The University of Manitoba

REGULATION OF TUMOR PROGRESSION:

IMPACT OF BIOLOGICAL RESPONSE MODIFIERS AND A PHORBOL ESTER TUMOR PROMOTER ON THE NATURAL DEFENSE RESISTANCE PHENOTYPE OF TUMORS

by

PAUL SANDSTROM

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This thesis and the years of effort
which went into it are dedicated to:

My wife, Lorraine
For her love and support.

My Grandfather, Austin Sandstrom
For his lessons and examples
There never was such a grandfather!!

The memory of my Grandmother, Stella Sandstrom.

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OBJECTIVES

The ability of a tumor cell population to become progressively more autonomous with respect to host mediated regulatory and surveillance mechanisms is dependent on both the frequency at which progressive variants arise within a tumor cell population as well as the nature of the selective pressures which are present. It would be predicted that one of the earliest phenotypic changes to be acquired by a neoplasm as it becomes more tumorigenic would be the decreased sensitivity to detection and destruction by those natural immune surveillance effectors which have been shown to defend against incipient neoplasia. However very little is known about the regulation of tumor progression with respect to natural resistance.

The objective of this investigation is two-fold.

i To investigate the contribution which natural resistance effectors play in the defence against incipient neoplasia as well as to examine their role in the selection of progressive natural immune resistant tumors.

ii To investigate the mechanisms involved in the generation of a natural defence resistant phenotype.

ABSTRACT

A critical aspect of tumor progression towards enhanced autonomy from host control is the generation and selection of progressive variants which are increasingly more resistant to detection and destruction by effectors of the natural immune system. Very little is known however about the regulation of tumor progression with respect to natural resistance. This thesis examines the subject of natural resistance associated tumor progression from the perspective of both the mechanisms involved in variant generation by tumor promoters and the processes of in vivo tumor selection. Initially, it was demonstrated that although natural defence mechanisms may effectively eliminate a threshold subcutaneous tumor inoculum in its early stages of development these same processes provide a selective pressure to direct and drive tumor progression once the neoplasm has overcome this defence, resulting in the generation of tumors exhibiting both augmented reductions in natural antibody (NAb) binding and reduced sensitivity to natural resistance measured in the ^{131}IdUrd tumor elimination assay. The biphasic modulation of natural resistance associated with biological response modifiers such as bacterial lipopolysaccharides and silica may markedly accelerate tumor progression by providing an initial period of reduced host defences permitting the less restrained proliferation and increased generation of variants in the tumor foci which may then undergo an exaggerated selection due to the subsequent stimulation of natural resistance.

The mechanisms involved in the generation of tumor variants displaying reduced susceptibility to natural resistance were also investigated. It was demonstrated that the exposure of lymphoma cell

lines to the phorbol ester tumor promoter 12-tetradecanoylphorbol-13-acetate (TPA) results in the generation of a reversible natural defense resistance phenotype, including decreased sensitivities to natural killer cells, activated macrophages and complement mediated lysis by natural antibodies as well as a marked enhancement of tumorigenicity. Although TPA treated cells demonstrate higher levels of serum natural antibody binding when assayed at 4°C, this increased binding appears to be very unstable and rapidly lost at 37°C. The alterations in NAb binding induced by TPA were shown to require an intact cytoskeleton and active energy metabolism. Inhibition by the protein kinase C (PKC) inhibitor, H7, suggests that the enhancement in NAb binding observed after treatment with TPA is dependent upon PKC. However, this phenotypic change is not associated with the exposure to a non-tumor promoting PKC activating diacylglycerol suggesting that PKC activation, while being necessary, may not be sufficient for enhanced NAb binding. These observations help to explain the mechanism of chemical tumor promotion as well as contribute to our understanding of how tumor variants capable of eluding natural resistance mediated destruction are generated during tumor progression in vivo.

(A) TUMOR PROGRESSION: AN OVERVIEW

Tumor progression describes the gradual evolution of a tumor cell population towards an increasingly malignant phenotype. Central to this process is the generation of tumor cell variants with unique survival characteristics, which in the competative microenvironment of the tumor and in the presence of host immune and non-immune selective pressures are enriched for and subsequently become dominant. Although initially tissue associated gene expression programs may minimize the differences between neoplastic cells within a given tumor, variant generation and selection of advantageous traits would tend to obscure these gene programs resulting in a more undifferentiated and malignant phenotype. Theoretically, all tumor associated properties are susceptible to independent variation and selection resulting in tumor progression in the direction of enhanced independence from host regulation and surveillance (1,2).

The concept that tumors progress through a series of stages towards enhanced autonomy from host controls was predicted by Foulds in the 1950's (3). He theorized that the phenotype of a tumor was determined by "numerous unit characters which were morphologically and functionally heterogeneous", and that tumor progression is defined by a sequence of irreversible phenotypic changes. Today it is believed that the heterogeneity which arises in tumors is dynamic and that those phenotypic changes associated with progression are in fact reversible (4,5). The dynamic heterogeneity model of tumor progression predicts that the frequency of progressive variants within a tumor population is achieved by their effective rates of generation

and loss. For example, in both the B16 F1 and B16 F10 melanoma lines the majority of cells are effectively non metastatic. The enhanced metastatic capacity observed with the B16 F10 line is believed to be a direct result of its increased rate of metastatic variant generation (5).

The regulation of tumor progression in vivo is a complicated and poorly understood phenomenon. It is dependent upon both the mechanisms and kinetics of tumor variant generation as well as the nature of the selective pressures which are present. The regulation of tumor progression, as it relates to the ability of a tumor to escape detection and/or destruction by immune surveillance effectors, may be particularly important, especially in the early stages of neoplastic development.

(B) TUMOR HETEROGENEITY

The vast majority of malignant neoplasms are believed to be monoclonal in nature, implying that tumor cells found in both the primary site as well as metastatic foci have all arisen from the same original cell. In apparent conflict with this monoclonal origin of cancer is the immense cellular heterogeneity found within any particular tumor (reviewed in 6). Tumor heterogeneity was initially observed histologically in the form of morphological diversity of the cellular composition of tumors (7), however, since then it has been shown that most tumor characteristics are susceptible to independent variation on a cell to cell basis.

1) Forms of Tumor Heterogeneity

Intraneoplastic diversity is generally considered to occur at several levels. At the most basic level, a tumor is considered to be heterogeneous simply because it includes not only neoplastic cells, but also normal host components such as lymphocytes, macrophages and connective tissue (7). Although this stroma tumor melange is an example of tumor heterogeneity in every sense of the word, it is usually not included in discussions of neoplastic diversity. This heterogeneity in tumor microenvironment may however affect malignant progression, as will be discussed later.

The presence of benign foci within a malignant tumor defines a second form of heterogeneity. Cancer cell derived benign foci have been documented for several unique tumor systems, most notably ganglioneuroblastomas and teratomas (7,8). It has been proposed at least in the case of teratomas that neoplastic totipotent stem cells give rise both to neoplastic cells as well as differentiate into a range of normal tissue types including bone, liver, skeletal muscle and spleen.

A third form of tumor diversity is the variation among the malignant neoplastic cells of a single tumor. Cancer cells within a particular tumor can vary widely with respect to a range of phenotypic characteristics including enzymatic, biochemical, biological (ie. metastatic), genetic, and immunological traits (1,6,8). It is important to note that tumor heterogeneity generally refers to genetic or epigenetic changes transmittable down a cell lineage. This is different from the diversity in tumors which arises from cell cycle

determined differences in cellular phenotype.

It is also important to note that cellular heterogeneity can not be regarded as a property unique to neoplastic tissue. Indeed, the cells of normal tissues are generally not homogeneous with respect to individual cellular characteristics, and if one reflects on embryonic development, all multicellular organisms are essentially monoclonal in origin (1). What is unique about tumors as opposed to normal cellular heterogeneity is that for any given cell type neoplastic heterogeneity is usually more remarkable.

Neoplastic subpopulations derived from the same tumor can differ from one another in a wide range of characteristics. An excellent example of the extent of intraneoplastic heterogeneity within a single tumor can be seen with the DLD-1 human colon carcinoma system (7,9). In this particular model, histopathological examination of a human colon tumor revealed the presence of two distinct regions classified as either anaplastic or containing acinar structures. Based on differences in colony morphology in soft agar, two morphologically unique subpopulations (A and D) were isolated. It is felt that these two subpopulations are responsible for the histological diversity observed in the intact tumor (7). Subsequent studies have revealed that in addition to morphological heterogeneity the DLD-1 cell line, and derivative clones are also karyotypically diverse (7) and demonstrate differences in cell surface antigen expression (carcino-embryonic antigen expression and H blood group substance) (10) biochemical markers (purine-metabolizing enzymes) (11) and sensitivity to various treatment modalities (chemotherapeutic drugs, hyperthermia

and x ray irradiation) (7).

2) Mechanisms of Tumor Heterogeneity Generation

a) Genetic Instability.

It is currently believed that the progression of a tumor towards an enhanced malignant state may be associated with the accumulation of genome changes produced by random mutational events (1,6,12,13). This enhanced genetic instability can be manifested in a number of different forms including changes in chromsome number (resulting from chromosome loss or duplication), changes in individual chromosome morphology (due to sister chromatid exchange or breakage) changes in chromosome banding patterns, and an increase in spontaneous mutation rates (1,2). Such genetic instability occuring in the presence of host immune and non immune selective pressures may result in the evolution of a tumor more able to survive and grow.

There are a number of studies which support the role of genetic instability in the progression of tumors towards increased malignancy. Progression associated changes in chromosome numbers can be identified in several systems. The sequential acquisition of chromosomes 7, 13 and 12 have been associated with the progression of Rous sarcoma virus transformed fibroblasts (1), and cells obtained from human malignant melanoma metastatic foci have been shown to exhibit chromosomal number abnormalities not found in cells of the primary tumor (14). It should be noted however that changes in chromosome number are not always associated with progression and occassionally primary tumors have been shown to be aneuploid while the metastatic cells are

diploid, as is the case in a rat mammary tumor model (1). In this system, however, metastatic cells demonstrated other forms of chromosomal alterations such as the possession of a unique set of Giemsa stained chromosomal markers not found in non metastatic cells. Progression associated changes in chromosomal banding are also seen in a human small cell lung cancer model, where variant cell lines which display an increased malignant phenotype, also display changes in chromosomal homogeneous staining regions (15).

Changes in gross chromosomal morphology are also seen with tumor progression. For example, the increase in malignancy associated with the previously mentioned human small cell cancer lines is associated with deletions to chromosome 3 (1).

Currently, it is controversial as to whether enhanced malignancy is associated with increased rates of spontaneous mutations. While Cifone et al. were able to demonstrate that the higher metastatic potentials of murine melanoma and fibrosarcoma cells correlated with a six to seven times higher spontaneous mutation rate (17), Yamishina et al. were unable to show a similar correlation with mouse mammary tumor cells (18).

(i) Reasons for Genetic Instability. There are several possible explanations for the increase in genetic instability observed with tumor progression. One suggestion is that an initial acquired genetic alteration might lead to enhance genetic lability (2). It is possible that the entire genome could be destabilized if genes coding for enzymes involved in DNA synthesis and repair or proteins of the mitotic machinery were mutated. Alternatively, it is possible that an

initial change in chromosome number and/or morphology might produce an environment for enhanced genomic instability as a result of an increased chance of non disjunction during mitosis.

It is also possible that the genetic instability observed in some tumors may arise not from an acquired but rather an inherited defect (2). Although rare, this could be the case for tumors arising in individuals with chromosomal fragility syndromes such as Fanconi's anemia and ataxia telangiectasia. Here as a result of inherited flaws in DNA repair and housekeeping rapidly proliferating tumors cells may express genetic instability. This enhanced genetic instability in combination with the suppression of the immune system may be responsible for the increase in tumor frequency observed with some of these syndromes (8).

It is also conceivable that external factors may come into play (2). Examples of these factors include carcinogenic chemical and radioisotopes including the mutagenic therapeutic agents used in cancer treatment whose persistent presence may lead to chronic genetic lability. Other examples of external stimuli are transforming oncogenic viruses whose incorporation into the host genome may lead to permanent genetic instability.

(ii) Genetic Instability and Oncogenes. It has been known for some time that oncogenes are extremely important elements in the initial transformation as well as later progression of malignancies, and it may be possible that genetic instability randomly acts on these loci resulting in the inappropriate expression, concentration or functioning of their gene products (12). There are several levels at which

this may potentially take place. One of the best studied of these is the amplification of the actual gene. There are a number of examples of gene amplification being associated with enhanced malignancy. Schimke (22) reported that amplification of the dihydrofolate reductase gene is associated with increased resistance to methyltrexate in a leukemia model. Little et al. (15) demonstrated that variant lines of a human small cell lung cancer which were identified as being more malignant also demonstrated a 20-78 fold amplification of the myc oncogene. Brodeur et al. (19) showed that while 50% of patients with stage III and IV neuroblastoma had an amplification of the myc loci no such increase could be found in stage I and stage II disease. In contrast, Filmus et al. have reported that while adenocarcinoma of the ovary is associated with c-K-ras amplification, the level of amplification is unchanged with the clinical progression of the disease (20). Gene amplification is manifested karyotypically as either double minute chromosomes or homogeneous staining regions (12).

Chromosomal translocations, insertion of enhancer promoter elements in the region of a oncogene and deletion of repressor elements may also be possible explanations for altered gene expression. For example, an 8:14 chromosomal translocation which results in the myc oncogene being placed under the control of the immunoglobulin enhancer is associated with the development of Burkitt's lymphoma (16). In addition to altered levels of gene expression, chromosomal translocation may result in the generation of hybrid proteins. Such is the case with the Philadelphia chromosome associated with chronic

myelogenous leukemia. Here an exchange between chromosome 9 and 22 results in the formation of chimeric protein containing the functional domain of the c-abl gene product with a much stronger enzymatic activity (23).

It has proven much more difficult to relate tumor development in humans with somatic mutations of oncogene coding and regulatory sequences. Most examples reported so far involve members of the ras family, most likely due to the availability of an NIH 3T3 transfection assay which easily detects activated ras (1).

One conclusion which has arisen from the study of either individual oncogenes or multiple oncogenes and tumor progression is that the relationship appears to be complex. Depending on the tumor progression model being used, some oncogenes show enhanced expression while others may show little if any change, and still others demonstrate diminished expression. It appears that while tumor progression is undoubtedly often associated with changes in oncogene expression it is currently uncertain which if any of these changes are obligatory for an increase in malignancy.

b) Epigenetic Instability

Tumor heterogeneity can not be attributed to genetic instability alone if one considers the rates of spontaneous mutations in comparison to the rates of variant generation. Although mutation rates in the order of 10^{-6} to 10^{-8} mutations per generation are common, rates as high as 7×10^{-5} have been reported. However, even mutation rates this high cannot account for the 10^{-2} to 10^{-3} variants per generation

seen with tumor cells (1,6,17). In order to explain the rapid generation of tumor variants and revertants proposed in the dynamic heterogeneity models discussed earlier, a number of non genetic or epigenetic mechanisms of generating tumor heterogeneity have been proposed. Two of the better characterized mechanisms are discussed below.

(i) DNA Hypomethylation. Biochemically, DNA hypomethylation describes the covalent linkage of a methyl group to the carbon five position of cytosine (24,26,27). Methylcytosine essentially represents a fifth nucleotide. Because the methyl group does not affect hydrogen bonding, methylation does not interfere with either DNA replication or transcription. Approximately 80% of mammalian DNA is methylated, the pattern of which appears to be inherited from one generation to the next.

This form of DNA modification has been reported to result in changes in the activation state of genes (24,25,26,27,28). In particular, it has been reported that hypermethylation of a structural gene in the 5' end of the coding sequence often results in the suppression of gene expression. In addition, so called "housekeeping" genes which are constitutively expressed are often hypomethylated. However, for some loci there does not appear to be a definite correlation between degree of methylation and the state of gene activation (26,27).

There are a number of reasons why DNA hypomethylation is an attractive explanation for at least some of the phenotypic variation seen in tumor cell populations (6). (i) DNA methylation patterns

frequently undergo rapid changes similar to variant generation. (ii) As mentioned previously, DNA methylation patterns are transmittable over at least several generations. (iii) DNA methylation has been implicated in the developmental programs of normal tissues possibly explaining observed similarities between tumor progression and tissue development. (iv) The inappropriate expression of some genes seen during tumor progression can be accounted for by DNA methylation. (v) Although DNA methylation patterns are heritable they are also reversible and thus consistent with the generation of tumor revertants predicted in the dynamic heterogeneity model of tumor progression.

Much of the support for DNA hypomethylation comes from the experimental manipulation of methylation patterns in tumor cells with the hypomethylating agent 5-azacytidine (25,28,29). Several groups have demonstrated that the treatment of tumor cell lines with 5-azacytidine results in changes in their tumorigenicity and in particular, metastatic potential (29,30). However, these studies can not discount possible genetic effects of 5-azacytidine. In addition, the attempt to correlate the methylation states of different cells with their tumorigenic phenotypes have been inconsistent and at least in one study metastatic capacity and state of DNA methylation appeared to be independent (reviewed in 1).

(iii) Somatic Cell Fusion. Somatic cell fusion describes an epigenetic event in which tumor cells fuse with each other or with normal host cells to achieve hybrid phenotypes (1,31,32). This initial epigenetic event results in subsequent chromosomal and genetic changes. Consequently, the phenotypic instability generated by

somatic cell fusion may be viewed as both epigenetic and genetic in origin. Although considered to be an exceptionally rare occurence, somatic cell fusion has been reported in several systems to result in tumors expressing enhanced malignant phenotypes. One of the best examples of this is the Eb murine lymphoma whose progression to a highly metastatic form has been attributed to the somatic cell fusion with a normal macrophage followed by extensive chromosomal segregation (32). In addition, it has been demonstrated experimentally that hybridomas formed between non metastatic murine plasmacytomas and murine B cells show a substantial rate of hepatic as well as splenic metastasis (32,33).

c) Influence of the Microenvironment

A great deal of evidence suggests that the microenvironment of a tumor is very important in the regulation of gene expression and phenotypic stability (34,35). Small intratumor differences in innervation, vascularization, stroma enzymes, as well as the availability of nutrients, oxygen, growth factors and ions can lead to cellular as well as zonal heterogeneity within a tumor (1). This heterogeneity would be expected to be especially marked if individual tumor cells exhibited a differential responsiveness to particular elements of the microenvironment.

One component of the tumor microenvironment which has received a great deal of attention, mainly due to its role in metastasis is the tumor associated extracellular matrix and stroma. Differences in the extracellular matrix components produced by parenchymal cells,

mesothelial cells, fibroblasts and endothelial cells are believed to be critical factors in normal and neoplastic tissue development (7), and changes in the extracellular matrix have been shown to result in altered gene expression and phenotype (36,37,38). For example, B casein secretion by murine mammary epithelial cells is influenced differently by various basement membrane components (37,38).

The availability within the microenvironment of various growth factors, hormones and differentiation inducers also has a great effect on the development of tumor heterogeneity. Some of the best examples of this form of microenvironmental influences are the demonstration that at least for some malignant tumors, implantation into mouse embryos results in benign reversion. For example, the implantation of murine embryonal carcinoma cells, the stem cells of teratocarcinoma, into a mouse blastocyst results in their benign reversion, which is dependent upon direct tumor contact with the blastocele surface of the trophectoderm (39,40,41). In another study, Gerschenson et. al. was able to show that the development of malignant lesions following the implantation of B16 melanoma cells into murine embryos was greatly diminished in embryos implanted during the stage of ontogeny when premelanocytes migrate into the embryonic skin. Subsequently it was shown that embryonic skin conditioned media was capable of inducing melanoma cells to revert to a more benign phenotype as indicated by irreversible growth inhibition and altered morphology (42).

Another aspect of the microenvironment which is suspected of being involved in the generation of tumor heterogeneity is that of

host cell infiltrates. These include immune effectors such as lymphocytes, granulocytes, macrophages and mast cells as well as non immune cells such as fibroblasts. The effects of such infiltrating cells can be inhibitory or stimulatory depending on the condition (1). An excellent example of the ability of infiltrating host cells to modulate tumor cell phenotype was demonstrated by Yamashina et al. (43). Here it was shown that mutagenic oxidants secreted by activated macrophages stimulated the generation of 6-thioguanine resistant mammary tumor variants.

In addition to inducing the generation of cellular or zonal heterogeneity within the primary tumor, these host factors would be expected to have a great influence over the differences observed between primary and metastatic cells as well as between cells from different metastatic sites. If one considers the different microenvironments encountered by metastatic cells in the various tissues and organs of implantation, it is easy to appreciate the generation of diversity in metastatic foci originating from the same primary tumor.

4) Stability of Phenotypic Diversity

Although malignant cells display extensive phenotypic and genotypic instability, it is still possible to obtain in vivo grown tumor populations expressing a stable selected phenotype. One would predict from the preceeding discussion concerning the generation of tumor heterogeneity that these highly malignant phenotypes would be rapidly lost due to the appearance of tumor variants. This however, does not appear to always be the case. Both metastatic and natural

immune resistant tumor populations have been isolated which vary markedly from the starting parental line and whose phenotypes can be maintained during extended tissue culture (44,45,46). The reason for this is currently unknown, however, it has been speculated that it may be the result of individual cells and subpopulations exerting a stabilizing influence on each other. There is a small amount of convincing data to support this hypothesis. It has been demonstrated that subclones of differing metastatic abilities isolated from the B16-F10 melanoma line are highly unstable when individually cultured, generating extensive heterogeneity in terms of metastatic potential within 20 to 40 passages. However, the original uncloned B16-F10 line demonstrated remarkable phenotypic stability over the same time frame. Even more interesting was the observation that when the individual subclones were mixed and cultured the resulting polyclonal populations stability was similar to that of the B16-F10 line (47).

Although the mechanism behind the phenotypic stabilizing effect of polyclonal tumor populations is not well understood, Nicholson has suggested that it may be dependent upon a cell-cell junctional communication system which is lost when tumor cells are dispersed and allowed to grow as separate clones (1). It is interesting to note that tumor promoting agents such as 12-tetradecanoylphorbol-13-acetate (TPA) are also capable of inhibiting contact dependent intracellular communication (48,49). For example, the exposure of benign or normal epithelial cells to TPA results in an uncoupling of junctional communication mimicing what is seen in highly metastatic mammary epithelial cells (1). In addition, NIH/3T3 cells transfected with the

src oncogene demonstrate both enhanced activation of protein kinase C (the receptor for tumor promoting phorbol esters) and the disruption of gap junction mediated intracellular communication (50). These observations may help explain the well characterized tumor promoting effects of phorbol esters on carcinogen initiated skin (53).

The concept that tumor populations impose regulatory constraints upon individual tumor cells is extremely important to our understanding of tumor progression. A theoretical extention of the concept that individual clones lack the stability of a polyclonal population is that strong selective pressures placed on a tumor population may result in its phenotypic destabilization (51). If this is correct, incomplete immune surveillance or therapeutic intervention may be involved in driving tumor progression.

(C) PHORBOL ESTER TUMOR PROMOTERS

1) Characteristics of Phorbol Esters

It has been known for some time that phorbol esters are capable of promoting the progression of cancer from the early initiation stages of tumorigenesis to the eventual appearance of fully malignant lesions. Phorbol esters themselves are obtained naturally from plants of the Euphorbiaceae family, in particular, croton tiglium the source of croton oil (52). Early investigations on skin tumor promotion utilized croton oil as a source of active phorbol ester tumor promoters. It was during these studies that some of the basic features of tumor promoters were first resolved. One of the most significant of these is that although a tumor promoter is not

carcinogenic, it will increase the rate of appearance of tumors if applied chronically to skin initiated with a suboptimal dose of carcinogen. In addition, although tumor initiation is irreversible, promotion is not. This is to say that the application of a promoter is still effective even up to several months after carcinogenic initiation, however, it has no effect if it is applied prior to initiation (53).

Tumor promoting phorbol esters are structurally relatively complicated molecules. Briefly, they consist of a polyfunctional seventeen carbon tetracyclic diterpene moeity to which may be attached lipophilic side chains at position 12 and 13 (54). It has been demonstrated that even small changes in the structure of tumor promoting phorbol esters can result in a marked reduction in tumor promoting activity. For example, methylation of the hydroxyl group at position 4 or the deletion of the hydroxyl group at position 20 results in a substantial decrease or complete loss of activity respectively. It was this observation coupled with the knowledge that phorbol ester tumor promoters are very active even at nanomolar concentrations which hinted at the existence of a specific phorbol ester receptor (52). However, due to elevated non-specific binding resulting from the lipophilic side chains of the most potent tumor promoters the identification of a phorbol ester receptor came only after the synthesis of phorbol-12, 13-dibutyrate, a derivative with an enhanced ratio of specific to non-specific binding (55).

Concurrent with the identification and characterization of a phorbol ester receptor was the identification of a new type of protein

kinase activity referred to as protein kinase C (56). It was the similarities in tissue and phylogenic distribution of the protein kinase C activity and phorbol ester binding as well as analogous binding and activation requirements which led to the speculation that these were different properties of the same protein. Today, this statement is recognized as being true and moreover it has been ascertained that phorbol esters are potent stimulators of protein kinase C activity (52).

2) Characteristics of Protein Kinase C

Protein kinase C is a ubiquitous threonine/serine kinase found in evolutionary distinct organisms (56). Measurement of protein kinase C activity levels in various tissues has demonstrated that while it is present to some degree in all tissues, it is by far highest in brain and lung tissue as well as neutrophils (52). When first discovered, it was thought to be a proteolytically activated kinase, and although this is not entirely false, subsequent studies have revealed that it is more generally activated as a result of inositol phospholipid breakdown (57,58).

The role of inositol phospholipid metabolism in signal transduction was suggested over a quarter century ago when it was demonstrated that acetylcholine stimulated the rapid incorporation of ^{32}P into the phospholipid fraction of pigeon pancreas slices (59). It was later resolved that the synthesis of phosphotidyl inositol was actually a subsequent event to the generation of second messengers resulting from inositol phospholipid breakdown (60). Today, it is known that for a

wide range of cell growth and differentiation stimulators that the initial step in signal transduction is the breakdown of inositol phospholipids. In most cases, the interaction of a specific cell surface receptor with its appropriate ligand results in a G protein mediated activation of phospholipase C. This membrane associated lipase selectively cleaves phosphotidyl inositol-4,5 bisphosphate resulting in the generation of two important second messenger molecules inositol-1,4,5 triphosphate and diacylglycerol (61). Both arms of this second messenger system act synergistically to activate protein kinase C. Inositol 1,4,5 trisphosphate liberates Ca^{+2} from internal storage sites via a receptor mediated process, while diacylglycerol acts directly on protein kinase C (56,63). This appears to be a highly specific interaction in that only 1,2-sn-diacylglycerols are capable of activating protein kinase C, both 2,3-sn-diacylglycerol and 1,3-diacylglycerols are entirely inactive (62). Protein kinase C is biochemically dependent upon both membrane phospholipids (phosphotidylserine) and Ca^{+2} for activation. It appears however that the interaction of diacylglycerol with protein kinase C on a 1:1 stoichiometry results in an increased affinity for Ca^{+2} such that the enzyme may be activated even in the presence of basal levels of cytoplasmic Ca^{+2}. However, the enzyme is activated very efficiently by the synergistic effects of diacylglycerol and inositol 1,4,5 trisphosphate (56).

Both products of inositol phospholipid breakdown are extremely transient in nature. Inositol-1,4,5-trisphosphate is rapidly converted to a inositol-1,4 diphosphate by a specific phosphatase

(56), while diacylglycerol is either resynthesized back into inositol phospholipids via phosphotidic acid or catabolized further into arachidonic acid which may be involved in further messenger production (56). In addition, cytoplasmic Ca^{+2} levels are rapidly returned to baseline either by the re-uptake into internal stores or the extracellular excretion by Na^{+}/Ca^{+2} exchange and Ca^{+2} transport adenosine triphosphatase (56,64).

Receptor mediated degradation of inositol phospholipids results in the translocation of protein kinase C from the cytosol where it normally exists in an inactive state, to the inner leaflet of the plasma membrane. Here upon interaction with membrane associated phosphotidylserine, active protein kinase C phosphorylates a range of target proteins including membrane receptors (transferrin and interleukin 2), transport proteins (Ca^{+2} transport ATPase, glucose transporter, Na^{+2}/H^{+} exchange system), enzymes (NADPH oxidase, cytochrome P450) and a number of proteins of miscellaneous function (fibrinogen, myelin basic protein, HLA antigens) just to name a few (56). Membrane associated protein kinase C is also more sensitive to the Ca^{+2} dependent proteolytic enzyme calpain. Limited proteolysis by calpain results in the cytosolic release of a lower molecular weight protein kinase C activity that is independent of diacylglycerol, phosphotidylserine and Ca^{+2} (56,65,66). Target proteins for this protein kinase C activity includes cytoskeletal elements (67).

3) Protein Kinase C Mediated Response Heterogeneity

One of the most intriguing questions pertaining to signal transduction by protein kinase C is how does this system mediate the vast heterogeneity of stimuli/response patterns which have been attributed to it. Within a single cell type, protein kinase C may be involved in the relaying of information from several independent receptors resulting in very different response patterns. For example, protein kinase C appears to play a role in both the relaxation and contraction of vascular smooth muscle (56).

Currently, at least five levels at which heterogeneity may be generated have been identified.

(i) The subcellular location of the activated protein kinase C will determine what target proteins are functionally modified by phosphorylation. It would be expected that this may be dictated by the location of the receptor/ligand interaction (56).

(ii) The membrane lipid environment of the protein kinase C molecule is believed to affect both the binding affinity and kinase activity of the enzyme (68,69). Protein kinase C has been shown to have a substantially higher binding affinity for phorbol esters when in the presence of phosphotidylserine as compared to neutral phospholipids, while other lipid analogues such as palmitoylcarnitine have been shown to suppress basal protein kinase C activity.

(iii) The duration of the protein kinase C activation arising from a particular stimulus may qualitatively affect the nature of a given response. It has been proposed that prolonged protein kinase C activation may result in phosphylation events not associated with

transient activation (70).

(iv) Recently, it has been demonstrated in cloning studies that PKC activity is not attributable to a single homogeneous molecular species. Rather it appears that protein kinase C is a group of at-least seven very similar subspecies designated α, βI, βII, γ, δ, $\in$ and L (71). The latter three have only recently been identified, however some potential differences in cell type distribution and activation requirements have been determined for the α, βI, βII and γ protein kinase C subspecies (72,73). For example, T cells are known to contain the α, βI and βII but not the γ isozyme which appears to be specific to brain tissue. In addition, the various subspecies appear to differ somewhat in both their activation requirements (71) and their ability to be proteolytically down regulated (66). It is very tempting to speculate that at least some of the response heterogeneity observed in the protein kinase C system is the result of differential activation of specific protein kinase C subspecies.

(v) Heterogeneity in protein kinase C mediated responses may also result from the interaction of the protein kinase C signal transduction pathway with other second messenger systems. Protein kinase C and cyclic adenosine monophosphate (cAMP) dependent protein kinase A represent the two major second messenger signal transduction pathways found in the majority of cells. Although these two serine/threonine kinases are known to relay information via different channels, they have been associated with similar responses including the phosphorylation of identical target proteins (56).

What is of most relevance to a discussion of response heterogene-

ity is the potential for interaction of these two pathways. It has been proposed that one pathway may either abrogate (bidirectional control) or potentiate (monodirectional control) the effect of the other. For example in platelets, neutrophils and lymphocytes activation of cAMP dependent protein kinase appears to block inositol phospholipid turnover and consequently, inhibits protein kinase C dependent responses while the opposite appears to be true (protein kinase C blocks the cAMP dependent kinase pathway) in hepatocytes, cardiac muscle and xenopus oocytes. Alternatively, activation of protein kinase C has been demonstrated to potentiate cAMP production in a variety of cell types (56). The interaction between the two major signal transduction systems would allow for further response diversity in protein kinase C mediated events.

4) Protein Kinase C Involvement in Phorbol Ester Mediated Responses

It appears that both phorbol esters and diacylglycerols bind to the same regulatory site on the enzyme. Evidence for this comes in the form of kinetic and stoichiometry studies which indicate that diacylglycerols are capable of competitively inhibiting the binding of phorbol esters to protein kinase C (74). It is for this reason that many phorbol ester mediated effects are mimiced by diacylglycerols. Treatment of cells with synthetic diacylglycerols that are readily intercalated into intact cell membranes or with phospholipase C which cleaves membrane phospholipids into diacylglycerols or with inhibitors of diglyceride breakdown or triglyceride synthesis which result in diacylglycerol accumulation all have shown remarkable similarities to

phorbol ester mediated responses (56). These analogous responses include the regulation of surface antigens and receptors as well as the phosphorylation of a variety of cellular proteins (75,76,77). The dependence of many TPA responses on activated protein kinase C have been demonstrated by several approaches. For example, it is possible to establish a positive correlation between the extent of some biological responses and the binding affinity of TPA to protein kinase C (52,55). It is also possible to down-regulate cellular levels of protein kinase C through the long term exposure to phorbol esters. Such a loss of protein kinase C activity has been shown to result in a parallel loss of mitogenic responsiveness to phorbol esters in Swiss 3T3 cells (56). Some of the most convincing evidence for the role of protein kinase C in phorbol ester mediated events comes from reconstitution studies. The microinjection of purified protein kinase C into the previously mentioned Swiss 3T3 cells has also been associated with the restoration of phorbol-12, 13-dibutyrate mediated mitogenicity in vivo (78). Similar studies have demonstrated that phorbol ester mediated activation of NADPH oxidase in vitro requires a plasma membrane fraction, ATP, NADPH, phospholipids and a critical cytosolic component that could be replaced by purified protein kinase C (79).

The development of a potent as well as specific inhibitor of protein kinase C has greatly aided the study of TPA mediation of cellular responses. Until relatively recently, most inhibitors of C type kinase activity also had inhibitory effects on other crucial cellular enzyme systems such as calmodulin (80). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) and N-(2-guanidino-

ethyl)-5-isoguinolinesulfonamide hydrochloride (HA1004) are capable of inhibiting a number of serine/threonine protein kinase activities through the competitive inhibition of ATP binding to its site on the enzymes (81). While both block several additional kinases only H7 blocks protein kinase C effectively. Thus, when both are used in concert they provide relatively decisive information about the role of protein kinase C in a particular response. H7 has been used extensively over the past several years to demonstrate protein kinase C mediation of a range of TPA associated responses including HL-60 cell differentiation (82), transferrin receptor down regulation in human leukemia cells (83), and decreased vincristine uptake by murine P388 leukemia cells (84).

It appears however that not all phorbol ester effects can be attributed to the activation of protein kinase C alone. Binding analysis has demonstrated the heterogeneous nature of the phorbol ester binding site. It has been observed that tumor promoting phorbol esters demonstrate a greater degree of binding heterogeneity than do phorbol esters which cause inflammation only (non-tumor promoting), implying that a particular phorbol ester receptor or family of receptors may be associated with tumor promotion (85). These studies as well as the observation that both complete and incomplete tumor promoters are capable of activating PKC (52) imply that more than one receptor may exist for tumor promoting phorbol esters. However, it is unclear whether this heterogeneity is the result of various isozymes of protein kinase C with different activation requirements or whether it is indicative of a novel receptor system.

Inhibition by H7 also provided strong evidence that phorbol esters may have effects entirely independent of protein kinase C activation. A number of phorbol ester responses in various cell types have been shown to be insensitive to H7 mediated inhibition. In at least one study, H7 was demonstrated to be incapable of blocking TPA induced neutrophil activation as measured by degranulation and the onset of a respiratory burst (86). Instead it was revealed that neutrophil activation by TPA could be blocked by W7 a calmodulin inhibitor implying that for at least some systems phorbol esters may activate calmodulin via a PKC independent pathway. Other investigators have found that only the degranulation response to phorbol esters is insensitive to H7, and that superoxide anion release is in fact PKC dependent (87). In addition TPA has been shown to substitute for the IL-1 requirement during anti-TCR-CD3 mediated activation of a murine T cell line. In this system, crosslinking of the TCR-CD3 complex alone results in prolonged activation of protein kinase C, while IL-1 is believed to act via a protein kinase C independent pathway (cited in 70, 88). These results imply that certain TPA induced responses may be independent of protein kinase C activation.

The existence of a phorbol ester responsive signal pathway in addition to protein kinase C may also help to explain why some but not all TPA responses can be mimiced by diacylglycerols. For example, synthetic diacylglycerols are ineffective activators of HL-60 differentiation (89,90) or anti-CD3 mediated proliferation of periferal T cells (91), two well studied TPA inducible models. Diacylglycerols have also proven to be incapable of substituting for

phorbol esters in the induction of IL-2 receptors or the down regulation of CD7, CD4 and CD3 on peripheral T cells (91,92,93). Differences between diacylglycerol and phorbol ester responses have been demonstrated even when cells are continuously restimulated with diacylglycerol ensuring that the disparity is not a result of the rapid disappearance of the kinase activator (89).

Although it is inviting to attribute observed differences in phorbol ester and diacylglycerol response patterns on the activation of unique receptors by TPA, differences in the regulation of protein kinase C may be a suitable alternative explanation. Recently, it has been reported that exogenous diacylglycerols but not phorbol esters are capable of activating a neural sphingomyelinase in GH_3 pituitary cells, an event believed to be independent of protein kinase C activation. The resulting products of sphingomyelin turnover are thought to inhibit protein kinase C activity and block its cytosol to plasma membrane translocation (94).

5) Role of TPA and Protein Kinase C in Tumor Promotion and Progression

Although it has been known for some time that phorbol esters such as TPA are potent promoters of tumor formation in carcinogen initiated murine skin, the mechanism of tumor promotion and the role played by TPA is still controversial. Most models agree that following initiation with a suboptimal dose of carcinogen, a step most likely associated with cytogenetic changes (possibly point mutations) (95), two additional stages are required for the development of a malignant

tumor. "Conversion" involves additional cytogenetic alteration to the genome of initiated cells resulting in their conversion from a benign to a malignant phenotype, while "promotion" describes a clonal expansion of cells which is most likely independent of any further mutational events.

Furstenberger et. al. advocate an initiation-conversion-promotion protocol for multistage tumor development, which is based on papilloma formation as being the end point of skin tumor promotion and clearly differentiates between complete and incomplete (second stage) tumor promoters (96). According to this scheme complete tumor promoters such as TPA are capable of mediating both conversion and promotion of initiated cells while second stage promoters such as mezerein can promote but lack the ability to convert. This model would explain the inferior ability of second stage tumor promoters in facilitating the development of papillomas in carcinogen initiated skin, except when the initiated skin is first treated with a convertogen such as TPA (97). Consistent with these observations it has been reported that clastogenic compounds, structurally and mechanistically unrelated to phorbol esters can replace TPA in this conversion stage of tumor development (ie. synergize with second stage promoters resulting in enhanced tumor formation) (96). These compounds directly mediate chromosomal alterations and damage but are unable to initiate tumorigenesis possibly as a result of their inefficient induction of point mutations in mammalian cells.

While convertogenic complete promoters have been observed to induce chromosomal aberrations, non-convertogenic second stage

promoters appear to lack this capacity (98). The chromosomal damage associated with TPA treatment appears to be mediated by endogenously produced clastogenic factors including arachidonic acid metabolites such as prostaglandin $F_{2\alpha}$. Consistent with their lack of convertogenic ability, second stage tumor promoters do not appear to stimulate the accumulation of these arachidonic acid metabolites (99).

Hennings et. al. supports an initiation-promotion-conversion protocol for multistage tumor development. This model considers the conversion of papillomas to carcinomas as a measure of skin tumor development, and therefore, may be more indicative of true tumor progression (100).

It is speculated that TPA induces the clonal expansion of initiated cells in the form of a papilloma. However, carcinoma formation requires exposure to an additional genotoxic agent such as urethane, which most likely mediates this conversion via a genetic mechanism. The role of TPA in tumor promotion would therefore be to clonally increase the number of initiated cells bearing critical mutations, thus providing a larger target population for a second genetic change induced by the converting agent. The inability of urethane to induce carcinomas in either the absence of chronic TPA exposure, or when TPA mediated papilloma formation is prevented by an inhibitor of promotion such as fluccinolone acetonide, indicates the importance of the papilloma stage in carcinoma development. As would be expected in this model, the application of a tumor promoter inhibitor after the formation of a papilloma does not block urethane mediated conversion.

This model also suggests that a heterogeneity exists for the papillomas formed during the promotion stage (100). While chronic TPA treatment of initiated skin results in the development of many papillomas only a portion appear to have the potential for conversion to carcinomas. These papillomas generally appear early on during TPA treatment and tend to persist even when treatment with the promoter is discontinued. In contrast, those papillomas which develop later during TPA exposure do not convert to carcinomas and generally regress when promoter treatment is stopped. Although incomplete promoters generate fewer papillomas, those which do form behave like the persistent papillomas observed during the early stages of TPA mediated promotion, and indeed both classes of promoters produce equal numbers of carcinomas. Consequently, this model predicts that there is very little functional difference between complete and incomplete tumor promoters.

The role of protein kinase C in multistage tumor development is not entirely clear (101). It has been established that in addition to inducing some of the same responses in murine skin (ie. acute hyperplastic and inflammatory activity) both complete and second stage tumor promoters bind to and activate protein kinase C (101). Although the kinetics of this activation appear to be somewhat different (ie. second stage tumor promoters such as bryostatin 1, may cause an accelerated degradation of protein kinase C relative to the rate of degradation seen in phorbol ester treated cells), the observation that both complete and incomplete tumor promoters appear to cause the clonal proliferation of cells which characterize the promotion steps

in either model of multistage tumor development implies the potential involvement of protein kinase C.

In addition to their effects on the promotion stage of tumor development, phorbol ester tumor promoters and activated protein kinase C have the ability to induce some of the tumor associated phenotypic changes observed in the later stages of progression. As previously mentioned, both TPA and the activation of protein kinase C have been associated with the inhibition of contact dependent intracellular junctional communication, an event believed to mediate the phenotypic destabilization of polyclonal tumor populations and result in the generation of tumor cell heterogeneity (1). Moreover, phorbol ester tumor promoter treatment of a variety of cell types has been shown to induce several specific phenotypic alterations associated with tumor progression. These include the induction of multidrug resistance (102), increased metastatic capacity (103), secretion of collagenase and other proteases (reviewed in 104), induction of anchorage independent growth (reviewed in 104) and increased resistance to various immune effectors (105,106).

The involvement of the protein kinase C signal transduction pathway in the progression of malignant neoplasia is highlighted by its apparent interaction with a number of oncogenes and oncogene products. At least four different transforming gene products are known to affect inositol phospholipid metabolism most likely resulting in the modulation of protein kinase C activity. For example, both the src product and the polyoma middle T antigen have been associated with increased levels of phosphatidylinositol 4,5 bisphosphate, the

substrate of phospholipase C (52). This is thought to be the result of enhanced phosphatidylinositol kinase activity. Ras expression has been reported to result in both elevated levels of diacylglycerol (107) and the down regulation of protein kinase C (108). Recently, it has been demonstrated that antibodies to phospholipase C can inhibit ras stimulated DNA synthesis, implying that the ras protein may be a upstream effector of phospholipase C activity (257). In addition, sis and erb B have also been linked to inositol phospholipid turnover (52). Alternatively, protein kinase C activation has been associated with the expression of nuclear oncogenes such as myc and fos, and recently it has been shown that the expression of a mutant protein kinase C gene in normal 3T3 fibroblasts results in cellular transformation (109).

(D) IMMUNOSURVEILLANCE

As early as 1909, Paul Ehrlich proposed the idea that if not for host defense mechanisms cancer would arise at a remarkable frequency (110). Research into transplantation immunity in the late 1950's led to the suggestion that the immune effectors involved in allograft rejection were also responsible for the rejection of malignant cells (111). This hypothesis was further defined by Burnet who coined the term immunosurveillance to describe this natural defence against incipient neoplasia (112). This theory was based on the concept that T cell mediated immune mechanisms are capable of responding to and eliminating immunogenic tumors. This was especially appealing since it assigned a function to T cell mediated immunity, which up to then

only appeared to be involved in experimental allograft rejection. It was even speculated that defense against malignant neoplasia was the major evolutionary selective pressure for the development of the T cell immune system.

Today, however, it is widely accepted that T cell immunity developed in response to the pressures of viral infections (113), and substantial evidence suggest that other immune effectors in addition to T cells may play a role in tumor immunosurveillance. This includes the observation that athymic mice demonstrate neither an increased rate of spontaneous tumor development nor a decreased ability to reject chemical carcinogen induced neoplasia (114). In addition, many tumors were shown to be non immunogenic in vivo even when co-injected along with lymph node cells from tumor bearing animals (115). Some investigators have also expressed skepticism as to whether the T cell immune system has the ability to respond fast enough to incipent neoplasia for it to be of any value in tumor immunosurveillance (116).

These observations coupled with the demonstration that lymphocytes from immunologically normal animals are capable of lysing a variety of tumor cells (117,118), lead to the proposal that tumor immunosurveillance may be based on a non-adaptive thymus independent immune response (119). Currently, this natural tumor immunosurveillance is attributed to three individual effector systems, natural killer (NK) cells, activated macrophages and natural antibodies (NAb).

1) Natural Antibodies

It has been known for some time that the serum of immunologically normal animals contains antibodies that are reactive with antigens to which the animal had never intentionally been immunized. Collectively, these antibodies are termed natural antibodies and they have been demonstrated in a variety of species including rodents, reptiles, birds and humans (120). Natural antibodies have been shown to react with synthetic haptens, viral antigens, autologous serum proteins such as albumin and transferrin as well as a variety of normal autologous cells including thymocytes, lymphocytes, brain tissue and neuraminidase treated erythrocytes (121,122, reviewed in 120). Normal murine serum has also been shown to react with a variety of allogeneic and syngeneic tumors (123,124,125,126). Accordingly, natural antibodies are suspected of being involved in tumor immunosurveillance (127), and there is considerable evidence suggesting that they are extremely potent against small tumor foci (128 and reviewed in 129).

a) Characterization and Regulation of Tumor Reactive Natural Antibodies

The origins of naturally occuring anti-tumor antibodies are uncertain. It is suspected that they may represent a population of cross reactive antibodies stimulated by either foreign agents (ie. microbiol intestinal flora) or endogenous stimulants (ie. cryptic self antigens). It has also been suggested that natural antibodies may either be the products of internally activated B cells in association with idiotypic networks and/or that they are representative of a germline V region sequence which codes for autoreactive specificities

(120,129).

Tumor reactive natural antibodies appear to be primarily of the IgM isotype and to a lesser degree of the IgG and IgA isotypes (120). The observation that both nude mice (130) and thymectomized bone marrow reconstituted mice (131) exhibit normal levels of natural antibodies implies that natural antibodies are a product of thymus independent immunity. In agreement with this it has been shown that mice bearing the xid mutation demonstrate both reduced humoral immunity to thymic independent type 2 antigens as well as decreased levels of circulating tumor reactive natural antibodies (128,132). This mutation is also accompanied by the absence of peritoneal $Ly\text{-}1^+$ B lymphocytes and Lyb $3^+5^+7^+$ and $IaW39^+$ splenic B cells, suggesting that one or both of these lymphocyte subpopulations may be involved in natural antibody production (128). It also appears that the ability to produce high levels of tumor reactive natural antibodies is a recessively inherited genetic trait (126). Levels of tumor reactive natural antibodies have been shown to increase both with age, and in response to immune adjuvants such as proteose peptone and bacterial lipopolysaccharides as well as with interferon γ inducers (126).

Investigations involving monoclonal natural antibodies have demonstrated some important features pertaining to the nature of the natural antibody/antigen interaction. Avrameas et al. has shown that monoclonal natural antibodies appear to exhibit a broad range of cross reactivity (133). In addition, a possible synergistic effect of polyclonal natural antibody binding to tumor cells has been observed with monoclonal natural antibodies. Colnaghi et al. using a panel of

hybridomas generated from fusions involving normal spleen cells has demonstrated that the binding of one monoclonal antibody population to a tumor target actually facilitated the enhanced binding of other monoclonal natural antibodies (134).

Several groups of tumor associated antigens including viral proteins, MHC molecules, autologous and developmental antigens and the carbohydrate moiety of glycolipids and glycoproteins are known to be reactive with natural antibodies (reviewed in 129). For example, the tumor reactive natural antibody activity of syngeneic murine serum can be absorbed by fetal tissue and autologous thymocytes (120). Although it has been demonstrated that the in vivo passage of tumor cells results in the selection of variants expressing a decreased natural antibody binding phenotype (135), the biochemistry, function, and regulation of those antigenic structures recognized and selected against during NAb mediated immunosurveillance are not clearly understood. It has been proposed however that immunoselection by natural antibodies may contribute to the appearance of altered surface glycosylation patterns found within progressing tumors (128).

b) Role of Natural Antibodies in Immunosurveillance

Although tumor immunosurveillance is most often attributed to cellular effectors such as natural killer cells and activated macrophages, there is a substantial and growing body of evidence supporting a role for natural humoral immunity in the defense against incipient neoplasia. Currently it appears that natural antibodies may provide potent resistance against small tumor foci. Evidence supporting this view includes:

i) Winn Type Assays - Tumor cells preincubated in syngeneic serum (containing natural antibodies) demonstrate a significant reduction in tumorigenicity when assayed in a threshold subcutaneous tumor inocula model (131).

ii) Selected Cells - Tumors obtained from the injection site of a threshold subcutaneous tumor inoculum demonstrate both a reduced sensitivity to complement mediated lysis by natural antibody and a decreased ability to bind syngeneic natural antibody (44,135,136). Correspondingly, these cells exhibit an enhanced tumor frequency when the tumorigenicity of threshold inocula was assayed in syngeneic animals and a reduced rate of elimination from syngeneic mice. In agreement with these observations is the demonstration that in vitro selection of tumor cells for resistance to complement mediated lysis by natural antibodies results in a tumor population expressing a reduced susceptibility not only to NAb in vitro but also to anti-tumor natural resistance measured in a tumor elimination assay in vivo (137).

iii) Differences in the Level of Circulating Natural Antibodies - It has been shown in a number of systems that tumor resistance in vivo corresponds to circulating tumor reactive natural antibody levels (128,131). Biological response modifiers such as bacterial lipopolysaccharides and proteose peptone result in both an increase in natural antibody levels in vivo, as well as a decrease in the frequency of tumors arising from threshold subcutaneous tumor inocula (131). The best evidence for the involvement of natural antibodies in tumor resistance is the increased frequency and decreased latency of tumors

developing from threshold subcutaneous inocula in xid bearing mice. While these mice exhibit normal or even slightly elevated levels of cytolysis by natural killer cells and activated macrophages they have substantially lower levels of circulating natural antibodies (128).

c) Mechanism of Natural Antibody Mediated Tumor Resistance

Although complement mediated cytolysis by natural antibodies has been studied extensively in vitro it is unlikely that this lytic mechanism is important in vivo, as indicated by the normal clearance rates of intraperitoneal tumors seen in C5 deficient and C3 depleted mice (129). Although there is a lack of good experimental evidence supporting any one particular mechanism of natural antibody mediated tumor cytolysis and/or cytostasis, several potential mechanisms have been proposed.

i) Opsonization - Natural antibodies are known to augment the phagocytosis of several non neoplastic cells, particularly those of hematopoietic origin, including thymocytes (138), ageing platelets (139) and effete red blood cells (140). It has been proposed that the opsonization of tumor cells by natural antibodies may facilitate their phagocytosis in vivo.

ii) Antibody Dependent Cellular Cytotoxicity - It has previously been demonstrated that a natural antibody directed natural killer cell effector mechanism may be involved in the rejection of bone marrow allografts (141). A possible role for antibody dependent cellular cytotoxicity has also been suggested for natural antibody mediated cell lysis (142).

iii) Interference with Cellular Functions - Natural autoimmune

antibodies directed towards cellular receptors are known to disrupt normal cellular metabolism and result in the manifestation of several autoimmune diseases, most notably myasthemia gravis (acetylcholine receptor) and atrophic autoimmune thyroditis (thyroid stimulating hormone receptor) (143). It has been suggested that a similar mechanism may function during natural antibody mediated natural resistance (128). In the same respect, it has been suggested that natural antibodies directed towards cell adhesion molecules may interfere with tumor cell dissemination and subsequent metastasis (128).

2) Natural Killer Cells

The existence of natural killer cells was first suggested in the early 1970's when it was observed that lymphoid cells from unsensitized animals and humans could lyse a variety of tumor cell lines (117,118,144). Although this phenomenon was first attributed to background or artifactual lysis, it has since become apparent that this unique lymphocyte population is important to both antitumor and antimicrobiol defence as well as playing a role in the regulation of hematopoiesis and immune functions (reviewed in 145, 146).

a) Characterization of Natural Killer Cells

Natural killer cells are a heterogeneous bone marrow derived large granular lymphoid population. Although it was earlier proposed that NK cells may be part of the T cell lineage, representing an immature T cell population, recent evidence suggests that NK cells are not pre-T cells and may develop along independent lines (145,146).

Morphologically these cells can be classified as large granular lymphocytes possessing slightly indented nuclei and distinctive azurophilic cytoplasmic granules, which are positive for acid phosphatase and β glucuronidase and arylsulfatase suggesting that they are primarily lysosomes. Analysis of NK cell surface marker composition has identified human NK cells as being positive for NKH-1 (leu 19) and CD16 Fc receptors although they are negative for surface immunoglobulin. Murine NK cells are positive for asialo GM1 as well as NK1.1 and/or NK1.2 (reviewed in 145 and 146).

Natural killer cells do not express the CD3 T cell receptor complex and do not demonstrate rearrangement of the α, β, γ and δ T cell receptor genes. NK cells may however express non functional β and δ T cell receptor transcripts (145) and have been reported to express the CD3 zeta chain (161). NK mediated cytotoxicity also appears to be MHC unrestricted meaning that it does not depend on the expression of class I or class II antigens for the functional recognition of a sensitive target cell (reviewed in 145 and 146). These findings imply that NK cells do not use the T cell receptor for target recognition.

b) Role of Natural Killer Cells in Tumor Defence

There is a substantial body of evidence supporting the role of NK cells in antitumor immune surveillance. In addition, research into LAK cells of which NK cells may be a major functional component has highlighted the clinical potential for NK cells in mediating the destruction of established malignant neoplasia.

The availability of mutant beige mice which demonstrate impaired

NK activity but normal T cell function has provided a good model for the study of NK involvement in tumor defense (147,148). Previously, it has been shown that NK sensitive tumors display a decreased induction time and enhanced rates of growth and metastasis in beige mice as compared to normal heterozygous littermates. This phenomenon could not be replicated with an NK resistant tumor (147), consistent with the concept that NK cells provide protection against various parameters of tumor growth in vivo.

Studies employing the adoptive transfer of NK cells into NK deficient mice also supports a role for this immune effector in tumor surveillance. One of the best examples of this involves the reconstitution of NK deficient γ-irradiated or mutant beige mice with an NK cell line. Such mice showed an enhanced rate of allogeneic bone marrow rejection, the suppression of lung tumor colony formation following the i.v. injection of B160/F10 melanoma cells, and a decreased incidence of radiation induced thymic leukemia as compared to unreconstituted controls (149). Human NK cell deficient conditions such as Chediak Higashi syndrome are associated with enhanced rates of lymphoid tumors, implying that natural killer cells may also be involved in tumor defence in humans (150).

Natural killer cells also play a role in antimicrobial defence in particular protection against viral infection. The depletion of NK cells following the treatment of mice with anti-GM1 and anti-NK1.1 antibodies is associated with enhanced susceptibility to encephalomyocarditis virus, influenza virus, Coxsackie B virus, vaccinia virus and murine cytomegalovirus (145). In addition, the adoptive transfer of

adult NK cells to young mice results in protection against murine cytomegalovirus infection (151). Similarly in humans, NK cell deficiencies appear to predispose individuals to the development of viral diseases such as varicella virus and cytomegalovirus infection (152).

The nature of natural killer cell mediated anti-viral defence is for the most part unknown, however, it appears that the killing of virus infected target cells may involve the secretion of tumor necrosis factor and at least in some cases involves HLA-DR^+ accessory cells (145).

c) Natural Killer Cell Recognition of Sensitive Targets

The nature of target cell recognition by NK cells is a problem which has confounded researchers for years. At the Fifth International Workshop on Natural Killer Cells (1988), it was once again reiterated that NK cells do not recognize sensitive targets via cell surface immunoglobulins or the T cell receptor complex, due to the apparent lack of these structures on the surface of functional NK cells (145). However, the presence of Fcγ receptors (CD16) on the cell surface does allow NK cells to mediate the antibody dependent cellular cytotoxicity of antibody coated targets (153). Lanier et. al. have recently suggested that the expression and signal transducing capacity of CD16 is dependent upon the coexpression of CD3 zeta chain, a membrane antigen thought to play a similar role in the expression and functioning of the T cell receptor complex (161). Recently, two biochemically dissimilar NK cell surface molecules have been identified which may be potential candidates for NK cell recognition

structures. Preliminary results demonstrated that antibodies directed against either of these molecules block effector/target binding as well as efficient lysis of sensitive targets (reviewed in 145). In addition, NK cells have been reported to express a laminin-like molecule on their cell surface which is believed to interact with matrix laminin or laminin receptors on sensitive targets (reviewed in 146). Although its precise role is uncertain, it has been suggested that this laminin-like molecule may function as a novel recognition/activation structure used by cells mediating non MHC restricted cytotoxicity.

Recently, it has been shown that a 60 kilodalton disulfide linked dimer on the surface of NK cells is capable of mediating transmembrane signalling. This molecule shows significant homology to C type animal lectins that share receptor characteristics. It has been proposed that this protein may function as a receptor able to selectively trigger the killing ability of NK cells (154).

There are several NK associated surface antigens which appear to provide an accessory function in NK cell mediated cytotoxicity. Although it is uncertain whether these structures have the signal transducing capacity required for NK cell triggering they may function to enhance the binding affinity of NK/target conjugates. These include the integrin LFA-1 which recognizes target associated adhesive proteins, as well as the receptor for C3bi complement fragments which may facilitate binding to complement coated targets (reviewed in 146).

Equally puzzling is the nature of the target structure which is recognized by functional NK cells. There appears to exist an inverse

correlation between the expression of class I MHC antigens on target cells and sensitivity to NK mediated lysis (155,156). However, this inverse relationship between MHC Ag expression and NK sensitivity was not observed in other studies (157).

It has been reported that protease digestion of cell surface associated molecules on NK cell sensitive targets results in enhanced resistance to NK mediated lysis. Early investigations utilizing detergent solubilized material revealed several potential NK cell target structures. Roder et al. demonstrated that the preincubation of NK cells with the detergent solubilized cell surface proteins of the NK sensitive YAC lymphoma inhibited subsequent binding to untreated YAC targets. At least three putative NK cell target structures, assigned molecular weights of 130 K, 160 K and 240 K, were identified by SDS PAGE. This inhibitory activity could be retained on concanavalin A sepharose columns implying that the target structures were glycosylated. NK resistant cell lines did not appear to express these NK target structures (158). Correlations between the level of expression of particular cell surface antigens on target cells and their sensitivity to NK cell lysis, as well as the blocking of NK cell mediated lysis by antibodies specific for target cell surface antigens has led to the identification of several potential target structures including CD15 (146), a 42 kDa phylogenetically primitive homodimer (160) and the transferrin receptor (although it is controversial) (146).

There is some evidence to support the role of target cell surface carbohydrates in natural killer cell recognition. There exists a

correlation between NK cell reactivity and the amount of neuraminidase releasable sialic acid (162), as well as the demonstration that certain monosaccharides can block NK cell mediated lysis (163). It has also been shown that a defect in the synthesis of asparagine linked oligosaccharides resulting in decreased mannose and increased fructose expression on a target cell surface correlated with enhanced recognition and lysis by NK cells (159).

It has been suggested that differentiation and oncofetal antigens may represent potential NK cell target structures. In several normal and tumor cell models undifferentiated cells tend to be more susceptible targets than their differentiated counterparts (164,165,166). For example, adult primary hematopoietic tissue is more resistant to NK mediated lysis than fetal thymic and bone marrow cells (166). In addition, tumor promoting phorbol ester mediated differentiation of an NK cell sensitive tumor cell line results in diminished NK cell recognition and lysis (164).

d) Target Cell Lysis

It has been proposed that NK cell lysis of sensitive targets takes place via several steps. After a Mg^{+2} dependent binding step target cells enter into a Ca^{+2} dependent lytic programming phase which in turn proceeds to Ca^{+2}/Mg^{+2} independent target cell disintegration. NK cells are believed to mediate this cytotoxicity through the secretion of several cytotoxic molecules found within cytoplasmic granules (reviewed in 146). A particularly important lytic event is the formation of lesions within the target cell membrane as a result of the insertion of the pore forming protein cytolysin. It has been

proposed that upon secretion cytolysin monomers polymerize to form ring like pores in the target cell membrane. This molecule appears to display some similarities with C9 which is also a pore forming molecule (reviewed in 167).

A number of enzymes have also been associated with NK mediated cytolysis. The use of specific inhibitors has demonstrated a role for serine esterase (168), arylsulfatase (169) and phospholipase A_2 (170) in target lysis, although it is uncertain at which stage these enzymes act. Recently, it has been shown that the addition of serine esterase greatly augments the lytic activity of cytolysin isolated from large granular lymphocyte granules (145).

Natural killer cells have also been shown to release cytokines including tumor necrosis factor (TNF) (145,146) and NK cell cytotoxic factor (NKCF), a 33-43 KDa effector proposed to have lytic activity distinct from TNF (145,146). Sevilla et al. following kinetic evaluation of NK cytotoxicity has proposed that two distinct mechanisms of NK cell mediated target cytolysis exist (171). Pore formation by proteins such as cytolysin comprise a rapid first order cytolytic mechanism which is followed by a slower secondary mechanism involving the release of target cell specific cytotoxic factors such as NKCF.

The triggering of target cell apotosis by NK cells has also been suggested. Similar to what is observed with cytotoxic T lymphocytes, NK cell mediated lysis of certain sensitive targets is associated with nuclear damage in the form of DNA fragmentation. The observation that the extent of DNA degradation is dependent upon which species the target cells are obtained from, implies that the degradation is most

likely due to the activation of target cell endogenous endonucleases (146).

3) Activated Macrophages

a) Characterization of Activated Macrophages

Macrophage mediated tumor cytolysis is another potential antineoplastic effector mechanism. Although macrophages from immunologically normal individuals demonstrate little if any cytotoxicity it is possible to recover activated macrophages following the in vivo stimulation with immune activating agents such as Bacillus Calmette Guerin (BCG), and soluble protein antigens (172,179). Immunologically activated macrophages have been shown to mediate the destruction of bacteria, single and multicellular parasites, fungi, viruses, and a variety of tumor cells (172,180). Activated macrophages appear to be largely non-specific in terms of their cytotoxicity. However some degree of selectivity must exist as evidenced by their inability to lyse normal non-malignant cells even when co-assayed with sensitive tumor targets (173). There are numerous examples of activated macrophage mediated tumor cytolysis and cytostasis as well as evidence supporting the involvement of activated macrophages in the defence against incipient neoplasia in vivo (174,175,176).

b) Activation and Regulation of Cytotoxic Macrophages

The activation of cytotoxic macrophages appears to require at least two steps, priming and triggering. Macrophage priming is mediated by one of several T cell derived lymphokines such as

interferon-γ. These lymphokines convey to the macrophage a transient short lived potential for the development of a cytotoxic phenotype. The actual onset of cytotoxicity requires an additional triggering signal which may be provided by a wide range of agents including heat killed gram positive bacteria, bacterial lipopolysaccharides or a number of lymphokines including interleukin-1 (IL-1) and tumor necrosis factor (TNF) (172,177,178).

During the course of an immune response the appearance of non-specific cytotoxic macrophages appears to be both transient and short lived. For example, tumoricidal macrophages can only be isolated from the peritoneal cavity of BCG immune mice 9 hours to 72 hours after challenge with PPD (179). This loss of macrophage mediated cytotoxicity is irreversible and may be dependent upon macrophage differentiation (172). The duration and intensity of cytotoxicity may be controlled in an autocrine manner by several macrophage derived factors. For example, both macrophage derived interferon-α and α-2-macroglobulin-protease complexes are able to suppress superoxide release by macrophages. In contrast, the secretion of reactive oxygen intermediates by macrophages is enhanced by tumor necrosis factor (180).

c) Mechanisms of Macrophage Mediated Cytotoxicity

Macrophage mediated tumor cytotoxicity is a complex process dependent on the secretion of a vast array of potentially toxic products (180). Target destruction seems to be dependent upon effector target contact, but not necessarily phagocytosis (181). Some macrophage derived secretory products that have been demonstrated to

be directly cytotoxic and/or inhibit cellular proliferation, include IL-1α, IL-1β, transforming growth factor-β (TGF-β), TNF, complement degradative products, hydrolytic enzymes (including proteases), reactive intermediates of oxygen and nitrogen and bioactive lipids such as prostaglandin E_2 (180).

Tumor necrosis factor appears to play a central role in the tumoricidal effects of activated macrophages and like cytotoxic macrophages the effects of TNF appear to be largely specific for transformed cells (neither usually shows significant toxicity toward normal cells) (182,183). Tumor cells selected for resistance to TNF concomitantly demonstrate a decreased sensitivity to activated macrophages (183). In addition, antibodies to TNF were shown in several investigations to block macrophage mediated cytolysis of sensitive tumor targets (183).

TNF is a 157 amino acid peptide produced by a variety of immune cells (184). Although the mechanism responsible for the direct tumoricidal potential of TNF is not entirely understood it does appear to depend on the expression of a TNF receptor on sensitive targets (185). The cytotoxic effect of TNF appears to be enhanced by protein synthesis inhibitors, leading to the suggestion that the synthesis of particular polypeptides may protect normal cells and TNF resistant tumor variants from TNF and consequently macrophage mediated killing (186). While TNF is known to be a secretory product of activated macrophages there is evidence to support the role of a membrane bound TNF species in some macrophage mediated killing. Anti-TNF antiserum has been shown to react with fixed activated macrophages and more

importantly, to block the lysis of sensitive tumor targets by fixed activated macrophages (187). In addition to its direct tumoricidal effects TNF is an important mediator of inflammation and may interfere with malignant neoplastic development by disrupting the vascularization of a tumor (188).

The production and secretion of reactive oxygen metabolites is another proposed mechanism of macrophage mediated tumor cytotoxicity. The perturbation of the macrophage membrane triggers the reduction of molecular oxygen (O_2) to a toxic reactive superoxide species (O_2^-). Superoxide molecules may then be further metabolized to hydrogen peroxide by superoxide dismutase. Other reactive oxygen metabolites produced during this respiratory burst include hypohalous acids and hydroxyl radicals (180,181). In addition to reactive oxygen, macrophages may secrete reactive nitrogen intermediates including nitrites and nitrates (180).

It is likely that there is no single mechanism responsible for macrophage mediated tumor cytotoxicity. Rather, target cell destruction is probably the result of the synergistic interaction of several separate pathways. For example, TNF in addition to its tumoricidal effects also facilitates the release of reactive oxygen metabolites from activated macrophages (180,189). Also, macrophage derived proteases secreted during cytotoxic attack synergize with reactive oxygen leading to target destruction (190).

(E) ESCAPE FROM IMMUNE SURVEILLANCE

The concept of immune surveillance proposes that cancer cells

continuously arise within an individual however, their outgrowth into fully malignant lesions is prevented by effectors of the host immune system. The emergence of tumors requiring clinical intervention is believed to be the consequence of their having successfully eluded host defences. This may occur at two different levels. The suppression of the host immune system by one of several mechanisms may provide an environment which is conducive to tumor growth. In addition, as has previously been mentioned, tumor cells are phenotypically and genotypically more unstable compared to normal cells. An important step in tumor progression may be the generation of tumor cell populations expressing reduced sensitivity to host immune effectors. In the following section, several proposed methods of tumor escape from the host defence mechanisms will be discussed.

1) Antigen Loss Variants

A non-immunogenic, non-rejectable tumor cell population would be expected to have great survival advantages during tumor progression. Selective pressures provided by host immune effectors would be anticipated to favor the eventual emergence of just such a population (136). As a result of the understandable difficulties in studying the antigenic phenotype of a single original cancer cell, most studies of the generation and selection of antigen loss variants are indirect, examining instead the change in antigenicity of established tumor cell lines. However, even indirect studies have yielded interesting results applicable to normal tumor progression.

One of the best studied tumor associated surface antigen systems

is that of the major histocompatability class I antigens. In several tumor models, the loss of MHC antigen expression has been associated with escape from host immunity (191-196). This is most often attributed to a loss in MHC restricted cytotoxic T cell recognition, an effector not normally included in discussion of natural resistance immune surveillance. It is however worth mentioning here, since it provides an excellent example of immune selection of antigen loss variants.

While SV40 transformed C3H fibroblasts initially demonstrate an increased level of MHC class I antigens, the in vivo passage of these cells results in outgrowth of a C3H population selected for decreased expression of H2-K (191). Further examination of these in vivo selected cells revealed that they were both resistant to and failed to induce SV40 specific cytotoxic T cells. Adenovirus type 12 is known to be both strongly oncogenic and to result in the loss of class I MHC expression when used to transform rat cells. Both of these activities have been mapped to the E1A region of the viral genome (195,196). In addition, a Gross leukemia virus induced thymoma arising in an AKR mouse was shown to be both highly tumorigenic and lack H-2K expression. The transfection of this tumor cell line with the H-2K gene was demonstrated to result in both H-2K expression and a marked decrease in its malignant phenotype (193).

Although the increased tumorigenicity associated with MHC class 1 antigens loss is most often attributed to the enhanced resistance to cytotoxic T cells, the involvement of similar antigen loss variants in the tumor progression associated decreases in sensitivity to natural

immune effectors has also been proposed (136). Tumors obtained from the injection site of threshold subcutaneous murine lymphoma inocula exhibited higher tumor frequencies than the parental cell line when the tumorigenicity of threshold inocula was examined in syngeneic animals. In vivo selected cells also demonstrated a reduced sensitivity to several effectors of natural resistance including cytolysis by natural killer cells and complement mediated lysis by syngeneic natural antibodies. Further investigations revealed that these tumor cells were less able to inhibit natural killer cell mediated cytolysis in cold target inhibition studies and bound less syngeneic NAb measured through fluorescence analysis, implying that they had been selected in vivo for a decreased expression of the tumor associated antigens involved in natural immune recognition (136). To date, these antigens have not been identified.

An interesting feature of the selection for antigen loss variants is that at least in some tumor models, there appears to be a hierarchy of immune recognition for multiple independent tumor antigens (197,199,200). This is to say that selection occurs in a stepwise fashion whereby only after a particular antigen is lost from the entire tumor population is there recognition and selection of subsequent antigens. This phenomenon has been described for the sequential loss of cytotoxic T cell defined antigens from a UV induced regressor tumor during in vivo progression towards an enhanced malignant phenotype (199,200), and for the immunodominance in the immune response towards multiple minor histocompatability antigens (200). The clarification of how a dominant tumor associated antigen

prevents the immune recognition of other antigens on a tumor cell surface may be of great value for future models of immune intervention into tumor progression.

Another interesting feature in the immune selection of antigen loss variants is that the process may be driven by the partial suppression of immunity in a manner similar to the generation of antibiotic resistant bacterial strains due to incomplete drug therapy (202). This may be particularly applicable to the more immunogenic regressor tumor phenotypes which are easily rejected from fully immunocompetent animals. The implantation of such tumors into fully immunosuppressed (athymic nude or anti-idiotypically suppressed) animals results in the high frequency outgrowth of tumors exhibiting no evidence of rejection antigen loss. However, implantation into partially immunosuppressed (UV irradiated) animals results in the high frequency outgrowth of antigen loss tumors (203). The mechanism behind this phenomenon is unclear. It is possible that partial suppression or delaying of the immune response allows a tumor to achieve a sufficient size such that it can no longer be rejected although it may still be recognized and selected for by the immune system. This concept is discussed more fully in CHAPTER 1 of this thesis.

It should be mentioned at this point that the selection of antigen loss variants is by no means the only mechanism by which a tumor may overcome immune restriction. Although the enhanced tumorigenicity following in vivo selection is commonly associated with decreased immune recognition, there is evidence that cells may also be

selected for reduced sensitivity to the lytic stage of immune destruction. For example, decreases in the natural killer cell and natural antibody mediated cytolysis of tumors obtained from the injection site of threshold subcutaneous lymphoma inocula, have been associated with both diminished immune recognition and a general decrease in sensitivity to lysis as measured by resistance to cellular disruption in a hypotonic solution (136).

2) Antigen Modulation

Another mechanism by which a tumor cell may facilitate inefficient immune recognition is antigen modulation. Rather than the tumor population being selected for a decrease in tumor associated antigen expression, the antigen loss is instead mediated by the actual recognition and binding of the immune effector. Antigenic modulation describes a process by which the interaction of an antibody with its appropriate surface antigen results in patching capping and eventual loss of the antibody/antigen complex by either internalization or shedding. This rapid post recognition loss of tumor associated antigens impedes active cellular lysis. There are a number of antigens which are easily mobilized and modulated from the surface of normal cells upon interaction with specific antisera or monoclonal antibodies including surface immunoglobulins, CD3, CD4 and CD8 (204,205,206).

The thymus leukemia (TL) antigen is a β_2 microglobulin associated class I MHC molecule found on thymocytes and thymic leukemia cells (207,208). TL^+ leukemia cells are actively lysed by anti-TL sera and

complement. However, preimmunization of TL^- animals against TL^+ tumor cells does not appear to yield any protective benefits. It is speculated that the TL antigen is rapidly modulated in vivo as a result of antibody binding (209).

3) Obstruction of Immune Recognition

It has been demonstrated in syngeneic systems that tumor bearing hosts may still possess active antitumor immune effectors (measured in vitro) even in the absence of any apparent antitumor immune response in vivo (210). It is believed that in some circumstances, the in vivo immune response towards a tumor although being present may be functionally blocked. Hellstrom et al. demonstrated that the in vitro measured antitumor cytotoxic response of lymphocytes derived from a tumor bearing host could be blocked by the sera of that same tumor bearing animal (210). The nature of these immune blocking agents are unknown, however, chemically, physically or enzymatically derived tumor membrane preparations have been shown to block antitumor immunity (211,119). Pretreatment of mice with disrupted allogeneic tumor cells effectively prevents the rejection of the same allogeneic tumor when challanged with viable cells (211). Tumor preparations generated by papain and KCl digestion of tumor cells have been shown to enhance the frequency of tumors arising from threshold subcutaneous syngeneic tumor inocula (119). It is possible that tumors may achieve a survival advantage through the release or secretion of surface antigens resulting in the blocking of tumor specific immunity.

4) Tumor Induced Immune Suppression

Malignant tumors may themselves be capable of suppressing host defences either generally or tumor specifically thus providing a more favourable environment for neoplastic growth. This may be achieved through the release of substances such as tumor derived gangliosides, L-alanine, or P15-E (a murine RNA tumor virus product) which can suppress the immune system directly or indirectly (235,236,237). In addition, immune complexes consisting of antibody and antigen have been implicated in the blocking of antitumor immunity (212). These complexes may be the consequence of antibody recognition of soluble tumor antigens or result from surface antigen modulation following antibody binding (mentioned previously). Although the mechanism is unknown it is believed that antibody/tumor antigen complexes may induce tumor antigen specific suppressor T cells (212,213).

It has also been demonstrated that macrophage derived PGE_2 exerts pansuppressor effects against the activation of several tumoricidal effector lineages in tumor bearing hosts. For example, syngeneic splenic macrophages from tumor bearing hosts were shown to significantly depress the generation of CTL as well as the Con A induced proliferation of normal splenocytes. This suppressive effect could be blocked by indomethacin, an inhibitor of prostaglandin synthesis. In addition, macrophages from tumor bearing animals have been shown to exert similar suppressor activity on cellular effectors of natural resistance such as NK cells and killer macrophages. The observation that at physiological doses PGE_2 suppresses antibody synthesis via an indirect effect on helper T cell, implies that NAb which are a product

of thymus independent immunity may be exempt from this form of suppression (239).

5) Defective Host Immunity

a) Self Tolerance and Genetic Unresponsiveness

Occasionally, tumor antigens are incapable of eliciting an immunological response in certain syngeneic hosts. This unresponsiveness may occur at one of two different levels. An animal may become tolerized to antigens which are presented during embryonic development or soon after birth. For example, there is a marked decrease in resistance to mammary tumor virus associated murine mammary carcinoma in mice infected neonatally with MTV (214). In addition, self tolerance may explain the relatively poor immune response toward embryonic tumor associated antigens.

Major histocompatability complex associated Ir genes are known to control the immune response to several infectious diseases including mycobacterium leprae and schistosomiasis (215,216). Certain antitumor immune responses may be regulated in the same way resulting in animals being predisposed to tumor development as a consequence of being genetic low responders for specific tumor antigens. The ability to generate a primary or secondary cell-mediated response to an AKR tumor cell antigenic determinant appears to be regulated by I region linked MHC genes (217). Susceptibility to certain hematopoietic malignancies in humans may also be associated with unique Ia allotypes. For example, the predominant allotypes of individuals with either chronic lymphocytic leukemia or hairy cell leukemia are similar

($DR5^+/IVD12^+/MT2^+$) and differ signficantly from a control population. As well, the presence of DR5 has been linked to an increased susceptibility to Hodgkin's Disease (218).

b) Clinically Induced Immunosuppression

Severe immune suppression is associated with several medical procedures, for example, as a valuable tool in organ transplantation or as an unwanted side-effect during cancer therapy. These immune compromised conditions are generated by a variety of agents acting at various sites. Both tumor cells and many immune cells share an acute sensitivity to chemotherapeutic radiomimetic drugs and ionizing radiation, thus the immune system is often damaged during cancer chemo/radiotherapy.

The ability of ionizing radiation to destroy the mature immune system and immune progenitor cells of an individual while leaving radiation resistant elements (such as thymic epithilium) intact, makes it useful in the preparation of a patient for organ and bone marrow grafting (219). In addition several immunosuppressive drugs are also used during allogeneic tissue transplantation including steroids, cancer chemotherapeutic drugs such as cyclophosphamide and site specific immunosuppressants such as cyclosporine and FK-506 (220,221).

The greatest disadvantage of clinically induced immunodeficient conditions is the enhanced susceptibility to infections (220). In addition, there is a significant increase in the occurence of cancer (220,222), in particular, tumors of the reticuloendothelial, lymphopoietic and hematopoietic systems. However, it is uncertain to what extent these malignancies are due to immune suppression considering

that ionizing radiation and many radiomimetric drugs are also carcinogenic.

c) Congenital and Acquired Immunodeficiencies

Congenital and acquired immunodeficiency syndromes are often associated with remarkable increases in the incidence of certain types of tumors (223), although the rates of many common cancers do not appear to be elevated. The absence of enhanced rates of the more common cancers may be indicative of the fact that most patients with immunodeficiency conditions seldom live long enough for these types of tumors to develop (223,224).

Wiskott Aldrich Syndrome is an X linked immune deficiency condition characterized by an inability to produce antipolysaccharide antibodies and later by a severe lymphopenia (223,225). Patients with this disorder who live long enough often develop lymphoreticular neoplasia occassionally as a result of B cell transformation by EBV virus (226). The autosomal recessive disorder Ataxia telangiectasia is known to affect both the T and B cell components of the immune system (227). Once again malignancies are a common cause of mortality among long lived patients, although in this case the increased tumor rate may be due in whole or in part to a defect in DNA repair characteristic of the syndrome (2).

Diminished tumor defence has also been demonstrated for immune deficiencies in species other than humans. One of the most applicable to discussions of natural immune surveillance is that of the xid mutation in CBA/N mice. This condition is associated with the absence of peritoneal Ly-1^+ B cells and splenic Lyb-3^+, 5^+, 7^+/IaW39^+ B cells

(228,229). The result of these cellular deficiencies is the lack of circulating IgG_3 and IgM immunoglobulins and the inability to mount a humoral response to thymic independent type 2 antigens similar to what is seen in the Wiscott Aldrich syndrome. Recently, it has been demonstrated that the xid mutation is also associated with an apparent decrease in circulating tumor reactive natural antibodies and an enhancement in the frequency of tumors arising from threshold subcutaneous tumor inocula (129).

Acquired immunodeficiency syndrome (AIDS) is associated with increased rates of Kaposi's sarcoma, a rare multicentric tumor of the lymphatic endothelium previously found primarily in immunosuppressed organ transplant recipients and in individuals from central Africa (230,231) (possibly due to generalized suppression of immunity resulting from malaria infection). Although recent studies have implicated growth factors produced by HIV infected T cells in tumor formation (232), it is speculated that the immune suppressed host provides an excellent environment for Kaposi's sarcoma growth (231). Other malignancies associated with AIDS are high grade lymphomas and squamous cell carcinomas (231).

d) Age

Superficially the marked increase in malignancies seen in elderly individuals would appear to be the result of a well established decrease in immune function (233). However, such a cause and effect relationship is difficult to prove in humans. It is possible that the increased tumor frequency observed with progressing age may be due to the extended latency periods of some cancers. However, experimentally

it has been demonstrated that UV induced tumors which are readily rejected in young mice grow progressively in old mice (234).

e) Environmental Immune Suppressive Agents

Exposure to environmental agents with known immunosuppressive or immunotoxic effects has been observed to correlate with enhanced frequencies of certain cancers as well as a decreased ability to reject experimental tumor implants.

Ultraviolet radiation has been shown to be an inducer of immune suppression. The adoptive transfer of lymphoid cells from UV irradiated mice can block the rejection of a number of UV induced syngeneic regressor tumor grafts, although they have no effect on the rejection of allogeneic tumors (240). These and other experiments involving the adoptive transfer of cells from UV irradiated animals imply that UV irradiation stimulates the production of a suppressor cell population, possibly of T cell origin (241). The cell type responsible for UV induced immunosuppression is however controversial. When UV irradiation is used to induce graft acceptance it appears to cause changes in splenic macrophage mediated functions, possibly resulting in impaired antigen presentation (242).

Chronic exposure to certain environmental mineral fibers, such as silica and asbestos, has been linked to the development of a number of tumors in humans. Although the mechanism by which these relatively inert particals contribute to tumorigenesis in vivo is not entirely understood, it is speculated that it may be the result of immune suppression or toxicity (243,244,245). For example, the occupational exposure to airborne asbestos dust has long been associated with the

development of several human tumors including mesothelioma, bronchogenic carcinoma, acute myelocytic leukemia and gastro intestinal cancer (reviewed in 246). It has been suggested that this enhanced rate of tumor development may be the result of an asbestos induced persistant impairment of the immune system, characterized by reduced NK cell activity, decreased mitogen responsiveness, fewer periferal blood monocytes, and an enhanced ratio of suppressor T cells to helper T cells (243).

6) Augmentation of Tumor Immunity

The ability of a tumor to evade host defences or to opportunize an immunosuppressed environment provides it with a definite survival advantage. It is therefore possible that the augmentation of antitumor immunity may be beneficial in the therapy and/or prophylaxis of cancer. To date, all attempts at the immunotherapy of human malignancies have met with only very limited success. Initial efforts of cancer treatment through the augmentation of antitumor defences involved the general stimulation of host immunity with bacterial products. Human tumors however, did not always respond to this so called "adjuvant therapy" and when they did, regressions were often incomplete and transient (247). At the time, it was not entirely clear how these bacterial products were capable of augmenting host defences, although this has been clarified by recent work. For example, the receptor mediated activation of macrophages by bacterial cell wall lipopolysaccharides is known to result in the release of several important regulatory molecules. These include tumor necrosis factor capable of being directly cytolytic for some neoplastic cells

and IL-1 which may trigger numerous cellular responses either directly or via a cascade of other lymphokines (248,249). In addition, LPS appears to be a polyclonal stimulator of B cells leading to a general stimulation of humoral immunity (253,254).

The characterization, cloning and production of large quantities of specific lymphokines has allowed for the development of more direct approaches to tumor immunotherapy. Currently the use of lymphokine activated killer (LAK) cells, adherent lymphokine activated killer (A-LAK) cells and tumor infiltrating lymphocytes (TIL) have been shown to provide beneficial effects with some specific tumors (250,251,252). A better understanding of LAK/TIL stimulation and tumor cytolysis as well as the potential for genetic manipulation (ie. the proposed insertion of the TNF gene) may make this a technique of more general use.

The ability of tumors to become progressively more autonomous with respect to host immune constraints is an obstacle to successful immunotherapy. This feature of tumor progression may be partially responsible for the limited success observed with previous and current approaches of immunotherapy and immunoprophylaxis and, if so, its clarification would be necessary before immune augmentation could become an accepted means of cancer treatment.

(F) COUNTER-SURVEILLANCE AND IMMUNOSTIMULATION

It has previously been proposed that the host immune system may in some situations behave as a promoter of tumor development. Termed counter-surveillance or immunostimulation this theory maintains that tumor specific immunity, by an as yet unresolved mechanism, actually

facilitates rather than inhibits oncogenesis (255,256).

The best support for the involvement of immunostimulation in tumor growth comes from experiments designed to titrate the in vivo antitumor immune response. For example, in a Winn type assay where tumor cell/spleen cell mixtures of varying proportions were implanted into immune compromized syngeneic hosts, optimal tumor growth was observed with a 1:1 ratio of specifically immune spleen cells, as compared to a similar ratio of normal spleen cells (257). In addition, when irradiated and thymectomized mice are immunologically reconstituted with graded numbers of normal spleen cells and are then challenged with a high dose of a chemical carcinogen, mice reconstituted with small numbers of splenocytes develop tumors faster than either unreconstituted animals or animals receiving a large number of cells (258). Similar results have also been reported for nude mice receiving various numbers of thymus or spleen cells (255). Prehn has proposed that partial (but not complete) immune depression may stimulate more highly immunogenic tumors, while the augmentation of antitumor immunity would facilitate the growth of less immunogenic tumors. It has also been suggested that all antitumor immune effectors may have immunostimulating capabilities (255).

This concept of counter-surveillance coupled with the observation that partial immune suppression may actually facilitate the in vivo generation of an antigen loss phenotype (203) may have direct implications for the design of immunotherapy strategies, and stresses the need for a more thorough understanding of immune response modifiers.

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CHAPTER 1

Regulation of Tumor Development:

The biphasic effects of silica and of lipopolysaccharide on natural resistance

ABSTRACT

The impact on tumor development of the biological response modifiers (BRM) silica and lipopolysaccharide (LPS) was assessed through the analysis of associated changes in natural immune resistance parameters in vivo and in vitro. The injection of the fumed silica Cab-o-sil 3 days before a threshold subcutaneous (sc) inoculum of L5178Y-F9 cells has been shown by others to increase the tumor frequency in syngeneic DBA/2 mice, consistent with its macrophage toxicity. In the current investigation we demonstrate that the tumors recovered from the silica treated animals exhibit augmented reductions in natural antibody (NAb) binding as well as decreased sensitivity to natural resistance measured in the ^{131}IdUrd tumor elimination assay when compared to tumors from control mice. Cab-o-sil injection increased host mediated antitumor NR measured in the ^{131}IdUrd tumor elimination assay providing a rationale for the augmented tumor selection in silica treated animals. In addition, silica induced a biphasic modulation of antitumor NAb and natural killer (NK) activities observed in the form of an initial decrease over the first 2 days, followed by an augmentation by days 5-7. While silica induced immune suppression may partially facilitate tumor development, the appearance of better selected, more autonomous tumors in Cab-o-sil treated mice in correspondence with a stimulation of NR parameters assayed in vivo and in vitro suggests that the adjuvant activity of silica may also contribute to its cocarcinogenic effect by accelerating tumor progression. Although it has previously been shown by others that an intraperitoneal (ip) injection of LPS 2-3 days prior to

a threshold sc tumor inoculum resulted in a reduced tumor incidence, the survival of threshold sc tumor inocula injected within 1 day of LPS was increased. A corresponding decrease in serum NAb antitumor activity was seen less than 24 hours after LPS injection, whereas increases in both NK cell and NAb levels had been observed after 5 days. This biphasic effect of LPS on the putative effectors of NR assayed in vitro was confirmed in the rapid tumor elimination assay of NR in vivo. Although their frequency of appearance was higher, the tumors that were initiated from threshold sc inocula during the period of LPS-induced NR abrogation exhibited greater reductions in NAb binding and sensitivity to NR than tumors from control mice. These data extend the support for NAb acting against tumor cells in vivo and reveal the dual nature of NR in tumor development, 1) defending against small tumor foci and 2) driving the progression of the surviving neoplasm. The latter activity very likely contributes to the tumor enhancing effects encountered with adjuvant therapy.

INTRODUCTION

Although a substantial body of evidence supports the role of antitumor immunity in determining the course of tumor development, the precise nature of this involvement appears to be complex. In the vast majority of studies the immune system and in particular non-adaptive natural resistance effectors appear to provide a potent defence against incipent neoplastic development. However, others suggest that the immune system rather than inhibiting, may actually stimulate tumor growth (1), while still others have proposed that the partial suppression of antitumor immunity may facilitate tumor progression towards an enhanced malignant phenotype (2). Insight into this complex relationship between a developing tumor and the antitumor immune response would be important in understanding the tumor promoting capacity of immunosuppressive agents and for the design of cancer immunotherapeutic regimens.

Although the harmful effects of environmental exposure to silica have been clearly evident in the association of silica dust inhalation and silicosis (3), evidence also suggests that silica contributes to tumor development. The relationship between exposure to silica and an increased risk for gastrointestinal (4) and esophageal tumors (5) in humans has largely been attributed to the fibrous nature of the mineral. However, the injection of crystalline quartz and amorphous fumed silica was associated respectively with the appearance of histiocytic lymphomas in rats (6) and a reduced survival of AKR mice dying with thymomas (7). In addition, Goldsmith and colleagues (8), after reviewing the literature on human and animal studies argued for

a link between occupational exposure to crystalline silica and lung cancer. Although the toxic effects of both quartz and fumed silica have been shown on macrophages in vitro (9,10 and personal communication from R.B. Baldwin and D.G. Hopper) the mechanisms of the cocarcinogenic activities of silica are not clear. Reductions in systemic phagocytic function following the intravenous injection of relatively massive doses of silica have characterized the detrimental effects of silica on phagocytic cells in vivo (9). However, more detailed analysis of macrophage function in silica treated rats revealed only transient decreases in a range of biochemical macrophage parameters (11), while reports of the adjuvant activity of silica (12,13,14) indicate the complexity of the response to this agent and confound attempts to formulate a simple rationale for its activity in vivo.

Conversely, microbial adjuvant treatment has been associated with prolonged remissions and reductions in tumor growth in humans (reviewed in 15) and suppression or slowing of tumor growth in numerous experimental animal models (reviewed in 16). However, repeated early attempts to control cancer by nonspecific immunological stimulation with microorganisms yielded both positive and negative results (15). More recent tests with microbial adjuvants (17,18) and initial trials with natural and recombinant interferon (IFN) and other biological response modifiers have similarly yielded only limited therapeutic effects (reviewed in 19). Extensive studies in animal tumor models showed that treatment with Bacillus Calmette-Guerin (BCG) could provide a benefit, have no effect or even enhance tumor growth

(18,20,21).

Attempts to dissect the complex immune response to BRM using animal models have shown that under certain conditions, suppressor cells capable of abrogating T-cell and adaptive humoral responses were generated (20, reviewed in 22). Single injections of a variety of BRM led to augmented natural killer (NK) cell activity 2-3 days later, however, subsequent decreases were frequently detected (reviewed in 23) and multiple adjuvant injections produced hyporesponsiveness to NK augmentation (19).

In order to assess the impact of immune response modulators on early tumor development, we have examined the effects of silica and of the immune adjuvant LPS on the progression of threshold tumor inocula in syngeneic mice. This paradigm of incipient neoplasia yields a more aggressive tumor which exhibits a unified natural defense resistant phenotype (24). The present report focuses on the less well-understood negative effects of LPS and positive effects of silica on natural resistance.

Abbreviations used: HBSS, Hanks balanced salt solution; 10% FFBS, Fischer's medium supplemented with 10% fetal bovine serum; NK, natural killer; NAb, natural antibody; NR, natural resistance; LPS, lipopolysaccharide; IdUrd, 5-iodo-2-deoxyuridine; RES, reticulo-endothelial system; E/T, effector to target ratio; NT, no treatment; Ig immunoglobulins, sc, subcutaneous; ip, intraperitoneal; C, complement.

MATERIALS AND METHODS

Mice and sera

DBA/2 mice were obtained from Canadian Breeders, Charles River, Quebec. CBA/J inbred mice used for NK cell assays only, were obtained from Jackson Laboratories, Bar Harbor, Maine. Serum NAb was assayed in blood samples obtained from axillary bleeding from normal mice or animals treated as indicated with LPS or silica. LPS-induced serum for comparative tumor analysis was obtained from mice stimulated 3-5 days previously with 100 ug LPS injected ip (25). This regime of LPS treatment has been shown to raise the level of antitumor NAb normally present in the serum in correspondence with a decrease in the tumor frequency of threshold sc tumor inocula given 2-3 days after LPS administration (26).

Tumor cells

The L5178Y-F9 and SL2-5 clones were obtained from their parental DBA/2 strain lymphomas L5178Y and SL2-5, through two successive clonings using a sloppy agar procedure. The $PX_2$16R5 was similarly derived from the P-815-X2 methylcholanthrene induced mastocytoma syngeneic to DBA/2 strain mice (26). These tumor lines were maintained in vitro using Fisher's medium containing 10% fetal bovine serum (10% FFBS) supplemented with penicillin and streptomycin (GIBCO Grand Island, N.Y.). The A/Sn strain lymphoma YAC-1 was maintained in RPMI 1640 containing fetal bovine serum and antibiotics as above. The cells were free of mycoplasma according to periodic testing performed by using the DNA staining technique.

Assessment of tumor frequency

Tumor cells were washed three times and serially diluted in Hank's balanced salt solution (HBSS) (GIBCO) for injection. An aliquot of 100 ul containing the desired number of cells to produce a tumor incidence of less than 100% was injected sc into a shaved area in the middle of the lower back of each mouse. Tumor frequency was assessed 45 days after tumor inoculation, at least 15 days after the last tumor had appeared. No regressions were observed.

Immunomodulators

Lipopolysaccharide (Sigma Chemical Co, St. Louis, Mo.) was a lyophilized powder prepared by the trichloracetic acid procedure from E. coli (serotype 026:B6). LPS was dissolved in HBSS and 100 ul aliquots containing the quantities indicated were injected ip.

Cab-o-sil (Cabot Corp., Boston, Mass.) is a fumed silica product of particle size 0.014 um. An ip injection of 100 ug/mouse given 24 h before a SRBC injection was shown to inhibit the plaque forming cell response (B. Carter, personal communication). Fumed silica has also been shown to be toxic to macrophages in vitro (R.W. Baldwin, D.G. Hopper, personal communication). Cab-o-sil was suspended in HBSS and the suspension was sonicated before intraperitoneal injection in 100 ul aliquots.

In vivo tumor selection

Cloned L5178Y-F9 cells maintained in vitro were washed three times and serially diluted in HBSS for injection into DBA/2 mice. A

100 ul aliquot containing the desired number of cells was injected sc into a shaved area in the middle of the lower back of normal DBA/2 mice or animals treated with LPS or Cab-o-sil as indicated for each experiment. Following growth in vivo, the tumor was removed sterilely from the injection site, teased apart with forceps and the cells were washed once and suspended in 10% FFBS. After three days in culture, they were usually growing at a rate comparable to that of the parental L5178Y-F9 lines.

Natural resistance assay

As described previously (27) tumor cells labelled in vitro with ^{131}I-5-iodo-2-deoxyuridine (^{131}IdUrd) (Edmonton Radiopharmaceutical Laboratories, Edmonton, Alberta) were washed and inocula of 10^7 cells were injected ip or sc into each of 5 mice. At time, 0,18 hrs and thereafter at intervals of 24 hrs the mice were whole body counted in an Ortec modular gamma counter (EG and G Instruments, Toronto, Canada). The amount of label remaining in the mice at each time point was expressed as a percentage of whole body counts at time 0 (%^{131}I retained). All of the mice in the label clearance experiments were maintained on drinking water supplemented with 0.1% KI to minimize thyroid uptake of released iodine. Previous studies have shown that the %^{131}I retained after ip injection of radiolabelled tumor cells correlated directly with the tumor cell numbers that could be recovered from the peritoneal cavity (27). The rate of tumor elimination observed in this assay is considered to be a measure of tumor sensitivity to thymus independent NR since similar rates of

tumor clearance were observed in normal and thymus deficient animals (28).

Natural antibody and complement mediated cytotoxicity assay

^{51}Cr-labelled tumor target cells were incubated with whole or diluted, syngeneic mouse serum as a source of NAb, followed by specifically absorbed rabbit complement as described previously (26).

NK cell cytotoxicity assay

Following an established procedure (27), tumor cells from in vitro cultures were washed and labelled with ^{51}Cr (Na_2CrO_4) (100 $\mu Ci/10^7$ cells) for 45 minutes at 37°C. The cells were washed three times with HBSS and adjusted to 10^5 cells/ml in 10% FFBS. Spleen or peritoneal cells obtained from the mice indicated in each experiment, were treated with 0.15 M ammonium chloride for 4 minutes at room temperature to lyse red blood cells and then washed 3 times in HBSS. Effector cells were combined with 100 μl aliquots of the tumor target cells at effector to target (E/T) ratios of 75:1 for spleen effectors and 50:1 for peritoneal effectors, at a final volume of 200 μl in V-bottom microtitre plate wells. Following a 4 minute centrifugation at 35 g the plates were incubated at 37°C in 5% CO_2 for 18 hours. The plates were centrifuged for 10 minutes at 180 g and 100 μl aliquots of supernatant were removed for gamma counting. In some tests, effector cells were pretreated with monoclonal rat antimouse Thy 1 antibodies (Dimension Laboratories, Mississauga, Ont., Canada) or rabbit anti-asialo-GM1 (Wako Pure Chemicals, Dallas, Tex.) and complement at

concentrations that killed 30% and 40% respectively of normal spleen leukocytes. Removal of silica induced peritoneal cells with carbonyl iron and a magnet (25) left 80% of the cells for assessment of their lytic activity.

NAb binding

Tumor cells (3×10^5) were washed in 10% FFBS (Hepes buffered) containing 0.1% sodium azide and incubated for 2 hrs in a 5% CO_2 atomosphere at 37°C in 200 μl of 3-4 day LPS stimulated serum, whole or diluted as indicated with the same medium containing 0.1% azide. The cells were then washed and re-incubated for 20 min in the dark in 100 ul of fluorescein isothiocyanate conjugated (FITC) goat 7S Ig anti-mouse IgG (Meloy, Springfield, Va.) and FITC goat $(Fab')_2$ anti-mouse IgM (Tago, Burlingame, Ca.) (each diluted 1:10 in Hepes buffered 10% FFBS containing azide). The cells were washed and fixed by incubating them for 5 minutes in 100 ul of 1% formaldehyde in HBSS, and the final sample volume was adjusted to 400 ul with Hepes buffered 10% FFBS containing 0.1% azide. Samples were analyzed at 4°C for linear fluorescence using a Coulter Epics V Multiparameter Sensor System. The control cells treated with medium followed by the fluorescein labelled antibody exhibited no fluorescence beyond that of untreated cells.

Determination of the phagocytic index

The phagocytic index of normal and silica treated mice was assayed based on the technique of Levy and Wheelock (9).

Statistics

The two-tailed student's t-dependent test was used to determine the statistical significance between differences observed in the various assays. T-dependent analyses were performed unless indicated otherwise. P values greater than 0.05 were not considered significant.

RESULTS

The effect of silica injection on tumor progression

The increase in tumor frequencies of threshold sc inocula of L5178Y-F9 cells injected into syngeneic DBA/2 mice 3 days after the initiation of treatment with fumed silica may have occurred due to a decrease in natural resistance in accord with the evidence in the literature that silica abrogates macrophage function (7). In order to study the NR abrogating effect of silica on the progression of small tumor foci, we examined the NR phenotypes of tumors that grew out at the injection site after the sc inoculation of 25 L5178Y-F9 cells into normal or silica treated syngeneic DBA/2 mice. Cab-o-sil was injected ip in 100 ug aliquots on 4 consecutive days beginning 3 days before the initiation of tumor growth and half of the treated mice were also given Cab-o-sil 3 times a week subsequent to the tumor inoculation for a total of 15 doses of silica. Consistent with previous experiments (7), the proportions of mice that developed tumors were 2/7 and 3/7 for the animals given 4 and 15 injections of Cab-o-sil respectively, compared with 1/7 for the control untreated mice. Contrary to what would be expected from the increased tumor frequencies that were

associated with this treatment, the tumors obtained after 3.5 or 5 weeks growth in animals given silica showed a marked reduction in sensitivity to natural resistance measured in the ^{131}IdUrd tumor elimination assay beyond that of the tumors from control animals (Table 1.1). The tumors from the silica treated mice also exhibited correlating augmented reductions in NAb binding measured through fluorescence analysis (Table 1.1).

The effect of silica on tumor elimination

In an attempt to further characterize the effects of silica on natural resistance in vivo, the rapid sc elimination of radiolabelled tumor cells was initially assessed in normal mice and animals given a daily ip dose of 100 ug silica beginning 1 or 3 days prior to and ending on the day of tumor injection. Although the rates of tumor clearance could not be distinguished over the first 24 hours, L5178Y-F9 were subsequently eliminated faster from mice given 4 silica injections than from the controls and the rate of clearance from mice given 2 injections of silica was intermediate (Fig. 1.1). A similar increased rate of clearance of large ip tumor inocula was seen in mice that had received two ip injections of 100 ug fumed silica either 11 and 12 days prior to the tumor injection or the day before and two hours prior to the tumor injection (data not shown). These data combine to suggest that a systemic stimulation of host natural defences occurs within 2 or 3 days of the first exposure to these small doses of fumed silica.

TABLE 1.1 - SENSITIVITY TO NR AND NAb BINDING OF TUMORS FROM SILICIA-TREATED MICE

Tumor source[1]	Tumor elimination % ^{131}I remaining ± SE day 3		NAb binding Mean channel fluorescence
	8 x 10^6 cells	6 x 10^6 cells	
In vitro control	28.9±10.8	11.7±1.9	72.9±1.6
Normal DBA/2	39.3±6.8	19.2±1.8	56.1±9.1
5 x silica DBA/2	58.0±3.7[3]	ND	28.1±2.7[2]
15 x silica DBA/2	ND	40.6±2.2[3]	29.8±7.7[2]

[1] Tumors removed from the tumor injection site 3.5, 3 or 5 weeks after the s.c. inoculum of 25 L5178Y-F9 cells into normal DBA/2, DBA/2 given 4 or 15 100 μg i.p. doses of Cab-o-sil respectively, were compared to L5178Y-F9 cells maintained in vitro.

[2] - $p < 0.05$ and

[3] - $p < 0.002$ when compared to the in vitro control tumor.

ND - Not done.

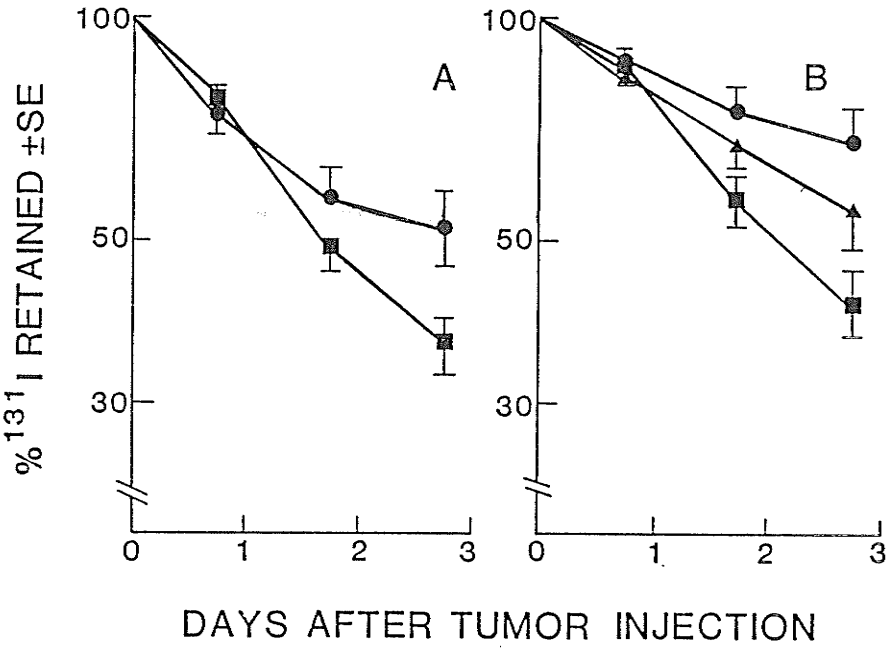

FIGURE 1.1 - Effect of fumed silica on tumor elimination. (A) Elimination of 5 x 10^6 ^{131}IdUrd-labelled L5178Y-F9 cells injected s.c. into DBA/2 mice given 100 μg i.p. injections of fumed silica on 4 consecutive days beginning 3 days before tumor inoculation (■) was more rapid than elimination from untreated animals (●), on day 3 ($p < 0.03$). (B) The level of s.c. elimination of 5 x 10^6 ^{131}IdUdr-labelled L5178Y-F9 cells from DBA/2 mice given 100 μg Cab-o-sil i.p. on 2 consecutive days beginning 1 day before the tumor injection (▲) was intermediate between values for animals not receiving silica (●) and for those receiving Cab-o-sil on 4 consecutive days (■), with the latter difference being significant on day 3 ($p < 0.01$).

The effect of silica on the RES

In order to determine whether the increases in tumor frequencies or in contrast, the increases in the elimination of large tumor inocula might be associated with silica-induced alterations in RES activity, the phagocytic index of both normal and silica treated animals was compared using the method of Levy and Wheelock (9). They had shown that a large iv injection of quartz silica produced a significant decrease in the phagocytic index and following their procedure we obtained values for the normal DBA/2 mice that were similar to their data for the same inbred strain (average phagocytic index of 4 experiments = .022). However, when daily ip injections of 100 ug Cab-o-sil were given for 2 or 4 days with the final dose given immediately before the assay, the ratio of the phagocytic indexes of silica treated to normal animals approximated one (0.9-1.1). These data indicated that neither augmentation nor abrogation of systemic phagocytic function were detectable over the first three days of exposure to these small ip doses of fumed silica.

The effect of silica on NAb and NK cell activity

Serum NAb levels were examined at several time intervals after exposure to Cab-o-sil in order to assess the possible role of NAb in the in vivo observations already outlined. Sera obtained from animals that received two ip injections of 100 ug fumed silica beginning 6 and 7 days previously exhibited small but significant increases in complement dependent NAb activity against the syngeneic L5178Y-F9 compared with sera from untreated animals (Fig. 1.2C) and a similar

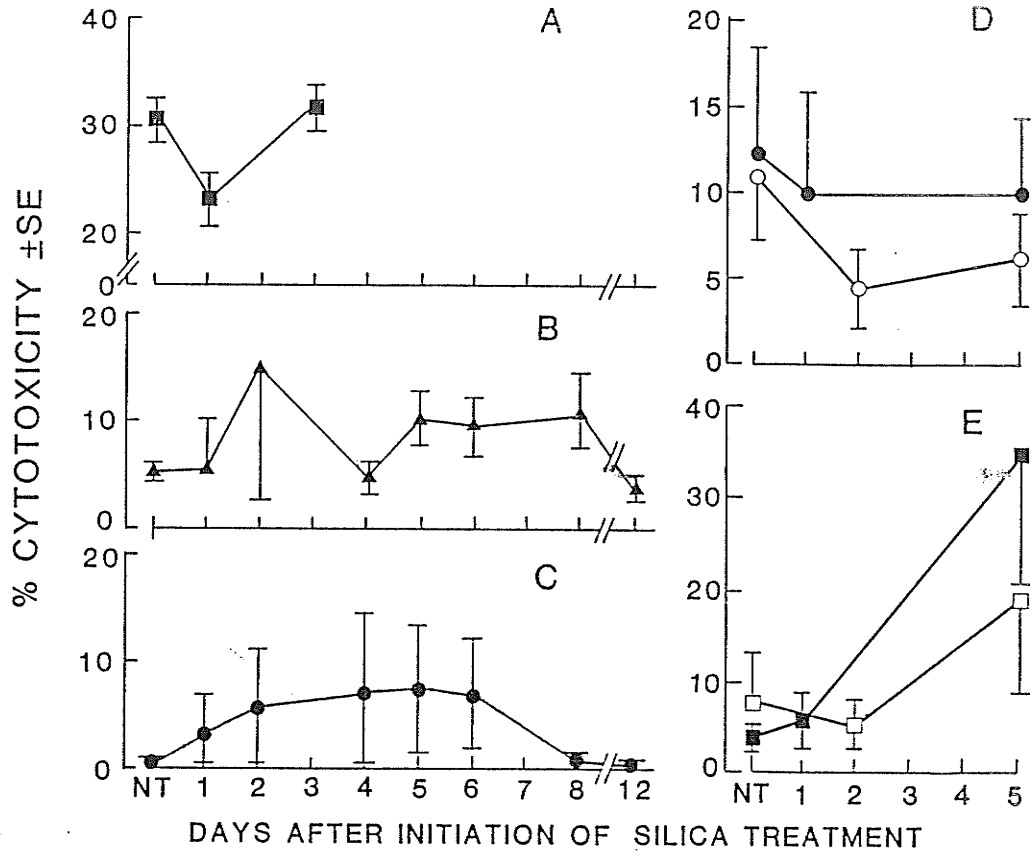

FIGURE 1.2 - Effect of silica on NAb and NK activity. Serum NAb as well as splenic and peritoneal NK activities were measured at different time intervals after the first of 2 injections of 100 μg Cab-o-sil given 24 hr apart. Whole syngeneic serum NAb from DBA/2 mice was generally increased after 2 days, with the cytolysis of serum from untreated mice being significantly different from that of animals given silica beginning 6 and 7 days previously for the L5178Y-F9 (c) and 6, 7 and 8 days previously for the SL2-5 (b) ($p < 0.05$). (a) Complement-dependent lysis of allogeneic YAC-1 targets in DBA/2 serum taken 2 days after the start of the silica treatment was lower than in untreated control sera and the difference was significant ($p < 0.05$). NK assays were carried out using YAC-1 targets and effector cells pooled from 3 DBA/2 mice (open symbols, 5-7 assays) or 3 CBA/J mice (closed symbols, 3 assays). (d) Spleen cells were assayed at an effector to target ratio of 75:1. (e) Peritoneal cells were assayed at a ratio of 50:1. The activity of peritoneal cells from CBA/J mice was significantly enhanced ($p < 0.04$) 5 days after silica treatment.

trend was observed using the syngeneic SL2-5 lymphoma as the target cell (Fig. 1.2B). While early decreases in NAb activity could not be consistently detected against these syngeneic tumors, probably because the normal levels were low, assays carried out against the allogeneic YAC-1 tumor revealed a small but significant reduction in NAb levels 2 days after the initiation of the silica treatment (Fig. 1.2A) confirming an early decrease in allogeneic NAb previously reported (29).

Although the L5178Y-F9 tumor is resistant to direct NK lysis in vitro, NK activity was examined because these cells may also act indirectly against tumor cells through NAb in an antibody-dependent cell mediated cytolytic mechanism (Chow et al, unpublished observation). The effects of the small ip injections of Cab-o-sil were examined on both splenic and peritoneal NK cells to assess their possible contributions to the alterations in tumor frequencies, the increased elimination of large tumor inocula and the augmented selection of small tumor inocula that had accompanied silica treatment. In initial studies with the low NK DBA/2 strain, the exposure to silica resulted in a decrease in splenic NK activity within 24 hours that persisted at least until day 5. In contrast, peritoneal NK activity which was only very slightly reduced in the first 24 hours rose markedly by day 5. These trends were confirmed using the high NK CBA/J strain (Fig. 1.2 D and E).

Characterization of the silica induced peritoneal effector cells

Carbonyl iron removal of phagocytic cells did not alter the level

of the antitumor activity seen in the peritoneal cells of silica treated mice tested at the same effector to target ratios (Table 1.2). This data suggested that both phagocytic and non-phagocytic cells contributed to the rise in cellular activity in the peritoneal cavity. In addition depletion of cells through complement-dependent lysis by anti-asialo-GM1 or anti-Thy 1 both yielded less active populations (Table 1.2).

Silica induced changes in spleen and peritoneal cellularity

Assessment of total spleen and peritoneal cell numbers 1,3 and 5 days following the first of 2 ip injections of Cab-o-sil given 24 hours apart revealed a significant early decrease in splenic leukocytes on days 1 and 3 followed by an increase in peritoneal cell numbers detectable on day 5 in correspondence with the observed early decrease in splenic NK activity and the gradual rise in peritoneal cell cytolysis associated with the silica treatment (Table 1.3).

The effect of LPS on tumor frequency

Bacterial endotoxins such as LPS have been shown in the past to augment natural immune effectors, NAb, NK cells and macrophages (25,30). Previous investigations demonstrated that LPS given 2 to 3 days prior to the sc injection of a threshold tumor inoculum decreased the tumor incidence in good accord with the increased activities of NK cells and NAb assayed 5 days after LPS treatment (7,26). The present more extensive analysis of the kinetics of LPS treatment revealed that injection of 1, 20 or 100 ug LPS ip either one

TABLE 1.2 - CHARACTERIZATION OF PERITONEAL CELL ACTIVITY FROM SILICA-TREATED MICE

Experiment number[1]	Effector treatment	% Cytolysis ± SE
1 (3)	None	55.6 ± 17.0
	carbonyl-iron/magnet	54.5 ± 15.0
2 (2)	None	71.9 ± 8.2
	anti-asialo-GM1 ± C	58.9 ± 1.1
3 (2)	None	23.6 ± 12.2
	anti-Thy 1 + C	12.8 ± 7.4

[1] The figures in brackets indicate the number of assays performed with peritoneal cells removed 5 days after the first of 2 i.p. injections of 100 μg Cab-o-sil given 24 hr apart. Experiments 1, 2 and 3 were carried out for 18 hr using YAC-1 targets at E/T ratios of 40-100/1, 50 or 100/1 and 20/1 respectively.

TABLE 1.3 - EFFECT OF SILICA ON SPLEEN AND PERITONEAL CELL NUMBERS

Expt. number[1]	Tissue site	Number of leukocytes[2]			
			Days after silica treatment		
		No treatment	Day 1	Day 3	Day 5
1	Spleen	$(1.2\pm.1)\times10^8$	$(0.9\pm.1)\times10^8$ [3]	$(0.9\pm.1)\times10^8$ [3]	ND
	Peritoneal cavity	$(2.0\pm.2)\times10^6$	$(1.5\pm.1)\times10^6$	$(2.4\pm.1)\times10^6$	ND
2	Spleen	$(1.2\pm.1)\times10^8$	ND	ND	$(1.2\pm.1)\times10^8$
	Peritoneal cavity	$(1.5\pm.1)\times10^6$	ND	ND	$(2.6\pm.2)\times10^6$ [4]

[1] Mice used in experiments 1 and 2 were 13 and 9 weeks old respectively.
[2] Cell counts were performed at the indicated intervals after the first of 2 100 μg Cab-o-sil injections given i.p. 24 hr apart.
[3] - $p < 0.05$
[4] - $p < 0.002$ compared to the non-treated control.
ND - Not done.

day preceeding, simultaneously or one day after the sc inoculation of a threshold dose of syngeneic L5178Y-F9 or $PX_2$16R5 cells, enhanced the tumor frequency compared to either no treatment or LPS administration 3 days prior to the threshold tumor inoculum (Table 1.4).

The effect of LPS on tumor elimination

The clearance of ^{131}I labelled tumor cells was examined in normal and LPS treated mice as a measure of the effect of this immune response modifier on NR in vivo. The tumor elimination rate over the first 18 hours was seen to be significantly slower in mice given 100 ug LPS two hours before the tumor cells ($p<0.006$) (Fig. 1.3), although by 72 hours the percentage ^{131}I remaining was virtually the same as that for animals given either HBSS or no treatment. These data depict a biphasic rate of tumor elimination from LPS treated mice with an initially reduced clearance rate gradually increasing after the first day to bring about the same level of elimination as control animals by day 3.

The effect of LPS on NAb and NK cell activities

In order to determine which effectors of natural resistance may be abrogated immediately after the injection of LPS, we examined the splenic and peritoneal NK cell activities and serum NAb levels of mice that received 100 ug LPS ip 2, 18 or 24 hours earlier in comparison with untreated controls. The NK activity at both sites was significantly enhanced by 2 hours and remained elevated for the test period of 24 hours (Fig. 1.4B). In contrast, the serum NAb levels were

TABLE 1.4 - EFFECTS OF LPS ON TUMOR FREQUENCY

Expt. number	Tumor inoculum		LPS injection		Tumor frequency	
	Cell	Dose	Dose	Day[1]	Number	Percent
1	L5178Y-F9	25 cells	100 μg	-3	5/16	31
			100 μg	0	8/16	50
			0 μg	-	6/16	38
2	L5178Y-F9	50 cells	1 μg	-2 or -4	2/16	13
			1 μg	-1,0 or +1	10/24	42
			0 μg	-	2/8	25
3	L5178Y-F9	300 cells	20 or 100 μg	-1	16/16	100
			0 μg	-	6/8	75
4	$PX_2$16R5	10 cells	20 μg	-1,0 or +1	6/24	25
			0 μg	-	0/8	0
5	$PX_2$16R5	10 cells	100 μg	-1	25/31	81
			0 μg	-	11/16	69
6	$PX_2$16R5	10 cells	20 μg	-1	11/23	48
			0 μg	-	5/24	21

[1] The time of i.p. injection is given relative to tumor inoculation.

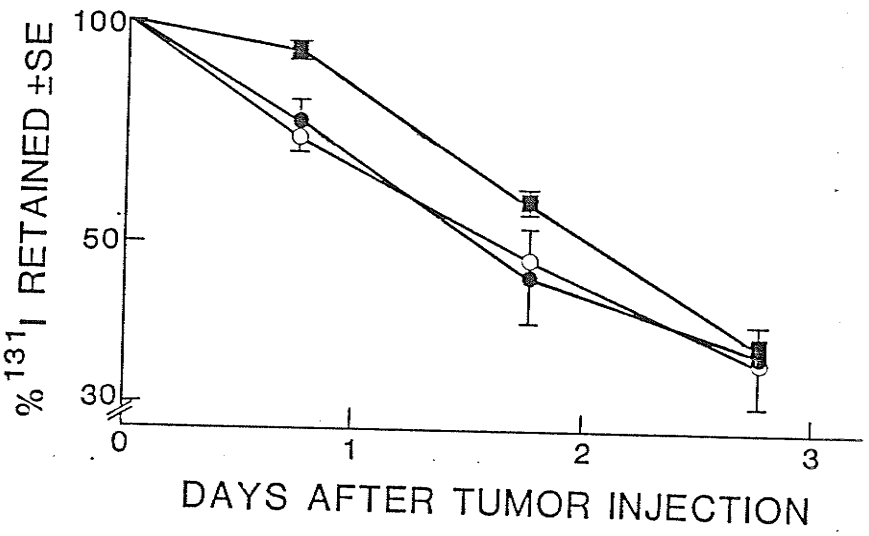

FIGURE 1.3 - Effect of LPS on tumor elimination. The i.p. clearance of 5 x 10^6 ^{131}IdUrd-labelled L5178Y-F9 cells was compared in normal syngeneic DBA/2 mice (●) and animals given 100 μg LPS i.p (■) or HBSS (○) 2 hr prior to the tumor inoculation. The tumor elimination rate over the first 18 hr was significantly reduced in mice treated with LPS ($p < 0.006$).

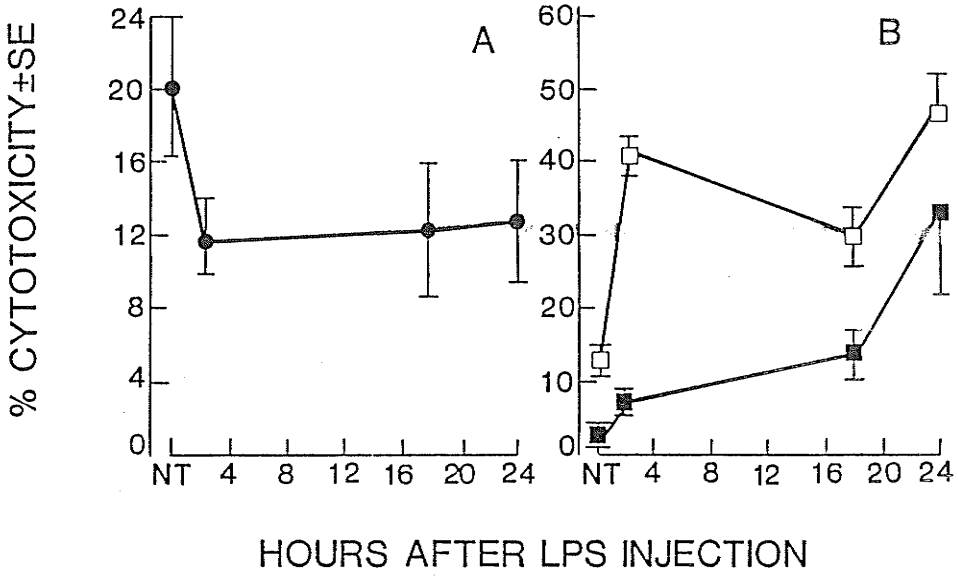

FIGURE 1.4 - Effect of LPS on NK cell and NAb activity. (a) The complement-dependent cytolysis of whole serum from normal DBA/2 mice and mice given 100 μg LPS i.p. 2, 18 and 24 hr earlier was assayed using the DBA/2 syngeneic SL2-5 lymphoma which is more NAb-sensitive than the L5178Y-F9. Serum NAb levels were significantly depressed by 2 hr after LPS treatment (●) ($p < 0.05$). (b) Normal DBA/2 mice and mice gien 100 μg LPS i.p. 2, 18 or 24 hr earlier were examined with respect to splenic (□) and peritoneal (■) NK-cell activity against the NK-sensitive YAC-1 lymphoma at effector to target ratios of 75:1 or 25:1 respectively. NK activity was significantly enhanced by 18-24 hr in both the spleen and the peritoneal cells with $p < 0.03$ and $p < 0.04$ respectively.

significantly depressed over the first 24 hours (Fig. 1.4A) appearing to correlate well with the reduction in tumor elimination seen shortly after LPS administration and with the increased tumor frequency of threshold sc tumor inocula injected within 24 hours of LPS.

The effect of LPS on tumor progression

In order to study the effect of LPS on natural immune selection against a neoplasm growing from a small number of tumor cells, we examined the NR phenotype of the tumors that developed from threshold sc inocula in normal and LPS treated mice. The tumors from animals given LPS 3 days before the threshold inocula had appeared at a lower frequency (Table 1.4, Expt. 1) and exhibited markedly reduced sensitivities to NR in the ^{131}IdUrd elimination assay compared with a tumor taken from an animal that had received no LPS (Table 1.5). NAb binding of these cells determined through fluorescence analysis was similarly reduced (Table 1.5). Threshold tumor inocula injected only 2 hours after LPS exhibited a higher survival rate than similar inocula in untreated mice (Table 1.4, Expt. 1) and the tumors that grew out exhibited similar reductions in ^{131}I-labelled tumor clearance (Table 1.5) and only slightly less reduced binding of NAb (Table 1.5) compared with the tumors from mice given LPS 3 days before tumor cells.

LPS inhibition of NAb binding

Considering the evidence for cross reactivity between bacterial and tumor antigens (31), we examined whether competative inhibition of

TABLE 1.5 - SENSITIVITY TO NR AND NAb BINDING OF TUMORS FROM LPS-TREATED MICE

Tumor source[1]	Tumor elimination[2] % ^{131}I retained ± SE day 3	NAb binding Mean channel fluorescence ± SE[3] (4)	(6)
In vitro control	13.1 ± 2.0	88.3 ± 7.7	ND
Normal DBA/2	18.8 ± 2.4	86.0 ± 8.7	82.5 ± 6.3
2 hr LPS DBA/2			
I	35.1 ± 1.9[5]	69.6 ± 8.7	61.7 ± 7.4[4]
II	33.0 ± 2.6[5]	73.7 ± 6.3	66.5 ± 5.9[4]
3 day LPS DBA/2			
I	27.8 ± 3.2[5]	57.6 ± 8.2[4]	ND
II	37.7 ± 6.6[5]	59.8 ± 6.0[4]	ND

[1] Tumors obtained from the injection site 3.5 weeks after s.c. inoculation of 25 L5178Y-F9 cells into DBA/2 mice given 100 μg LPS i.p. 2 hr previously, 3 days previously or into normal mice, were compared with L5178Y-F9 cells maintained in vitro.
[2] I.P. elimination of 5×10^6 cells.
[3] The number of tests are given in brackets.
[4] $p < 0.03$
[5] $p < 0.01$ when compared to the tumor from a normal DBA/2.
ND = Not done.

antitumor NAb by LPS may account for the reductions in NAb levels seen shortly after LPS injection. Because ontogeny studies had shown that NAb levels increased slightly with advancing age (26), serum from retired breeders was employed to provide a high level of unstimulated serum NAb. Although LPS did not decrease the NAb binding of high concentrations of DBA/2 serum to the SL2-5 tumor determined through fluorescence analysis, when a 1:60 dilution of serum was preincubated for 20 min at 37°C with 1600 ug LPS/ml the mean fluorscence channel minus background fluorescence was reduced to 2.5 ± 3.2, compared to 13.6 ± 2.1 for untreated serum and the difference was significant ($p<0.02$).

DISCUSSION

Although exposure to silica has been associated with an increased incidence of "spontaneous" (6,7), carcinogen-induced (32) and transplanted tumors (7,11) in experimental animals, the mechanisms of its cocarcinogenic effect have not been well studied. The macrophage toxicity of this agent has, however, been well documented using both in vivo and in vitro studies and the harmful effects of silica in vivo have largely been attributed to this property. Our current investigations clearly point out the importance of the adjuvant effects of silica as a contributing factor in tumor progression.

In the present and previous studies (7), Cab-o-sil treatment increased the tumor frequency of threshold sc tumor inocula in vivo. Since silica has frequently been applied in experimental animal models to abrogate accessory cell function and systemic phagocytosis we

considered that it had interfered with anti-tumor natural resistance against these tumor foci. Nevertheless, the tumors that survived exhibited an augmented reduction in NAb binding and sensitivity to NR in vivo suggesting that they had undergone a more rigorous selection by host natural defense mechanisms compared with the tumors from untreated mice. The effects of silica on the host immune system therefore appeared to follow biphasic kinetics, whereby the early abrogation of natural resistance associated with the increased tumor frequencies was followed by an enhancement of constituents that contributed to an increased clearance of radiolabelled tumor cells and an augmented tumor selection. In retrospect, this stimulation was suggested by small decreases in the tumor frequencies of threshold tumor inocula injected 7 days after the initiation of Cab-osil treatment (7). The observations are consistent with a silica induced stimulation of NR supporting our contention that a more resistant tumor could have evolved in animals exposed to Cab-o-sil through the increased selection pressure exerted by silica stimulated NR.

The increased tumor frequencies of threshold sc tumor inocula injected within 24 hours of LPS administration, in conjunction with the reduced tumor elimination rates and NAb levels seen 24 hours after LPS treatment, suggest that a period of attenuated NR occurs shortly after exposure to this immune adjuvant. However, we have previously shown that the tumor frequencies were reduced in animals given the same threshold sc tumor inocula 2-3 days after LPS while NAb and NK levels showed correlating increases measured at 5 days (7). Thus our present and previous observations combine to suggest that LPS also has

a biphasic effect upon NR which differentially alters the fate of small tumor inocula injected at different times. The evidence for a biphasic effect of LPS was extended by the ^{131}IdUrd labelled tumor elimination assay which revealed an initial period of reduced tumor clearance followed by an augmented elimination rate in LPS treated mice. Vaage (21) has reported similar kinetics in the detrimental before beneficial effects of BCG on the outgrowth of mammary carcinoma transplants in syngeneic mice. While prophylactic exposure to treatment with BCG before the tumor inoculation reduced the tumor incidence, initiation of the BCG therapy simultaneously with the mammary carcinoma transplant increased the tumor frequency in comparison with control animals.

Previous investigators have described agents with both stimulatory and inhibitory effects on NK cells; pyran copolymer, BCG, C. parvum and adriamycin (cited in 23). The increases however, were usually observed first at approximately day 3 for spleen and peritoneal cells and the decreases were observed subsequently, day 10-15 in the splenic effectors, in association with suppressor cells either macrophages or non-adherent cells lacking easily detectable markers. In our experiments, the biphasic effects consisted of an early abrogation in both in vivo and in vitro parameters of NR followed by increases that occurred in approximately the same time frame as the augmentation in NK activity observed by others. Although the generation of suppressor cells has been proposed to explain the reduced NK levels reported at 10-15 days after exposure to a variety of adjuvants (23), the brief intervals between our LPS or silica

treatment and the observed reductions in NR argue that induced specific suppression is an unlikely rationale. Furthermore, the initial tumor enhancing phase of BCG infection seen on the growth of mammary carcinoma transplants in mice (21) could not be transferred with spleen and lymph node cells from BCG treated animals in accord with the idea that the early inhibitory phase of microbial modulators is not the result of suppressor cell activity.

Individual parameters of NR including RES, NAb and NK activities were assessed to identify the mediators that might contribute to the sequential alterations in host resistance to tumors following exposure to silica. Early decreases were observed in the NAb and splenic NK antitumor activities following exposure to silica, suggesting that reductions in these reactants may have contributed to the increased tumor frequencies of threshold tumor inocula in Cab-o-sil treated mice. In addition, subsequent increases were seen in NAb and in peritoneal cell activities which correlated with the stimulatory effects of silica on NR measured as reduced tumor frequencies (7) and as an increased elimination of radiolabelled tumor cells. Characterization of the silica induced peritoneal cell activity suggested that phagocytic cells and cells that exhibit asialo-GM1 and the Thy-1 marker consistent with the antigenic profile of NK cells (33,34,35), both contributed to the dramatic rise in the cytolytic capacity of the peritoneal population. Furthermore, the apparent inverse alterations in splenic and peritoneal activity and cellularity suggested that movement of effector cells may have been an underlying factor in the biphasic response of silica.

Although a decrease in systemic phagocytic function can not be implicated in the early response to silica, more subtle alterations in macrophage activities influencing the action of other mediators may still have contributed to the increased outgrowth of threshold tumor inocula in Cab-o-sil treated mice (7). In this regard macrophage regulation of NK activity has been proposed (36,37) so that even slight alterations in macrophage function following exposure to silica may lead to a rapid increase in their suppressive effect upon NK cell activity. For example, macrophage derived prostaglandin E_2 has been shown to suppress NK cell activity (38). Alternatively, considering that large granular lymphocytes exhibit a chemotactic response (39,40) our data is consistent with a marked exodus of NK cells from the spleen directed toward the peritoneal cavity where there is a dramatic increase in cell numbers and spontaneous tumoricidal activity seen by ourselves and others within 5 days of silica injection into that environment (11). Such a homing of NK activity to the peritoneal cavity may at least transiently reduce NR in other tissue sites. Although we can only speculate at this time on the processes underlying the early reduction in detectable antitumor NAb after silica injection, macrophage release of FcR (40,41) or the appearance of cross-reacting antigens in the circulation possibly as a result of cell stimulation or tissue damage may be contributing factors. This could temporarily decrease the effective level of NAb, one element in the transient reduction in NR.

The marked stimulation of NR seen as reduced tumor frequencies and increases in tumor elimination, NAb and cytolysis by peritoneal NK

and phagocytic cells, is consistent with the adjuvant effect that silica has exerted on adaptive immune responses (12,13,14). Since macrophages exposed to silica exhibit small increments in intracellular IL-1 and show increases in IL-1 release (43) the antitumor effects are likely pleiotropic (reviewed in 44) and similar to the nonspecific adjuvant effects induced by microbiol adjuvants such as LPS. Macrophage activation may contribute directly to increased anti-tumor resistance through cytolysis by phagocytic cells for example those detected in the peritoneal population. In addition, IL-1 released from the macrophages may initiate a cascade of NK augmenting lymphokines including IL-2 (reviewed in 45) and IFN (reviewed in 46) which could activate NK cells alone (reviewed in 47) or in synergy with IL-1 (48). IL-1 may act directly against sensitive tumor targets (49). Finally, IL-1 released by macrophages exposed to silica may provide a necessary stimulus for B cell activation (50) synergizing with T cell derived B cell growth and differentiation factors (51,52) or possibly acting directly on pre-B cells (53) to increase serum NAb and thus contributing to the rise in NR. It is also possible that the adjuvant effect of silica on antitumor defence may be partially mediated by tumor necrosis factor. Recently it has been demonstrated that alveolar macrophages incubated with silica particles or asbestos fibers release tumor necrosis factor in a concentration dependent fashion (63).

The LPS induced augmentation of NR including increases in NAb and NK levels and the correlating decreases in tumor incidence of threshold sc tumor inocula demonstrated the adjuvant effect of this

agent on nonadaptive immunity (26). This may occur to a large extent through stimulation of macrophages to release IL-1 or tumor necrosis factor (54,60) similar to the effects outlined for silica or as a result of the enhanced production of reactive oxygen metabolites (61). In addition bacterial lipopolysaccharides are known to augment IL-6 secretion by endothelial cells. A number of important immunological effects have been attributed to IL-6 including the stimulation of Ig synthesis by B cells, the enhancement of T cell responses, and the proliferation of pluripotent hematopoietic stem cells (cited in 62).

While the stimulatory effects of microbial products have been extensively studied, the inhibitory activity has not. However several alternatives have been proposed for their early suppressive effects. Vaage (21) has argued that antigenic competition by BCG during a critical inductive phase of the primary response against a mammary carcinoma transplant may account for the early detrimental effect of this adjuvant. Flemming and Nothdurft (55) reported an early reduction and subsequent increase in reticulo-endothelial system function in response to iv doses of LPS which were comparable in size to our ip injections. They contend that the early depression in phagocytic activity was based on haemodynamic changes and direct damage to macrophages. Intravenous injection of LPS has also been associated with the production of a factor which was detrimental to cell mediated immunity assayed in the mixed lymphocyte reaction (56) and the data was consistent with interferon-β acting as the suppressive agent. Furthermore the down regulation of intracellular and secreted lysozyme 24 hours after macrophage exposure to LPS or

interferon α/β has been cited as an indication that constitutive macrophage function may be compromised during increased synthesis of induced activities (57).

Our experiments suggest that the early reduction in NR seen as increased tumor frequencies of threshold tumor inocula and reduced tumor clearance in LPS treated mice may also be based at least in part on the correlating decrease in NAb activity. Although competitive inhibition by LPS was detected only at a low concentration of retired breeder serum, the ability of LPS to reduce fluorescence detected NAb binding suggests that in addition to Fc receptor or cross reactive antigen release due to cell stimulation, rapid antibody absorption by the injected LPS could contribute to the reduction in that mediator. Transient decreases in preformed NAb may be a generalized phenomenon in microbial therapy considering the apparent cross-reactivity between bacteria and tumor cells. The concept of overlapping specificities supported by the demonstration of bacterial reactive antitumor NAb (31) is also evident in the proposal that specific stimulation may contribute to the enhanced antitumor NAb levels that arise in time after exposure to microbial products (25,58) and in the speculation that BCG may elicit specific responses against certain tumors; hepatoma, melanoma and leukemic cells due to cross-reactivity (59).

The initial decrease and subsequent augmentation of NAb and/or NK activities in silica and in LPS treated mice corresponding inversely with the changes in tumor frequencies of threshold tumor inocula extended the support for NK cells and NAb acting against tumors in vivo and for the biphasic nature of the effects of these agents on

NR. The increases in NAb and peritoneal NK cell reactivity in LPS treated mice and also peritoneal phagocytic activity in Cab-o-sil treated mice taken in conjunction with the outgrowth of tumors that expressed correlating reductions in their elimination rates and NAb binding beyond the decreases exhibited by tumors from untreated animals support a role for all of these mediators and NAb in particular, acting against tumors in vivo.

Our data clearly reveal the dual nature of NR in tumor progression. Although natural defence mechanisms may effectively eliminate a tumor in its early stages of development these same processes provide a selective pressure to direct and drive tumor progression once the neoplasm has overcome this defence. Under conditions of augmented resistance, once the tumor has escaped, the selection pressure will be more severe and the rate of progression therefore accelerated. This could contribute to the sustained detrimental effects of adjuvant treatment seen as enhanced tumor growth (20,21). Furthermore, threshold tumor inocula injected during the phase of reduced NR shortly after LPS treatment yielded higher tumor frequencies and more highly resistant tumors than similar inocula in control animals, suggesting that this combination of reduced NR followed by an augmented response is particularly lethal. Others have also proposed that partial immunosuppression may actually drive tumor progression by providing an environment favorable to the outgrowth of antigenic tumor variants. Previously, it has been demonstrated that a highly immunogenic UV induced regressor tumor which is readily rejected from immunocompetent normal mice, is capable of forming tumors in mice

partially immune suppressed by UV irradiation. Tumor cell lines reisolated from UV treated recipients demonstrated a heritable loss of tumor specific antigens and a change to a progressor tumor phenotype as indicated by their ability to form tumors in normal mice. In contrast, although the parental tumor readily grew in severely immune compromised nude mice, reisolated tumor cell lines showed no change in antigenicity or regressor phenotype (2). The authors suggest that partial immune suppression may allow more time for the tumor to "generate and expand" antigenic variants which could then be selected for by antitumor immune effectors. These observations emphasize the hazards of therapeutic intervention that might initially decrease the host's natural or innate resistance to a developing tumor. This could temporarily allow a period of more extensive tumor proliferation and variant generation followed by a subsequent increase in the host's natural defence which would focus a more vigorous selection process upon the expanded tumor population thus increasing the rate of progression toward a more autonomous tumor. Similarly, the impact of environmental exposure to silica on an incipient tumor is at least twofold. An initial period of reduced host natural defences would permit a less restrained proliferation and increased generation of variants in the tumor foci which would then undergo an exaggerated selection due to the subsequent stimulation of NR. The repetition of this sequence of events through chronic exposure to low doses of silica would be expected to markedly accelerate tumor development.

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CHAPTER 2

Tumor Progression in Vitro: Tumor Promotor Induced Reversable Decrease in Natural Immune Susceptibility

ABSTRACT

Growth of established murine tumor lines in media containing the phorbol ester tumor promotor 12-0-tetradecanoylphorbol-13-acetate (TPA), was associated with reversible reductions in sensitivity to in vitro and in vivo parameters of natural resistance. L5178Y-F9 cells exposed to 100 ng TPA/ml for 2 days and returned to culture without TPA for 0-2 days, exhibited reductions in sensitivity to complement mediated lysis by natural antibodies (NAb), activated macrophages and hypotonic lysis. The natural killer (NK) cell sensitive SL2-5 lymphoma was less sensitive to NK cells, complement dependent NAb and hypotonic lysis after 2 days growth in 2 or 3 μg TPA/ml. Although TPA treated L5178Y-F9 cells could bind higher levels of serum NAb in vitro, this was complicated by the instability of the binding at 37°C resulting in an effectively reduced capacity to bind NAb which was also demonstrated by TPA treated SL2-5 cells. The tumor frequency of threshold subcutaneous inocula and the intravenous metastatic potential of the TPA treated tumors was increaed in syngeneic DBA/2 mice revealing possible correlations between reductions in the cellular characteristics assayed in vitro and decreased susceptibility to host mediated defenses in vivo. Continued growth of the TPA treated cells for a total of 2-8 days without TPA produced a reversal in the in vitro parameters, in the tumor frequency and in the metastatic potential indicating the requirement for TPA to maintain the resistant phenotype. These data are consitent with the initial reversible nature of the promotion phase of multistage carcinogenesis. The reversible TPA induced reductions in sensitivity to mediators of

natural resistance may be an integral component of promotion, contributing to tumor survival in vivo and increasing the probability that the tumor will progress to a more malignant phenotype.

INTRODUCTION

The concept of immune surveillance, proposes that immune mechanisms can provide a defense against neoplasia by recognizing and eliminating the aberrant cells. This theory was initially based on the adaptive immune response, in particular, cytotoxic T lymphocytes (reviewed in 1). Substantial evidence, however, has resulted in the subsequent rejection of T cell mediated tumor immunosurveillance against incipient neoplasia and the metastatic spread of disease in favor of a non-adaptive thymus independent natural immunity. Cellular and humoral natural immune effectors such as natural killer (NK) cells, macrophages and natural antibodies (NAb) appear to provide a potent defense against small tumor foci (2-6). Successful tumor development in vivo would be anticipated to require the initial escape from these immune effectors. This may be accomplished either through the suppression of immunosurveillance (as discussed in Chapter 1) or as a result of variants within the tumor cell population acquiring a natural defense resistant phenotype. In current models of multistage carcinogenesis (reviewed in 7,8), in vivo application of a chemical tumor promotor 12-0-tetradecanoylphorbol-13-acetate to initiated mouse skin produced promotor-associated biochemical changes and palpable tumors. Extensive experimentation has also shown that exposure to the same promotor in tissue culture yields pleiotropic effects on cells including an array of early membrane associated changes followed by macromolecular synthesis dependent alterations (reviewed in 9). It seemed reasonable therefore to examine the effect of this phorbol ester tumor promotor on tumor susceptibility to the

parameters of NR in vivo and in vitro in order to assess the extent to which changes in tumor sensitivity to NR may contribute to promotion. Furthermore, we predicted that any effects of the promoting agent on tumor susceptibility to NR might be reversible at least initially, consistent with the early reversibility of murine skin tumor promotion (7,8).

Abbrevations: NR, natural resistance, TPA, 12-0-tetradecanoylphorbol-13-acetate; NAb, natural antibodies; NK, natural killer; LPS, lipopolysaccaride; HBSS, Hank's balanced salt solution; 10% FFBS, Fischers' medium containing 10% fetal bovine serum; IP, intraperitoneal; SC, subcutaneous; DMSO, dimethyl sulfoxide; 4-0-Me-TPA, 4-0-methyl-12-0-tetradecanoyl-phorbol-13-acetate; E/T, effector/target ratio; PEC, peritoneal exudate cells; C. parvum, Corynebacterium parvum; FACS, fluorescence-activated cell-sorter; FITC, fluorescein isothiocynate; P, probability; Pt_i, P t-independent; Pt_d, P t-dependent; S.C., subcutaneous; i.p., intraperitoneal.

MATERIALS AND METHODS

Mice, sera and tumor cells

Male DBA/2 mice were obtained from Canadian Breeders, Charles River, Quebec, and from the University of Manitoba Vivarium at Gunton, Manitoba. Sera were bled per axilla from lipopolysaccharide (LPS) stimulated DBA/2 mice 3-5 days after the intraperitoneal (i.p.) injection of 100 ug LPS (Sigma Serotype 0127:B8) dissolved in Hank's Balanced Salt Solution (HBSS). LPS stimulation has been shown to increase the levels of anti-tumor NAb and to decrease the tumor

fequencies of small subcutaneous (SC) inocula of the same tumor cells (10).

The NK resistant L5178Y-F9 and the NK sensitive SL2-5 tumor lines were obtained from their parental DBA/2 strain T-cell lymphomas L5178Y and SL2-5 through two successive clonings in sloppy agar (10). These tumor lines were maintained in vitro using Fisher's medium containing 10% fetal bovine serum (10% FFBS) supplemented with penicillin and streptomycin (GIBCO Grand Island, N.Y.).

In vitro drug treatment

Clone L5178Y-F9 cells were grown for 2 days from a concentration of 2 x 10^4 cells/ml, in 10% FFBS or the same medium containing a) 100 ng/ml TPA (Consolidated Midland Corporation, Brewster, New York) and 0.1% or 0.01% dimethyl sulfoxide (DMSO) (Fisher Scientific Canada), b) 100 ng 4-0-methyl-12-0-tetradecanoylphorbol-13-acetate (4-0-Me-TPA) and 0.1% DMSO, or c) 0.1% or 0.01% DMSO. The cells were then either tested or washed once in 25 ml 10% FFBS and returned to standard tissue culture. SL2-5 cells were also similarly grown in 2 or 3 ug/ml TPA and 0.2 or 0.3% DMSO respectively.

Tumor frequency

Tumor cells were washed three times and serially diluted in HBSS for injection. An aliquot of 0.1 ml containing the desired number of cells was injected s.c. into a shaved area in the the middle of the lower back of each mouse. Tumor frequency was assessed by the appearance of a palpable lump at the injection site.

Metastasis

Tumor cells were washed three times and diluted to 2.5 X 10^6/ml in HBSS. Aliquots of .1 ml containing 2.5 X10^5 cells were injected into a tail vein. Mice were sacrificed after 14-16 days and the white surface tumor nodules on the liver were counted using an inverted microscope.

Cytolysis Assays:

NK cells - Briefly tumor cells from in vitro cultures were washed and labelled with ^{51}Cr (Na_2CRO_4) (100 uCi/10^7 cells) for 45 min at 37°C. The cells were washed three times with HBSS and adjusted to 10^5 cells/ml in 10% FFBS. Spleen cells obtained from polyinosinic :polycytidylic acid stimulated mice (100 ug i.p. 20 hours previously) were treated with 0.15 M ammonium chloride for 4 min at room temperature to lyse the red blood cells and then washed 3 times in HBSS. Splenic effector cells were combined with 100 ul aliquots of the tumor target cells at three effector to target (E/T) ratios, 150/1, 75/1 and 37.5/1, in a final volume of 200 ul in V-bottom microtitre plate wells. Following a 4 min centrifugation at 35 g the plates were incubated at 37°C in 3% CO_2 for 6 hr. The plates were centrifuged for 10 min at 180 g and 100 ul aliquots of supernate were removed for gamma counting.

Natural antibody and complement - This two-step microcytotoxicity assay employed an incubation of ^{51}Cr-labelled tumor target cells with whole LPS-induced serum as a source of NAb (200 ul/3-5 x 10^5 cells) for 1 hr at 37°C, followed by one wash and an incubation at 37°C for 1

hour with a 1/12 dilution of specifically absorbed rabbit complement (10).

Activated macrophages - As previously reported (11), murine peritoneal exudate cells (PEC) were collected 4 days after i.p. injection of 100 ug Corynebacterium parvum (C. parvum). Red blood cells were lysed with 0.15M ammonium chloride and the PEC washed twice with HBSS and resuspended in 10% FFBS. Equal aliquots of PEC were allowed to adhere to flat bottomed microtitre plate wells for 75 min at 37°C in 6% CO_2. Adherent macrophages were washed 3x with HBSS and aliquots of 10^4 ^{51}Cr labelled tumor cells were added in 200 ul 10% FFBS so that each tumor target was tested at the same, single E/T in each experiment and this ranged from 15/1-50/1 over 4 experiments. Plates were incubated for 18 hrs at 37°C in 6% CO_2, centrifuged for 10 min at 200 g and an aliquot of the supernatant was removed from each well for gamma counting.

Hypotonic lysis - In a modification of the assay described by Russel et al (12), ^{51}Cr labelled tumor cells were incubated in either 15% isotonic 10% FFBS or isotonic 10% FFBS for 1 hr at 37°C in V-bottom microwell plates. The plates were centrifuged for 10 min at 200 g and an aliquot of the supernatant removed for gamma counting.

The percentage lysis in all of the microcytotoxicity assays was calculated as:

$$\% \text{ specific cytolysis} = \frac{\% \text{ experimental } ^{51}Cr \text{ release} - \% \text{ spontaneous } ^{51}Cr \text{ release}}{100 - \% \text{ spontaneous } ^{51}Cr \text{ release}} \times 10$$

Flourescence-activated cell-sorter (FACS) analysis of NAb binding

Aliquots of 5 x 10^5 L5178Y-F9 or SL2-5 tumor cells were washed in Hepes buffered 10% FFBS containing 0.1% sodium azide and incubated for 1-2 hrs in a 3% CO_2 atmosphere at 37°C in 200 ul of LPS-stimulated serum (whole or 1/2, 1/4, 1/8 dilutions) or medium containing 0.1% azide. The cells were then washed and incubated in 100ul of fluorescein isothiocyanate (FITC) conjugated goat IgG (7S) anti-mouse IgG (Meloy, Springfield, VA.) and goat $F(ab^1)_2$ anti-mouse IgM (Tago, Burlingame, Ca.) each diluted 1:10 in Hepes buffered 10% FFBS containing azide for 20 minutes at room temperature in the dark. The cells were washed and fixed by incubating them for 5 minutes in 100 ul of 1% formaldehyde in HBSS, and the final sample volume was adjusted to 0.5 ml with Hepes buffered 10% FFBS containing 0.1% sodium azide. Samples were stored on ice until analyzed for linear fluorescence using a Coulter Epics V Multiparameter Sensor System. The control cells treated with medium followed by the fluorescein labelled antibody exhibited very little fluorescence.

Statistics

The two tailed student's t-test was used to determine the statistical significance between differences observed in the various assays. P values for independent (Pt_i) and paired or dependent (Pt_d) analyses greater than 0.05 were not considered significant.

RESULTS

Susceptibility to complement mediated NAb lysis

L5178Y-F9 cells were grown for 2 days in 10% FFBS containing 100 ng/ml TPA, a dose which had previously been shown to generate consistent reductions in complement mediated NAb lysis (13). In the current study this dose of TPA resulted in a significant, greater than 50% reduction in complement mediated NAb lysis (Table 2.1). Similar treatment with 4-0-Me-TPA, an incomplete, first stage promotor (7), produced a significant but smaller reduction, approximately 25%, in sensitivity to NAb and complement. Growth in the solvent DMSO used to dissolve the phorbol esters produced no change in NAb mediated lysis suggesting a relationship between promotor activity and the extent of the reduction in cytolysis. Similar exposure of a second murine T cell lymphoma, the SL2-5, to 100 ng TPA/ml for 2 days, DMSO alone or 4-0-Me-TPA did not produce a decrease in NAb and complement lysis (Table 2.1, expts. 46). Increasing the concentration of TPA to 3 ug/ml produced a significant decrease in sensitivity of SL2-5 cells to NAb and complement (Table 2.1, expt 7) indicating the variation in susceptibility of even similar tumor types to TPA modification.

The return of the promotor treated tumors to standard tissue culture produced a reversal of the NAb and complement resistant phenotype within 2-4 days (Fig. 2.1A and B).

Susceptibility to NK mediated cytolysis

Similar studies using the NK sensitive SL2-5 lymphoma demon

Table 2.1 - Reduced sensitivity of TPA treated tumors to NAb and complement

Expt #[a]	Tumor	Treatment[b]	% Cytolysis ± SE	% of Control Cytolysis	Pt_d
1.(6)	L5178Y-F9	none	15.3 ± 1.8		
		100ng TPA + 0.1 or 0.01% DMSO	6.2 ± 0.9	40.5	<0.002
2.(6)	L5178Y-F9	none	15.5 ± 2.1		
		0.1 or 0.01% DMSO	14.7 ± 1.1	94.8	NS
3.(6)	L5178Y-F9	none	13.9 ± 1.0		
		100ng 4-O-ME-TPA + 0.1 or 0.01% or DMSO	10.7 ± 0.4	77.0	<0.03
4.(6)	SL2-5	none	39.7 ± 1.1		
		100ng TPA + 0.1 or 0.01% DMSO	45.9 ± 3.3	115.6	NS
5.(14)	SL2-5	none	37.0 ± 1.9		
		0.1 or 0.01% DMSO	42.8 ± 4.6	115.6	NS
6.(4)	SL2-5	none	39.8 ± 1.3		
		100ng 4-O-Me-TPA + 0.1 or 0.01% DMSO	41.6 ± 2.8	104.5	NS
7.(5)	SL2-5	none	45.5 ± 8.1		
		2ug TPA + 0.2% DMSO	40.9 ± 10.2	89.9	NS
		3ug TPA + 0.3% DMSO	34.9 ± 5.8	76.7	<0.03

[a] The number of tests performed is shown in parenthesis.
[b] Tumors were grown for 2 days in media containing the agents indicated.

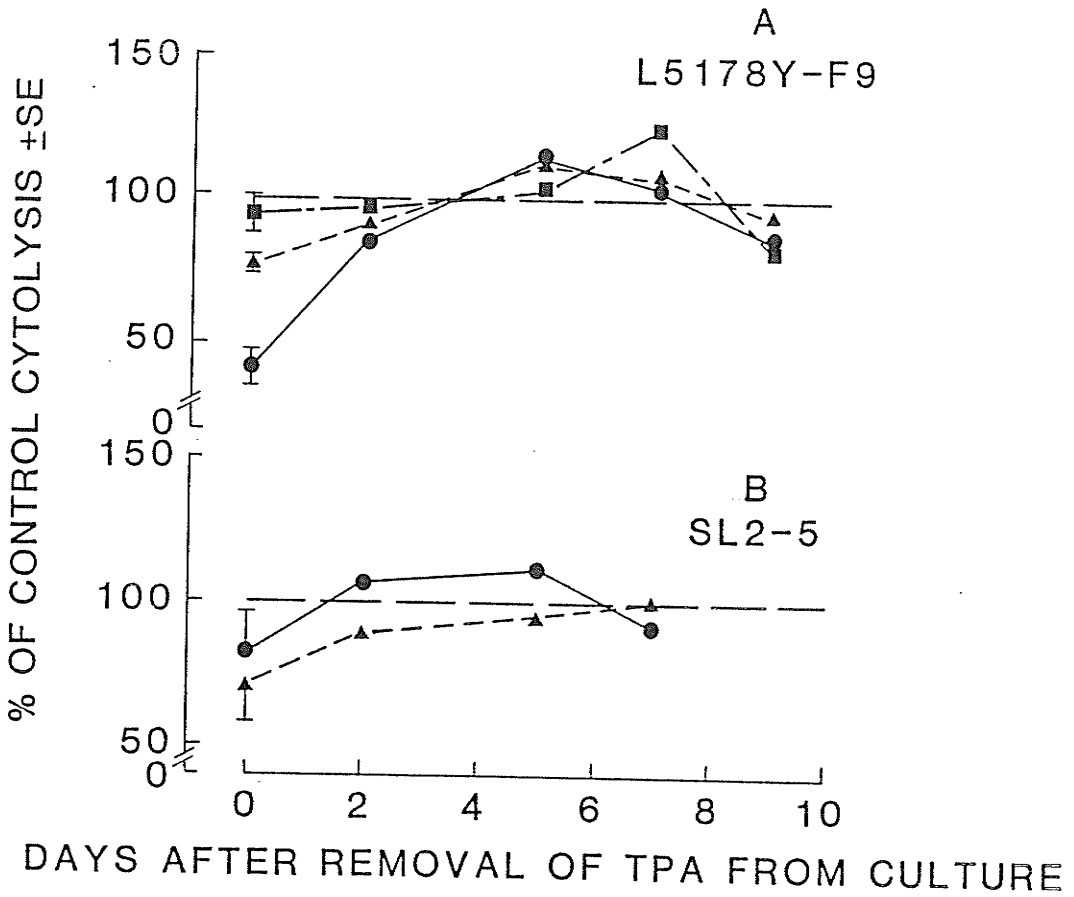

Figure 2.1. Reversible reduction in sensitivity of TPA-treated cells to NAb and complement and to NK cells. Tumor cells grown for two days in a phorbol ester and/or DMSO were washed, returned to standard culture for the periods indicated and tested for their sensitivity to NAb and complement. Susceptibility is expressed as a percentage of the cytolysis of untreated control cells of the same clone. A) L5178Y-F9 cells were grown in 100 ng TPA/ml with DMSO (0.1 or 0.01%/ml) (●)($Pt_d < 0.002$ at time 0 vs control), 100 ng 4-0-Me-TPA/ml with DMSO (▲) ($Pt_d < 0.03$ at time 0 vs control) or DMSO (■) B) SL2-5 cells were grown in 2 or 3 ug TPA/ml with 0.2 or 0.3% DMSO respectively (●) ($Pt_d < 0.05$ at time 0 vs control). Sensitivity of the TPA/DMSO treated SL2-5 cells to NK cytolysis was also assessed and lysis at E/T of 150/1 expressed similarly (▲)($Pt_d < 0.001$ at time 0 vs control). The numbers of lytic units capable of lysing 20% of the time 0 vs the control cells, $8.85 \pm 1.71 \times 10^3$ vs $13.67 \pm 1.30 \times 10^3/10^6$ spleen cells respectively, were significantly different ($Pt_d < 0.02$).

strated that 2 days growth in 10% FFBS containing 2 or 3 ug TPA/ml also induced a significant decrease in NK susceptibility. This phenotype was also reversible upon removal of TPA from culture, with the NK sensitivity being equivalent to the control values 7 days after return to normal tissue culture (Figure 2.1B).

Susceptibility to macrophage mediated cytolysis

L5178Y-F9 cells grown in 100 ng TPA/ml for 2 days showed an initial significant 50% reduction in sensitivity to C. parvum activated macrophages. Cells examined 6 days after their return to standard tissue culture could not be distinguished from non-treated control L5178Y-F9 (Table 2.2).

NAb binding

Since susceptibility to mediators of natural resistance involves at least recognition and lysis, NAb binding of TPA treated tumors was assessed through indirect fluorescence analysis in order to dissect the TPA-induced reduction in tumor sensitivity to NAb and complement. The effect of TPA on NAb binding depended on the conditions of the assay. Tests performed using serum NAb dilutions of 1/4-1/6 and a lower incubation temperature, 4°C, revealed generally moderate levels of control L5178Y-F9 binding with increases as high as 50% in NAb binding at day 0 or 2 days after the exposure to TPA (Table 2.3A) and an increased heterogeneity in the NAb binding profile of the TPA treated cells (Figure 2.2). Under such assay conditions, 100 ng TPA/ml was observed to produce an optimal increase in NAb binding

Table 2.2 - Reversible reduction in cytolysis of TPA-treated L5178Y-F9 cells by activated macrophages

Tumor Treatment[a] TPA	Days after TPA	% Cytolysis ± SE[b]	Pt_d[c]
-	-	55.2 ± 9.9	
+	0	20.3 ± 8.3	< 0.03
+	6	61.6 ± 7.2[d]	NS

[a] Cells grown in media containing 100ng TPA/ml for 2 days were tested immediately or after 6 days in standard culture with no TPA.

[b] Four experiments were performed using a single E/T in each experiment which ranged from 15/1-50/1.

[c] Compared with no TPA treatment.

[d] Ptd < 0.01 compared with 0 days after TPA treatment.

Table 2.3 - Differential Nab binding of TPA-treated L5178Y-F9 tumor cells depending on the binding conditions

Expt #[a]	TPA[b]	Days After TPA	Serum Nab Incubation				Mean Fluorescence Channel Nab Binding ± SE
			Dilution	Temperature	Time	Cycles	
A 1.(4)	-	-	1/6	37°C	1 hr	1	48.1 ± 3.2
	+	0	1/6	37°C	1 hr	1	54.4 ± 5.1
	+	2	1/6	37°C	1 hr	1	69.0 ± 4.5[e]
2.(5)	-	-	1/6	4°C	30 min	1	41.0 ± 3.5
	+	0	1/6	4°C	30 min	1	63.0 ± 5.0[f]
B 1.(2)	-	-	whole serum	37°C	30 min	1	111.5 ± 21.8
	+	0	whole serum	37°C	30 min	1	54.9 ± 50.2
	+	2	whole serum	37°C	30 min	1	40.0 ± 30.7
2.(1)	-	-	1/4	37°C	2 hr	1	52.9
	+	0	1/4	37°C	2 hr	1	51.6
	-	-	1/4	37°C	1 hr	2[c]	75.7
	+	0	1/4	37°C	1 hr	2[c]	50.9
3.(1)	-	-	1/4	37°C	1 hr	2[c]	82.0
	+	0	1/4	37°C	1 hr	2[c]	46.8
	+	2	1/4	37°C	1 hr	2[c]	29.5
			4th and Final Serum NAb Incubation				
			Wash	Dilution	Temperature	Time	Cycles
C[d].(3)	+	2	-	prev 1/4	4°C	1 hr	1	78.8 ± 10.0
	+	2	+	fresh 1/4	4°C	1 hr	1	82.2 ± 5.0
	+	2	-	prev 1/4	37°C	1 hr	1	35.7 ± 3.6[g]

[a] The number of tests performed is shown in parenthesis.
[b] L5178Y-F9 were grown in 100 ng TPA/ml for 2 days.
[c] Cells were incubated in a fresh aliquot of serum for the second cycle.
[d] In experiment C, TPA treated L5187Y-F9 cells were washed and incubated in fresh aliquots of a 1/4 dilution of serum NAb for 1 hour at 4°C, 3 times. These cells were then divided and without washing incubated in the third aliquot of NAb (prev 1/4) for 1 additional hour at 4°C or at 37°C or washed and incubated in a fresh aliquot of NAb (fresh 1/4) at 4°C for 1 hour.
[e] Pt_d <0.02 compared with no TPA treatment control.
[f] Pt_d <0.002.
[g] Pt <0.002 and Pt <0.002 compared with prev 1/4 at 4°C and fresh 1/4 at 4°C respectively.

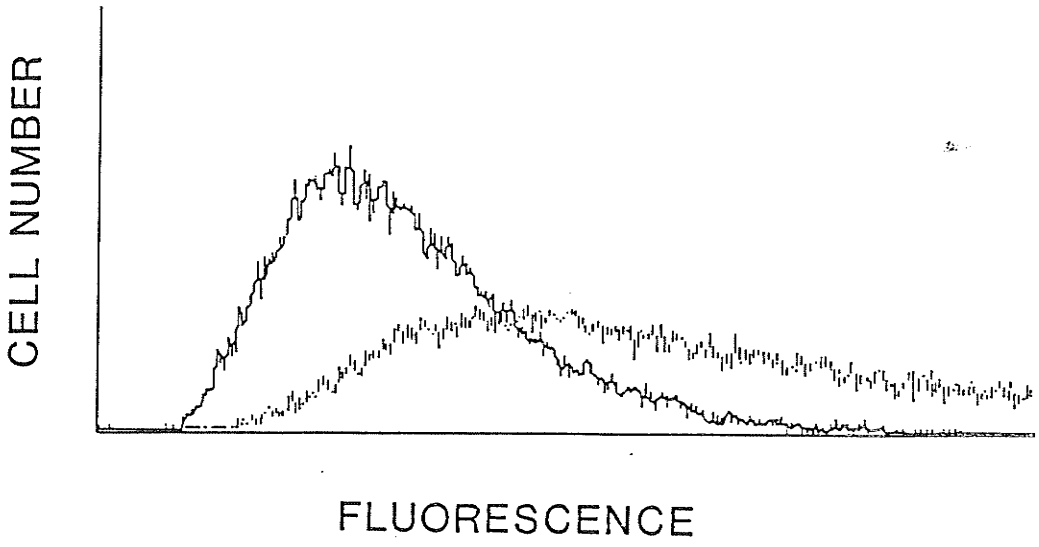

Figure 2.2. Indirect fluorescence-detected NAb binding profile of TPA treated L5178Y-F9 cells. The NAb binding capacity of L5178Y-F9 cells grown im 100 ng/ml TPA for 36 hrs (----) was assayed in comparison with untreated control L5178Y-F9 cells (——).

(Fig. 2.3). However, assays employing a higher incubation temperature, 37°C , and higher concentrations of LPS-induced serum NAb or repeated cycles of incubation in fresh aliquots of diluted serum NAb, produced higher levels of control L5178Y-F9 NAb binding with significantly and markedly reduced NAb binding (50%) on day 0 or 2 days after the TPA treatment (Table 2.3B).

A possible relationship between the high and low NAb binding exhibited by TPA-treated L5178Y-F9 was examined by incubating the cells at 4°C in 3 cycles of fresh diluted serum NAb to increase the NAb binding and then splitting the cells into aliquots for subsequent incubation at 4°C or 37°C to assess the binding stability. Three cycles of exposure to a fresh 1/4 dilution of NAb at 4°C for 1 hr each followed by an additional hour incubation at 4°C in the third aliquot of serum produced consistently high binding and this was not improved by exposure to fresh serum during the fourth hr at 4°C (Table 2.3C). However an additional hour incubation or as little as 5 minutes in the third aliquot of serum at 37°C produced a marked and significant reduction in NAb binding (Table 2.3C). UV light microscopic examination of the NAb binding pattern of the TPA treated cells incubated in NAb only at 4°C revealed tiny points of fluorescence over the entire surface of the cells with a few brighter spots. Fewer and only very faint fluorescent points were seen on the cells following the subsequent incubation at 37°C. A similar higher NAb binding of TPA treated cells was observed for IgM and for IgG assayed separately at 4°C, with a decrease upon incubation at 37°C (data not shown).

Examination of tumor cells grown in TPA and returned to standard

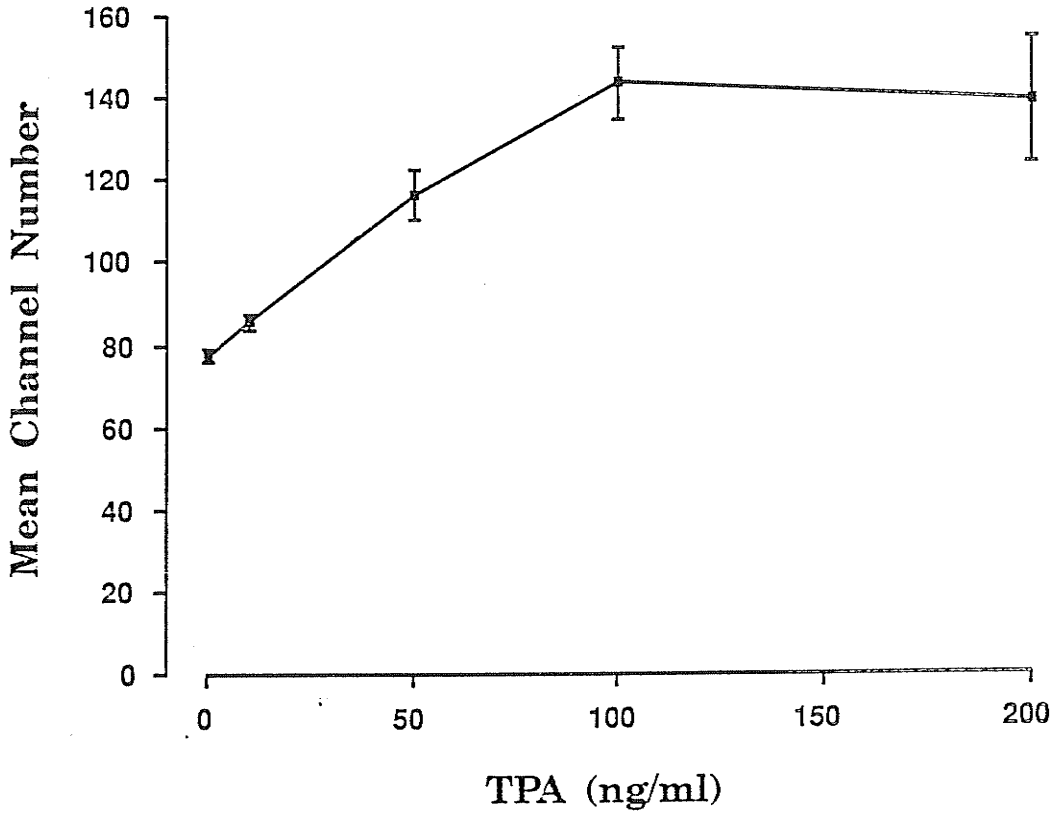

Figure 2.3. Effect of TPA on NAb Binding. L5178Y-F9 cells were grown for two days in the presence of TPA at the concentration indicated, washed and returned to standard culture for an additional 48 hours. NAb binding assays were performed using a 1:6 serum NAb dilution and at 4°C, conditions aimed at stabilizing the tumor cell membrane.

tissue culture showed that the NAb binding of TPA-treated L5178Y-F9 cells assayed at 1/6 serum dilution and 37°C had returned to normal levels 4-6 days after their return to culture without TPA (Table 2.4, expt 1). SL2-5 cells grown in 2 ug TPA for 2 days also exhibited a reversible change in NAb binding. Assayed with concentrated serum at 37°C they revealed an initial reduction in NAb binding seen after 2 days out of TPA culture followed by a return to control NAb binding levels after 2 more days in standard culture (Table 2.4, expt 2).

Susceptibility to hypotonic lysis

Evidence of metabolic control in tumor cell lysis by murine antibodies and complement (14) or by NK cells (15-17) coupled with the correlating sensitivities of tumors to NK cells, activated macrophages and hypotonic lysis (17) suggested the importance of osmotic fragility in susceptibility to NR mediators. Therefore, the sensitivity to hypotonic lysis of L5178Y-F9 cells grown in TPA for 2 days was assessed in 15% isotonic medium. The cells exposed to TPA exhibited a significant reduction in susceptibility to hypotonic lysis which returned to control levels within 2 to 4 days following their return to standard tissue culture with no TPA (Table 2.5). A similar reversible decrease in sensitivity to hypotonic stress was seen for TPA treated SL2-5 cells, however, the reduction occurred 2 days later than for the L5178Y-F9. These observations support a direct alteration in tumor cell sensitivity to lysis by TPA rather than an indirect effect through TPA released from the tumor cell altering an effector activity.

Table 2.4 - Reversible alteration in NAb binding by TPA-treated tumor cells

Expt #[a]	Tumor Cells	TPA[b]	Days After TPA	Serum NAb Incubation				Mean Fluorescence Channel NAb Binding ± SE
				Dilution	Temperature	Time	Cycles	
1.(3)	L5178Y-F9	-	-	1/6	37°C	1 hr	1	44.9 ± 2.6
		+	0	1/6	37°C	1 hr	1	52.7 ± 7.8
		+	2	1/6	37°C	1 hr	1	67.3 ± 6.6[c]
		+	4	1/6	37°C	1 hr	1	36.3 ± 8.4
		+	6	1/6	37°C	1 hr	1	40.5 ± 5.4
		+	8	1/6	37°C	1 hr	1	40.9 ± 2.0
2.(2)	SL2-5	-	-	whole serum	37°C	30 min	1	112.1 ± 8.3
		+	0	whole serum	37°C	30 min	1	121.9 ±31.1
		+	2	whole serum	37°C	30 min	1	43.1 ± 7.0
		+	4	whole serum	37°C	30 min	1	112.7 ±18.0

[a] The number of tests performed is shown in parenthesis.
[b] L5178Y-F9 and SL2-5 cells were grown in 100 ng and 2 ug TPA respectively for 2 days.
[c] Pt_d <0.02 compared with no TPA treatment control.

Table 2.5 - Reversible reduction in sensitivity to hypotonic lysis of TPA-treated tumor cells

Expt #[a]	Tumor	Treatment[b] TPA	Days after TPA	%Cytolysis ± SE
1.(4)	L5178Y-F9	-	-	56.3 ± 5.0
		+	0	40.7 ± 5.7[c]
		+	2	52.8 ± 9.4
		+	4	64.4 ± 5.5
2.(1)	SL2-5	-	-	41.5
		+	0	45.3
		+	2	22.8
		+	4	28.4
		+	8	38.6

[a] The number of tests perfomed is shown in parenthesis.
[b] Cells grown in TPA for 2 days, 100 ng/ml in experiment 1 and 2 ug/ml in experiment 2, were returned to standard culture for the periods indicated.
[c] $Pt_d < 0.02$ compared with no TPA treatment.

Tumor frequency of TPA treated tumors

In order to determine whether the transient TPA-induced natural defense resistant phenotype detected in vitro was reflected in the fate of the cells in vivo, their tumorigenicity was assessed in the threshold s.c. tumor inoculum assay of NR. L5178Y-F9 cells grown in 100 ng/ml TPA for 2 days consistently exhibited increased tumor frequencies which amounted to 3 times the incidence arising from the same inoculum of untreated cells (Table 2.6). The TPA-treated L5178Y-F9 cells grown in the absence of TPA for 8 days revealed an intermediate tumor frequency in parallel assays. While the differences were small a similar reversible trend was observed with SL2-5 cells grown in 2 ug/ml TPA (Table 2.6).

Metastatic potential of TPA-treated tumors

Since mediators of natural immune resistance have been implicated in the defense against circulating tumor cells (3,18) the intravenous or "experimental" metastatic capability of the TPA-treated, natural defense resistant tumors was examined. L5178Y-F9 cells grown in 100 ng/ml TPA for 2 days and tested immediately or after 1 day in standard tissue culture without TPA exhibited an approximately seven-fold increase in their ability to form liver foci (Table 2.7). Continued growth of the TPA treated cells in standard culture for 8 days was associated with a metastatic capacity that could not be statistically distinguished from control untreated L5178Y-F9 cells.

Table 2.6 - Reversible increase in tumor frequency of TPA-treated tumor cells

Expt #[a]	Inoculum[b]				Tumor Frequency	
	Cell	TPA	Days after TPA	Number	Number	%
1.(4)	L5178Y-F9	-	-	25	6/28	21.4
	L5178Y-F9	+	0	25	17/27	63.0
2.(2)	L5178Y-F9	-	-	25	4/14	28.6
	L5178Y-F9	+	0	25	12/14	85.7
	L5178Y-F9	+	8	25	6/13	46.2
3.(1)	SL2-5	-	-	10^4	1/6	16.7
	SL2-5	+	0	10^4	2/6	33.3
	SL2-5	+	6	10^4	1/6	16.7

[a] The number of tests performed is shown in parenthesis

[b] Cells grown in TPA for 2 days, 100 ng/ml in experiments 1 and 2 and 2 ug/ml in experiment 3, were returned to standard culture for the periods indicated.

Table 2.7 - Reversible increase in experimental metastasis of TPA-treated L5178Y-F9

Expt #	TPA	Days After TPA	Surface Liver Nodules[a] per Mouse	Mean ± SE
1.	-	-	5,0,0	1.7 ± 1.7
	+	1	25,15,1	13.7 ± 7.0
	+	8	0,0,0	0.0 ± 0.0
2.	-	-	15,9,8,0,0,0	5.3 ± 2.6[b]
	+	0	61,43,25,25,24	35.6 ± 7.3
	+	8	19,19,13,7,2,1	10.2 ± 3.3[b]

[a] L5178Y-F9 cells grown in standard media or in 100 ng TPA/ml for 2 days and returned to tissue culture without TPA for the number of days indicated, were washed and injected IV in a dose of 2.5×10^5 cells. Surface liver nodules were counted 15 and 14 days following the injections for experiments 1 and 2 respectively.

[b] $Pt_i < 0.01$ compared with cells just grown in TPA for 2 days.

DISCUSSION

Previous investigators have described the suppressive effect of phorbol ester tumor promotors on the natural immune system, in particular NK cells (19) and macrophages (20,21). The present data supports the idea that these agents may also contribute to tumor development through a second mode, the generation of a natural defense resistant tumor phenotype. We observed that two days growth of an NK sensitive or an NK resistant murine T cell lymphoma in TPA reduced their sensitivity to cytolysis by the mediators of NR tested; complement dependent NAb for both tumors, activated macrophages for the L5178Y-F9 and NK cells for the NK sensitive SL2-5. In addition, TPA-treated L5178Y-F9 cells exhibited an increased SC tumor forming capacity and upon IV injection produced more liver metastasis. TPA treatment increased the tumorigenicity of SL2-5 cells slightly. Subsequent tumor growth in culture medium containing no TPA demonstrated the reversibility of the resistant phenotype. The concurrent changes in tumor sensitivity to the in vitro parameters of NR, in the tumorigenicity of threshold inocula and in IV metastasis formation support our contention that promotor induced changes in tumor NR phenotype contribute to tumor development. Furthermore, they are consistent with the early reversibility of TPA induced promotion of initiated mouse skin in vivo (8).

Dissection of the reversible reduction in sensitivity to complement dependent NAb lysis revealed corresponding changes in susceptibility to hypotonic lysis and in NAb binding under conditions simulating physiologic. Our indirect fluorescence-detected NAb binding

assays carried out under conditions of low binding efficiency and/or at 4°C to facilitate the stabilization of the tumor cell membrane, demonstrated a reversible TPA-induced increase in NAb binding by L5178Y-F9. This suggests that TPA regulates the expression of molecules associated with NAb binding whether through specific antigen/antibody reaction, Fc receptor or lectin-like binding. Whether the augmented NAb binding detected at 4°C on cells grown in TPA for 2 days is in any way associated with a TPA-induced increase in IL-2 receptor expression as seen with a variety of human T cell types (22,23) is currently under investigation. In contrast, reversible reductions in NAb binding were seen with the TPA treated cells under conditions of greater exposure to NAb at a higher temperature, 37°C. These opposing observations appear to arise from the instability of high NAb binding to the TPA treated tumor because TPA treated L5178Y-F9 cells which had acquired a high level of surface NAb through repeated incubation in fresh aliquots of serum at 4°C exhibited a rapid reduction in bound NAb following a subsequent incubation at 37°C. This suggests a loss of surface Ig possibly due to increased shedding or endocytosis associated with TPA-induced increased membrane fluidity (24) or augumented receptor-like internalization upon NAb binding similar to the rapid effects of TPA on unoccupied epidermal growth factor receptors (25), C3b receptors (26) and transferrin receptors (27). These observations coupled with previous demonstrations of high levels of NAb acquisition in vivo (28) and the strong inverse association consistently seen for NAb binding and tumorigenicity (4,10,11) suggest that a rapid loss of surface bound NAb

following high NAb binding under physiological conditions in vivo probably contributes to the increased tumorigenicity and metastasis of the TPA treated cells.

It is also possible that the shedding of NAb/antigen complexes from the surface of TPA treated cells under physiological conditions may lead to the induction of tumor specific suppression. Previously, it has been reported that the i.p. injection of tumor cells or KCI extracted tumor antigens together with antitumor antibodies (sequentially or in the form of immune complexes) into allogeneic mice results in a specific immune suppression. One of the most significant features of this suppression is the inability of macrophages from immune suppressed animals to bind specific tumor cells even in the presence of cytophilic antibodies. It is speculated that antigen/antibody complexes may result in the generation of a $Lyt1^+$ suppressor inducer population which then induces $Lyl23^+$ suppressor effector cells (29-30). The induction of specific suppression resulting from the rapid shedding of NAb/antigen complexes from the surface of TPA treated cells in vivo would be entirely consistent with and help explain the observed increase in tumorigenicity of TPA treated cells.

Since osmotic lysis has been implicated as a final process in cell death initiated by complement dependent antibodies, NK cells and by activated macrophages (12,14-17) the TPA induced reduction in susceptibility to hypotonic stress may explain to a large extent the reversible reductions in tumor cell sensitivity to all three mediators and to host mediated NR in vivo. Although TPA induced normal human

lymphocytes to be killed by NK cells (31) arguing that promotion of normal cells may be associated with increased sensitivity to NR, reductions have been reported in susceptibility of TPA-treated tumor and embryonic cells to NK lysis (32-34) and exposure to TPA reduced macrophage cytolysis of a rat fibrosarcoma (20). While the TPA induced NK resistance of the HL-60 was attributed to a decrease in NK binding (34), the NK resistance of TPA-treated trophoblast cells which was associated with increased tumorigenicity was not (33). This NK resistance was however reversed by inhibition of protein synthesis, reminiscent of our previous studies of tumor resistance to hypotonic lysis which could be reversed by cycloheximide (4). The evidence to date therefore suggests that at least a second phenotypic change, decreased sensitivity to osmotic lysis in addition to reduced effector binding, can account for TPA induced reductions in sensitivity to NK cells, activated macrophages and complement-dependent NAb.

There are a number of implications in these data. First the reduction and subsequent restoration of tumor susceptibility to the mediators of natural resistance associated with exposure to and removal from TPA suggests that this promotor reversably regulates tumor sensitivity to these mediators. The correspondence in the kinetics of the in vitro changes with the rise and fall of the tumorgenicity and metastasis of the TPA treated cells argues that these mediators may play a role in the defense against the tumors in vivo. Furthermore, the underlying reversible reduction in sensitivity to hypotonic lysis, may rationalize to a great extent the changes in mediator susceptibility observed in vitro and the alterations in

tumorigenicity and metastatic potential in vivo. Our observations are consistent with and may help to explain the initially reversible nature of promotion seen in the in vivo mouse skin model of multistage carcinogenesis. The reversible TPA-induced reductions in sensitivity to mediators of NR may be an integral component of promotion, contributing to tumor survival in vivo and increasing the probability that the tumor will progress to a more malignant phenotype.

The TPA induced increased potential for NAb binding is interesting. While associated with an effective reduction in NAb binding under conditions intended to simulate the physiological environment, it nevertheless indicates that the response to a promotor includes an increased reactivity with a mediator of natural immune resistance, NAb, consistent with predictions that arise from a consideration of immune surveillance acting against tumor promotion. Furthermore the increased NAb reactivity may provide a marker for promotion and an opportunity for eventual therapeutic manipulation.

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CHAPTER 3

Tumor Promoter Regulation of Natural Antibody Binding

ABSTRACT

In this report we continue to examine the previously described unstable increase in natural antibody (NAb) binding observed with L5178Y-F9 cells treated for 48 hours with the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) followed by 48 hours in normal media. Here we demonstrate that the non-tumor promoting, protein kinase C (PKC) activating diacylglycerol, 1-oleoyl-2-acetyl-sn-glycerol (OAG) is unable to mimic the TPA induced increase in NAb binding. In fact, OAG resulted in a significant decrease in binding, in contrast also with the lack of change produced with the non tumor promoting, non-PKC activating TPA analogue 4-0-Me-TPA. These observations coupled with the ability of the PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) but not N-(2-guanidino-ethyl)-5-isoquinolinesulfonamide (HA1004) to block the TPA induced increase in NAb binding implies that PKC activation may be required but is not sufficient to generate this phenotype. While both TPA and OAG were able to induce similar decreases in NAb binding after 22 hours of treatment, the non-PKC activating 4-0-Me-TPA had no effect. This suggests that the differences in NAb binding observed with cells grown for longer periods in either TPA or OAG may be the result of events which occur subsequent to the initial activation of PKC by either agent. In addition, while disruption of the cytoskeleton or blocking of energy production resulted in the abrogation of the TPA mediated increase in binding, similar treatments had little effect on normal NAb binding. These data imply that the increase in NAb binding seen with TPA requires both a functional cytoskeleton and active energy production.

INTRODUCTION

The term immunosurveillance was first used by Burnet (1) to describe the host mediated immune defence against incipient neoplasia. In addition to natural killer (NK) cells (2) and macrophages (3,4) natural antibodies are important components of natural immune surveillance as evidenced by correlative in vivo and in vitro experiments (5,7), Winn type assays in syngeneic mice (6) and recent studies involving xid bearing mice which are genetically predisposed for low serum NAb levels (8). It has been suggested that natural antibodies provide a potent defence against small tumor foci, by mediating opsonization and/or antibody dependent cellular cytotoxicity or possibly by interfering with critical tumor cell surface events (8). Although several groups of tumor associated antigens including developmental epitopes, major histocompatability molecules, viral proteins and the carbohydrate moieties of cell surface glycolipids and glycoproteins are known to react with NAb (reviewed in 5) the nature of those antigens recognized during NAb mediated tumor surveillance is not well understood.

The increase in tumorigenicity observed during the in vivo growth of tumor cells is characterized by a number of progressive phenotypic changes including decreased reactivity with syngeneic NAb (9). In addition tumor cells grown in the presence of a tumor promoting phorbol ester and then selected in vitro for reduced sensitivity to complemented mediated lysis by NAb, demonstrate a significant reduction in sensitivity to natural resistance as measured in a tumor elimination assay in vivo (10).

These observations suggest that changes in the NAb binding ability of tumor cells may play an important role in tumor progression. However, very little is known about the regulation of NAb binding. 12-0-tetradecanoylphorbol-13-acetate (TPA) has been widely used in multistage carcinogenesis studies as a potent promoter of tumor development. Although it is not carcinogenic, the chronic application of TPA to skin previously initiated with suboptimal doses of a carcinogen will result in an enhanced rate of tumor development. While TPA can still provide effective tumor promotion even up to several months after carcinogenic initiation it has no effect on tumor development if applied prior to initiation suggesting that the TPA induced changes which are critical to tumor promotion are in fact also reversible (11,12,13). In addition to their effects on the promotion stage of tumor development, phorbol ester tumor promoters have been shown to induce several specific phenotypic alterations associated with enhanced tumorigenicity and progression. These include the induction of anchorage independent growth (14), secretion of collagenase and other proteases (14), increased metastatic capacity (15) and the induction of multidrug resistance (16). TPA may also enhance tumor development by facilitating the escape of neoplastic cells from immune surveillance effectors. It has previously been demonstrated that TPA has suppressive effects on various mediators of natural immunity, in particular NK cells and macrophages (17,18,19). More recently, we have demonstrated that treatment of a murine T cell lymphoma line with TPA was also associated with a reversible decrease in susceptibility to a variety of natural immune effectors including

NAb and complement, NK cells and macrophages (20). Both decreased tumor susceptibility and natural immune effector activity may contribute to the tumor promoting ability of TPA.

In apparent opposition to an increased resistance to natural immunity we have previously reported that TPA exposure for 48 hours was associated with an increase in NAb binding when assayed under conditions aimed at minimizing cell surface antigen mobility and modulation (4°C and high serum NAb dilutions). NAb binding was further enhanced by an additional 48 hour incubation in standard tissue culture. This phenotype was shown to be highly unstable in that the enhanced NAb binding achieved through repeated incubations in fresh aliquots of serum at 4°C was rapidly lost when the temperature was increased to 37°C. Moreover, TPA treated cells actually displayed a decreased ability to bind NAb when assayed under conditions which were closer to physiological (37°C and higher serum concentrations) (20).

The mechanisms by which TPA mediates decreases in susceptibility to natural resistance and in particular regulates NAb binding are unknown. Protein kinase C is believed to be the major cytoplasmic receptor for tumor promoting phorbol esters, and the role of this kinase in many TPA generated phenotypic changes has been well established (13,21,22). Protein kinase C is intrinsically involved in a variety of cellular activation and differentiation events. Diacylglycerol generated by phospholipase C mediated hydrolysis of phosphotidylinositol-4,5-bisphosphate functions as the endogenous activator of protein kinase C in receptor mediated signal transduction

(21,23). As a result of a common receptor system TPA and diacylglycerols have been shown to have many similar effects on cells. However, recently it has become evident that not all of the effects of TPA can be ascribed to the activation of protein kinase C (13). Synthetic diacylglycerols have proven ineffective in mimicking TPA in the induction of anti-CD3 mediated proliferation of periferal T cells (24) as well as the maturation of HL-60 cells (25,26). These results have been interpreted to mean that in these systems TPA induced maturation and proliferation is either independent of, or at the very least, not solely dependent on PKC activation. In addition, the inability of diacylglycerols to replace TPA in the induction of the IL-2 receptor (24) or the down regulation of the CD7, CD4 and CD3 differentiation antigens on peripheral T cells (27,28) has led to the suggestion that PKC activation may not be independently responsible for mediating all of the effects of TPA on antigen expression.

In the current investigation, we examined the mechanisms by which TPA regulates NAb binding to L5178Y-F9 T leukemic cells with particular attention payed to the role of PKC. The regulation of a number of cell surface antigens by TPA appears to be mediated by cytoskeletal changes or are dependent upon functional energy metabolism (29,30). For this reason, the cytoskeletal and energy requirements of TPA induced changes in NAb binding were also examined.

Abbreviations used: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; NAb, natural antibody; NK, natural killer cells; LPS, lipopolysaccharide; HBSS, Hank's balanced slat solution; 10% FFBS, Fischers' medium containing 10% fetal bovine serum; FITC,

fluorescein isothiocynate; S.C., subcutaneous; 4-O-Me-TPA, 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; H7, 1-(5-ioquinolinesulfonyl)-2-methylpiperazine; HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide; P, probability.

MATERIALS AND METHODS

Mice and Sera

Male DBA/2 mice were obtained from the University of Manitoba Vivarium, Manitoba. Sera for comparative tumor analysis were bled per axilla from lipopolysaccharide (LPS)-stimulated DBA/2 mice 3-5 days after the i.p. injection of 100 μg LPS (Sigma Serotype 0127:B8) dissolved in Hank's Balanced Salt Solution (HBSS). LPS stimulation has been shown to increase the levels of anti-tumor NAb and to decrease the tumor frequencies of small subcutaneous (s.c.) inocula of the same tumor cells (6).

Cell Lines

The NK-resistant L5178Y-F9 was obtained from the parental DBA/2 strain T-cell lymphoma L5178Y through two successive clonings in sloppy agar (6). This tumor line was maintained in vitro using Fischer's medium containing 10% fetal bovine serum (10% FFBS) supplemented with penicillin and streptomycin (Gibco, Grand Island, NY).

In Vitro Drug Treatment

Cloned L5178Y-F9 cells were grown for 2 days from a concentration of 2 x 10^4 cells/ml, in 10% FFBS or in the same medium containing (i) 100 ng/ml TPA (Consolidated Midland Corporation, Brewster, NY), (ii)

100 μg/ml 4-0-Me TPA (Sigma Chemical Co.) or (iii) 100 μg/ml OAG (Sigma Chemical Co.). After 2 days the cells were washed once in 25 ml 10% FFBS and returned to standard tissue culture for an additional two days before being assayed. Alternatively, L5178Y-F9 cells were grown for 22 hours from a concentration of 1 x 10^5 cells/ml, in 10% FFBS or in the same medium containing (i) 100 μg/ml TPA, (ii) 100 μg/ml 4-0-Me-TPA or (iii) 100 μg/ml OAG, washed and assayed directly.

For experiments examining the early effects of TPA cloned L5178Y-F9 cells were washed and resuspended in 10% FFBS or the same medium containing 100 μg/ml TPA for various stated lengths of time prior to being assayed for NAb binding.

Inhibitors

H7 and HA1004 (Seikagaku America, Inc.) were dissolved in distilled water at the concentration of 10 mM and stored at 4°C in the dark. Sodium azide, cholchicine, and cytochalasin B (Aldrich Chemical Co., Milwaukee, WI) were dissolved in HBSS and stored at 4°C in the dark.

NAb Binding

Tumor cells (3 x 10^5) were washed in 10% FFBS (Hepes buffered) containing 0.1% sodium azide and incubated twice for 1 hour in a 5% CO_2 atmosphere at 4°C in 200 μl of LPS-stimulated serum, diluted with the same medium containing 0.1% azide. The cells were then washed and re-incubated for 20 min in the dark at 4°C in 100 μl of fluorescein-isothiocyanate-conjugated (FITC) goat $(Fab')_2$ anti-mouse IgG (Meloy,

Springfield, VA) and FITC goat $(Fab')_2$ anti-mouse IgM (Tago, Burlingame, CA) (each diluted 1:10 in Hepes-buffered 10% FFBS containing azide). The cells were washed and fixed by incubation for 5 min in 100 μl of 1% formaldehyde in HBSS, and the final sample volume was adjusted to 400 μl with Hepes-buffered 10% FFBS containing 0.1% azide. Samples were analyzed at 4°C for linear fluorescence using a Coulter Epics V Multiparameter Sensor System. The control cells treated with medium followed by the fluorescein-labelled antibody exhibited no fluorescence beyond that of untreated cells.

Statistics

The Student's t-dependent test was used to determine the statistical significance between differences observed in the various assays. T-dependent analyses were performed unless otherwise indicated. P values greater than 0.05 were not considered significant.

RESULTS

Effect of diacylglycerol and 4-0-Me-TPA on NAb binding. Protein kinase C is widely believed to be the major cytoplasmic receptor for TPA. If the changes in the NAb binding phenotype associated with TPA treatment are regulated by PKC activation, other PKC activators should be able to mimic the effect of tumor promoting phorbol esters. Certain synthetic diacylglycerols, such as OAG are known to be readily intercalated into the plasma membranes of intact cells resulting in the direct activation of PKC (reviewed in 21). Previously, it has been demonstrated that growth of L5178Y-F9 cells in the presence of TPA for 48 hours followed by 48 hours in standard tissue culture resulted in a significant increase in NAb binding when assayed at 4°C (20). However, when L5178Y-F9 cells were grown in the presence of a range of OAG concentrations, the same treatment protocol failed to induce a similar increase in NAb binding (Fig. 3.1). In fact, OAG resulted in a significant decrease in binding at 100 μg/ml, also in contrast with the lack of any alteration in this phenotype following treatment with the non tumor promoting non-PKC-activating methylated analogue of TPA, 4-0-Me-TPA (Fig. 3.2). The inability of 4-0-Me-TPA to mediate any change in NAb binding implies the TPA induced increase in binding is not the result of a non-specific effect of phorbol ester exposure, such as the hydrophobic interaction of TPA with the plasma membrane. This is consistent with our previous observation that 4-0-Me-TPA was significantly less efficient than TPA in mediating a decrease in sensitivity to NAb and complement (20).

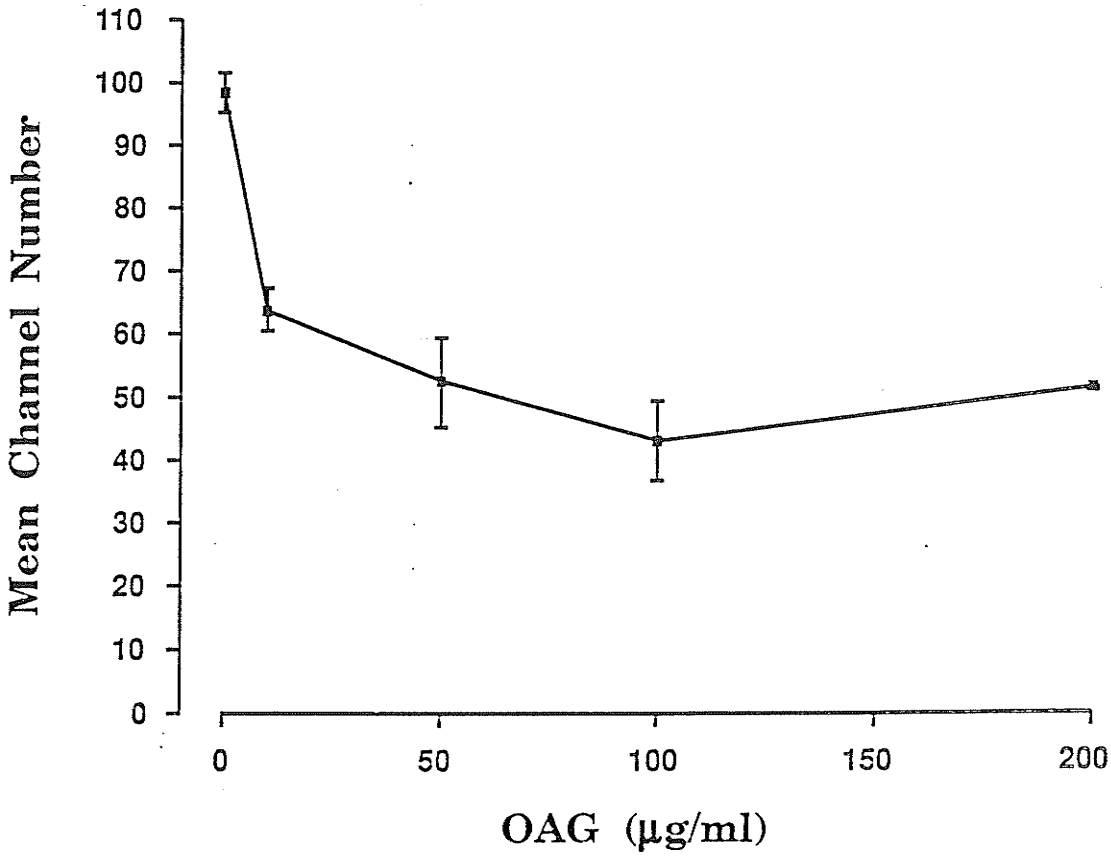

Figure 3.1 - Effect of OAG on NAb binding. L5178Y-F9 cells were grown for 2 days in the presence of OAG at the concentrations indicated, washed and returned to standard culture for an additional 48 hours. Cells were assayed for NAb binding as described. An OAG concentration of 100 µg/ml was selected for all subsequent experiments.

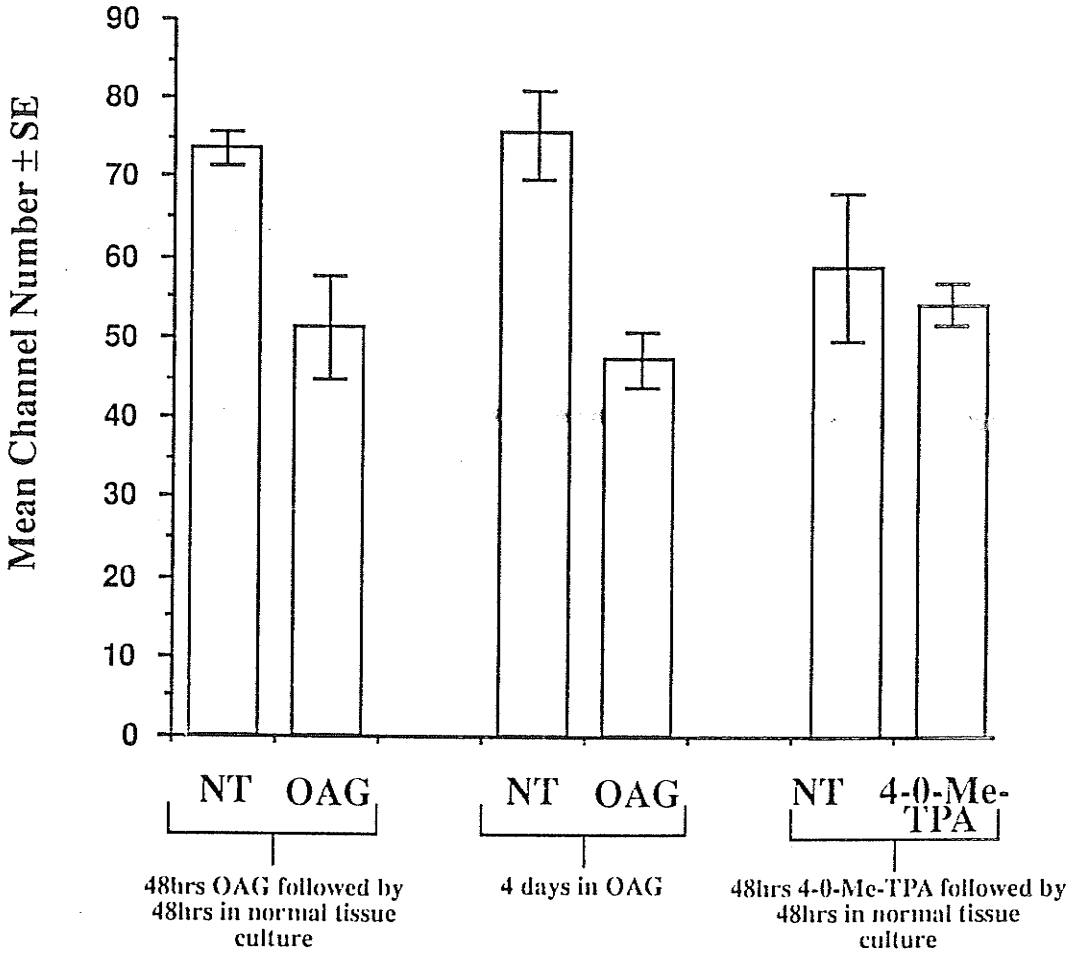

Figure 3.2 - Effect of prolonged growth in the presence of OAG and 4-0-Me-TPA on the NAb binding phenotype of cells. Incubation of L5178Y-F9 cells with OAG (100 μg/ml) for 48 hours followed by an additional 48 hours in normal 10% FFBS, or 4 days continuously in OAG results in a decrease in NAb binding. In contrast, 48 hours growth in the presence of 4-0-Me-TPA (100 ng/ml) followed by an additional 48 hours in normal 10% FFBS had no effect on NAb binding. Cells were assayed for NAb binding as described. OAG resulted in a significant decrease ($p < .05$) for both drug treatment conditions.

OAG differs from TPA in that the former is metabolized much more rapidly in the cell (21). The observation that cells cultured in OAG (100 μg/ml) for 48 hours and then exposed to the same drug for an additional 48 hours do not demonstrate the enhanced NAb binding phenotype seen with TPA (Fig. 3.2) argues against the metabolism of OAG being responsible for the differences seen with diacylglycerol and TPA.

Effect of PKC inhibition on TPA mediated increases in NAb binding. H-7 is a potent and relatively specific inhibitor of PKC mediated events (31). H7 was shown to inhibit the increase in NAb binding resulting from the growth of L5178Y-F9 cells for 48 hours in TPA, followed by an additional 48 hours in standard tissue culture (Fig. 3.3). Interestingly, co-culturing in the presence of H7 (12 μM) not only blocked the TPA induced increase in NAb binding but also a portion of the NAb binding in untreated cells, implying that protein kinase C may be involved in the regulation of both. HA1004, a non-PKC inhibiting control for H-7 had no effect on NAb binding to either TPA treated cells or non-treated controls (Fig. 3.4).

Early effects of TPA on NAb binding. Previously we have reported that L5178Y-F9 cells exposed to 100 μg/ml TPA for 48 hours demonstrated a marked increase in NAb binding which is enhanced by an additional 48 hour incubation in normal tissue culture when assayed under conditions which minimize antigen mobility (4°C and dilute serum). This increase was shown to be transient returning to untreated control levels by 6 days out of TPA. However, nothing was known about the effect which TPA had on NAb binding over the first 24 hours of treatment. Here we

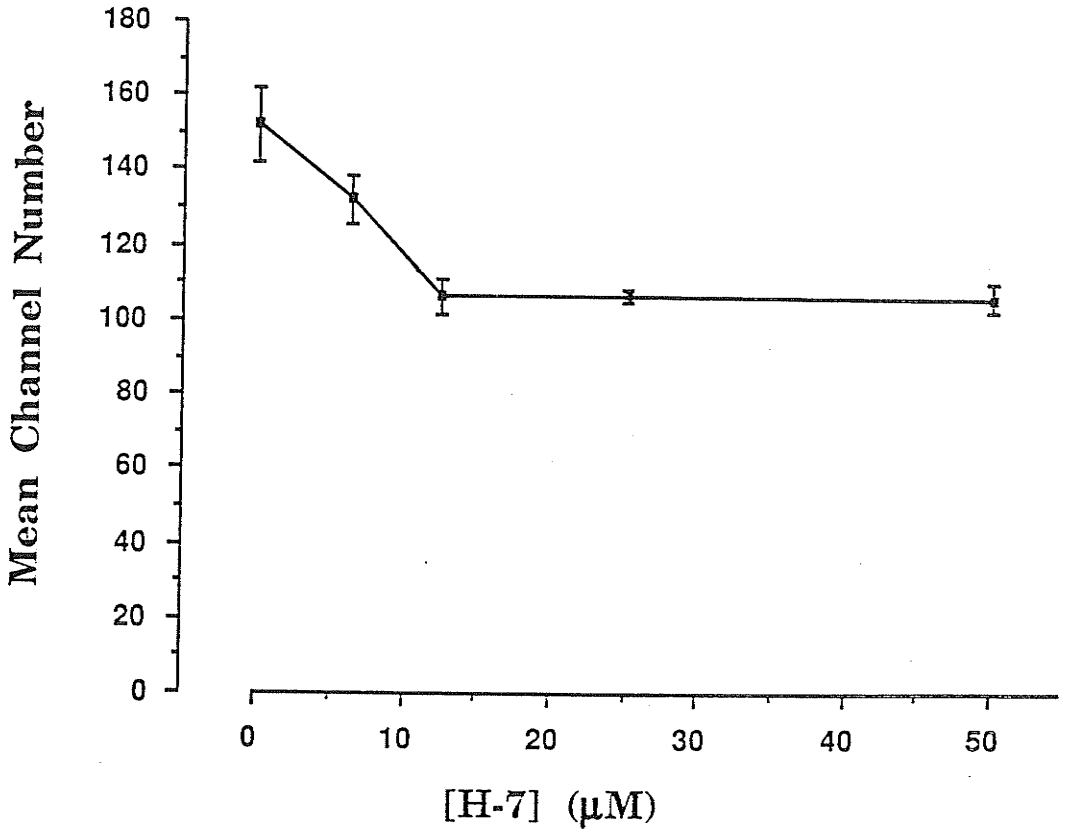

Figure 3.3 - Effect of H7 on TPA induced increase in NAb binding. L5178Y-F9 cells were preincubated in either 10% FFBS or the same media containing varying concentrations of H7, for 15 mintues prior to the addition of TPA (100 μg/ml). After 48 hours, the cells were washed and re-established at 3 x 10^4 cell/ml in either 10% FFBS or the same media containing H7 for an additional 48 hours. Cells were assayed for NAb binding as described. An H7 concentration of 12 μM was selected for all subsequent experiments. H7 concentrations greater than 50 μM were found to be toxic.

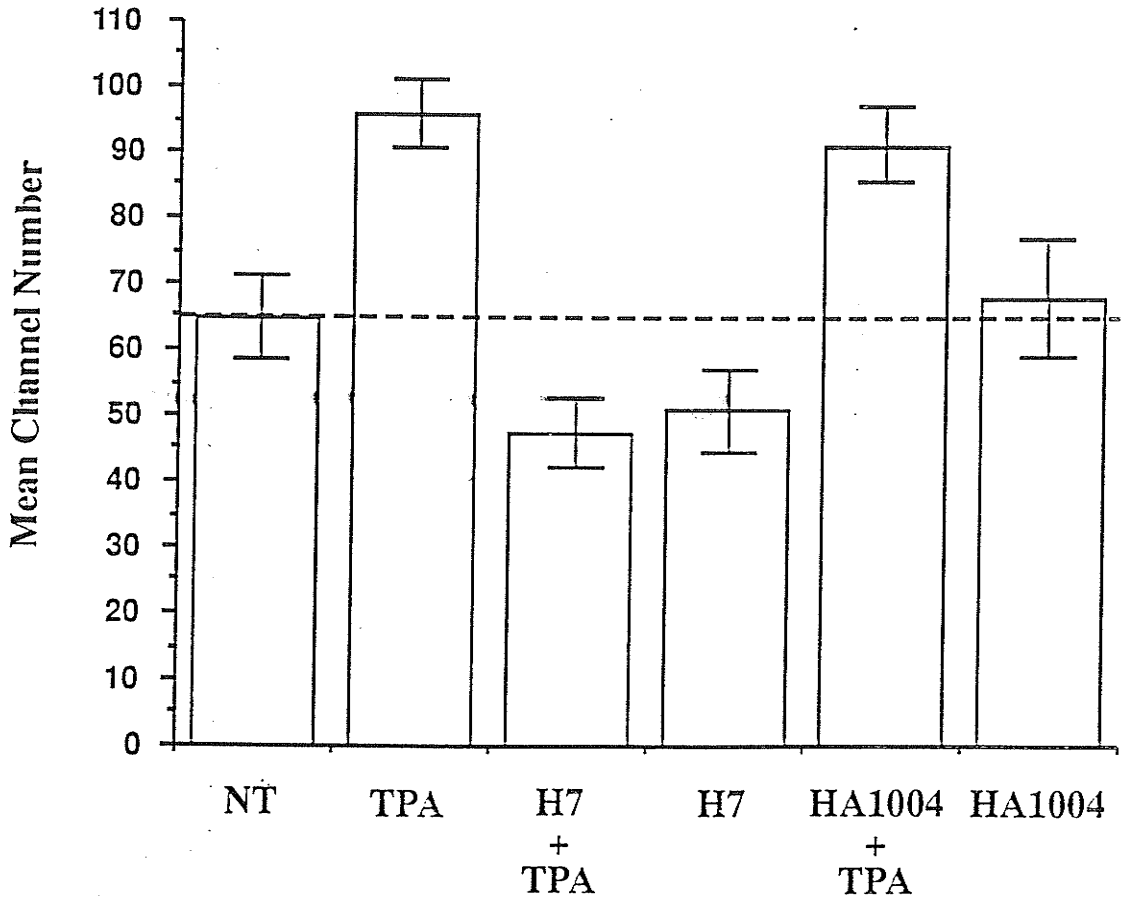

Figure 3.4 - H7 but not HA1004 is capable of inhibiting the TPA mediated increase in NAb binding as well as a portion of control NAb binding. L5178Y-F9 cells were set up at 3 x 10^4 cells/ml in either 10% FFBS or the same media containing either H7 (12 μM) or HA1004 (12 μM). After 15 minutes TPA (100 μg/ml) was added to the appropriate samples. After 48 hours the cells were washed and set up again at 3 x 10^4 cells/ml in 10% FFBS or the same media containing either H7 (12 μM) or HA1004 (12 μM). After an additional 48 hours the cells were assayed for NAb binding as described. H7 resulted in a significant decrease in binding with both TPA treated and non-TPA treated controls ($p < .05$). --- represents level of control binding.

report that TPA treatment resulted in a significant decrease in NAb binding within 15 minutes, and that this decrease persisted over the first 22 hours of treatment (Fig. 3.5).

Effect on NAb binding phenotype of 22 hour treatment with TPA, OAG and 4-O-Me-TPA. TPA is widely believed to mediate many of its effects through the activation of PKC. In order to ensure that the changes in NAb binding associated with TPA were not the result of some secondary non-specific effect of phorbol ester exposure, we examined what effect 4-O-Me-TPA had on NAb binding. 4-O-Me-TPA is essentially a non-tumor promoting, non-PKC activating methylated derivative of TPA (32). The finding that 4-O-Me-TPA also had no effect on NAb binding at 22 hours further supported the hypothesis that the changes in NAb reactivity associated with TPA are the result of a specific as opposed to non-specific mechanism (Fig. 3.6). Cells grown in the presence of the PKC stimulator OAG (100 μg/ml) for 22 hours did however demonstrate the reduced NAb binding phenotype seen following 22 hours of growth in TPA (Fig. 3.6), implying that PKC activation may be involved in the generation of this phenotype.

Effect of metabolic inhibitors on NAb binding. TPA is known to induce marked changes in the organization of the cellular cytoskeleton (33,34,35). In addition, a number of antigens which are regulated by TPA, appear to be associated with coated pits on the cell surface (36). Previously, it has been suggested that antigen internalization

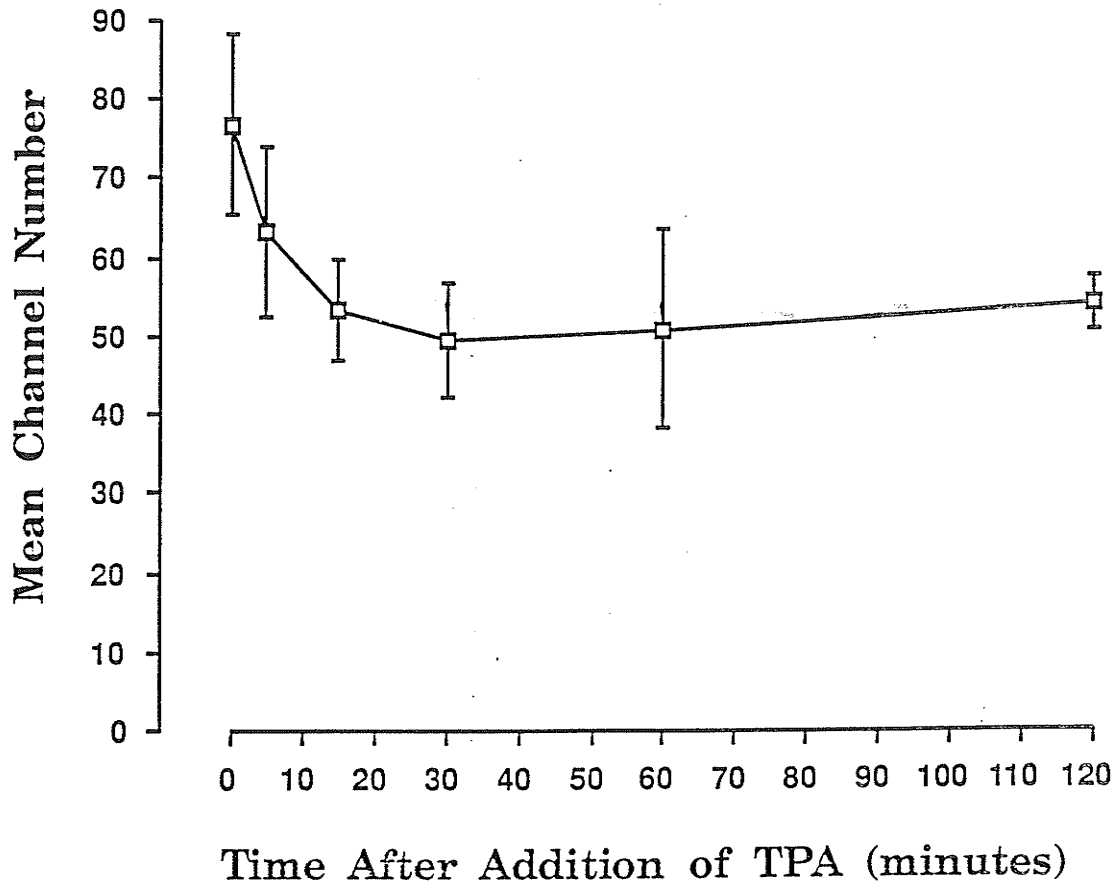

Figure 3.5 - Initial decreases in NAb binding induced by TPA. L5178Y-F9 cells were washed and set up at a concentration of 3 x 10^5 cells/ml in either 10% FFBS or the same media containing 100 μl/ml TPA. Cells were assayed for NAb binding as described. TPA resulted in a significant decrease in NAb binding as early as 15 minutes, and persisting at least to 120 minutes ($p < .05$ for 15, 30, 60 and 120 minutes).

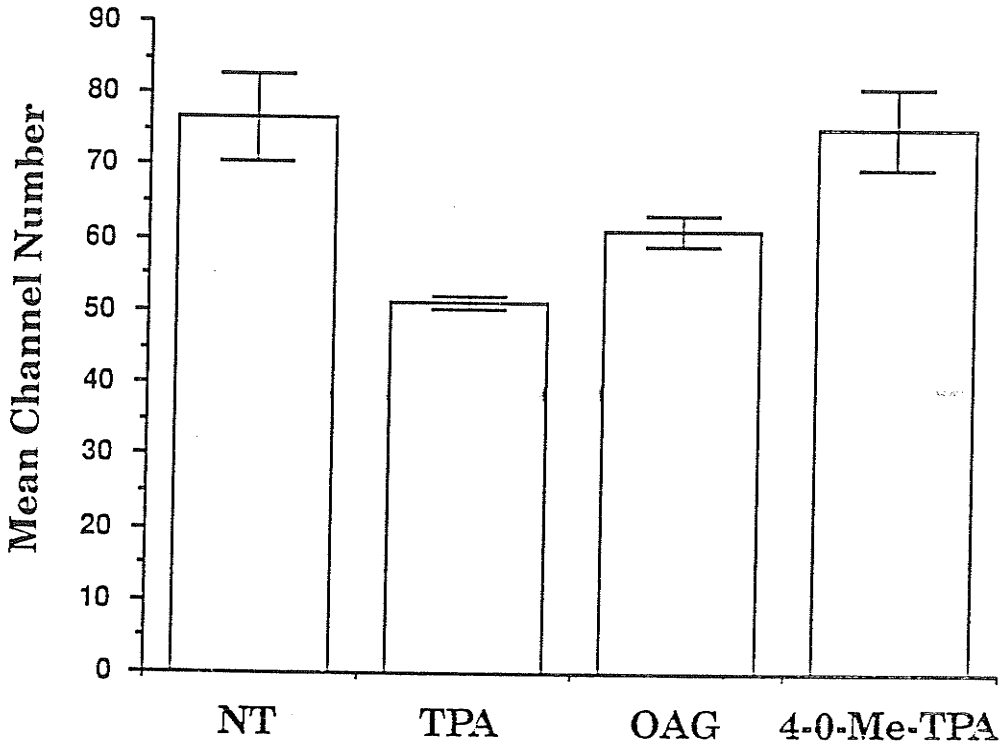

Figure 3.6 - Effect on NAb binding phenotype of 22 hour treatment with TPA, OAG and 4-0-Me TPA. L5178Y-F9 cells were set up at 1 x 10^5 cells/ml in 10% FFBS or the same media containing 100 μg/ml TPA or 100 μg/ml 4-0-Me TPA. After 22 hours the cells were assayed for NAb binding as described. TPA but not 4-0-Me TPA resulted in a significant decrease in NAb binding at 22 hours ($p < .05$).

via coated pits may be a cytoskeletal dependent process (37), and at least in the case of the transferrin receptor, TPA induced internalization can be inhibited by cytoskeletal disruption (29). In order to further dissect the mechanisms involved in the increase in NAb binding observed with TPA treatment, including its instability, the effect which cytoskeletal disruption and energy metabolism inhibition had on the NAb binding phenotype of untreated and TPA treated cells was examined. It was found that while disruption of the cytoskeleton by colchicine and cytochalasin B resulted in a marked reduction in NAb binding associated with cells treated for 48 hours with TPA followed by an additional 48 hours in standard tissue culture, the inhibitors had no significant effect on NAb binding to untreated controls (Fig. 3.7A and 3.7B). However, the blocking of energy metabolism by sodium azide resulted in both a marked reduction in binding to TPA treated cells as well as a small reduction in binding to otherwise untreated cells.

DISCUSSION

The role of NAb in the defence against incipent neoplasia has been well established (5-8). However, very little is known about the mechanisms involved in the regulation of NAb binding to the surface of syngeneic tumor targets. Recently, we have reported that TPA treatment for 48 hours followed by an additional 48 hours in normal tissue culture resulted in a significant increase in NAb binding at 4°C (20), implying that PKC may be involved in the regulation of NAb binding. The synthetic diacylglycerol, OAG, readily intercalates

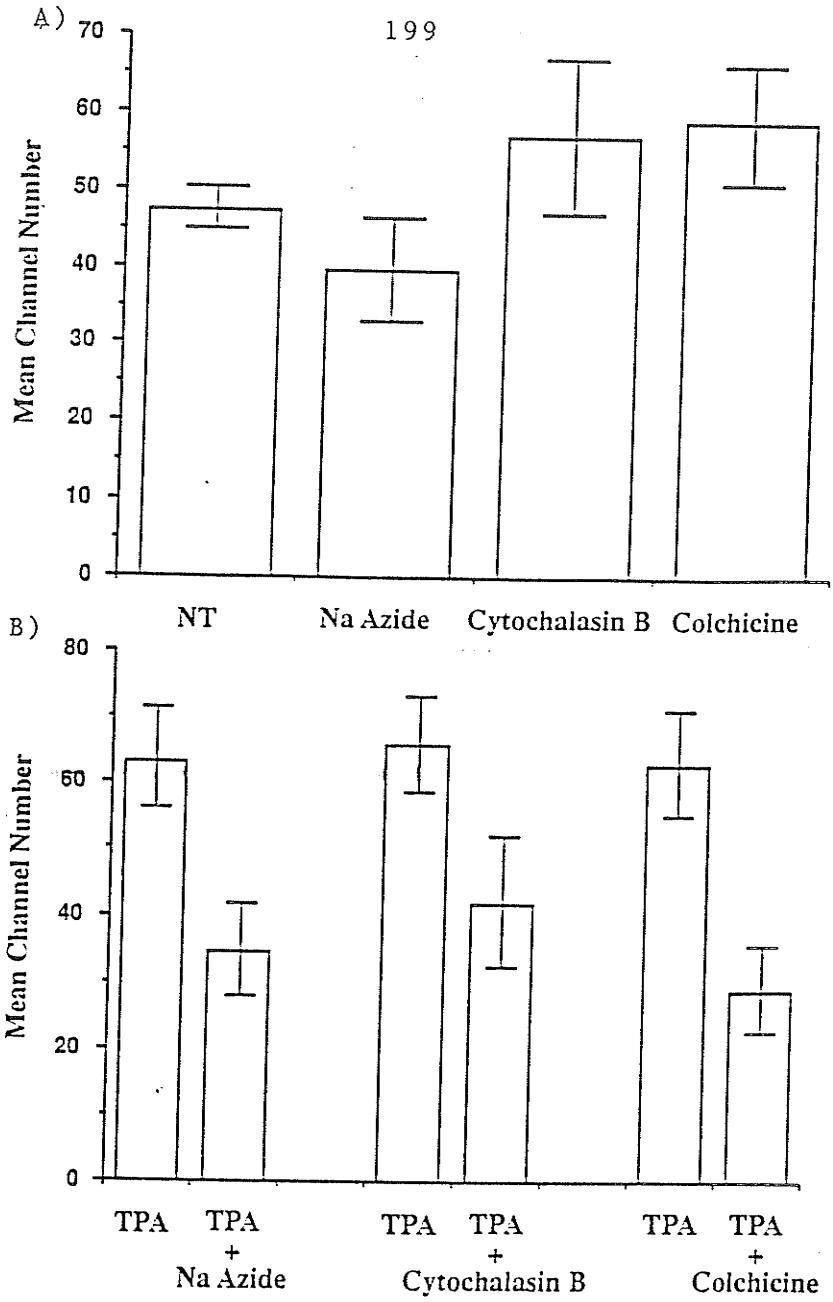

Figure 3.7 - Effect of cytoskeleton disruption and energy metabolism inhibition on NAb binding to TPA treated and non-treated cells. L5178Y-F9 cells were set up at a concentration of 3 x 10^4 in (A) 10% FFBS or (B) the same media containing TPA (100 μg/ml). After 48 hours, the cells were washed and set up in normal 10% FFBS for an additional 48 hours. Cells were pre-incubated in the presence of the appropriate metabolic inhibitor for 1 hour prior to being assayed. In addition, the inhibitors were present during the NAb binding step of the assay. Cells were assayed for NAb binding as described. While cholchicine (10 mM), cytochalasin B (50 mM) and sodium azide (10 mM) all resulted in significant reductions in the elevated levels of NAb binding observed after TPA treatment ($p < .05$) they had no effect on control NAb binding levels.

into the plasma membranes of cells directly activating PKC (21). A range of OAG concentrations were unable to mimic the TPA induced increase in NAb binding seen with 48 hours of drug treatment followed by 48 hours in normal tissue culture, and in fact resulted in a significant decrease in binding. This was also in contrast with the lack of any change in NAb binding following a similar treatment with the non tumor promoting non PKC activating TPA analogue, 4-0-Me-TPA. The relatively rapid metabolism of OAG compared to TPA does not appear to be responsible for the inability of OAG to mimic TPA, since restimulation with OAG after 48 hours did not result in enhanced binding. This would imply that while the increase in NAb binding observed after several days growth in the presence of TPA is not a non specific effect of phorbol ester exposure, it does appear to be either independent of or not solely dependent upon the activation of protein kinase C. However, the initial result of TPA treatment is a rapid decrease in NAb binding at 4°C which is maintained over the first 22 hours. The observation that this phenotype can be mimicked by a 22 hour OAG treatment, but not by treatment with 4-0-Me-TPA, implies that it may possibly be the direct consequence of PKC activation and not the result of a non-specific event. It is possible, therefore, that one of the initial events upon treatment of L5178Y cells with either TPA or OAG is a rapid PKC dependent down regulation of NAb binding sites. However, as a consequence of differences in the regulation of PKC activity or possibly as a result of the subsequent activation of other non-PKC dependent pathways, TPA, but not OAG results in the re-expression and eventual enhanced expression of NAb binding sites on

the cell surface.

H7 is a competitive inhibitor of the ATP binding site on PKC resulting in a relatively selective inhibition of PKC activity (31). The observation that H7 completely inhibited the increase in NAb binding seen after prolonged exposure to TPA, while HA1004 (a non-PKC inhibiting control) had no effect suggests a role for protein kinase C in the enhanced NAb binding. This data coupled with the observations made with OAG implies that PKC activation while being necessary may not be sufficient for enhanced NAb binding.

Alternatively, it is also possible that the differences in the regulation of PKC activity by these two compounds may result in the observed differences in NAb binding. Diacylglycerols, in addition to being potent PKC activators, may also be involved in the inhibition of this kinase. Recently, it has been speculated that the PKC independent activation of a neutral sphingomyelinase by diacylglycerol but not phorbol esters may be responsible for at least some of the reported differences which these agents have on cellular functions (38). It is believed that the products of sphingomyelin turnover may attenuate PKC activity and later PKC membrane translocation. If the growth of L5178Y-F9 cells in the presence of OAG for 4 days does result in the eventual inhibition of PKC activity, the associated decrease in NAb binding would be in agreement with the diminished NAb binding observed when the PKC activity of normal and TPA treated cells is blocked for a similar period of time by H7.

Interestingly, protein kinase C may play a similar role in skin tumor promotion by TPA. Although complete tumor promoters such as TPA

are potent activators of PKC, other PKC activating compounds, such as bryostatin display only weak second stage promoter activity (13). A second stage tumor promoter when used alone has little, if any, skin tumor promoting activity, although it can enhance the effects of a complete promoter such as TPA (13). These results may imply that while PKC activation may be required during the promotion step of multistage carcinogenesis, other TPA induced changes in addition to PKC activation may also be critical.

As previously eluded to, H7 appears to be capable of inhibiting a portion of NAb binding in untreated control cells, implying that even in the absence of TPA, PKC may directly or indirectly control the expression of certain antigen recognized by NAb. Currently, it is not known if the enhanced NAb binding seen with TPA is the result of an increase in the expression of the PKC dependent antigen pool seen in untreated cells or if it is the result of the expression of antigens not seen on untreated cells.

It is interesting to note that TPA appears to alter the expression of several activationally regulated antigens in a similar biphasic fashion. For example, while TPA treatment has been reported to result in an increased expression of both the T cell antigen receptor (39) and the transferrin receptor (40) several days post treatment, it has also been demonstrated that both these antigens are rapidly internalized soon after the inital exposure to TPA (41,42). As a result of similarities in the kinetics of TPA induced alterations in NAb binding and activation associated antigen expression, it may be speculated that serum NAb is recognizing activationally regulated

epitopes.

TPA treatment is known to be associated with marked changes in the organization of the cellular cytoskeleton. These changes include reorganization of actin microfilaments leading to their specific association with plasma membrane sites (33), the dynamic association of lymphocyte integrins with the cytoskeletal protein talin and possibly with actin microfilaments (34) as well as the phosphorylation and proteolytic modification of particular cytoskeletal protein such as myosin light chains (35) and microtubule associated proteins (21). Here we report that the unstable increase in NAb binding observed with cells treated with TPA for 2 days followed by normal tissue culture for two days is dependent upon an intact cytoskeleton as well as functional energy production. The pretreatment of TPA treated cells with cytoskeletal and energy metabolism inhibitors one hour prior to their being assayed for NAb binding resulted in a significant decrease in the amount of NAb bound at 4°C, but had no significant effect on binding to untreated cells. Thus, while TPA appeared to induce an increase in NAb binding when assayed at 4°C, the observation that this phenotype could be reversed by sodium azide, cholchicine and cytochalasin B suggested that it is a dynamic process requiring both continuous energy production and cytoskeletal involvement.

There are several potential mechanisms by which TPA may mediate cytoskeletal-dependent changes in NAb binding. The structural architecture of the surface of a cell appears to be contingent upon the organization of the cytoskeleton (43). It is possible that TPA

results in a general unfolding or reorganization of the plasma membrane of treated cells, leading to the exposure of cryptic antigens recognized by NAb, a condition which is maintained by a net energy expenditure and the active participation of cytoskeletal elements. The disruption of the cytoskeleton or the blocking of energy production would be expected to short circuit a TPA induced increase in NAb binding by allowing the membrane to return to its original state, with an associated loss of cryptic binding sites.

It could also be envisioned that the enhanced expression of NAb binding sites on the surface of TPA treated cells is the result of, and is maintained by, an increased rate of their synthesis and export to the cell surface. This continuous cytoskeletal and energy dependent externalization of new binding sites would be balanced against their loss from the cell surface possibly due to shedding or perhaps as a result of internalization and degradation within the lysosome compartment. The previous observation that TPA treated L5178Y-F9 cells which had acquired a high level of surface NAb at 4°C, exhibited a rapid reduction in bound NAb when the temperature was increased to 37°C may be to some degree a reflection of a TPA induced dynamic turnover of binding sites on the cell surface occuring at physiological temperatures but not at 4°C.

Alternatively, TPA may alter the cycling rather than the synthesis of NAb binding sites, changing their steady state distribution between the cell surface and the internal presumably endosomal compartment, without altering the actual size of the cycling pool. This mechanism has been implicated in the regulation of several cell

surface antigens by TPA, including the T cell antigen receptor (TCR) (41), epidermal growth factor receptors (44), T4 antigen (30), asialoglycoprotein receptor (45), tumor necrosis factor receptor (46) and possibly the transferrin receptor (29,42), although the response being investigated is usually the decrease in antigen expression induced by a short term exposure to TPA. One could speculate that although the NAb binding sites on both TPA treated and control cells are constantly cycling, the residency time of the binding sites on the cell surface is increased after TPA treatment. This may be the result of either an increased externalization rate, a decreased internalization rate, or a combination of the two.

The early redistribution of internal and external antigen pools induced by TPA bears a number of similarities to the regulation of NAb binding associated with TPA. For example, active TCR cycling induced by TPA is PKC dependent and inhibited at 4°C (41), while the proposed TPA induced translocation of the T4 antigen or transferrin receptor from the cell surface to the cytoplasmic pool is blocked by sodium azide and colchicine respectively (30,29).

It is also interesting to note that for T cell receptor cycling the rate of internalization appears to be accelerated with increased antibody mediated T cell receptor crosslinking, dramatically altering receptor distribution between the internal and cell surface pools and resulting in a net decrease in receptor expression on the cell surface (41). A similar phenomena may occur during the binding of NAb to TPA treated cells at 37°C. Previously, we have reported that the TPA induced increase in NAb binding can be observed at 37°C only when

assayed with dilute serum concentrations. Assays employing higher concentrations of serum NAb or repeated cycles of incubation in fresh aliquots of diluted serum NAb, conditions which would be expected to result in increased crosslinking of the NAb binding sites on the cell surface, actually produce a significant reduction in NAb binding to TPA treated cells at 37°C. If the antigens recognized by NAb are cycling at 37°C, it is possible that extensive crosslinking by NAb may increase the ratio of internalization to externalization, resulting in a net decrease in NAb binding on the cell surface.

The fact that the NAb activity found in LPS induced serum is most likely polyspecific recognizing a number of different antigens on the tumor cell surface implies that more than one mechanism may be involved in the regulation of NAb binding by TPA. However, the observation that serum NAb binding to TPA treated cells may be lost as a result of the active turnover of its binding sites has important implications. Previously, we have reported that TPA treated L5178Y-F9 cells demonstrate a reversible increase in their in vivo tumorigenicity as indicated by both an augmented metastatic potential, and an enhanced tumor frequency arising from threshold s.c. tumor inocula. Bearing in mind the weight of evidence implicating NAb in the defence against small tumor foci (5,6,7,8) it is possible that the shedding or internalization of bound NAb prevents the "functional" immune recognition of TPA treated tumor cells, and represents a critical event in the escape of a tumor from immune surveillance. PKC activation is required but not sufficient for the generation of this phenotypic state.

In summary, it appears that TPA treatment has a biphasic effect on the NAb binding capacity of L5178Y-F9 cells, whereby an initial decrease in NAb binding is followed by an increase sometime later. This later increase in binding which has previously been shown to be highly unstable at physiological temperatures, is also dependent upon an intact cytoskeleton as well as functional energy metabolism. The role of PKC in TPA mediated changes in NAb binding appears to be more complex. Although inhibition by H7 suggesting that the enhancement in NAb binding observed after treatment with a tumor promoting phorbol ester is dependent upon PKC activation, this phenotypic change is not associated with the exposure to a non-tumor promoting endogenous PKC activator such as OAG, suggesting that PKC activation, while being necessary, may not be sufficient for enhanced NAb binding. Knowledge of the mechanisms by which TPA mediates changes in NAb binding will contribute to our understanding of how tumor variants capable of eluding NAb mediated destruction are generated during tumor progression in vivo.

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CHAPTER 4

Conclusion

The rate at which a tumor cell population progresses towards enhanced autonomy from host mediated regulatory and surveillance mechanisms depends upon the interaction of two independent events. Tumor progression is determined by both the frequency at which progresive tumor variants arise within a tumor cell population as well as the nature and intensity of the selective pressures acting upon the heterogeneous tumor cell population resulting in the enrichment of those phenotypic characteristics which are advantageous to survival. Although virtually any characteristic of neoplastic cells can be subject to independent variation, selection and consequently progression, it might be expected that one of the earliest progressive changes to occur during the development of a tumor may involve the generation of a tumor cell population which is less sensitive to detection and/or destruction by the tumor specific immune surveillance effectors which defend against incipient neoplasia. Having overcome this initial barrier a tumor cell population could proceed to acquire other characteristics of enhanced malignancy such as the ability to invade adjacent tissue and metastasize to distant sites. This thesis has examined the regulation of tumor progression with respect to natural resistance and immunosurveillance from both the perspective of the mechanism by which natural immune resistant variants are generated as well as the role which natural immunity itself plays in driving tumor progression.

Chapter I dealt with the effects which immune modulation of natural resistance have upon tumor progression. Our data clearly revealed the dual natural of natural resistance on tumor progression.

Although natural defence mechanisms may effectively eliminate a tumor during the early stages of development, these same processes provide selective pressures to direct and drive tumor progression once the neoplasm has overcome this defence. These results suggest that biphasic modulation (ie. suppression followed by augmentation) of natural resistance may actually promote tumor progression by temporarily allowing a period of more extensive tumor proliferation and variant generation followed by a subsequent increase in the host's natural defences which would focus a more vigorous selection process upon the expanded tumor population thus increasing the rate of progression towards a more autonomous tumors. These results complement the observations of Urban et al. (1). Using an immunogenic regressor tumor which is easily rejected from immunocompetent animals, this group demonstrated that while implantation of such tumors into fully immunosuppressed (athymic nude) animals results in a high frequency outgrowth of tumors exhibiting no evidence of rejection antigen loss, implantation into partially immunosuppressed (uv irradiated) animals results in the high frequency outgrowth of antigen loss tumors. The authors suggest that partial immune suppression may allow more time for the tumor to generate and expand antigenic variants which could then be selected for by antitumor immune effectors.

Although this data may be open to alternative explanations, such as the possibility that silica and LPS are either acting directly or indirectly (ie. as a result of lymphokines induced by the biological response modifier treatment) to effect tumor cell growth, keeping in

mind that the rejection of small tumor inocula is the result of thymus independent non-adaptive tumor specific immune mechanisms one would predict that the biphasic regulation of NR would result in and is consistent with the changes in tumor frequency we observed. Our results not only provide a possible explanation of how the repeated exposure to various immunotoxic agents such as silica and asbestos may promote tumor development but also emphasizes the hazards of therapeutic or other forms of intervention that might initially decrease the host's natural or innate resistance to a developing tumor.

As mentioned earlier, the rate of tumor progression is at least partially determined by the ability of a tumor cell population to generate tumor variants which are less sensitive to the effectors of natural immunity. In Chapter II of this thesis the regulation of tumor progression is examined from the perspective of what effect the generation of natural defence resistant variants has on the in vivo growth characteristics of a tumor. We observed that two days growth of an NK sensitive or an NK resistant murine T cell lymphoma in TPA reduced their sensitivity to cytolysis by the mediators of natural resistance tested; complement dependent NAb for both tumors, activated macrophages for the L5178Y-F9 and NK cells for the NK sensitive SL2-5. In addition TPA treatment was observed to result in an increase in tumorigenicity as demonstrated by an enhanced subcutaneous tumor forming capacity and an increased ability to form liver metastasis. Subsequent tumor growth in culture medium containing no TPA demonstrated the reversibility of this TPA induced natural defense resistant tumor phenotype. This study does not exclude the possi-

bility that TPA induced changes which are independent of the increased resistance to natural immune effectors, such as an increased secretion of collagenase or the enhanced affinity for matrix proteins, may contribute to the enhancement of tumorigenicity. However, the concurrent changes in tumor sensitivity to the in vitro parameters of natural resistance, in the tumorigenicity of threshold inocula and in intraveneous metastasis formation is consistent with and helps to support the view that changes in sensitivity to natural resistance effectors can contribute to tumor development and progression. These results suggest that an important step in successful tumor development may be the generation of tumor variants capable of evading destruction by natural resistance effectors, and that an increased ability to generate such variants may facilitate an enhanced rate of tumor progression and growth. Furthermore, the reversible nature of these TPA induced reductions in sensitivity to mediators of natural resistance is consistent with the initial reversibility of the promotion phase of multistage carcinogenesis. These changes may therefore be an integral component of tumor promotion by phorbol esters, contributing to tumor survival in vivo and increasing the probability that the tumor will progress to a more malignant phenotype.

In this respect it is interesting to note that others have reported the suppressive effects of phorbol ester tumor promoters on the natural immune system in particular NK cells (2) and macrophages (3,4). In view of what was reported in Chapter I of this thesis pertaining to the augmentation of tumor progression by transient

periods of immune suppression, it is conceivable that to some degree the skin tumor promoting capacity of phorbol esters is attributable to both their ability to generate natural defense resistant tumor variants as well as by providing an immune suppressed environment which allows more time for the tumor to generate and expand such variants.

We have also reported that although TPA treated L5178Y-F9 cells could acquire higher levels of serum NAb in vitro, this increased binding appears to be very unstable and rapidly lost at 37°C. The kinetics and possible mechanisms of such binding are discussed in detail in Chapter 2, however it is interesting to reiterate the potential role this phenotypic change may play in tumor progression. It is possible that the shedding of NAb/antigen complexes from the surface of TPA treated cells under physiological conditions may lead to the induction of tumor specific suppression. Previously, it has been reported that the i.p. injection of tumor cells or KCI extracted tumor antigens together with antitumor antibodies (sequentially or in the form of immune complexes) into allogeneic mice results in a specific immune suppression (5). One of the most significant features of this suppression is the inability of macrophages from immune suppressed animals to bind specific tumor cells even in the presence of cytophilic antibodies. It is speculated that antigen/antibody complexes may result in the generation of a $Lyt1^+$ suppressor inducer population which then induces $Lyl23^+$ suppressor effector cells.

Although it has not yet been tested it is possible that the rapid shedding of NAb/antigen complexes from the surface of TPA treated

cells in vivo may result in the generation of an immune suppressed state which in light of conclusions of Chapter I may be both condusive to the expansion of the tumor and allow more time for variant generation. Again, such a phenotypic change may contribute to the tumor promoting capacity of TPA.

The final chapter of this thesis dealt with the mechanism by which TPA generates this unstable increase in NAb binding. We reported that this increase in binding induced by TPA appears to be dependent upon an intact cytoskeleton as well as functional energy metabolism. The role of PKC in TPA mediated changes in NAb binding appears to be more complex. Although inhibition by H7 suggesting that the enhancement in NAb binding observed after treatment with a tumor promoting phorbol ester is dependent upon PKC activation, this phenotypic change is not associated with the exposure to a non-tumor promoting endogenous PKC activator such as OAG, suggesting that PKC activation, while being necessary, may not be sufficient for enhanced NAb binding. Knowledge of the mechanisms by which TPA mediates changes in NAb binding will contribute to our understanding of how tumor variants capable of eluding NAb mediated destruction are generated during tumor progression in vivo.

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Original Contributions

This thesis is an entirely original investigation conducted by myself, with the technical assistance of Dr. E. Rector (operation of the flow cytometer) and Rupinder Singal (NAb binding assays on cells treated with cytoskeletal and energy metabolism inhibitors).

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