy neoplastic cells. It appeared therefore, that the sensitivity of the in vivo grown SL2-5 lymphoma to NK cells and NAb was superimposed upon a framework of counterlytic processes. In accordance with this hypothesis, NK cells and NAb as putative effectors of NR may have directed the selective elimination of the tumor variants most vulnerable to these mediators on the basis of their counterlytic capacity. The fact that the ability of a tumor to resist or repair lytic damage was observed in a wide variety of tumor models

suggested that this mechanism may play a crucial role in determining the net susceptibility of many tumors to the mediators of NR and ultimately, the outcome of host-tumor interactions.

In summary, the generation of a range of tumor variants, and the subsequent selective survival of the cells least sensitive to the effectors of host mediated anti-tumor defences could best account for the reductions in susceptibility to NR and the increased heterogeneity for sensitivity to syngeneic NR and serum NAb observed for the in vivo passaged SL2-5 lymphoma. It appeared that the net change in the SL2-5 NR-sensitive phenotype may have been dependent upon a balance between the presence of sensitive and resistant variants in combination with the ability of the host to impose selective pressures at the in vivo site of tumor growth. In addition, correlations between reductions in susceptibility to NR and to sensitivity to NK cell and NAb activity were detected for the SC passaged tumors. Furthermore, it appeared that the decreased susceptibility of the in vivo grown SL2-5 cells to NR was thymus independent. These observations were consistent with the hypothesis that NK cells and NAb are effectors of host mediated NR and thus contributed to the progression of the SL2-5 lymphoma. Finally, the fact that a reduction in sensitivity to NR for the in vivo passaged cells correlated with a decreased susceptibility to hypotonic lysis and that the reduced osmotic fragility of this population was reversed by inhibitors of tumor cell metabolism and cytoskeletal assembly suggested that an augmentation of counterlytic processes may be a major factor underlying the in

vivo development of the SL2-5 and possibly all tumors.

CHAPTER VI

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BIBLIOGRAPHY

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