NATURAL ANTIBODIES IN TUMOR SURVEILLANCE: DEFENSE AGAINST TRANSPLANTED SYNGENEIC TUMOR FOCI AND RECOGNITION OF *RAS* ONCOGENE TRANSFORMED CELLS

BY

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To my parents, for everything.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytolysis
Anti-Gal	Anti-α-galactosyl
CP	*Corynebacterium parvum*
DMH	Dimethylhydrazine
E/T	Effector-to-target ratio
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FFBS	Fischer's medium containing 10% fetal bovine serum
FITC	Fluorescein isothiocyanate-conjugated
G418	Geneticin
GAP	GTPase activating protein
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid
$hygr^r$	Hygromycin resistance
i.p	Intraperitoneal
i.v.	Intravenous
LPS	Lipopolysaccharide
MCF	Mean channel fluorescence
MOI	Multiplicity of infection
NAb	Natural antibodies
NAb^+	High Natural antibody binding
NAb^-	Low Natural antibody binding
neo^r	Neomycin resistance
NK	Natural killer
NK^r	Natural killer resistant

LIST OF ABBREVIATIONS (continued)

NK^s	Natural killer sensitive
NR	Natural resistance
PEC	Peritoneal exudate cells
PKC	Protein kinase C
s.c.	subcutaneous
SFD	Serum-free Dulbecco's Modified Eagle Medium/ F12 (Ham) (1:1)
t_d	*t*-dependent Student's *t* test
t_i	*t*-independent Student's *t* test
TPA	12-*O*-tetradecanoylphorbol-13-acetate
xid	X-linked immunodeficiency

ABSTRACT

Natural antibodies (NAb) able to bind to the surface of tumor cells have been detected in the circulation of many vertebrate species, including humans. Because of their existence in normal, unimmunized individuals it has been proposed that anti-tumor NAb contribute to a first line of defense against incipient neoplasia. Two requirements arising from this proposal are: (1) tumor cells which bind more NAb should have a lower ability to grow *in vivo* than similar tumor cells which bind less NAb and; (2) NAb should be able to recognize and bind to newly emerging tumor cells.

The first question was addressed by selecting murine lymphomas *in vitro* for altered NAb binding. Fluorescence activated cell sorting was used to select spontaneous high and low NAb binding variants from a heterogeneous L5178Y-F9 lymphoma and to select high NAb binding variants from 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-treated L5178Y-F9 and SL2-5 lymphoma clones. In both cases, cells selected for increased NAb binding *in vitro* had a reduced ability to grow when threshold inocula were injected subcutaneously into syngeneic mice. High NAb binding cells obtained following TPA treatment were further shown to have no consistent alterations in sensitivity to other parameters of natural resistance which could account for their reduced tumorigenicity. These results support the hypothesis that NAb binding is an important parameter in determining the ability of a small transplanted tumor focus to grow.

The second question was addressed by expressing oncogenes in non-tumorigenic murine fibroblast cell lines. An increase in NAb binding was associated with the expression of an activated H-*ras* oncogene in 10T½

cells following its introduction through transfection or retroviral infection or after Zn-promotion of an inducible gene. However, increased NAb binding was not observed in certain *ras* expressing 10T½ clones selected on the basis of their ability to form foci in the presence of untransformed cells, nor in v-H-*ras*-infected NIH 3T3 cells. Increased NAb binding was also not observed following v-*myc* introduction into 10T½ or v-*src* transformation of 10T½. The results show that expression of the *ras* oncogene leads to cell surface changes which culminate in increased NAb binding. However, these changes are dependent upon other genes in the target cell and are not associated strictly with neoplastic transformation. While the results suggest that the targets of NAb-mediated surveillance may be restricted to certain tumor types and/or particular stages of tumor development, the evidence that increased NAb binding is induced following expression of a transforming oncogene supports the hypothesis that NAb are important in the defense against incipient neoplasia.

CHAPTER 1

INTRODUCTION

PART I. LITERATURE REVIEW

(A) Multistep Nature of Tumor Development

Since the original proposal by Foulds over thirty years ago that "the structure and behavior of tumors are determined by numerous unit characters which, within wide limits, are independently variable, capable of highly varied combination, and liable to independent progression" (Foulds, 1958), it has become a widely held view that tumorigenesis is a multistep process. The conversion of a normal cell to a fully malignant tumor cell is thought to involve the sequential acquisition of a variety of traits which endow the cell with increasingly altered growth characteristics. Early observations from statistical studies were consistent with a process of tumor development involving multiple rate-limiting steps. An analysis of the age-dependent incidence of cancer in humans indicated kinetics dependent upon the fifth or sixth power of elapsed time, which suggested that five or six steps were required for the development of a clinically detectable tumor (Peto et al., 1975). More direct support for multistep tumor develpment was provided by animal models of carcinogenesis, which divided the tumorigenic process into three distinct steps: initiation, promotion and progression (Farber and Cameron, 1980). A frequently employed animal model is the two-step model of mouse skin carcinogenesis which provided the first demonstration of multistage carcinogenesis *in vivo* (reviewed in Yuspa and Poirier, 1988). In this system, mouse skin is initially exposed to a carcinogen at a low dose which by itself does not result in tumor formation. This is followed

by repeated treatment with a tumor promoting agent, which is most commonly the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). This treatment, like the low dose carcinogen application, does not induce tumors on its own. The combined protocol, however, results in the appearance of multiple squamous papillomas, demonstrating a requirement for two independent steps in tumor induction. A requirement for a third step in the carcinogenic process was suggested by the observation that only a low percentage of these benign tumors progressed to a carcinoma phenotype, while the remainder either persisted or regressed. The conversion of papillomas to carcinomas could be increased by further treatment with ionizing radiation (Jaffe et al., 1987) or chemical carcinogens but not with tumor promoters (Hemmings et al., 1983). Since both initiation and progression were induced by agents with the potential to mutate DNA, it seemed likely that genetic alterations were involved in the process of tumorigenesis. This was consistent with numerous observations from diverse lines of study which suggested that genetic damage may be responsible for cancer (reviewed in Bishop, 1987). In recent years studies at the molecular level have borne out the original hypothesis of cancer as a multistep disease and have begun to elucidate the nature of the multiple alterations which are required to convert a normal cell to a fully malignant tumor cell.

(B) Molecular Basis of Multistep Carcinogenesis

A major breakthrough in the understanding of the molecular basis of multistep tumor development began with the discovery of a class of genes called oncogenes, whose importance in tumorigenesis was highlighted by

their identification in two different approaches aimed at investigating the genetic basis of cancer (reviewed in Land et al., 1983b). One line of studies was aimed at determining the genes responsible for the tumor-inducing capacity of animal retroviruses, while the other utilized the technique of gene transfer (transfection) to detect transforming genes present in the DNA isolated from human tumors of non-viral origin. The transforming genes in both cases were found to be mutated versions of normal cellular genes, which were termed proto-oncogenes. The growing family of oncogenes now includes sixty or more members (Varmus, 1989). The potential of proto-oncogenes to be converted to genes with transforming activity suggested that their gene products must have important functions in the regulation of normal cell growth and proliferation. Indeed, evidence is accumulating that proto-oncogene proteins occupy important positions along the signalling pathways which stimulate cell growth and their conversion to oncogenes, by a variety of mechanisms, results in the inappropriate expression of that growth promoting activity (reviewed in Hunter, 1991).

The relevance of oncogenes to the multistep process of tumorigenesis became evident in studies which showed that although single transforming oncogenes could by themselves convert some established (immortalized, non-senescing) cell lines to tumor cells, neoplastic transformation of primary cells required the introduction of more than one oncogene. Studies with DNA tumor viruses provided the first evidence that conversion of cells to a tumorigenic state could involve two or more oncogenes. It was shown that while neither the middle T nor large T genes of polyomavirus were able to transform rat embryo fibroblasts on their own, the two together elicited

a fully tumorigenic phenotype (Rassoulzadegan et al., 1982). Similar results were subsequently observed with a number of oncogenes of cellular origin. For example, it was shown that cotransfection into rat embryo fibroblasts of the *myc* and *ras* oncogenes, but not introduction of either gene alone, resulted in neoplastic transformation (Land et al., 1983a). The *ras* oncogene has been similarly shown to cooperate with DNA tumor virus-derived oncogenes, such as the E1a gene of adenovirus, in the transformation of primary cells (Ruley, 1983). In addition, treatment of *ras* oncogene-transfected rat embryo fibroblasts with the tumor promoter TPA allowed for the focal outgrowth of transformed cells (Dotto et al., 1985). Thus a parallel was observed between oncogene-induced neoplastic transformation *in vitro* and the two-step induction of tumors in mouse skin. The demonstration that transformation of primary cells *in vitro* required the action of more than one oncogene raised the possibility that the multistep development of tumors *in vivo* may reflect multiple genetic alterations which involve the activation of oncogenes.

More recent evidence suggests that alterations in another class of genes, the tumor supressor genes (alternatively referred to as anti-oncogenes, growth suppressor genes, or recessive oncogenes) are likely to be equally important as those in oncogenes in the process of tumor development. In contrast to the dominantly acting oncogenes, whose normal cellular counterparts are thought to play positive roles in cell growth and proliferation, tumor suppressor genes are thought to function normally through a variety of mechanisms in the inhibition of inappropriate cell proliferation (reviewed in Boyd and Barrett, 1990). Hence it is the loss of their function which is associated with neoplastic transformation.

Normally this involves the inactivation of both alleles of the tumor suppressor gene, so that the phenotype associated with alterations of these genes is expressed in a recessive manner at the cellular level. However, mutations in one tumor suppressor gene, p53, can act in a dominant manner, likely through the ability of the mutant p53 protein to inactivate the product of the normal allele (reviewed in Marshall, 1991). In some instances there may also be a phenotypic effect of loss of one allele of a tumor suppressor gene, presumably due to a reduction in the dose of the gene product (Fearon and Vogelstein, 1990).

Evidence for the involvement of tumor suppressor gene inactivation in multistep tumorigenesis *in vitro* comes from studies which showed that mutant p53 genes could cooperate with *ras* in the transformation of rat embryo fibroblasts (Eliyahu et al., 1984; Parada et al., 1984). Although it was originally believed that p53 was acting as an oncogene in this system, subsequent studies have provided strong evidence that the activity of the mutant p53 gene product in transformation, at least in part, involves blocking the normal function of the wild type p53 protein (for review, see Marshall, 1991). In contrast, overexpression of the wild type p53 gene has been shown to inhibit the *ras* plus Ela-, *ras* plus *myc*- or *ras* plus mutant p53-induced transformation of rat embryo fibroblasts (Eliyahu et al., 1989; Finlay et al., 1989). Some mutant p53 oncogenes may also possess dominant activity independent of their ability to interfere with the normal function of wild type p53 since introduction of a mutant p53 gene into a cell that had deleted both p53 alleles enhanced its tumorigenicity (Wolf et al., 1984). Thus *in vitro* studies have provided a model for multistep tumorigenesis which involves both the activation of

dominantly acting oncogenes and the loss of tumor suppressor gene function. Although the number of tumor suppressor genes identified to date are relatively small in comparison to the vast oncogene family, their importance in tumor development is clear from the high frequency with which known and suspected tumor suppressor genes have been shown to be inactivated in human tumors (reviewed in Sager, 1989). In fact, the accumulating data suggest that p53 is the gene which is most frequently altered in human cancer (reviewed in Marshall, 1991).

A relationship between oncogene-mediated multistep tumorigenesis *in vitro* and multistage carcinogenesis *in vivo* was further suggested by studies which showed that mouse skin tumors initiated by a number of different chemical carcinogens, including 7,12-dimethylbenz[a]anthracene, N-methyl-N'-nitro-N-nitrosoguanidine, methylnitrosourea, 3 methylcholanthrene and urethane, frequently contained activated *ras* oncogenes which were mutated in a carcinogen specific manner (Quintanilla et al., 1986; Bonham et al., 1989; Brown et al., 1990; reviewed in Sukumar, 1990). The concordance between the nature of the activating mutation in *ras* and the chemical specificity of the initiating carcinogen implied a direct interaction of the carcinogen with *ras* gene sequences and hence a causative role for *ras* in the initiated cell phenotype. Further, it has been shown that activated *ras* genes introduced into mouse skin by transgenic mouse technology (Leder et al., 1990) or by retroviral infection (Brown et al., 1986b) could substitute for the initiating carcinogen in the two-step model.

While these studies implicated genetic mutations, particularly in oncogenes, in the initiating step of multistage mouse skin carcinogenesis

consistent with the genotoxic properties of the initiating carcinogens, the mechanism of tumor promotion is less well understood. In contrast to initiating agents, phorbol ester tumor promoters such as TPA do not bind to DNA, and must therefore exert their effects initially at the epigenetic level. TPA has been shown to induce pleotropic effects in a variety of cell types, including changes in cell morphology, membrane transport, cell-cell communication, growth factor receptors, protein phosphorylation, phospholipid metabolism, cell proliferation, cell differentiation and the induction of cellular genes (reviewed in Krauss et al., 1989). The ability of TPA to induce these pleiotropic effects appears to be largely due to its effects on a key cellular regulatory enzyme, protein kinase C (PKC). PKC, which is in fact a family of proteins that includes several subspecies, is a serine/theonine protein kinase which is dependent upon Ca^{++} and phospholipid (particularly phosphatidylserine) for its activation (Nishizuka, 1988). The physiologic activator of PKC is diacylglyerol, which dramatically increases the affinity of the enzyme for Ca^{++} and renders it fully active without an increase in Ca^{++} concentration (Kaibuchi et al., 1988). A major mechanism for the production of diacylglycerol is through the action of phospholipase C, which is stimulated following the interaction of several cell-surface receptors with their specific ligands and catalyzes the hydrolysis of phosphatidylinositol-4,5 bisphosphate (Rhee et al., 1989). The other product of phosphatidylinositol-4,5 bisphosphate hydrolysis, inositol-1,4,5 trisphosphate, liberates Ca^{++} from intracellular stores and the increased cytosolic Ca^{++} synergizes with diacylglycerol in the activation of PKC, as well as acting as an intracellular second messenger in the activation of other signal

transduction pathways (Streb et al., 1983; Nishizuka, 1986; Berridge, 1987). Castagna et al (1982) demonstrated that TPA directly activates PKC and it has been subsequently shown that PKC is the major cellular target for TPA (reviewed in Blumberg, 1988). TPA appears to substitute for diacylglycerol at very low concentrations, and like diacylglycerol, TPA dramatically increases the affinity of the enzyme for Ca^{++} (Nishizuka, 1984). In contrast to diacylglycerol, which disappears within a few seconds or at most a few minutes of its formation due to anabolic and catabolic processes in the cell, TPA is not rapidly degraded and persists in the cell (Nishizuka, 1986). Therefore, TPA may extend a usually limited phase of a cellular response and disrupt the normal sequence of events. That TPA is mediating many of its cellular effects through the activation of PKC was suggested by studies in which artificial elevation of cellular diacylglycerol, and more recently, overexpression of PKC could also induce many of the responses seen with phorbol esters (Blumberg, 1988; Krauss et al., 1989). Increased PKC activity may also be involved in the tumor promoting activity of another class of tumor promoters, exemplified by okadaic acid, which are potent inhibitors of the phosphoserine/threonine phosphatases that reverse the action of many protein kinases including PKC (reviewed in Cohen et al., 1990).

Although it is well accepted that TPA can induce many cellular changes through the activation of PKC, the relevance of these cellular alterations to tumor promotion is unknown. Two effects of TPA which are thought to be important in its tumor promoting activity, and which can also be induced by alternate means of PKC activation, are the induction of cell proliferation and a reduction in gap-junctional commmunication

between normal and initiated cells (Weinberg, 1989). Some effects of TPA appear to be irreversible, since even after cessation of TPA treatment some skin papillomas do not regress, and transformed foci in cell culture remain stably transformed (Weinstein, 1988). This suggests that the critical effects of tumor promoters involve not just clonal expansion but the acquisition of some heritable alteration. Nevertheless, cell proliferation appears to be an important aspect of tumor promotion since it has been shown that a physiologic growth promoting stimulus, wound healing, could provide tumor promoting activity in a two step model of carcinogenesis. Wound healing was an obligate requirement for tumor formation both in v-*jun* oncogene-transgenic mice (Schuh et al., 1990), and in v-*src* oncogene-infected chickens (Dolberg et al., 1985). In addition, sites of skin abrasion, and hence wound healing, were associated with papilloma formation in H-*ras* transgenic mice (Leder et al., 1990; Bailleul et al., 1990). It has been proposed that the action of tumor promoters in carcinogenesis is to stimulate growth of the population of initiated cells and allow a greater chance of a second spontaneous mutation essential for progression to occur during DNA replication (see Hunter, 1991).

The action of TPA in tumor promotion may not be restricted to its effects on PKC. A number of phorbol ester responses in various cell types were shown to be insensitive to inhibitors of PKC, including neutrophil activation (Wright and Hoffman, 1986) and the down-regulation of T cell surface antigens (Jung et al., 1988). These TPA-induced effects were suppressed by inhibitors of calmodulin, suggesting that TPA may activate a calmodulin-dependent pathway. Tumor promotion on mouse skin can be divided into 2 phases and TPA is considered a complete promoter because it

can mediate both phases (reviewed in Weinstein, 1988). Since other compounds, such as mezerein, can also activate PKC but are only able to accomplish the second stage of promotion, it has been suggested that the ability of TPA to act as a first stage promoter is due to an activity besides activation of PKC. One phenomenon observed following treatment of cells with TPA but not with second stage promoters is the induction of chromosomal abnormalities (Farber and Kinzel, 1990). However, neither the mechanism by which the chromosomal abnormalities are induced nor their relevance to the action of TPA as a complete promoter are known.

(C) Multiple Genetic Alterations in Tumorigenesis *In Vivo*

Genetic analysis of human tumors has provided direct evidence for the existence of multiple genetic alterations, including the activation of oncogenes and the inactivation of tumor suppressor genes within a number of tumor types (reviewed in Bishop, 1991). The study of human colon carcinoma has been particularly useful for elucidating the molecular basis of multistep tumorigenesis because tumors at various stages of development can be isolated for genetic analysis (reviewed in Fearon and Vogelstein, 1990). Mutations in at least four to five genes were detected in malignant carcinomas, while fewer changes were observed in the pre-malignant adenomas from which the malignant tumors are thought to arise. Genetic alterations included the mutational activation of an oncogene, K-*ras*, and multiple allelic losses which resulted in the deletion of several known and suspected tumor suppressor genes. Although the genetic alterations observed usually occurred at characteristic phases of colorectal tumor progression, the accumulation of the changes, rather than their order of

appearance seemed to be responsible for determining the biologic properties of the tumor. In addition to genetic alterations, an epigenetic change, DNA hypomethylation, was also consistently observed very early in colorectal tumorigenesis. Although other human tumor types do not allow for a direct genetic comparison of different stages of the same tumor, molecular analysis of many tumor types has revealed evidence of multiple genetic alterations (reviewed in Fearon and Vogelstein, 1990). Thus it appears that the multistep nature of tumor development can be explained, at least in part, by a requirement for multiple genetic alterations which result in both the activation of oncogenes and the inactivation of tumor suppressor genes. Epigenetic changes, although less well studied, likely also play a role in tumor development.

(D) *Ras* Oncogenes

The *ras* family of oncogenes have been extensively studied due to their frequent detection in human and animal tumors by gene transfer. Research has focussed both on the transforming activity of oncogenic *ras* and upon the normal function of its proto-oncogenic counterpart in cell biology.

1) *Ras* Genes

There are 3 functional *ras* genes, H-*ras*-1, K-*ras*-2, and N-*ras* (hereafter referred to as H-*ras*, K-*ras* and N-*ras*) in the mammalian genome (Barbacid, 1987). H-*ras* and K-*ras* were originally identified as the transforming genes present in Harvey and Kirsten murine sarcoma viruses respectively (Harvey, 1964; Kirsten and Mayer, 1967). A viral counterpart

has not been identified for N-*ras*, which was originally detected in a human neuroblastoma (Shimuzu et al., 1983a). These genes encode highly related proteins with molecular weights of 21,000, generically referred to as p21. The coding sequences of each of the genes are divided into 4 exons. The K-*ras* gene possesses two alternate fourth exons, A and B (Shimuzu et al., 1983b). Exon 4B is included in the major form of $p21^{K\text{-}\underline{ras}}$ expressed in mammalian cells, while the viral oncogene encodes a protein which utilizes exon 4A (Capon et al., 1983).

Cellular *ras* oncogenes have been shown to differ from their proto-oncogenic counterparts by point mutations in their coding sequences, which results in single amino acid changes in $p21^{\underline{ras}}$. Mutations have been detected in codons 12, 13 and 61 in naturally occurring cellular *ras* oncogenes (Barbacid, 1987). Substitution of the glycine residue at position 12 for any other amino acid (except for proline) produces a transforming protein, as does deletion of gly^{12} or insertion of additional amino acids between ala^{11} and gly^{12}. Replacement of gly^{13} with valine or aspartic acid but not serine results in oncogenic activation of *ras*. Mutations in codon 61 which convert gln^{61} to any other amino acid except proline or glutamic acid (and to a lesser extent, glycine) also yields *ras* oncogenes. While *ras* oncogenes derived from cellular genes carry single activating mutations, four retroviruses which have been shown to harbour *ras* oncogenes each contain genes with 2 mutations. For example, the mutations in the *ras* oncogenes of Harvey and Kirsten murine sarcoma viruses result in the conversion of glycine and alanine at residues 12 and 59 to arginine and threonine or serine and threonine respectively. In addition to the mutations found in naturally occurring *ras* oncogenes, *in*

vitro mutagenesis studies have shown that mutations at positions 116 or 119 can also confer transforming activity upon a *ras* protein (Barbacid, 1987).

2) Biochemical Properties of *ras* p21

The *ras* proteins are initially synthesized in a precursor form of 189 ($p21^{c\text{-}H\text{-}\underline{ras}}$ and $p21^{c\text{-}N\text{-}\underline{ras}}$) or 188 amino acid residues ($p21^{c\text{-}K\text{-}\underline{ras}}$). The N-terminal 164 amino acids are highly homologous between the 3 *ras* proteins, differing by a maximum of 15 residues (Santos and Nebreda, 1989). Eight of these differences occur between amino acids 121 and 135. There is almost no sequence similarity between the 3 *ras* p21 proteins over the remainder of the molecule with the exception of the 4 C-terminal amino acids. Each protein ends with the sequence cys-A-A-X, where A is any aliphatic amino acid and X is any amino acid. This sequence is also present in several other proteins, including the α and γ subunits of several G proteins (Santos and Nebreda, 1989). Other domains of *ras* proteins also exhibit significant sequence homology with the α- (Barbacid, 1987) and γ- (Gautam et al., 1989) subunits of G proteins. The cys-A-A-X motif of *ras* p21 is the target for a series of post-translational modifications in which the cysteine residue at position 186 (185 in $p21^{c\text{-}K\text{-}\underline{ras}}$) is polyisoprenylated and carboxymethylated and the 3 C-terminal amino acids are proteolytically removed (Hancock et al, 1989). Cysteine residues 181 and 184 of $p21^{H\text{-}\underline{ras}}$, and 181 of $p21^{N\text{-}\underline{ras}}$ are subsequently palmitoylated. The major expressed form of cellular $p21^{K\text{-}\underline{ras}}$ (including exon 4B) does not contain these upstream cysteines and is not palmitoylated, while the virally expressed form contains a cysteine at amino acid 180 which is palmitoylated (Buss and

Sefton, 1986; Hancock et al., 1989).

Ras proteins are localized to the inner surface of the plasma membrane (Willingham et al., 1980; Willumsen et al., 1984a), and it has been shown that this membrane attachment is required for the transforming activity of oncogenic p21 (Willumsen et al., 1984a; Willumsen et al., 1984b). Polyisoprenylation of cys^{186} (or cys^{185} for $p21^{c\text{-}K\text{-}\underline{ras}}$) is essential for membrane localization of p21, while palmitoylation of other cysteines (see above), although not essential, increased the avidity of the binding and enhanced the biological activity of oncogenic *ras* p21 (Hancock et al., 1989). Although $p21^{c\text{-}K\text{-}\underline{ras}}$ is not palmitoylated, the C-terminus of this protein contains a sequence of 6 positively charged lysine residues that may increase the avidity of the membrane binding through interactions with negatively charged membrane lipid head groups.

Ras proteins bind and exchange guanine nucleotides and exist in the cell bound to either GDP or GTP (Scolmick et al., 1979; Shih et al., 1980). It has been shown that GTP-bound *ras* is biologically active while the GDP-bound form is inactive (Trahey and McCormick, 1987; Field et al., 1987; Satoh et al., 1987). *Ras* proteins also possess an intrinsic GTPase activity which converts bound GTP to GDP (Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1984). In addition to the effects of this intrinsic GTPase activity, the hydrolysis of bound GTP is stimulated over 200 fold by interaction of *ras* with the GTPase activating protein (GAP) (Trahey and McCormick, 1987).

It has been demonstrated that oncogenic *ras* proteins are altered in their guanine nucleotide-binding properties. Activating mutations which result in amino acid substitutions at positions 12, 13 or 61 have been

shown to reduce the intrinsic (Gibbs et al., 1984; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1985) and/or GAP-stimulated (Trahey and McCormick, 1987) GTPase activity of p21. The reduction in GTPase activity would be expected to result in an increased proportion of cellular *ras* in the GTP-bound active form, likely accounting for the biological activity of oncogenic p21. The substitution of threonine for alanine at amino acid 59 which occurs in the viral H-*ras* and K-*ras* oncogenes confers upon *ras* p21 an autophosphorylating activity, for which thr^{59} is the phosphate acceptor (Barbacid, 1987). The biological significance of this phosphorylation is not known. Activated *ras* proteins with mutations produced in residues 116 or 119 by *in vitro* mutagenesis have greatly diminished affinity for GDP and GTP. Some of the substitutions result in only reduced affinity for GTP and GDP and may favor formation of the active GTP bound protein due to a higher availabilty of GTP in the cell (Barbacid, 1987). Other mutations at these positions which create p21 proteins with negligible guanine nucleotide binding may produce a constitutive conformational activation mimicking that produced by GTP binding (Santos et al., 1988).

3) Functional Domains of *ras* p21

Mutational analysis and comparison with partially related proteins were used to assign certain functions to specific domains within p21 (reviewed in Barbacid, 1987). Analysis of deletion mutants of oncogenic *ras* identified five non-contiguous domains of p21 (residues 5-63, 77-92, 109-123, 139-165 and the C-terminal cys-A-A-X sequence) which were essential for *ras* transforming function (Willumsen et al., 1986; Sigal.,

1986). The remaining regions could be deleted without drastically affecting the transforming activity and were referred to as dispensable domains. Most of the essential internal domains were hydrophobic and appeared to be involved in binding guanine nucleotides. However, the hydrophilic region including amino acids 32-40, although essential for *ras* function, was not required for GDP binding, GTPase activity or membrane attatchment. For this reason, it was speculated that this region may be required for *ras* protein interaction with its putative downstream target, and this portion of the protein is commonly referred to as the effector domain. This region has also been shown to be essential for the interaction between *ras* p21 and GAP (Cales et al., 1988; Adari et al., 1988; Vogel et al., 1988), raising the possibility that GAP may be a downstream effector of *ras* (see below).

More recently, clues to the structural bases of the functional domains have come from X-ray crystallographic studies of genetically engineered H-*ras* proteins containing amino acids 1-166 or 1-171. The three-dimensional crystal structure has been determined for this portion of proto-oncogenic p21, both in the GDP-bound form (de Vos et al., 1988; corrected in Tong et al., 1989) and in the GTP-bound conformation (Pai et al., 1989), and for a similar portion of an oncogenic *ras* protein (containing a $gly^{12} \rightarrow val^{12}$ substitution) in the GDP-bound form (Tong et al., 1989b). The *ras* protein consists of six β-strands, four α-helices and nine connecting loops. The essential regions for *ras* function include all strands of the central β-sheet as well as some of the adjacent helices and connecting loops. Four of the loops appear to be involved in interactions with bound guanine nucleotides. One of these interacts with the

phosphates, another with ribose and two with the guanine base. The mutations found in naturally occurring *ras* oncogenes produce amino acid substitutions in regions found to be important for the binding of the guanine nucleotide. Residues 12 and 13 are located in a loop which is in close approximation with the phosphate residues, the effector loop and the loop containing gln^{61} (Pai et al., 1989). Amino acids gly^{12} and gln^{61}, which are the residues most often mutated in human tumors, are in close proximity in the three dimensional structure of *ras* generated in the triphospate conformation. A comparison of the crystal structures of oncogenic (val^{12}) and proto-oncogenic (gly^{12}) p21 revealed that the phosphate binding loop (loop 1) was much larger in the former and lacked two hydrogen bonds present in the proto-oncogenic protein (Tong et al., 1989b). Since it has been suggested that this loop includes the catalytic site for GTP hydrolysis (de Vos et al., 1988), this conformational alteration may account for the reduced GTPase activity of the mutant protein. Gln^{61} has been shown to be close to the γ-phosphate in the triphosphate conformation. Replacing it with an amino acid with a hydrophobic side chain may also affect the catalytic site by destabilizing interactions with either the phosphate or with neighboring amino acids. The structural basis for the observed autophosphorylation of the viral H-*ras* and K-*ras* p21 proteins was also suggested from the crystal structures. The location of residue 59 near the γ-phosphate suggested that substitution of threonine for alanine at residue 59 would place the hydroxyl group on its side chain in close enough proximity to accept the γ-phosphate of GTP. Amino acids 32-36 of the putative effector region are situated in another looped region while the remainder of the amino acids

(37-40) of this functional domain are part of the second β-strand. The looped region involving the effector region appears to be detached from an otherwise compact molecule and would therefore be easily accessible to other proteins. A comparison of the crystal structures of GTP- and GDP-bound proto-oncogenic p21 showed major conformational differences in the region including amino acids 30-38, consistent with this domain being involved in interactions with an effector molecule(s) (Jurnak et al, 1990; Milburn et al., 1990; Schlichting et al., 1990). A major conformational change was also observed in a second region spanning residues 60-76 of loop 4 and α-helix 2. A monoclonal antibody, Y13-259, which binds to this region, was shown to neutralize the transforming activity of oncogenic *ras* protiens (Barbacid, 1987). However, *in vitro* mutagenesis showed that this region was dispensable for transforming activity (Willumsen et al., 1986). Milburn et al (1990) propose that this region is the recognition site for a putative upstream regulator which stimulates exchange of bound GDP for GTP (see below). The observation that this region was non-essential for transformation could then be explained by an independence of oncogenic *ras* p21 from upstream regulators, and the neutralizing effect of Y13-259 by an ability of the antibody to block GDP-GTP exchange, locking *ras* in the GDP-bound inactive form. The two regions which undergo conformational alterations between the GDP- and GTP-bound forms of p21 are both located on the surface of the molecule, and occupy a contiguous stretch of the surface.

4) Biochemical Model of *ras* p21 Function

Ras proteins show similarities, both in their biochemical properties

and in their structure, to the guanine nucleotide binding G proteins involved in the modulation of signal transduction through transmembrane signalling systems. For this reason, it has been hypothesized that proto-oncogenic p21 may perform a similar role as a transducing protein (Tanabe et al., 1985). According to the current view, upon binding its extracellular ligand, a presumed transmembrane receptor protein (or proteins) directly or indirectly induces the exchange of GDP for GTP on *ras* p21, converting the protein to its active form. GTP-bound p21 then interacts with an effector protein (or proteins) which further transmits the growth signal. After interaction with its effector, bound GTP is hydrolyzed to GDP, inactivating *ras* and terminating the signal. Activating mutations which reduce the GTPase activity of p21 therefore maintain the protein in the active conformation and cause a sustained unregulated transmission of the signal.

Support for the proposed role for *ras* p21 as an intermediary in signal transduction has come from studies which showed that the function of *ras* is required for cells to respond to certain extracellular stimuli. The microinjection of a neutralizing anti-*ras* antibody blocked the serum-induced initiation of DNA synthesis in NIH 3T3 cells (Mulcahy et al., 1985), the proliferation of NIH 3T3 cells transformed by growth factor receptor-like oncogenes with tyrosine kinase activity (Smith et al., 1986), the nerve growth factor-induced neuronal differentiation of PC12 cells (Hagag et al., 1986), and the insulin-induced maturation of *Xenopus* oocytes (Deshapande and Kung, 1987; Korn et al., 1987). That *ras* p21 was playing an active role in the transmission of these signals was suggested by the fact that microinjection of oncogenic *ras* proteins into cells had

effects similar to that of the stimulus, inducing the proliferation of quiescent fibroblasts (Feramisco et al., 1984), stimulating differentiation of PC12 cells (Bar-Sagi and Feramisco, 1985), and promoting oocyte maturation (Deshpande and Kung, 1987; Korn et al., 1987). Further support for the hypothesis that *ras* is involved in the transmission of signals through membrane receptors was provided by studies which demonstrated an increased proportion of *ras* p21 in the GTP-bound active state following the stimulation of cells through a number of different cell surface receptors. Increased GTP-p21 has been observed following the stimulation of cells bearing the appropriate receptor with anti-CD3 or T cell mitogens (Downward et al., 1990a), anti-CD2 (Graves et al., 1991), platelet derived growth factor (Satoh et al., 1990a; Gibbs et al., 1990), epidermal growth factor (Satoh et al., 1990b), interleukin 2, interleukin 3, and granulocyte/macrophage colony-stimulating factor (Satoh et al., 1991). *Ras* proteins have also been found to co-cap with surface immunoglobulins in B lymphocytes, implicating *ras* p21 in the signalling pathway which is initiated by the cross-linking of surface immunoglobulins (Graziadei et al., 1990). A common element among the stimuli which have been shown to affect *ras* p21 may be a signal transduction pathway involving tyrosine kinase activity. The receptors for platelet-derived growth factor and epidermal growth factor both contain tyrosine kinase domains (Hanks et al., 1988), while tyrosine phosphorylation has been implicated in signal transduction from cytokines, including interleukins 2, 3 and 4 and granulocyte/macrophage colony stimulating factor (Koyasu et al., 1987; Morla et al., 1988; Isfort et al., 1988; Saltzman et al., 1988; Gomez-Cambronero et al., 1989), and in signalling through CD2 and the T

cell and B cell antigen receptors (Samelson et al., 1986; Patel et al., 1987; Campbell et al., 1990; Monostori et al., 1990; Gold et al., 1991). In addition, the expression of oncogene products with constitutively enhanced tyrosine kinase activity, including both receptor (*erbB-2/neu*) and non-receptor (v-*src*, v-*abl*) proteins, was associated with increased amounts of GTP-bound *ras* p21 (Satoh et al., 1990b; Gibbs et al., 1990).

The events linking membrane receptor stimulation to the conversion of *ras* p21 from the GDP- to GTP-bound form are not known. One level at which the ratio of GTP- to GDP-bound p21 may be regulated is through changes in the rate of guanine nucleotide exchange. In yeast, the CDC25 protein stimulates the exchange of RAS-bound GDP for GTP (Robinson et al., 1987; Broek et al., 1987). Three apparently distinct mammalian proteins have been isolated which promote guanine nucleotide exchange by *ras* p21 (Wolfman and Macara, 1990; Downward et al., 1990b; Huang et al., 1990). How the activity of these putative exchange factors may be regulated by transmembrane receptors is not known.

A second level at which *ras* protein activity is regulated is by the hydrolysis of bound GTP which deactivates p21. Modifications in the rate at which this process occurs would similarly modify the activation state of p21. A possible link between receptor stimulation and modification of GTPase activity was suggested by reports that stimuli which were shown to result in increased GTP-bound *ras* p21 (see above) also produced alterations in GAP. The increased proportion of GTP-bound p21 upon stimulation of T cells through the T cell receptor (CD3) or CD2 could be mimicked by phorbol ester treatment, suggesting that a PKC-dependent pathway was involved (Downward et al., 1990a). It was further shown that

phorbol ester treatment inhibited GAP activity, suggesting that this may be the mechanism through which *ras* activity was increased. It was also demonstrated that GAP was phosphorylated on tyrosine residues in response to cell stimulation with platelet-derived growth factor (Molloy et al., 1990; Kaplan et al., 1990) and epidermal growth factor (Bouton et al., 1991; Moran et al., 1991) and in cells transformed by *src* or *fps*, two oncogenes encoding cytoplasmic proteins with tyrosine kinase activity (Ellis et al., 1990; Bouton et al., 1991; Moran et al., 1991). It was observed that GAP was additionally phosphorylated on serine residues in cells expressing the v-*src*-encoded oncoprotein and in cells stimulated with epidermal growth factor (Moran et al., 1991). In addition to phosphorylation, GAP was altered in its subcellular distribution, and in its interaction with other proteins. GAP was shown to be associated with tyrosine phosphorylated proteins of 190 and 62 KD, or in one report 64 KD, in response to epidermal growth factor stimulation or in response to transformation by oncogenes with tyrosine kinase activity (Ellis et al., 1990; Bouton et al., 1991; Moran et al., 1991). The complex with the 190 KD protein was shown to be exclusively cytosolic and had reduced GAP activity (Moran et al., 1990). The significance of the formation of these complexes to the signal transduction process was further suggested by the observation that mutations in the *src* protein which rendered this oncogene defective for transformation also inhibited the efficient formation of complexes between GAP and the phosphorylated proteins (Bouton et al., 1991). GAP has also been detected in complexes with proto-oncogenic and oncogenic *src* proteins in cell lysates (Brott et al., 1991). Following stimulation of cells with platelet-derived growth factor, GAP was shown to

physically associate with the platelet-derived growth factor receptor as part of a multi-subunit complex, which also included phospholipase C-γ, phosphatidylinositol-3 kinase and the 74 KD product of the *raf*-1 proto-oncogene (Kazlauskas et al., 1990; Kaplan et al., 1990). How the observed effects on GAP relate to a putative *ras*-inclusive signal transduction pathway is not known. One possible but unproven hypothesis is that a reduction in GAP activity due to its phosphorylation, association with other proteins, or altered subcellular localization would result in an increased proportion of *ras* p21 in the GTP-bound form and thus lead to increased signalling. An alternate interpretation is raised by the possibility that in addition to, or instead of acting as a negative regulator, GAP may be a downstream effector of *ras* activity (McCormick, 1990). If GAP were an effector of *ras* activity, the resultant changes in GAP following growth factor stimulation or tyrosine kinase oncogene activation may in fact be related to its transmission of the signal from p21. For example, phosphorylated GAP may be interacting with, and altering the activity of a downstream target.

That GAP may be an effector of *ras* activity was originally suggested from studies which showed that mutations in the putative effector binding region of p21 prevented the GAP-mediated stimulation of normal *ras* p21 (Cales et al., 1988; Adari et al., 1988). The most direct evidence that GAP may act as an effector was provided by a study which showed that *ras* p21 and GAP similarly blocked the G protein-mediated coupling of a muscarinic receptor to potassium channels in atrial cell membranes, and the effect of each protein was dependent upon the presence of the other (Yatani et al., 1990). However, other studies have yielded results not

consistent with an effector function of GAP and direct proof that GAP acts either as an effector or as a negative regulator of *ras* activity is still lacking (for review, see McCormick, 1990). Recently, it has been shown that the protein product of the NF1 gene shares many properties with GAP (reviewed in Marshall, 1991). The NF1 protein can act as a GTPase activating protein for *ras* p21, does not stimulate the GTPase activity of oncogenically activated *ras* p21 proteins and interacts with p21 through the same effector domain. It has been shown that mammalian cells contain both GAP and the NF1 protein, and that the two proteins differ with regard to some of their properties (Bollag and McCormick, 1991). For example, NF1 bound to p21 proteins up to 300 times more efficiently than did GAP, and the activity of NF1, but not GAP was inhibited by micromolar concentrations of certain lipids. It is possible that one or both of GAP or NF-1 may be the effector of *ras*, while the other is a negative regulator of *ras* protein activity. Conversely, there may be another as yet unidentified protein (or protein) which is the true downstream effector. Consistent with this latter hypothesis is the report by DeClue et al (1991) which described a v-H-*ras* effector domain mutant which was defective for transformation at 39.5°C, but bound guanine nucleotides and demonstrated a normal increase in GTPase activity when stimulated by GAP or NF1 at the same temperature.

5) *Ras* p21 and Signal Transduction

The type of signal transduction pathway in which *ras* p21 may be involved is also not known. Because related RAS proteins in yeast have been shown to act as positive regulators of adenylate cyclase, and

mammalian *ras* could substitute for yeast RAS in this activity (Kataoka et al., 1985), it was originally suggested that *ras* proteins would perform a similar role in mammalian cells (Nurse, 1985). However, no such activity has ever been demonstrated in mammalian cells (Beckner et al., 1985). More recent studies have focussed on a possible role of *ras* proteins in the phosphoinositide signalling pathway. Increased turnover of phosphatidylinositol (Fleischman et al., 1986; Huang et al., 1988; Pan and Cooper, 1990) or phosphatidylcholine (Price et al., 1989; Lacal, 1990) and increased levels of diacylglycerol (Preiss et al., 1986; Macara, 1987; Lacal et al., 1987; Huang et al., 1988) have been detected in mammalian cells or *Xenopus laevis* oocytes microinjected or scrape loaded with oncogenic *ras* proteins, and in cells transfected with activated *ras* oncogenes. It has been suggested that *ras* may be directly involved in regulating either phospholipase C (Fleischman et al., 1986; Preiss et al., 1986; Wolfman and Macara, 1987; Marshall, 1987), which catalyzes the hydrolysis of phosphatidylinositol to yield diacylglycerol and inositol trisphosphate, or phospholipase A_2 (Bar-Sagi and Feramisco, 1986), which catalyzes the hydrolysis of the 2 acyl fatty acid ester of glycerophospholipids to yield free arachidonic acid and lysophospholipids. However, a role for *ras* in the direct regulation of these enzymes remains uncertain. Yu et al (1988) demonstrated that proliferation of NIH 3T3 cells induced by mimics of phospholipase C (phorbol ester plus a calcium ionophore) or phospholipase A_2 (prostaglandin F2α) activity was blocked by microinjection of a neutralizing anti-*ras* monoclonal antibody. This result suggested that *ras* proteins do not function specifically to control the action of phospholipases C or A_2 but must instead act subsequently to them.

The results from another study, however, yielded different results (Smith et al., 1990). It was observed that co-injection of anti-phospholipase C antibodies with either oncogenic *ras* p21 or phospholipase C into quiescent NIH 3T3 cells inhibited the induction of DNA synthesis normally stimulated by the injection of p21 or phospholipase C. In contrast, co-injection of anti-*ras* antibodies inhibited the DNA synthesis induced by *ras* p21 but not that stimulated by phospholipase C. On the basis of these results it was suggested that the *ras* protein is an upstream effector of phospholipase C, and that phospholipase C activity is necessary for *ras*-mediated induction of DNA synthesis. Others have reported that increased levels of diacylglycerol observed following both constitutive expression of *ras* oncogenes in NIH 3T3 cells and microinjection of oncogenic *ras* protein into *Xenopus* oocytes occurred without concomitant increases in phosphatidylinositol metabolism, but rather were associated with, and preceded by, rapid alterations in phosphatidylcholine metabolism (Lacal et al., 1987a; Wolfman and Macara, 1987; Macara, 1989; Lacal, 1990). From these studies it was suggested that the increased diacylglycerol levels observed in these cells originated from phosphatidylcholine metabolism and that *ras* may directly or indirectly activate choline kinases (Lacal, 1990). Another group reported that increases in phosphatidylcholine metabolism and diacylglycerol levels, which occurred within 10-20 minutes following scrape-loading of oncogenic p21 into Swiss 3T3 cells, were blocked by prior down-regulation of protein kinase C (Price et al., 1989). These authors suggested that *ras* rapidly activates protein kinase C, which in turn activates a number of cellular signalling systems and leads to a sustained increase in diacylglycerol levels. The conflicting results from

different studies likely reflect the limitations of the ability to determine the biochemical activity of a specific molecule on the basis of phenomena observed in the context of a whole cell system.

6) *Ras* p21 and PKC

Regardless of its source, the presence of elevated diacylglycerol levels has been consistently observed in *ras*-transformed cells and suggested that *ras* activity may be mediated through a pathway involving PKC. *Ras* oncogene expression has been shown to affect the expression of PKC in a number of studies. Partial down-regulation of PKC in H-*ras* or K-*ras* transformed NIH 3T3 fibroblasts was suggested by reduced responsiveness to phorbol esters and reduced phorbol ester binding (Wolfman and Macara, 1987). C3H10T½ mouse fibroblasts expressing a *ras* oncogene constitutively (Weyman, et al., 1988) or conditionally (Haliotis et al., 1990) exhibited reductions in both PKC activity and protein level. Chiarugi et al (1990) reported that PKC activity was reduced in the cytosol but increased in the nuclear fraction of *ras*-transformed Balb/3T3 fibroblasts. Borner et al (1990) found that in rat embryo fibroblasts and liver epithelial cell lines expressing two isoforms of PKC, constitutive or Zn-inducible expression of an activated *ras* oncogene was associated with both a reduction in expression of PKC_ϵ at the mRNA and protein levels, and with an increase in expression of PKC_α.

There is also evidence that some of the cellular effects induced by *ras* may be mediated through PKC-dependent signalling pathways. Down-regulation of endogenous PKC by pretreatment of Swiss 3T3 cells with a phorbol ester inhibited more than 80% of the mitogenic activity of

microinjected oncogenic *ras* p21 (Lacal et al.,1987b). The observation that co-injection of PKC and p21, but not PKC alone, reconstituted the mitogenic response of these cells strongly suggested that *ras* required PKC activity for its affect on cell proliferation. v-*src* and v-*fps*, two tyrosine kinase oncogenes whose affects on cell proliferation were blocked by antibodies to the *ras* protein (Smith et al., 1986), were shown to be dependent upon PKC for induction of a transformation-related gene (Spangler et al., 1989). Further evidence for a PKC-inclusive pathway of *ras* p21 signalling was provided by studies which showed that *ras*-induced transcriptional activation of c-*fos* (Stacey et al., 1987) and c-*jun* (Sassone-Corsi et al., 1989) was mediated by the same sis-acting DNA-regulatory sequences that confer responsiveness to TPA. Transcriptional activation of genes carrying these regulatory sequences (TPA-response elements) is mediated by the transcription factor AP1, which consists of the proto-oncogene c-*jun* protein complexed either to another member of the jun family or to a member of the fos family (reviewed in Binetruy et al., 1991). *Ras* oncogene expression and TPA have both been shown to induce dephosphorylation of the C-terminal sites of c-*jun* and increase its activity (Boyle et al., 1991; Binetruy et al., 1991). However, *ras* p21 expression was additionally associated with phosphorylation of c-*jun* at N-terminal sites, suggesting the existence of a PKC-independent effect (Binetruy et al., 1991). *Ras* appears to induce other cellular effects independently of PKC activity, including morphological transformation and c-*myc* expression, which were both induced following introduction of purified oncogenic p21 into Swiss 3T3 cells in which PKC had been removed by phorbol ester pretreatment (Lloyd et al., 1989). Also, PKC-down-

regulation did not affect K-*ras*- or v-*fps*-induced expression of the glucose transporter gene in rat fibroblasts (Hiraki et al., 1989) or *ras* induction of c-fos expression in NIH 3T3 cells (Cai et al., 1990). Thus, *ras* p21 appears to act through at least two distinct pathways, one dependent upon, and one independent of PKC. The PKC-independent pathway appears to be required for the induction of cell proliferation since PKC-activation is not sufficient to stimulate proliferation of NIH 3T3 cells in which *ras* activity has been blocked by microinjection of a neutralizing anti-*ras* antibody (Yu et al., 1988) or by expression of a *ras* mutant which dominantly inhibits normal *ras* p21 activity (Cai et al., 1990). Synergy between *ras* p21 and PKC in transformation was suggested by the ability of TPA to enhance the transformation of rodent fibroblasts induced by a transfected H-*ras* oncogene (Hsiao et al., 1984; Dotto et al., 1985; Hsiao et al., 1986). More direct support for this hypothesis was provided by a study which showed that overexpression of the β_1 form of PKC in a rat fibroblast cell line was associated with an increased susceptibility to transformation by a *ras* oncogene (Hsiao et al., 1989).

The search for other downstream elements of a *ras* p21-inclusive signal transduction pathway have largely focussed on the proteins encoded by other proto-oncogenes. One such protein which has been implicated in this pathway is the serine/threonine kinase c-*raf*-1. c-*raf-1* was shown to be hyperphosphorylated and enzymatically activated in cells which are transformed by *src*, *fms* or *ras*, and in cells which have been treated with platelet-derived growth factor or TPA (Morrison et al., 1988). In addition, expression of either c-*raf-1* antisense RNA or a kinase-defective Raf-1 mutant in NIH 3T3 cells blocked proliferation induced by serum

growth factors, TPA or K- and H-*ras* oncogenes (Kolch et al., 1991). As discussed above, *ras* expression induces c-*jun* expression and leads to increased activity of the c-*jun* protein as part of the AP1 complex (Binetruy et al., 1991). *Ras* oncogene expression also induces a rapid, but transient increase in the protein levels of another element of the AP1 complex, c-*fos* (Stacey et al., 1987). The importance of c-*fos* and the AP1 complex in *ras*-induced transformation was suggested by the observation that cells resistant to transformation by v-*fos* were also resistant to transformation by Ha-*ras* (Zarbl et al., 1987) and c-jun (Wisdom and Verma, 1990). Further, c-*fos* antisense RNA expression in EJ c-H-*ras* oncogene-transformed NIH 3T3 cells caused partial reversion of the transformed phenotype (Ledwith et al., 1990). *Ras* oncogene p21 induction of c-*fos* expression in REF-52 fibroblasts, which was necessary for the induction of their proliferation, was shown to be mediated through another transcriptional activator, the serum response factor (SRF), in a PKC-dependent manner (Gauthier-Rouviere et al., 1990). A third transcription factor shown to be activated following *ras* oncogene expression is PEA3 (Wasylyk et al., 1989). In contrast to AP1, c-*fos* expression was not required for the induction of PEA3 activity. As discussed above, the introduction of oncogenic $p21^{H\text{-}ras}$ into quiescent Swiss 3T3 cells by scrape-loading leads to increased levels of c-*myc* mRNA (Lloyd et al., 1989), and *ras* oncogene expression in the Rat-1 cell line was also reported to be associated with increased expression of c-*myc* (Godwin and Lieberman, 1990). c-*myc* encodes a DNA-binding protein which is thought to play a role either as a transcriptional regulator or as a controlling factor in DNA synthesis (reviewed in Luscher and Eisenman, 1990). The interaction of a

ras p21 signalling pathway with known and suspected transcription factors suggests that the pleotropic changes in cell phenotype observed following *ras* oncogene-induced neoplastic transformation (see below) may be due to an altered pattern of gene expression. Indeed, it has been shown that the expression of numerous genes is altered in response to *ras* p21 expression, some of which occur early while others show more delayed kinetics (reviewed in Godwin and Lieberman, 1990).

7) *Ras* Transformation *In Vitro*

The transforming activity of an oncogene has been traditionally assayed by *in vitro* introduction of the gene into a recipient cell line, most commonly the immortalized mouse embryo cell line NIH 3T3, followed by an assessment of either the ability of the recipient cells to produce tumors upon subsequent injection into immune-deficient nude mice, or the formation of areas of dense multilayer cell growth (foci) in the normally monolayer distribution of the recipient cells. Cellular transformation *in vitro* is generally accompanied by pleiotropic changes encompassing morphological, biochemical and growth-related properties. Some of the cellular alterations frequently associated with *ras*-induced transformation include loss of cell surface fibronectin, altered patterns of membrane lipid and glycolipid expression, novel processing of saccharides on glycoproteins, decreased adhesion to substratum, increased hexose uptake, loss of density-dependent growth, increased anchorage-independent growth, reduced binding of growth factors and increased secretion of autocrine growth factors (Rosenfelder et al., 1977; Santer et al., 1984; Collard et al., 1985; Matyas et al., 1987; Nanus et al., 1989; Godwin and Lieberman,

1990; Hirakawa et al., 1991). Although morphological alterations generally accompany *ras*-induced tumorigenic conversion, the biochemical pathways leading to these two manifestations appear to be at least partially distinct, since it has been shown that fusion of transformed cells to normal counterparts, or transfection with DNA from normal cells sometimes suppresses only morphological transformation or only tumorigenicity (reviewed in Santos and Nebreda, 1989).

It has been shown that expression of an activated *ras* oncogene is sufficient for tumorigenic conversion of numerous established rodent cell lines (Barbacid, 1987), as well as human cells immortalized by a variety of methods (reviewed in Amstad et al., 1988). In addition, overexpression of proto-oncogenic *ras* can transform NIH 3T3 cells, but a very high level of expression is required (reviewed in Barbacid, 1987). Although Spandidos and coworkers (Spandidos and Wilkie, 1988; Spandidos et al., 1990) reported that overexpression of normal *ras* p21 in a rat fibroblast cell line could suppress transformation by oncogenic *ras*, the activity of the *ras* oncogene has generally been shown to be dominant at the cellular level, since transformed recipients also expressed the endogenous *ras* proto-oncogene and co-transfection with the normal allele did not affect the transformation frequency (Barbacid, 1987). Individual established cell lines show differing susceptibilities to *ras*-induced transformation. For example, transfection of equivalent amounts of *ras* oncogene DNA into two murine fibroblast cell lines, NIH 3T3 and 10T½, resulted in the formation of 10-90 fold more foci in the former cell line (Hsiao et al., 1984; Taparowski et al., 1987). In addition, some established rodent cell lines are not transformed by *ras* alone but require the presence of a

cooperating oncogene (see below) (Ruley, 1990).

8) *Ras* Oncogenes and Multistep Transformation *In Vitro*

Ras oncogene expression is generally insufficient for the neoplastic transformation of primary cells, except under special conditions in which there is a high expression level of oncogenic p21 and adjacent normal cells are absent (see below) (Land et al., 1986). Although insufficient for tumorigneic conversion, expression of an activated *ras* oncogene in primary cells does in some instances produce phenotypic alterations such as changes in morphology and cell proliferation (Nanus et al., 1989; Lemoine et al., 1990). *Ras* transformation *in vitro* has been shown to be influenced by the expression of other genes in the target cell. The introduction and expression of a second, complementing oncogene allowed *ras* to transform primary cells (Ruley, 1990). Generally, oncogenes which have been shown to cooperate with *ras* in the transformation of primary cells have belonged to a subset of oncogenes classified as nuclear based on the location in the cell of their gene product. These include three different *myc* genes (c-*myc*, L-*myc* and N-*myc*), *jun*, *fos* and mutant p53, which likely acts in a dominant negative manner (Herskowitz, 1987) to inhibit normal p53 protein function (see above), as well as several DNA-tumor virus-derived oncogenes whose gene products are expressed in the nucleus. However, nuclear location is not a requirement for cooperation with *ras* since the *BCL2* oncogene, which encodes a membrane-associated, guanine nucleotide-binding protein, also cooperated with activated *ras* in the transformation of early-passage rodent fibroblasts (Reed et al., 1990). However, unlike *ras*, *BCL2* was unable to cooperate with c-*myc* or the

adenovirus Ela gene in this process. Although *BCL2* and *myc* did not cooperate in the transformation of early passage rodent fibroblasts, the two oncogenes have previously been shown to cooperate in the tumorigenic conversion of lymphoid cells, demonstrating that oncogene effects are dependent upon the genetic context in which they are expressed (Vaux et al., 1988; Nunez et al., 1989). The results suggested that the product of *BCL2*, although exhibiting some similar characteristics to *ras* p21, regulates a distinct cellular pathway involved in the transformation process. The basis of oncogene cooperation with *ras* appears to be related to the stimulation of a cell signalling pathway which cooperates with that induced by *ras* to subvert the normal growth regulatory processes of the cell (reviewed in Hunter, 1991). Although oncogenes capable of cooperating with *ras* frequently have the ability to immortalize cells, their contribution to transformation goes beyond cell immortalization since *ras* fails to transform some immortalized cell lines and expression of cooperating oncogenes in these cells, including v-*myc* and Ela, allowed for *ras* transformation (Ruley, 1990). *Ras* transformation of primary cells may require other genetic alterations in addition to a single cooperating oncogene, since non-random genetic alterations have been observed in cells transformed by *myc* + *ras*. These include the loss of a specific chromosome in Syrian hamster fibroblast cells (Oshimura et al., 1988) and a site-specific integration of H-*ras* in rat embryo fibroblast cells (McKenna et al., 1988). Further, the ability of *ras* + *myc* to transform diploid cells derived from rodent fetuses was observed to decrease with increasing age of the fetus, suggesting that early embryo cells have a pattern of gene expression which cooperates with *myc* and *ras* in cell transformation (Yagi

et al., 1989). Consistent with the hypothesis that *ras* transformation of primary cells may require additional genetic changes, Peacock et al (1990) reported that the frequency of *ras* transformation of rat embryo fibroblasts was higher when two additional cooperating oncogenes were utilized instead of one. In addition to their ability to cooperate with *ras* in the transformation of primary cells, the same oncogenes have been shown to enhance *ras* transformation of cells which *ras* transforms only weakly on its own (Taparowsky et al., 1987; Rawson et al., 1991). Although the biochemical basis of oncogene cooperation with *ras* is not known, proposed mechanisms include the control of transcription, collaboration in cell cycle progression and effects on DNA replication (Ruley, 1990).

Ras transformation *in vitro* can also be suppressed by expression of certain genes. Introduction of a *ras*-related gene called Krev-1 (also known as *rap* 1A and *smg* p21) caused phenotypic reversion of v-K-*ras* transformed NIH 3T3 cells without affecting K-*ras* p21 expression levels (Noda et al., 1989; Kitayama et al., 1989). Overexpression of wild type p53 suppressed transformation of rat embryo fibroblasts by E1a + *ras*, *myc* + *ras* and mutant p53 + *ras* (Finlay et al., 1989; Eliyahu et al., 1989).

Thus, as shown by the differential susceptibility of recipient cells to *ras* transformation, and the ability of additional genes to enhance or suppress *ras* transformation, the genetic context in which *ras* is expressed is central to its affect on cell behavior. This is further exemplified by the fact that expression of *ras* in certain cell types induced not transformation, but differentiation (Bar-Sagi and Feramisco, 1985; Yamaguchi-Iwai et al, 1990), meiotic maturation (Birchmeier et al., 1985) or cell cycle arrest (Franza et al., 1986; Ridley et al., 1988). In

addition, *ras*-induction of transformation and differentation may not be mutually exclusive since expression of H-*ras* or N-*ras* oncogenes in Epstein-Barr virus-immortalized human B cells produced both malignant transformation and terminal differentiation (Seremetis et al., 1989).

Ras transformation *in vitro* can also be influenced by exogenous stimuli. Factors which have been shown to enable *ras* to transform primary cells, or enhance *ras* transformation of established cell lines include TPA (Hsiao et al., 1984; Dotto et al., 1985; Hsiao et al., 1986), a low molecular weight factor present in fetal calf serum (Hsiao et al., 1987), glucocorticoid hormones (Marshall et al., 1991), mezerein, 1-oleoyl-2-acetylglycerol, phospholipase C and low concentrations of retinoic acid (Lopez et al., 1989). In addition, treatment with the hypomethylating agent 5-aza-2'-deoxycytidine resulted in the transformation of non-tumorigenic revertants of NIH 3T3 cells expressing *ras* oncogenes (Rimoldi et al., 1991). Conversely, other environmental factors can also suppress *ras* transformation *in vitro*. It has been shown that while transfection of an activated *ras* oncogene into rat embryo fibroblasts yielded no foci of transformed cells, cointroduction of *ras* and a neomycin resistance (neo^r) gene, followed by growth in medium containing the neomycin analogue geneticin (G418), allowed for an outgrowth of *ras* oncogene-expressing transfectants which were tumorigenic (Spandidos and Wilkie, 1984; Land et al., 1986). Since the removal of surrounding normal cells was associated with the outgrowth of tumorigenic cells, it was proposed that the normal cells in some way suppressed the growth of neighboring cells (normal and *ras*-transfected), and expression of a *ras* oncogene was not sufficient to confer cell autonomy from this regulatory influence. The mechanism of

suppression by surrounding normal cells is not known but could involve their secretion of growth inhibitory cytokines, an inhibitory signal delivered via cell-cell contact, or the direct intercellular transmission of a negative regulator (Hunter, 1991). The addition of protease inhibitors to the culture medium following transfection was shown to inhibit T24-H-*ras* mediated transformation of NIH 3T3 cells (Garte et al., 1987). Protease inhibitors have also been shown to supress the promoting activity of TPA *in vivo* and *in vitro* (reviewed in Chang et al., 1985). Several chemicals have been shown to inhibit H-*ras* oncogene-induced transformation of established rodent cell lines, including the calcium ionophore A23187, indomethacin, ϵ-amino-n-caproic acid and retinoic acid at higher concentrations than that which showed an enhancement of *ras* transformation (see above) (Lopez et al., 1989; Cox et al., 1991).

Studies of the *in vitro* transforming activity of *ras* implicate this oncogene in either early or late events in the multistep process of tumorigenesis, depending upon the cell type into which it is introduced. Expression of a *ras* oncogene in primary cells appears analagous to the initiation step in mouse skin carcinogenesis, since a subsequent treatment with a chemical tumor promoter or a cooperating oncogene is required for tumorigenic conversion. However, *ras* oncogene transformation of established cell lines, which have presumably undergone some "initiating" event during the process of immortalization, implicates *ras* in the later step of cell conversion from the preneoplastic to neoplastic state. Further, *ras* oncogene expression has been shown to convert some immortalized, non-tumorigenic cell lines, as well as some tumorigenic but non-metastatic cell lines to fully malignant, metastatic tumor cells

(reviewed in Liotta, 1988). The ability of oncogenic *ras* to confer metastatic ability upon a cell, like its ability to promote tumorigenic conversion, is dependent upon the particular recipient (Muschel, 1985). The expression of several endogenous genes, including c-*jun* and c-*fos* were found to differ between one cell line which became metastatic following transfection and expression of activated *ras* and another which did not (Tuck et al., 1991). Other genes have been shown to directly influence the effect of *ras* on the metastatic phenotype, including the adenovirus Ela gene which reduced the metastatic potential of *ras*-transformed rat embryo cells (Pozzatti et al.,1988). The metastasis-inhibitory activity of the Ela gene was associated with increased expression of a cellular gene, NM23 (Steeg et al., 1988), whose expression level has been shown to inversely correlate with metastatic ability (reviewed in Sobel, 1990). The nature of external stimuli present at the time of cell transformation may also influence the abiltity of *ras* to induce metastatic behavior. Egan et al (1989) reported that while 25% of the cell lines produced following the introduction of *ras* and *myc* oncogenes into the murine fibroblast cell line 10T½ in the presence of fetal calf serum were both tumorigenic and metastatic, cell lines produced following the introduction of the same oncogenes in the presence of dialyzed calf serum were tumorigenic but not metastatic. Thus, the introduction of a *ras* oncogene *in vitro* can drive the progression of recipient cells at many different stages, depending upon the genetic and environmental context of that cell.

9) *Ras* Oncogenes and Multistep Tumorigenesis *In Vivo*

While *in vitro* studies demonstrated that introduction and expression

of exogenous *ras* oncogenes led to the transformation of recipient cells, further evidence that *ras* has a causative role in tumorigenesis has been provided by experiments designed to more closely approximate the physiological conditions under which tumor formation would normally occur. One method used to simulate *in vivo* tumorigenesis was the *in vitro* introduction of *ras* oncogenes into primary cells followed by their re-transplantation into mice. By this method *ras* oncogenes were introduced into mouse skin keratinocytes (Roop et al., 1986), cells of the urogenital sinus which in male mice develop into the prostate gland (Thompson et al., 1989) and mouse mammary epithelial cells (Strange et al., 1989; Miyamoto et al., 1990). In each case, the grafted cells exhibited displastic growth resembling premalignant lesions. When the *myc* oncogene was introduced together with *ras* into the mouse urogenital sinus, rapidly growing carcinomas were observed in the developing organ, demonstrating that *ras*-induced neoplastic transformation *in vivo* required the action of a cooperating oncogene (Thompson et al., 1989). In addition, the tumors which arose were clonal in origin and exhibited chromosomal abnormalities, suggesting that additional genetic changes besides *myc* and *ras* were required for tumor formation. Another approach which was used to examine the ability of *ras* oncogenes to induce tumors *in vivo* was infection with replication-defective retroviruses containing *ras* oncogenes. v-H-*ras* infection of mouse skin epidermal cells followed by TPA treatment induced benign papillomas (Brown et al., 1986b). Some of the papillomas subsequently progressed to invasive carcinomas which expressed v-H-*ras* p21 and were clonal in origin. Twenty percent of the mice derived from midgestation mouse embryos infected with a defective retrovirus containing

the v-H-*ras* oncogene developed tumors of the skin with a latency of 4-8 weeks (Compere et al., 1989). In contrast, infection of embryos with a *ras/myc* double oncogene virus resulted in 27% of the animals developing rapidly growing tumors in a variety of tissues after a latency of 2-3 weeks. Thus *myc* cooperated with *ras* both to increase the frequency of tumor induction and to broaden the range of cell types which were targets for transformation. The tumors which developed were clonal in origin, again suggesting that *myc* and *ras* were not sufficient for malignant transformation *in vivo*.

A less invasive technique used to assess the carcinogenic capacity of *ras* oncogenes *in vivo* is transgenic mouse technology, which has been frequently employed to introduce *ras* oncogenes into the germ line of mice. Introduction of either v-H-*ras* (Sinn et al., 1987; Sandgren et al., 1989; Leder et al., 1990), c-H-*ras* (Quaife et al., 1987; Efrat et al., 1990; Bailleul et al., 1990) or N-*ras* (Mangues et al., 1990) oncogenes linked to a variety of promoters have each induced disturbances in cell growth in an organ specific manner consistent with the tissue-specificity of the promoter. When expression of the transgenic H-*ras* oncogene was directed to mouse skin, benign papillomas developed on the skin surface at areas of epidermal abrasion (Bailleul et al., 1990; Leder et al., 1990). In one study, papillomas were also induced by TPA treatment and some of these progressed to squamous cell carcinomas or sarcomas (Leder et al., 1990). v-H-*ras* and N-*ras* transgenes under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer induced difuse hyperplasia of the Hardarian gland as well as the focal development of malignancies of mammary and salivary tissue (Sinn et al., 1987; Mangues et al., 1990). Mating MMTV/v-

H-*ras* transgenic mice to MMTV/c-*myc* transgenics resulted in an accelerated formation of tumors, but these tumors were still clonal in nature (Sinn et al., 1987). Transgenic mice expressing low levels of the c-H-*ras* oncogene under the control of the albumin enhancer/promoter exhibited mild hepatic displasia and occasionally developed focal liver nodules between 4 and 5 months of age (Sandgren et al., 1989). Transgenic mice expressing c-*myc* or SV40 virus small and large T antigens linked to the same promoter also exhibited hepatic dysplasia and developed focal liver tumors, and mating these mice to the c-H-*ras* transgenics accelerated the tumor development. All of these studies with transgenic mice suggested that the *ras* oncogene contributed to tumorigenesis *in vivo* but was not sufficient for malignant conversion. However, one study showed that in transgenic mice expressing the c-H-*ras* oncogene under the control of the rat elastase 1 promoter massive tumors involving all the pancreatic acinar cells developed within a few days of pancreatic differentiation (Quaife et al., 1987). This result suggested that a *ras* oncogene alone was sufficient to induce pancreatic neoplasia. The reason for this apparent contradiction to a multistep process of tumor development is not known, but the results may be related to the particular cell type in which the *ras* oncogene was being expressed, as well as its stage of differentiation and pattern of gene expression. Alternatively, it may be related to the particular gene construct used to produce the transgenic mice. Another factor which may predispose to single step, *ras*-induced transformation and which is a limitation in all of the transgeic mouse studies, is the fact that the *ras* oncogene is expressed in all cells of the targeted organ. This may simulate the removal of surrounding normal cells and, combined with the

high levels of *ras* expression driven by artificial (for *ras*) promoters, the conditions may resemble those shown to permit *ras* transformation of primary cells *in vitro*. In spite of these limitations, the *in vitro* and *in vivo* studies have shown that introduction of a *ras* oncogene into many cell types promoted their conversion to tumor cells and provided strong evidence that the activation of a *ras* oncogene can contribute to the multistep process of tumor development.

10) *Ras* Oncogene Activation in Spontaneous and Chemically-Induced Carcinogenesis

Evidence that mutation of endogenous *ras* genes contibutes to tumor development has been provided by numerous studies of carcinogen-induced tumors in animals. Mutationally activated *ras* oncogenes have been frequently detected in a variety of carcinogen-induced rodent tumors (reviewed in Sukumar, 1990). A number of different chemicals have been utilyzed in these studies, and the nature of the *ras* mutations observed in the different model systems reflects the chemical specificity of the carcinogen, implying that a direct interaction between the carcinogen and the *ras* gene was responsible for the observed mutation. It then follows that the *ras* mutation likely had a causative role in the carcinogen-induced tumor development. In several studies, *ras* mutation has been shown to be an early event in chemically-induced carcinogenesis. Mutationally activated H-*ras* and K-*ras* oncogenes were detected in rat mammary glands 2 weeks after s.c. injection of nitrosomethylurea, preceding the onset of preneoplasia or cancerous growth (Kumar et al., 1990). Although the rats were treated with carcinogen at 2 days of age, tumors did not develop

until puberty and occurred very rarely in ovarectomized animals, implying that physiological processes associated with sexual maturation were required to cooperate with *ras* in the tumorigenic process. A role for estrogen as a tumor promoter was suggested, since injection of estrogen into ovarectomized animals resulted in tumor formation. Activated *ras* oncogenes have also been detected in carcinogen-induced premalignant lesions in mouse skin (Quintanilla et al., 1986; Bonham et al., 1989; Brown et al., 1990), liver (Buchman et al., 1989; Dragani et al., 1991) and lung (You et al., 1989). There is also evidence suggesting that mutational activation of *ras* can contribute to the later stages of tumor progression in carcinogen-induced tumors. While *ras* mutations were heterozygous in dimethylbenzanthracene-induced, TPA-promoted mouse skin papillomas, the mutation was homozygous or amplified in emergent carcinomas (Quintanilla et al., 1986). Bremner and Balmain (1990) similarly observed that loss of heterozygosity on mouse chromosome 7, which contains the H-*ras* gene, occurred frequently in those skin carcinomas which also contained H-*ras* oncogenes. Activated H-*ras* oncogenes were also detected in dimethylbenzanthracene-induced mammary tumors derived from preneoplastic hyperplastic outgrowth lines lacking *ras* mutations (Cardiff et al., 1988).

In addition to carcinogen-induced tumors, activating *ras* mutations have been detected in spontaneously occurring animal tumors. 91% of spontaneous lung tumors in strain A mice, which exhibit a high incidence of these neoplasms, contained an activated K-*ras* oncogene (You et al., 1989). In another strain of mice which develops frequent spontaneously occurring tumors, the C57BL/6 X C3H F1, 66% of spontaneous liver tumors

contained activated H-*ras* genes (reviewed in Candrian et al., 1991). However, an examination of 49 spontaneous non-liver tumors arising in this same strain of mice revealed only 5 containing activated *ras* oncogenes, suggesting either a differential susceptibilty to *ras* mutations in different tissues, or a differential ability of *ras* mutations to induce tumors in different cell types (Candrian et al., 1991).

11) Importance of *Ras* Oncogenes in Human Cancer

Activation of *ras* oncogenes has been implicated in the etiology of human cancer based on the high frequency with which mutations in one of the three *ras* genes have been detected in various tumor types (reviewed in Bos, 1989). Amplification of the K-*ras* proto-oncogene has also been reported (Pulciani et al., 1985). The incidence of *ras* mutations varies among different tumor types, with the highest frequency of activating mutations detected among adenocarcinomas of the exocrine pancreas. An activated K-*ras* oncogene has been detected in about 90% of these tumors (Almoguera et al., 1988; Smit et al., 1988; Grunewald et al., 1989). Activated *ras* genes have also been detected at a relatively high frequency (30-60% of tumors examined) among several other tumor types, including adenocarcinomas of the lung (Rodenhuis et al., 1988) and colon (Bos et al., 1987; Forrester et al., 1987; Vogelstein et al., 1988), acute myeloid leukemia (reviewed in Bos, 1989) and follicular and undifferentiated carcinomas of the thyroid (Lemoine et al., 1989). In several tumor types a *ras* oncogene has been detected only occasionally, while in others no mutated *ras* genes have been reported (Bos, 1989).

There is also evidence suggesting that mutational activation of *ras*

oncogenes may frequently be an early event in tumor development in humans. In the study of human colorectal tumorigenesis, activated K-*ras* genes have been detected in approximately 50% of carcinomas (Bos et al., 1987; Forrester et al., 1987) and in a similar percentage of premalignant adenomas greater than 1 cm in size (Vogelstein et al., 1988). Since the analysis of adenomatous and carcinomatous regions of individual tumors showed that the latter tissue was derived from, and not simply adjacent to the former, the results indicated that activation of *ras* preceded malignant conversion (Bos et al., 1987; Vogelstein et al., 1988). Activated *ras* oncogenes have also been detected in other premalignant lesions including preleukemias (reviewed in Bos, 1989), benign (micro-) follicular thyroid adenomas which are considered to progress at low frequency to follicular carcinomas (Lemoine et al., 1989) and the dysplastic nevi thought to be precursors for melanomas (Shukla et al., 1989).

Additionally, *ras* oncogenes have been implicated in malignant progression in human tumors. Although *ras* mutations are frequently detected in both premalignant colon adenomas and the carcinomas thought to arise from them, in some cases the mutations are only observed in the carcinomas, indicating that the timing of the mutation is not invariant and that *ras* oncogene expression will have different biological effects depending upon what other genetic changes have taken place in the target cell (Bos, 1989). A correlation was observed between the presence of activated *ras* oncogenes in lung adenocarcinomas of smokers and the malignancy of the tumors (Reynolds et al., 1991). 8 of 10 adenocarcinomas which had metastasized at the time of surgery contained activated *ras*

genes compared to 5 of 18 tumors which had not metastasized. *Ras* activation was also implicated in the progression of chronic myelogenous leukemia by a study in which *ras* genes with activating mutations were detected at a higher frequency during blast crisis than during the chronic phase of the disease (Liu et al., 1988).

In summary, *in vitro* and *in vivo* studies have provided strong evidence that the *ras* oncogene can have a causative role in the multistep process of tumor development. *Ras* appears able to act at many different stages of tumorigenesis and the biological consequences of activating mutations in *ras* are dependent upon the genetic charactaristics of the cell in which the oncogene is expressed as well as the environment of the cell.

(E) Raison d'etre For Multistep Tumorigenesis

The requirement for multiple alterations presumably reflects the existence of multiple barriers to cell growth which must be overcome during the course of tumorigenesis. Neoplastic transformation *in vitro* appears to involve alterations in both the inherent growth characteristics of cells and in their response to the surrounding environment. Cell transformation is associated with such traits as continuous cell cycle progression (proliferation) and an infinite replication capacity (immortalization) and conversely a loss of differentiation potential (Hunter, 1991). While *in vitro* cell transformation, for the main part, involves the breach of intracellular growth control, potential suppressive effects of the environment are also evident. As discussed earlier, surrounding normal cells appear to exert an inhibitory effect on *ras*

transformation of primary rat embryo fibroblasts *in vitro*, and this suppression could be overcome by the effects of tumor promoters or cooperating oncogenes. Thus, stimulation of cell proliferation, and liberation from the suppressive effects of surrounding normal cells appear to be independent requirements for tumorigenesis *in vitro*.

A similar inhibitory effect of normal cells on the growth of *ras*-transformed cells *in vivo* was observed in studies by Dotto and coworkers (Dotto et al., 1988). While *ras* transformed mouse skin keratinocytes implanted alone onto the back of a mouse produced rapidly growing squamous carcinomas, reimplantation of the same cells together with a four-fold excess of normal dermal fibroblasts resulted in the appearance of only small, non-progressing nodules. Tumor promoters appear to have a similar ability to allow *ras*-expressing cells to overcome the inhibitory effects of surrounding cells *in vivo*, since TPA treatment led to the appearance of macroscopic papillomas in mouse skin initiated by infection with a defective retrovirus carrying the *ras* oncogene (Brown et al., 1986b). In addition to the influences of surrounding normal cells, the multistep process which leads to the development of a fully malignant, metastatic tumor *in vivo* will be subject to numerous other factors which act to restrict cell expansion. The microenvironment in which the tumor develops will be much more complex than that encountered *in vitro* (Nicolson, 1987). Cells will be incorporated into the three dimensional structure of an organ, which will in itself impart additional constraints on cell growth. Also, instead of the homogeneous cell populations encountered *in vitro*, the *in vivo* microenvironment will include multiple cell types, each of which may secrete extracellular matrix components and/or soluble mediators

which can effect cell growth. Tumor growth *in vivo* will also be subject to the limits of oxygen and nutrient diffusion and will thus additionally require the formation of new blood vessels in order to expand in three dimensions (reviewed in Liotta et al., 1991). In order for the tumor to extend its growth beyond the limits of the organ in which it originates and invade adjacent tissues, the cells must acquire the abilities which allow it to penetrate basement membranes, including proteolytic activities and motility. The process of metastasis, which is generally defined as the endpoint in the multistep tumorigenic process, involves a cascade of linked, sequential steps culminating in the outgrowth of a secondary tumor at a site distant from the primary neoplasm (Fidler and Hart, 1982; Liotta et al., 1983). To accomplish this, the tumor cell must be able to leave the primary tumor, invade the local host tissue, enter the circulation, arrest at the distant vascular bed, extravasate into the target organ interstitium and proliferate at the new site. Each step in the metastatic cascade will require the tumor cell to have specific properties. At all stages of tumor development, from the first appearance of a single "initiated" cell in an otherwise normal tissue to the metastatic spread of a fully malignant tumor, cells will be exposed to humoral and cellular components of the immune system, which may perform a surveillance function (see below).

(F) Immune Surveillance and Antitumor Natural Resistance

A fundamental tenet underlying the function of the immune system is the discrimination of self from non-self. Thus, under normal, non-pathologic conditions an immune response is not produced against self

tissues. Since numerous phenotypic changes occur during the conversion of a normal cell to a fully malignant tumor cell, it is possible that at some point the cell will express characteristics recognized as non-self and will become the target of an immune response. This is the basis of the hypothesis of immune surveillance which proposes that one function of the immune system is to recognize and eliminate neoplastic cells and thus maintain the integrity of self (Burnet, 1971). The term surveillance implies that this defense mechanism acts on altered cells early in the process of tumor development, before the appearance of a clinically detectable tumor.

Many different components of the immune system have been put forward as effectors in the proposed antitumor surveillance. The classical T cell-dependent immune response was originally proposed as the major mechanism responsible for defense against neoplasia. Histological studies have provided findings consistent with such a role for immune lymphocytes (Ioachim, 1976). Infiltrates of lymphoid cells were present at the tumor site in most neoplasms, predominantly and sometimes exclusively in the early stages of tumor growth. In some neoplasms, the lymphoid infiltrates were associated with tumor regression. Support for the hypothesis that a functional immune system plays a role in the prevention of malignancies in humans has come from epidemiological surveys of individuals with naturally occurring or induced immunodepression (Herberman, 1984; Penn, 1989). Such studies have revealed that patients whose immune system has been therapeutically suppressed to prevent rejection of organ grafts or unintentionally suppressed as a side effect of cancer chemotherapy or radiotherapy, as well as individuals with primary or acquired

immunodeficiency disorders all exhibit an increased incidence of cancer compared to the general population. The types of malignancies for which an increased frequency has been observed in association with immune suppression include some cancers which are relatively rare in the general population. In particular, the frequencies of non-Hodgkin's lymphoma and Kaposi's sarcomas were markedly increased in association with each of the immune depressed states mentioned above with the exception of primary immunodeficiency disorders, for which no increase in Kaposi's sarcomas was observed (Penn, 1989). In addition, skin and lip cancers were increased in organ transplant recipients living in areas with high exposure to sunlight, while leukemias showed an increased incidence in patients with primary immunodeficiency disorders. Although other factors besides the immune suppression likely contribute to the observed increased incidence of cancer, including direct carcinogenic effects of the immunosuppressive treatments and a possible genetic susceptibility to cancer among individuals with primary immune disorders, the results are consistent with a role for the immune system in tumor defense.

However, in the 1970's criticisms of the immune surveillance hypothesis were raised by a number of investigators (Moller and Moller, 1976; Rygaard and Povlsen, 1976). Among the main criticisms was the suggestion that if the immune system was responsible for tumor surveillance, an increase in the frequency of all cancers, rather than only certain tumors, should be observed in immmunodepressed patients. In addition to the failure to see a generalized increase in cancer in immune depressed humans, these criticisms were also prompted by observations in mice. Although immune suppressed mice were more susceptible to polyoma

virus-induced tumors, athymic (nude), T cell-deficient mice did not exhibit an increased rate of spontaneous tumor development. However, Ioachim (1976) countered that an increased rate of spontaneous tumors was not observed in nude mice because the animals had a high susceptibility to various infections and consequently had too short a lifespan to observe the outcome of the relatively prolonged process of tumor development. A similar argument was used to account for the absence of a generalized increase in the incidence of all tumors in immune deficient humans, since the lifespan of these patients was similarly shortened due to the occurence of infections and certain cancers. Consistent with this hypothesis, nude (nu/nu) mice raised under germ-free conditions had a high incidence of lymphoreticular tumors which was not observed in their non-immunodeficient (nu/+) littermates, and these tumors occurred on average at an age not attained by nude mice raised under conventional conditions (Outzen et al., 1975). However, very few spontaneous tumors of other types were observed in germ-free nu/nu or nu/+ mice, and there were no differences in the incidence or latent period of sarcoma formation following s.c. implantation of 3-methylcholanthrene pellets into either type of mouse.

The suitability of a classical T cell-mediated immune response to a tumor surveillance function has also been questioned on the basis that, in order for T cells to be effective in this role, a prior exposure to antigen would be required (Moller and Moller, 1976). A primary T cell response would likely not be effective in surveillance against tumors since: antigen levels would have to surpass a certain threshold for immunization; the antigens would need to be presented on specialized

antigen presenting cells; and there would be a lag time between the initiation of the response and the proliferation and recruitment of effectors to the site of action. These factors would dictate that a substantial tumor load would accumulate before immune defense mechanisms would begin to act against the aberrant cells, which is contrary to the principle of surveillance.

The apparent inability of the T cell-dependent immune response to satisfy all of the predictions of the tumor surveillance hypothesis led to the examination of other aspects of the immune system as possible participants in tumor defense. Greenberg and Green (1976) provided evidence which suggested the existence of an innate resistance to tumor growth which was thymus-independent. Small numbers of murine tumor cells, which grew well *in vitro* or when injected intraperitoneally into syngeneic recipients, were frequently rejected when transplated s.c., and the same rate of rejection was observed in mice which had been thymectomized, lethally irradiated and bone marrow reconstituted. The tumor elimination process appeared to be rapid, since no palpable tumor mass was detectable in animals rejecting the tumor, even though theoretically only a very short time would be required for the tumor to obtain such a size (based on *in vitro* growth rates). Further, no memory was involved, since mice which had rejected a small tumor inoculum were no more resistant to a subsequent challenge with a large tumor dose than were naive recipients. The rejection mechanism appeared to involve some specificity, since the simultaneous i.v. injection of syngeneic tumor membrane extracts enhanced the tumor frequency of small s.c. inocula. The rapid rejection of tumor cells observed in this study was consistent with the existence of a first

line of defense which could act before an adaptive immune response could be generated. Such a mechanism would overcome the criticisms of the tumor surveillance hypothesis based on the lag time required for the generation of an adaptive response. Accordingly, the identification of the mechanisms responsible for this thymus-independent, antitumor "natural resistance" has subsequently become the focus of much research aimed at the investigation of the tumor surveillance hypothesis.

The three effector candidates of antitumor natural resistance which have received the most attention are natural killer (NK) cells, macrophages and natural antibodies (NAb). Each of these have been classified as natural immune mediators based on the fact that expression of their immune activity does not require prior sensitization with antigen. Since each of these mediators has been shown to exhibit reactivity to tumor cells to which they have not been previously exposed, they would appear to be ideal candidates for participants in a rapid first line of defense against incipient neoplasia. Numerous studies have been carried out aimed at investigating the involvement of each of these effectors in tumor defense.

1) Macrophages

Macrophages have many biological properties which make them candidates to function in tumor surveillance (reviewed in Hibbs et al., 1972; Hibbs et al., 1978; Adams and Snyderman, 1979). Macrophages are the differentiated tissue phase of a system of long-lived mononuclear phagocytes which includes the less mature circulating blood monocytes as well as the promonocytes and their precursors in the bone marrow.

Macrophages are widely distributed in the body as the mobile components of all normal connective tissue. Their ubiquity and motility give macrophages the potential to monitor and respond to subtle changes in the local environment of all tissues. In addition, the number of macrophages present in a given tissue can be increased by recruitment from a dividing pool of precursor cells. Most importantly, activated macrophages have been shown to display a potent cytolytic activity against a variety of tumor cells *in vitro*, while being concomitantly unreactive against normal cells.

In order to acquire cytolytic activity against tumor cells, macrophages must be activated either *in vivo* or *in vitro*. Analysis of macrophage activation *in vitro* has demonstrated the requirement for at least two stimuli in this process (Meltzer, 1981; Adams and Hamilton, 1984; Chen et al., 1987). The first of these stimuli is provided by one of several T cell derived lymphokines including interferon-γ, while the second can be supplied by a range of agents including heat-killed gram positive bacteria, bacterial lipopolysaccharide (LPS) or a number of lymphokines. It is possible to obtain murine macrophages at distinct stages of activation by *in vivo* stimulation with different agents (Johnson et al., 1983). Sterile inflammation induced by agents such as starch or thioglycollate broth produced "responsive" macrophages, whose cytolytic activity could be stimulated by exposure to lymphokines and LPS; mice treated with pyran copolymer yielded "primed" macrophages which became directly cytolytic when treated with LPS; while injection of heat-killed or viable bacteria produced macrophages which were fully activated for tumor cytolysis. Although the requirement for *in vitro* or *in vivo* activation for optimal cytolytic activity might appear to limit the

potential for macrophages to perform a tumor surveillance role, the activating factors are not antigen specific and macrophages with spontaneously activated tumor lytic ability have been isolated from normal animals, including nude mice (Keller, 1978; Pels and Otter, 1979). Further, resident macrophages were able to lyse neoplastic targets to a considerable degree if previously cultured with those or other tumor cells for 2 days (Adams and Snyderman, 1979). One study which examined the selective lysis of tumor cells demonstrated that activated macrophages, while inactive against normal mouse embryo fibroblasts, were cytolytic against the same cells following their spontaneous transformation *in vitro* (Hibbs et al., 1972). This result suggested that macrophages may be effective against transformed cells early in the process of tumor development.

In addition to their direct cytolytic activity, macrophages have been shown to interact with tumor cells in other ways. Murine macrophages, elicited following the injection of sterile starch, have been shown to induce tumor cell cytostasis *in vitro*, inhibiting DNA synthesis without detectable tumor cell lysis (Mitani et al., 1989). Macrophages can also interact with antibody-bound tumor cells and participate in antibody-dependent cell-mediated cytolysis (ADCC), which can occur by two apparently distinct mechanisms (reviewed in Adams et al., 1984). A rapid form of ADCC, which occurs over 5-6 hours, was dependent upon polyclonal antibodies while a slower process, requiring 24-72 hours for completion, was mediated by IgG2a monoclonal antibodies. It has been shown that the slower form of ADCC by murine macrophages could also be mediated by monoclonal antibodies of the IgG1, IgG2b and IgG3 isotypes (Johnson et

al., 1986). The various tumor-lytic activities appear to be mediated by macrophages at a different stages of activation, since responsive or primed macrophages were effective in mediating the slow form of ADCC, while macrophages fully activated for direct cytolysis by administration of Bacillus Calmette-Guerin or *Propionibacterium acnes in vivo* or by lymphokines plus LPS *in vitro* had a diminished capacity for this form of ADCC (Johnson et al., 1986). In contrast, the rapid ADCC interaction was mediated most efficiently by macrophages activated for direct cell killing. Although the normal assessment of ADCC has been through the measurement of tumor cell cytolysis, macrophages have also been shown to kill antibody-bound tumor cells by phagocytosis (Munn et al., 1991). Thus macrophages have the ability to inhibit tumor growth by a variety of mechanisms which suggests that this may be a normal function of these cells *in vivo*.

2) Macrophages in Antitumor Defense

Evidence consistent with the hypothesis that macrophages do play an important role in defense against tumors has been provided by a variety of studies. Systemic administration of agents known to stimulate macrophage activation, including Bacillus Calmette-Guerin and *Corynebacterium parvum*, were shown to delay the appearance and reduce the incidence of spontaneous and chemically-induced tumors in mice (reviewed in Adams and Snyderman, 1979). An increased latency and decreased frequency of U.V.-induced skin tumors was observed in mice treated i.p. with pyran copolymer (Norbury and Kripke, 1979). The pyran-treated mice were also more resistant to the growth of a transplanted syngeneic tumor, and a similar increased

resistance to small s.c. tumor inocula was observed in mice pretreated i.p. with *mycobacterium butyricum* or proteose peptone (Chow et al., 1979). The induction of enhanced resistance to transplanted syngeneic tumors by reticuloendothelial activators was also observed in rats (reviewed in Keller, 1990). Conversely, treatment of rodents with agents which can depress the function of macrophages, such as silica particles or carrageenan, reduced their ability to reject transplanted tumors (Keller, 1976; Chow et al., 1979), and abrogated the tumor protective effect triggered by micro-organisms (Keller et al., 1990). Silica treatment was also shown to reduce the latency of U.V.-induced skin cancer in mice (Norbury and Kripke, 1979). Another approach aimed at assessing the effect of macrophages on tumor growth *in vivo* was utilized by Keller (1976), who demonstrated that simultaneous injection of peptone-induced macrophages, even at a site away from that of the tumor inoculum, reduced the rate of tumor growth following s.c. injection of a syngeneic tumor. A more pronounced reduction in tumor growth was observed when the tumor cells were mixed with macrophages before injection. Finally, consistent with escape from macrophage-mediated lysis being an important factor in determining the ability of tumor to grow, macrophage-resistant variants have been obtained following the growth of syngeneic tumors in mice (Urban and Schreiber, 1983; Urban et al., 1986). The U.V.-induced fibrosarcomas utilized in these studies were immunogenic, and it was observed that the cells also lost tumor-specific antigens defined by cytolytic T cells. The reductions in sensitivities to macrophages and T cells occurred in a sequential manner, with macrophage-resistance occurring first. The earlier appearance of macrophage-resistance is consistent with macrophages having

a role in the first line of tumor defense. In addition, macrophage-resistant tumor cells which retained the antigens recognized by cytotoxic T cells grew faster than their macrophage-sensitive counterparts during the first 10 days after tumor implantation.

In addition to their direct and antibody-dependent effects on tumor cells, macrophages may also have a role in regulating the activity of other natural immune mediators. Interferon-γ and prostaglandin-E2, which respectively stimulate or inhibit the cytotoxic activity of NK cells, are produced by apparently distinct subsets of macrophages (Nelson et al., 1990). Interestingly, the increased tumor-resistance induced by injection of *C. parvum* or *L. monocytogenes* into rats could be abrogated by injection of monoclonal antibodies reactive against rat interferon-γ (Keller et al., 1990). It is not known whether the antibodies were blocking activation of macrophages, NK cells or both. Macrophages have also been implicated in the regulation of natural antibodies, since the level of syngeneic tumor-reactive NAb was shown to increase following the i.p. injection of *Mycobacterium butyricium* or proteose peptone and to decrease following the injection of silica (Greenberg et al., 1980). Thus macrophages appear to be important participants in antitumor natural resistance.

3) NK Cells

NK cells are a discrete subpopulation of large granular lymphocytes which are non-adherent, non-phagocytic and have been shown to have spontaneous cytolytic activity against a variety of tumor cell lines and primary tumors, as well as against virus-infected cells and some normal cells (Herberman, 1984). Although no surface antigens have yet been

described which are unique to human NK cells, these cells have been shown to express a unique combination of antigens, each of which are shared by other cell types (Trinchieri, 1989). A series of alloantigens designated NK-1, NK-2 and NK-3 appear to be specifically expressed on murine NK cells. The cytolytic activity expressed by NK cells is non-MHC-restricted, and they can lyse both allogeneic and syngeneic tumor cells. The basis of the ability of NK cells to lyse a variety of tumor cells in an MHC-unrestricted manner is not known since, with the exception of the Fcγ receptor used in ADCC (see below), there is no definitive information on the type of receptor used by NK cells for target cell recognition and killing. NK cells do not rearrange T cell receptor genes and do not express surface T cell receptor molecules (Trinchieri, 1989). Molecules which have properties consistent with antigen receptors have been identified on rat (Giorda et al., 1990) and human (Harris et al., 1991) NK cells. A murine monoclonal antibody precipitated a molecule of 60 KD from rat NK cells which, under reducing conditions, migrated on SDS-PAGE gels as a single 30 KD band. This molecule, designated NKR-P1, was highly expressed on rat NK cells and was capable of mediating signal transduction. The gene encoding NKR-P1 was cloned and the predicted protein sequence exhibited significant homology to C-type animal lectins. The message for the gene was highly expressed in NK cells and a tumor cell line with NK characteristics, but was absent in T cells and other cell lines of rat origin that do not express NK activity. Recently, 3 closely related but distinct genes homologous to NKR-P1 were found to be expressed in murine NK cells (Giorda and Trucco, 1991). The putative receptor identified on human NK cells is a heterodimer consisting of subunits of 43

and 38 KD which are distinct from the T cell receptor. The antigenic determinants expressed by this structure are evolutionarily conserved, since monoclonal antibodies directed against the putative human NK receptor bound to the equivalent of NK cells in teleost fish (called nonspecific cytotoxic cells). The antibody could activate NK cells for cytolysis and lymphokine secretion, and also inhibited NK lysis of tumor cells by blocking conjugate formation, but did not inhibit ADCC. Recently, another candidate NK receptor was reported in a review of the VIIth International Workshop on Natural Killer Cells (Karre et al., 1991). A 150 KD glycoprotein designated NK-R1 was described (J. Ortaldo, J. Roder, S. Anderson) which was expressed on virtually all $CD3^-$ large granular lymphocytes and on very few cytotoxic T lymphocytes. The gene encoding this protein maps to human chromosome 3 in the region 3p21-3p42, and the majority of patients with von Hippel Lindau Syndrome, which have a deletion in this region of chromosome 3, had reduced NK activity. Further, high expression of an antisense construct of this gene abrogated the NK-like activity of a T cell clone.

NK cells are thymus-independent, since high levels of activity are detectable in nude mice and in neonatally thymectomized mice and rats (reviewed in Herberman and Ortaldo, 1981). Mouse NK activity is under polygenic control, and high responsiveness is usually dominant over low responsiveness (reviewed in Trinchieri, 1989). Crosses between two mouse strains with low NK activity can yield mice with high or intermediate NK activity, demonstrating that the low NK phenotype can have different genetic bases. Mice exhibit peak NK activity between 6-10 weeks of age which afterwards continually declines in most strains (Kiessling et al.,

1975). A particularly useful murine model for investigating the importance of NK cells *in vivo* is the beige mouse. Beige mice have strongly depressed NK activity, while other B and T lymphocyte functions are almost normal (Roder, 1979; Roder and Duwe, 1979; Roder et al., 1979). A similar deficiency in NK cell activity is associated with Chediak-Higashi syndrome, a rare autosomal recessive disease in humans (Roder et al., 1980).

Resting NK cells are functionally active and can lyse susceptible tumor target cells without prior exposure to that cell. The cytolytic activity of NK cells is also rapidly enhanced by exposure to cytokines such as interferon and interleukin-2 (Trinchieri and Santoli, 1978; Trinchieri et al., 1978; Henney et al., 1981). The enhancement of cytotoxic activity is demonstrable after 3-6 hours of incubation and does not require cell proliferation (Trinchieri et al., 1984). A variety of agents have been shown to increase NK activity *in vivo* when injected into mice, including the interferon inducer polyinosinic polycytidylic acid (Djeu et al., 1979), various bacterial strains and viruses (Wolfe et al., 1976; Herberman et al., 1977; Kearns and Leu, 1984), pyran copolymer (Santoni et al., 1979), proteose peptone and LPS (Chow et al., 1981b). NK cells are also capable of secreting several lymphokines, including interferon-γ and B cell growth factors, suggesting that they have the potential to regulate other mediators of natural resistance (Trinchieri, 1989). In addition to their direct cytolytic effects, both murine and human NK cells express receptors for the Fc portion of IgG and can mediate ADCC against tumor cells (Trinchieri, 1989).

4) NK Cells in Antitumor Defense

Their spontaneous tumor-lytic activity makes NK cells ideal candidates for effectors of a first line of resistance against neoplasia and a substantial body of evidence supports the hypothesis that one role of NK cells is in antitumor natural resistance. A frequently utilized approach has been to look for correlations between resistance to the growth of transplanted tumor cell lines and the levels of NK activity in the recipient animal (reviewed in Herberman and Ortaldo, 1981). In a variety of situations, transplanted tumors have been shown to grow less well in mice with high NK activity than in those with low NK activity. For example, there was an increased frequency and decreased latency of tumor formation following s.c. implantation of syngeneic tumors into low NK beige (bg/bg) mice compared to their heterozygous (bg/+) littermates (Karre et al., 1980). Talmadge et al (1980) demonstrated a similarly reduced latency following injection of an NK-sensitive, but not an NK-resistant tumor cell line into beige mice. More recently, it was shown that the formation of tumors following the injection of NK-sensitive pre-malignant B cell lines into mice correlated inversely with the level of host NK activity (Felsher et al. 1990). While *in vivo* elimination of T helper cells did not alter the tumorigenicity of the cell lines, depletion of NK cells with anti-asialo-GM1 strongly diminished host resistance to tumor growth. Conversely, treatment of the mice with the interferon inducer polyinosinic-polycytidylic acid enhanced host resistance. This result suggested that NK cells may be important in the elimination of cells at an early stage of tumor development. Further, tumors which formed following injection of a pre-malignant B cell line into mice which had not

been depleted of NK activity were >20 fold more resistant to NK lysis than the parental line. A similar result was obtained by Brown and Chow (1985) who reported that tumors arising in syngeneic mice following the s.c. injection of low numbers of an NK-sensitive lymphoma had reduced sensitivity to NK lysis compared to the parental line. The *in vivo*-passaged tumors, which were also reduced in sensitivity to natural antibodies, were less sensitive to natural resistance *in vivo* than the parental line, as assessed by their reduced rate of clearance following i.p. injection into syngeneic mice. These results suggested that escape from NK-mediated defense was necessary for tumor survival, consistent with NK cells performing a tumor surveillance role.

In addition to the evidence that NK cells have a role in defense against transplanted tumors, there is also support for the hypothesis that NK cells are important in mediating resistance to chemically-induced or spontaneous tumors. Altmann et al (1990) studied the initial phase of 1,2-dimethylhydrazine (DMH)-induced carcinogenesis in the mouse, which is manifested as a hyperplasia of the duodenal crypt. Complete ablation of splenic NK activity by treatment of the mice with anti-asialo GM1 enhanced the hyperplasia while treatment of mice with polyinosinic-polycytidylic acid caused regression of the DMH-induced hyperplasia. Also, there have been reports of an increased incidence of lymphomas in beige mice (Loutit et al., 1980), and of lymphoproliferative disorders in patients with Chediak-Higashi syndrome (Dent et al, 1966). Mice treated with γ-irradiation showed both a severe depression of NK activity and a high incidence of thymic leukemia (Warner and Dennert, 1982). Reconstitution of the mice with an NK cell line immediatedly after cessation of γ-

irradiation markedly reduced the incidence of thymic leukemia.

Another approach aimed at investigating the involvement of NK cells in tumor surveillance has been to examine the ability of NK cells to react against tumor cells which had been transformed *in vitro* by oncogenes. The rationale behind these experiments was that if NK cells are to play a role in surveillance, host cells should become susceptible to NK-mediated cytotoxicity during transformation to the malignant phenotype. Consistent with this hypothesis, an increased sensitivity to NK cell lysis *in vitro* has been demonstrated following constitutive or conditional *ras* oncogene expression in the murine fibroblast cell line 10T½ (Trimble et al., 1986; Greenberg et al., 1987a), and following constitutive expression of the same oncogene in the rat fibroblast cell line rat-1 (Johnson et al., 1985). However, Anderson et al (1989), who utilized a 10T½ clone containing an inducible *ras* oncogene, suggested that increased NK sensitivity was not associated with increased steady-state levels of oncogenic p21, but rather was associated with the time during which the levels of p21 were either increasing or decreasing in the cell. Further, neither *src* transformation of 10T½ (Anderson et al., 1989) nor *ras* transformation of NIH 3T3 cells (Greenberg et al., 1987a) produced increased sensitivity to NK cell lysis. Thus, the potential for NK cells to recognize cell changes associated with oncogene-induced transformation is still somewhat uncertain.

In addition to the evidence supporting a role for NK cells in the defense against primary tumors, there is strong evidence in favor of NK cells participating in the prevention of the metastatic spread of tumor cells. A variety of studies have shown that reduced NK activity was

associated with increased metastasis. For example, it has been shown that NK-sensitive, but not NK-resistant tumors displayed enhanced rates of spontaneous metastasis following s.c. injection into beige (bg/bg) mice compared to that observed in bg/+ mice (Talmadge et al., 1980). Depletion of NK cells in mice with specific antisera or with cytotoxic drugs has also been shown to increase the rate of experimental metastasis produced following i.v. injection of NK-sensitive tumor cells (reviewed in Smithson et al., 1991). Futher, the decreased resistance to metastasis in the mice depleted of NK cells by antisera or cytotoxic drugs could be reversed by adoptive transfer of normal spleen cells or spleen cells enriched for NK cells. Administration of interferon or interferon inducers prior to i.v. tumor challenge was shown to decrease the number of lung metastases (Hanna and Fidler, 1980; Brunda et al., 1984). The protective effect appeared to be due to NK cells, since treatment of mice with anti-asialo-GM1 prior to administration of interferon abolished the observed antimetastatic effect (Yokoyama et al., 1986). Further support for a role for NK cells in the elimination of metastasizing tumor cells was provided by a study which showed that the sensitivity to NK cell lysis *in vitro* of a series of *ras*-transformed cell lines correlated inversely with their ability to arrest and survive in the lung for the first 48 hours after intravenous inoculation (Greenberg et al., 1987b). This apparent regulatory role observed in the experimental metastasis assay suggested that NK cells may be active against disseminating tumor cells in the circulation and capillary beds, or in tissues at sites of extravasation.

Thus, there is strong evidence that NK cells participate in the defense against neoplastic development, both in a first line of resistance

against incipient neoplasia and in the prevention of metastatic spread in the later stages of the disease.

5) Natural Antibodies

Immunoglobulins exist in normal unimmunized individuals, and have been found to bind to a variety of "antigens" to which the host has not been intentionally immunized. These immunoglobulins are commonly referred to as natural antibodies (NAb), and NAb which bind to the surface of zenogeneic, allogeneic and/or syngeneic tumor cells have been detected in a number of vertebrate species (Aoki et al., 1966; Herberman and Aoki, 1972; Martin and Martin, 1975a: Pierroti and Colnaghi, 1976; Menard et al., 1977; Wolosin and Greenberg, 1979; Gronberg et al., 1980; Houghton et al., 1980; Chow et al., 1981b; Colnaghi et al., 1982; Itaya et al., 1982). Further, tumor lines have been shown to acquire bound NAb rapidly *in vivo*, between 3 and 18 hours after intraperitoneal implantation into syngeneic mice (Wolosin and Greenberg, 1979). Their existence in normal individuals without a requirement for previous exposure to the tumor and ability to combine rapidly with tumor cells *in vivo* are properties which make NAb suitable to participate in a first line of defense against tumor development.

Tumor-reactive NAb have generally been detected by one of two methods. One procedure which has been frequently employed is the measurement of cytolysis following the addition of an exogenous source of complement, usually derived from rat or guinea pig serum, to NAb-exposed, ^{51}Cr-labelled target cells. A second method that has been used is indirect fluorescence, in which tumor bound NAb is detected by subsequent exposure

to fluorochrome-labelled anti-immunoglobulins. The former method has the disadvantage of detecting only NAb capable of mediating complement-dependent lysis, while the latter procedure can be used to measure any tumor-bound NAb, regardless of isotype or manner of binding. Analysis of normal mouse sera by precipitation with anti-immunoglobulin subclass antibodies or fractionation on staphlococcal protein A columns has shown that NAb capable of mediating complement-dependent cytolysis of tumor cells may be either IgM or IgG (Martin and Martin, 1975a; Chow et al., 1981a; Gronberg et al., 1985). Indirect immunofluorescence has revealed tumor-reactive murine NAb of the IgM isotype as well as all subclasses of IgG (Chow and Chan, 1987). Tumor-reactive monoclonal antibodies secreted by hybridomas formed between myelomas and non-immunized mouse splenocytes have also been produced which are either IgG or IgM (Colnaghi et al., 1982; Agassy-Caholon et al., 1988).

6) Specificities and Source of Tumor-Reactive NAb

While numerous studies have shown the presence of NAb which can bind to the surface of tumor cells, very few of the antigens to which tumor-reactive NAb bind have been characterized. There is evidence to suggest that different antitumor NAb bind to distinct antigens, and that the polyclonality of this reactivity may be important. Colnaghi et al (1982) characterized four monoclonal natural antibodies and concluded that each had different specificities based on the pattern of complement-dependent lysis exhibited against a panel of different cell types. Further, a mixture of all four of the monoclonal NAb produced a greater level of tumor cytolysis than was expected by the sum of the individual antibodies,

suggesting that the NAb in some way synergized in their tumor cell binding. One approach designed to elucidate the nature of the antigens to which tumor-reactive NAb can bind has been to determine the distribution of the antigens by examining the ability of different cell types to absorb tumor reactive NAb. Such absorption studies have suggested that some tumor-binding NAb may be reactive with differentiation antigens. Two antigens expressed on human malignant melanoma cell lines were classified as differentiation antigens based on the ability of fetal, but not adult fibroblasts to absorb reactive NAb (Houghton et al., 1980). Similarly, human NAb reactive with cultured astrocytoma cells were absorbed by fetal but not adult brain tissue (Pfreundschuh et al., 1982). Miller et al (1989) reported that a large proportion of murine NAb reactive with two syngeneic lymphomas could be absorbed by thymocytes, and that the majority of monoclonal NAb selected on the basis of reactivity to the lymphomas also bound to thymocytes.

Another group of antigens to which tumor-reactive NAb may bind are viral antigens. Antibodies directed against C-type viral antigens are produced in many mouse strains as a response to endogenously expressed murine leukemia virus (Ihle and Hanna, 1977). Colnaghi et al (1982) demonstrated that two monoclonal NAb reactive with chemically-induced murine lymphomas bound to mouse embryo fibroblasts infected with an ecotropic C-type virus but not to uninfected cells. Gronberg et al (1985) were able to inhibit syngeneic NAb-mediated complement lysis of a murine leukemia virus-induced lymphoma by serum absorption with C-type virus particles. In addition, four systems of surface antigens related to a glycoprotein product (gp70) of endogenous murine leukemia viruses are

expressed on thymocytes in many strains of mice and NAb reactivity to each of these antigens has been detected (Obata et al., 1976; Obata et al., 1978; Stockert et al., 1979; Obata et al., 1981). These antigens have also been detected on spontaneous, X-ray-induced, chemically-induced and murine leukemia virus-induced murine leukemias, as well as on some methylcholanthrene-induced sarcomas (Obata et al., 1978; Stockert et al., 1979; Obata et al., 1981).

A bias towards carbohydrate reactivity is apparent among antitumor NAb (reviewed in Miller et al., 1989). It has been reported that treatment of a murine lymphoma cell line with tunicamycin, which prevents N-linked glycosylation of cell surface proteins, reduced its sensitivity to NAb-dependent complement lysis by 30-60% (Gronberg et al, 1985). Gil et al (1990) demonstrated the presence of IgM class NAb reactive with tumor cell surface carbohydrates in all mice strains examined. Specific carbohydrate epitopes to which tumor-reactive NAb bind have been described, including the glycolipid determinants 3-fucosyllactosamine, expressed on murine teratocarcinomas and human carcinomas (Umeda et al., 1986), and lactoneotetraglycosyl ceramide, expressed on a rat fibrosarcoma (Takimoto et al., 1985). In humans, Anti-α-galactosyl (anti-Gal) NAb, which react with the oligosaccharide residue Galα1-3Galβ1-4GlcNAc-R, are present in unusually large amounts in the serum of healthy individuals and constitute 1% of circulating IgG (reviewed in Galili and Macher, 1989). While the epitope recognized by this NAb has not been detected on normal cells in humans, several human tumor cell lines have been shown to express this molecule on their surface and to bind anti-Gal NAb. In addition, inappropriate blood group antigens have been detected on human tumors, and

are targets for NAb (reviewed in Miller et al., 1989).

It has been shown that a common property of many NAb is multi-reactivity (reviewed in Schwartz, 1988). Monoclonal NAb obtained from mice or humans and screened in solid phase assays against a panel of self and non-self molecules have frequently bound to more than one antigen. Self antigens to which these polyreactive NAb frequently bind include cytoskeletal proteins, nucleic acids, IgG Fc fragments and serum albumin, while foreign antigens which have been employed in these assays and which are frequently bound by NAb include ovalbumin, hen egg lysozyme, bacterial lipopolysaccharides and polysaccharides and chemical haptens (Ternynck and Avrameas, 1986; Ragimbeau and Avrameas, 1987; Hartman et al., 1989; Rousseau, et al., 1989; Lydyard et al., 1990). The "functional affinity" (avidity) of some murine polyreactive NAb was investigated by Ternynck and Avrameas (1986) based on the dissociation constants (K_D) observed in solid phase immunoassays. It was found that a given monoclonal NAb exhibited a range of functional affinities for different antigens. The affinities for some antigens were similar to those of induced antibodies for the same antigen, although repeated immunizations with heterologous proteins induced the formation of antibodies with lower K_D's. This study suggested that the binding of these polyspecific NAb may be of sufficient avidity to be physiologically relevant. While the relationship between these multi-reactive NAb and antitumor NAb is not known, there is one report of a murine monoclonal NAb which reacts with both tumor cells and with single stranded DNA (Smorodinsky et al., 1988).

The majority of NAb-secreting cells are short-lived and the progeny of cycling lymphocytes, as shown by the elimination of 50-90% of those

cells 2-3 days after one cycle of treatment with hydroxyurea (Levy et al., 1987), an antimitotic drug which selectively eliminates cycling cells *in vivo* (reviewed in Rocha et al., 1983). Outbred, athymic nude mice (nu/nu) were shown to have levels of serum antitumor NAb which were similar to those in normal heterozygous littermates (nu/+) as assessed by complement-mediated cytotoxicity against tumor cell lines, suggesting that the production of these NAb is thymus-independent (Martin and Martin, 1974; Chow et al., 1981a). Also supporting this hypothesis is the report that syngeneic tumor cells rapidly acquired similar levels of complement-fixing NAb when injected i.p. into thymectomized, irradiated, bone marrow reconstituted mice as in sham-thymectomized controls (Wolosin and Greenberg, 1979). Low NK beige mice (bg/bg) and their normal heterozygous littermates (bg/+) were also shown to exhibit similar levels of NAb-dependent complement-mediated cytotoxicity against an allogeneic lymphoma (Chow et al., 1981b). In contrast, mice bearing the X-linked immunodeficiency (xid) mutation were shown to have very low levels of circulating antitumor NAb as assessed by complement-mediated lysis of tumor cells (Martin and Martin, 1975b; Chow and Bennet, 1989), or by measurement of fluorescence-detected NAb binding to a syngeneic lymphoma (Chow and Bennet, 1989). The latter study revealed marked reductions in binding of both IgM and IgG NAb. Animals inheriting the xid defect fail to respond to a number of thymus-independent type-2 antigens, including several bacterial carbohydrates, and have reduced levels of circulating IgM and IgG3 immunoglobulins (Perlmutter et al., 1970). These mice have been shown to lack peritoneal Ly-1$^+$ B lymphocytes and Lyb-3$^+$,5$^+$,7$^+$ and IaW39$^+$ B cells (Scher, 1982; Hayakawa et al., 1986), indicating that the

majority of antitumor NAb are derived from one or more of these subsets. Ly-1$^+$ B cells have been proposed as the major source of serum IgM in unstimulated normal mice (Forster and Rajewski, 1987), including the polyreactive subset (Hayakawa et al., 1984; Painter et al., 1988). The levels of tumor-reactive NAb differ in different mouse strains, and it has been reported that high serum NAb is inherited in a recessive manner (Chow et al., 1981b). Levels of antitumor NAb in mice were shown to increase with age (Menard et al., 1977; Chow et al., 1981a; Ehrlich et al., 1984) and were elevated following treatment of mice with the adjuvents proteose peptone and LPS or by interferon-inducers such as polyinosinic polycyticylic acid and 2-amino-5-bromo-6-methyl-4-pyrimidinol (U-25) (Chow et al., 1981b).

The natural stimuli which induce secretion of NAb are not completely understood but are thought to derive from both exogenous and endogenous sources (reviewed in Bandeira et al., 1988). Exogenous stimuli may include normal enteric bacteria or subclinical bacterial infections, endogenous viruses, or macromolecules present in food or in the air. Possible endogenous sources of NAb stimulation include idiotype-anti-idiotype interactions, self antigens and cryptic self antigens revealed on damaged cells. External stimuli seem to be largely responsible for the production of IgG and IgA class NAb, since mice raised under germ-free conditions and fed an ultrafiltered, chemically defined diet had normal serum IgM levels, but IgG and IgA levels were approximately 5% of those in conventionally raised littermates (Bos et al., 1989). This study also revealed that most NAb against carbohydrate antigens of bacterial origin found in conventional mice were caused by exogeneous stimulation. Nevertheless,

substantial quantities of NAb were present in these "antigen-free" mice, indicating that endogenous stimuli are responsible for the production of a portion of the NAb pool. Bandeira et al (1988) refer to antibodies arising due to endogenous stimuli as "internal" antibodies to differentiate them from "natural" antibodies which are produced in response to environmental stimuli. The apparent bias of tumor-reactive NAb specificity towards carbohydrate antigens suggests that these antibodies may be primarily induced by exogenous stimuli, including bacteria. NAb have been shown to bind to numerous gram-positive and gram-negative bacteria (Miller et al., 1989; Lydyard et al., 1990), bacterial polysaccharides (Borradori et al., 1990; Hansen and Jackson, 1990; Le Moli, et al., 1991) and bacterial flagellin (Rouseau et al., 1989). Consistent with the idea that antibodies elicited by bacterial stimulation may also bind to tumor cells is the report that a monoclonal antibody raised against polysialic acid reacted with invasive pathogenic bacteria, a differentiation antigen expressed on rat embryonic kidney, and with a human nephroblastoma (Bitter-Suermann and Roth, 1987). That tumor-reactive NAb may cross-react with bacterial antigens was suggested by the report that preincubation of normal mouse serum with bacterial sonicates prepared from *E. coli* and *L. monocytogenes* reduced the complement-detected NAb reactivity against a syngeneic lymphoma (Gronberg et al., 1985). Also, the anti-Gal NAb found in human sera appear to be the result of constant stimulation by gastrointestinal bacteria which express α-galactosyl epitopes on their lipopolysaccharides and other cell wall components (Galili et al., 1988). Similarly, certain anti-blood group NAb have been shown to be produced in response to endogenous bacteria (reviewed in

Miller et al., 1989). However, levels of a tumor reactive NAb specific for anti-3-fucosyllactosamine were comparable in germ-free mice fed a chemically defined, ultrafiltered diet and their conventionally reared littermates, suggesting that not all anti-carbohydrate, tumor-reactive NAb are induced by exogenous stimuli (Bos et al., 1989). Damaged red blood cells have been proposed as one possible endogenous source of stimulation for tumor-reactive NAb, since a monoclonal NAb which reacts with bromelain- or papain-treated mouse erythrocytes also reacted with several murine lymphoma cell lines (Serbon and Witz, 1988).

In summary, the specificities of tumor-reactive NAb are for the most part unknown, but likely include many different antigens and this polyclonality may be functionally important. Accordingly, the stimuli which induce secretion of NAb likely also derive from multiple sources including both endogenous and exogenous antigens.

7) NAb in Antitumor Defense

The existence in normal individuals of NAb which reacted with tumor cells, and the demonstration that these NAb could bind rapidly to tumor cells *in vivo* suggested that NAb may participate in the first line of defense against tumor development. A variety of approaches have been employed to test this hypothesis. For the most part, these have been aimed at testing 3 predictions arising from this theory, namely: (1) levels of circulating NAb should correlate with resistance to tumor development; (2) tumors which develop must necessarily have escaped NAb-mediated surveillance and; (3) the sensitivity of a tumor cell to NAb should correlate inversely with its tumorigenicity. Although NAb is proposed to

be only one of several mediators involved in antitumor resistance, and therefore strict adherence to these 3 predictions would not be expected, results have been obtained which are consistent with each of these predictions and thus lend support to the hypothesis that NAb is involved in tumor surveillance.

Experiments aimed at testing the first prediction have involved comparing the level of tumor binding NAb present in an animal to its ability to resist the growth of a transplanted tumor cell line. Menard et al (1977) reported an inverse correlation between the level of complement-dependent, NAb-mediated cytotoxicity towards a syngeneic fibrosarcoma in Balb/c mice and the growth of this fibrosarcoma when transplanted s.c. into these mice. Subsequently, other investigators have confirmed this inverse correlation upon examination of tumor frequencies following s.c. injection of small tumor inocula into young and old mice (Chow et al., 1981a; Ehrlich et al., 1984). Old mice had higher levels of cytotoxic antitumor NAb than young mice and both NK-sensitive (NK^s) and NK-resistant (NK^r) syngeneic murine lymphomas produced lower tumor frequencies when injected s.c. into the former (Chow et al., 1981a). A similar inverse correlation between levels of tumor-reactive NAb and susceptibility to transplanted tumor growth has been observed in mice of differing genetic backgrounds (Gil et al., 1990), and also upon injection of syngeneic tumors into rats (Itaya et al., 1982). Further, the administration of adjuvents, including lipopolysaccharide and proteose peptone, increased the level of circulating tumor-reactive NAb in mice and also increased their resistance to s.c. transplanted NK^r syngeneic tumors (Chow et al., 1981a). More recently it has been shown that normal (CBA/N X CBA/J) F1

xid/+ female mice demonstrated twice the level of fluorescence-detected serum antitumor NAb reactivity against a syngeneic lymphoma and half the tumor frequency upon s.c. transplantation of this tumor compared to the B cell-deficient (CBA/N X CBA/J) F1 xid/- male mice (Chow and Bennet, 1989). The xid/- and xid/+ mice exhibited similar levels of NK and activated macrophage lysis against the lymphoma cell line, strongly suggesting that the higher tumor susceptability of the xid/- mice was due to the low levels of antitumor NAb. The correlation observed with transplanted tumor cell lines was also extended to a carcinogen-induced tumor model in rats (Itaya et al., 1982). When 3-methylcholanthrene was inoculated into rats, the tumor incidence in a group of rats with high NAb levels was significantly lower than that in a group of rats with low NAb levels.

Results consistent with the hypothesis that evasion of NAb-mediated defense is an important parameter in determining the ability of a tumor to grow have been provided by Chow and coworkers (Chow, 1984a; Brown and Chow, 1985; Brown et al., 1986) who examined the NAb binding of tumors arising following the injection of small tumor foci into mice. The injection of small tumor foci, which yield tumors in less than 100% of recipients, is meant to simulate the evolution of nascent tumors *in vivo*, and rejection of these implants has been shown to be thymus-independent (Greenberg and Greene, 1976). Tumors obtained after the s.c. injection of low numbers of NK^r or NK^s murine lymphomas into syngeneic mice exhibited reduced sensitivity to NAb-dependent complement lysis (Chow, 1984a; Brown and Chow, 1985; Brown et al., 1986) or reduced fluorescence-detected NAb binding (Brown et al., 1986) *in vitro* compared to their starting cell lines. Tumors obtained following the growth of the NK^r murine lymphoma

L5178Y-F9 at an s.c. site in syngeneic DBA/2 mice for 3.5 weeks or 3 months also showed a reduced ability to acquire complement fixing NAb in the peritoneal cavity compared to the parental line (Chow, 1984a). In addition, L5178Y-F9-derived tumors obtained following the growth of a large cell inoculum at the i.p. site, as well as experimental metastases found in spleen, brain and lungs following i.v. inoculation all exhibited reduced sensitivity to NAb and complement compared to the parental line (Chow, 1984a). These results suggested that NAb may participate in tumor defense at diverse anatomical sites. The reduced sensitivities of tumors obtained from various organs following i.v. inoculation was consistent either with the presence of NAb-mediated defense at these sites or with NAb acting against tumor cells in the circulation. The latter possibility suggests that NAb may contribute to the control of the metastatic process.

The third prediction, that is that the sensitivity of a tumor cell to NAb should correlate inversely with its tumorigenicity, has also been tested in a variety of studies. The sensitivity to natural resistance of tumor cell lines has generally been compared either by assessing the ability of the cells to form tumors when injected s.c. at a dose which yields less than 100% tumor frequency, or by measuring the rate of elimination of radio-labelled tumor cells occurring early (18 hours to 3 days) following i.p. or i.v. inoculation (reviewed in Miller et al., 1989). Consistent with the predicted inverse relationship between sensitivity to NAb and tumorigenicity, two clones derived from an NK^r lymphoma which exhibited similar susceptibilities to lysis by activated macrophages but markedly different sensitivities to NAb-dependent complement lysis *in vitro* produced tumors upon s.c. inoculation into

syngeneic mice with frequencies which correlated inversely with their NAb reactivity (Chow et al., 1981a). The tumor cells selected following *in vivo* growth described above exhibited both reduced sensitivity to NAb *in vitro* and increased tumorigenicity compared to their respective starting lines when reinjected into syngeneic mice, as demonstrated either by their reduced rate of clearance following i.p. injection (Chow, 1984a; Brown and Chow, 1985; Brown et al., 1986a) or by their increased tumor frequency following s.c. challenge (Brown and Chow, 1985). Interferon treatment of an NK^r murine lymphoma increased its sensitivity to NAb-dependent complement lysis *in vitro* and its *in vivo* acquisition of NAb in the peritoneal cavity while concomitantly producing an increased rate of clearance following i.p. injection into syngeneic mice (Miller et al., 1983). Since interferon treatment did not alter the NK^r phenotype of the tumor or its sensitivity to activated macrophage lysis *in vitro*, the results strongly suggested that interferon treatment was increasing the susceptibility of the cell to natural resistance by increasing its sensitivity to NAb. A more complex relationship between NAb reactivity *in vitro* and tumor cell behavior *in vivo* was suggested by an examination of tumor cell variants generated *in vitro* by treatment with TPA followed by selection for resistance to NAb plus complement. The selected cells demonstrated reduced sensitvity to NAb-dependent complement lysis *in vitro* and a reduced rate of clearance following i.p. injection into syngeneic mice (Chow and Chan, 1987). However, these same cells exhibited increased fluorescence-detected NAb binding *in vitro* and a reduced tumor frequency following s.c. injection. This result suggested that NAb with different functional characteristics (i.e. complement fixing vs non-complement

fixing) may be involved in tumor defense at different anatomical sites, perhaps dependent upon the availability of other effectors (see below). An inverse correlation between NAb reactivity and tumorigenicity was also observed in a study of *in vitro* transformed cells (Aggasy-Cahalon et al., 1988). An allogeneic monoclonal NAb showed slightly higher binding to low rather than high tumorigenic variants of c-H-*ras*-transformed NIH 3T3 cells which was statistically significant.

In addition to the above mentioned correlative studies, the influence of NAb binding on the fate of tumor cells *in vivo* has also been more directly assessed. Murine lymphomas preincubated with serum NAb prior to their s.c. injection into syngeneic mice produced a lower tumor frequency than untreated cells or cells treated with specifically preabsorbed serum (Chow et al., 1981a). The effect of passive immunization with NAb has also been examined (Agassy-Cahalon et al., 1988). In this study, two natural monoclonal IgM antibodies, which were shown to react with an allogeneic lymphoma *in vitro*, were injected into urethane-treated Balb/c mice before the appearance of tumors. Inoculation with one of the monoclonal IgM NAb was associated with a significant decrease, and the other with a significant increase in the number of lung tumors observed compared to mice receiving no IgM. However, the mechanism by which the antibody injections altered urethane-induced tumor formation is not known, and was likely indirect, since the monoclonal antibodies did not bind to adenoma tissue taken from the urethane-treated mice.

Although much of the work related to tumor-reactive NAb has been done in rodent models, there is also some evidence that NAb may play a role in tumor defense in humans. A role for anti-Gal NAb in defense

against metastasis was suggested by Castronovo et al (1989), who demonstrated that the purified antibody inhibited the *in vitro* attachment of human tumor cells to umbilical vein endothelium and to isolated laminin. Further, pre-exposure of malignant murine tumor cells to human anti-Gal antibodies was shown to reduce lung colonization in an experimental metastasis assay (Castronovo et al., 1987). Epidemiological studies have implicated other human NAb, such as the anti-A blood group antibody, in antitumor resistance. Individuals with anti-A blood group antibody were found to have a 10%-20% lower incidence of adenocarcinoma compared to those without anti-A blood group antibody (Race and Sanger, 1962; Hakkinem, 1970), and about 15% of adenocarcinoma cells derived from blood group O or B patients were found to express incompatible blood group A antigen (Clausen et al., 1986).

For NAb which directly bind to tumor cells, there are several potential mechanisms by which the bound NAb may inhibit growth of that cell. Studies with specifically induced antibodies have shown that antitumor antibodies can act in a variety of ways including opsonization leading to phagocytosis by macrophages, complement-dependent cytolysis, ADCC effected by NK cells or macrophages, or direct action on the tumor cells in the absence of other effectors. One of these mechanisms may act alone, or in concert with others depending on the availability of effectors, the isotype of NAb bound, the nature and density of the NAb binding, and the antigen bound. IgG antibodies were the most efficient class of murine antibodies in mediating ADCC of tumor cells by murine macrophages and splenocytes and by human non-adherent peripheral blood lymphocytes or monocytes (Herlyn et al., 1985; Ralph and Nakoinz, 1983;

Ralph and Nakoinz, 1984; Seto et al., 1983; Kawase et al., 1985; Shen et al., 1981, Perlmann et al., 1981; Zoller et al., 1982), while IgM was inactive in inducing ADCC by macrophages (Herlyn et al., 1985, Ralph and Nakoinz, 1983; Ralph and Nakoinz, 1984; Seto et al., 1983; Kawase et al., 1985), and either synergistic with IgG (Shen et al., 1981; Perlmann et al., 1981) or slightly active on its own (Zoller et al., 1982) with human peripheral blood lymphocytes. IgM, however, was much more efficient in complement-dependent lysis of tumor cells than IgG (Herlyn et al., 1985; Ralph and Nakoinz, 1983). Antibodies directed against growth factor receptors may be able to exert direct effects on tumor cells in the absence of other effectors. For example, IgG and IgA monoclonal antibodies against the human transferrin receptor (Taetle et al., 1983), and an IgM monoclonal to the murine transferrin receptor (Trowbridge et al., 1982) inhibited growth of malignant cells in culture. Antibodies to glycolipids may be able to similarly affect tumor growth in the absence of other effectors since it was recently reported that IgM monoclonal antibodies directed against the ganglioside G_{M2} produced central necrosis when added to spheroid cultures of a human glioma cell line which expressed high levels of G_{M2} (Bjerkvig et al., 1991). Cultures established from the antibody-treated cells expressed 50% lower G_{M2} content and were markedly reduced in sensitivity to anti-G_{M2}. In addition to their effects which lead to tumor cell death, NAb may also affect the invasive properties of tumor cells as suggested by the observed inhibition of adhesion to vascular endothelium and extracellular matrix by human-derived anti-Gal antibodies (Castronovo et al., 1989).

While several potential antibody-mediated actions have been

demonstrated *in vitro*, the mechansims by which antibodies act *in vivo* against tumor cells have not been directly demonstrated. IgG2a and IgG1 monoclonals against the receptor for epidermal growth factor similarly inhibited tumor growth in athymic mice (Masui et al., 1986). However, only the IgG2a antibodies exhibited complement-mediated cytotoxicity or antibody-dependent macrophage-mediated cytotoxicity against the tumor cells *in vitro*, again demonstrating the differential activities of antibody subclasses and suggesting that neither complement nor effector cells may be necessary for anti-growth factor receptor antibody-mediated inhibition of tumor growth *in vivo*. Recently, i.p. injection into mice of a monoclonal IgM antibody recognizing tumor cell surface carbohydrates was shown to afford protection against subsequent i.p. or s.c. challenge with the tumor to which the antibody was raised; and the data suggested that a complement-independent, macrophage-dependent mechanism was responsible for the observed protective effect, since protection against tumor challenge was observed in complement deficient mice but not in silica-treated animals (Gil et al., 1990). NAb may act in a similar manner to the immune antibodies since it was also shown that levels of IgM, anti-carbohydrate NAb in mice correlated with natural resistance to the same tumor and this resistance also appeared to be complement-independent and macrophage dependent. Greenberg et al (1983) previously suggested that complement-mediated lysis was not a major mechanism through which NAb act against tumor cells in mice, since a reduced rate of i.p. tumor cell clearance was not observed in C5 deficient B10.D2o mice compared with the C5 replete B10.D2n, and cobra venom factor treatment of C5-deficient mice, which depletes C3, also did not alter the rate at which tumor cells were

eliminated. Because of the reported inefficiency of IgM class antibodies in promoting ADCC by macrophages, Gil et al (1990) raise the possibility that the IgM may be acting in a lectin-like manner, binding macrophages to tumor cells by means of common carbohydrates expressed both on the tumor and on macrophages. Supporting this hypothesis is the fact that certain lectins which recognize cell surface carbohydrates have been shown to induce macrophage-mediated lysis of tumor cells and to inhibit tumor growth *in vivo* (reviewed in Gil et al., 1990). The multivalency of IgM may allow these antibodies to function similarly.

In summary, tumor-reactive NAb possess a number of characteristics which make them suitable to participate in a first line of defense against incipient neoplasia and a variety of studies have provided evidence supporting this hypothesis. NAb appear to be only one component of a natural resistance system which also includes macrophages and NK cells, and the potential exists for extensive interactions between these putative mediators, relating both to the regulation of their activity and to the execution of their effector function against tumor cells. This antitumor natural resistance system may allow the host to respond rapidly to the appearance of newly arising neoplastic cells before a potentially more vigorous adaptive immune response can be mounted, and the two phases of the tumor response likely combine to provide a barrier to tumor growth which must be surmounted during the multistep process of tumor development.

PART II. OBJECTIVES

The objective of this study was to further investigate the role of NAb in natural antitumor defense. Although there is a substantial body of evidence supporting a role for NAb in tumor defense, NAb is only one of several mediators proposed to be involved in this process. This fact makes it difficult to assess the relative contribution of NAb-mediated mechanisms to the alterations in tumor growth which have been observed in studies of animals with differing NAb levels or in studies with NAb binding tumor variants, since changes related to other putative effectors of natural resistance may have simultaneously been present and may have contributed to the altered *in vivo* behavior of the tumor cells. Therefore, an *in vitro* model which allowed for direct selection of tumor cells for altered NAb binding was employed to more directly assess the contribution of NAb to antitumor resistance.

A further goal, for which the majority of this thesis is devoted, was the investigation of a prediction arising from the hypothesized tumor surveillance role of NAb which has heretofore not been addressed. This prediction is that sensitivity to NAb should arise during the course of tumor development. That is, the genetic changes which alter the behavior of a cell and ultimately result in its tumorigenic phenotype should be associated with an increased recognition by NAb. While it has been shown that transplantable tumor cell lines bind NAb, and the accumulated evidence suggests that sensitivity to NAb is an important parameter in determining the ability of these cells to grow when transplanted into

syngeneic mice, these cell lines arose *in vivo* and therefore have progressed far along the multistep pathway of tumor development. These end-stage tumors are likely not representative of the cells which would be targets of a surveillance mechanism. In order to investigate the relationship between NAb-reactivity and the early steps of tumorigenesis, an *in vitro* model of cell transformation was employed in which the expression of oncogenes was used to simulate individual steps in the multistep process.

CHAPTER 2

TUMORIGENICITY OF MURINE LYMPHOMAS SELECTED THROUGH FLUORESCENCE-DETECTED NATURAL ANTIBODY BINDING

ABSTRACT

Fluorescence-activated cell sorting was used to isolate high and low IgM natural antibody binding populations from a heterogeneous line of the L5178Y-F9 murine lymphoma. The ranking of NAb binding and complement-dependent NAb lysis of the selected and starting lines were the same and opposite to that of their tumorigenicity in syngeneic DBA/2 mice. L5178Y-F9 and SL2-5 clones repeatedly treated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate and selected by fluorescence-activated cell sorting for high NAb binding exhibited increases in NAb binding and sensitivity to complement-dependent NAb lysis which corresponded with reduced tumor frequencies of threshold inocula. Although the high NAb binding SL2-5 line was slightly more sensitive to natural killer cell cytolysis, changes in susceptibility to activated macrophages or hypotonic lysis were not consistent with the observed reductions in tumor frequency so that the selected alterations in NAb binding corresponded best with tumorigenicity. These data confirm the same inverse relationship exhibited previously by *in vivo* and *in vitro* selected tumor variants and provide more precise evidence supporting a role for NAb in host resistance against tumor foci.

INTRODUCTION

Natural antibodies, NK cells and macrophages have all been proposed as effectors in antitumor natural resistance. While studies utilizing a variety of approaches have provided results consistent with NAb performing such a function, the fact that other effectors are likely also

contributing to tumor defense has made it difficult to assess the importance of NAb. One approach which has been utilized to investigate the contribution of natural immune mediators to antitumor natural resistance is the analysis of tumor variants with altered sensitivity to these effectors. Tumor growth *in vivo* has yielded cells with an altered sensitivity to natural defense mediators. NK^r variants were derived by sequential passage of an NK^s transformed fibroblast cell line in thymectomized, irradiated, fetal liver-reconstituted mice followed by normal mice (Collins et al., 1981) and an NK^r L5178Yc/27av line was isolated after passage of the NK^s L5178Yc/27v lymphoma as ascites in syngeneic normal mice for a year (Durdik et al., 1980). Tumors obtained from the injection site of threshold s.c. tumor inocula consistently expressed a unified natural defense-resistant phenotype including decreased sensitivities to NK, complement-dependent NAb cytolysis, and hypotonic lysis as well as a reduced rate of clearance in the [^{131}I]iododeoxyuridine-labeled tumor elimination assay, lower NAb binding, and an increased tumor frequency (Chow et al., 1983; Chow, 1984a; Brown and Chow, 1985; Brown et al., 1986a). In addition, *in vitro* tumor selection has yielded cells with altered natural resistance (NR) phenotypes including a concanavalin A-resistant Chinese hamster ovary mutant with increased sensitivity to NK, reduced sensitivity to macrophages, and reduced tumorigenicity (Pohajdak et al., 1984; Pohajdak et al., 1986) and a similar macrophage-resistant, NK^s nonmetastic variant derived from the macrophage-sensitive NK^r metastatic MDAY-D2 tumor mutagenized with ethyl-methanesulfonate and selected with wheat germ agglutinin (Nestel et al., 1984). Another NK^s, poorly tumorigenic cell line

was produced by mutagenization and selection with anti-*H-2*^{d} and complement (Piontek et al., 1985; Karre et al., 1986). In our *in vitro* model of tumor progression, murine tumor cells were exposed to TPA, a phorbol ester tumor promoter known to have pleiotropic effects on cultured cells including increased cellular heterogeneity and alterations in sensitivities to natural effectors (Chow, 1984b; Werkmeister et al., 1982a; Werkmeister et al., 1982b; Gidlund et al., 1981). Subsequent selection with NAb and complement yielded cells which were less sensitive to complement-dependent NAb lysis and to radiolabeled tumor clearance but which bound more NAb and had a decreased tumor frequency (Chow and Chan, 1988).

Presently, we have used direct *in vitro* selection for high NAb binding in a more discriminating approach to assess the contribution of NAb in NR. Fluorescence-activated cell sorting was applied to TPA-treated L5178Y-F9 and SL2-5 cells, and to an L5178Y-F9 line that appeared to have generated a variant in culture, in order to select for cells with increased or decreased binding of NAb. The relationship between NAb binding and tumorigenicity was examined with these cells.

MATERIALS AND METHODS

Mice and Sera

DBA/2 mice were obtained from the University of Manitoba vivarium at Gunton, Manitoba, or from Canadian Breeders, Charles River, Quebec. Whole serum NAb was bled per axilla from mice that had received an i.p. injection of 100 μg lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO; serotype 055:B5 or 0127:B8) 3-4 days previously. *In vivo*

stimulation with the polyclonal B cell activator LPS has previously been associated with increases in autoantibodies seen in untreated mice (Dziarski, 1982; Dziarski, 1984) although the fine specificity representation may be different (Van Smick and Coulie, 1982). Our studies have shown that LPS stimulation raised the level of antitumor NAb normally present in the serum and decreased the tumor frequencies of threshold s.c inocula of the same tumor cells (Chow et al., 1981a).

Tumor Cells

The L5178Y-F9 and SL2-5 clones were obtained from the DBA/2 strain lymphomas L5178Y and SL2 respectively through two successive clonings using a sloppy agar procedure which has been described previously (Chow and Greenberg, 1980). Cells were maintained in Fischer's medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (10% FFBS) supplemented with penicillin and streptomycin. The cells were free of mycoplasma according to periodic testing performed using the DNA staining technique.

Fluorescence-activated Cell Sorting

Heterogeneous L5178Y-F9 Population. L5178Y-F9 cells maintained continuously *in vitro* for an extended period of time appeared to be heterogeneous for fluorescence-detected IgM NAb binding at 4°C. An aliquot of 2 X 10^6 cells was incubated with 800 μl whole LPS-induced mouse serum for 15 min at 37°C in a 3% CO_2 atmosphere, washed, and then resuspended in HEPES-buffered 10% FFBS containing a 1/5 dilution (0.1 mg/ml) of fluoresceine isothiocyanate conjugated (FITC) goat $F(ab')_2$ anti-mouse IgM

(Tago, Burlingame, CA). The cells were then washed, resuspended, and sorted at 4°C into IgM NAb$^-$ and IgM NAb$^+$ populations using a Coulter Epics V multiparameter sensor system (Coulter Electronics, Inc., Hialeah, Fla).

TPA-treated Cells. Initially, 100 ng TPA/ml and 0.1% dimethyl sulfoxide, or 1 μg TPA/ml and 1% dimethyl sulfoxide were added to 7.5 X 10^5 L5178Y-F9 cells or 1 X 10^6 SL2-5 cells respectively. The concentrations of TPA chosen are those which have been shown previously to be the minimum required in order to generate distinguishable variants of the respective lymphomas after subsequent selection with NAb plus complement (Chow, 1984b; Chow and Chan, 1987). Cells were grown for 2-4 days in the presence of TPA, which did not adversely affect their viability. Pellets of 2.5-4 X 10^6 TPA-treated cells were resuspended in 750-1000 μl syngeneic serum NAb diluted 1:1 with HEPES-buffered 10% FFBS and incubated for 1h at 4°C. The cells were washed and incubated for 20 min at room temperature in 450-650 μl of the same HEPES-buffered medium containing a 1/10 dilution (0.5 mg/ml) of FITC goat IgG (7S) anti-mouse IgG (Meloy, Springfield, VA) and a 1/10 dilution (0.05 mg/ml) of the FITC goat F(ab')$_2$ anti-mouse IgM. The cells were washed, resuspended in 1 ml medium, and the most fluorescent 10% (SL2-5) or 3% (L5178Y-F9) of the cells were collected by cell sorting at 4°C. Three successive cycles of TPA treatment followed by sorting yielded the final selected cells: L5178Y-F9 TPA/NAb$^+$3 and SL2-5 TPA/NAb$^+$3. Following the first and second sortings, the cells were grown in medium containing TPA until the next selection. Following the final selection, the cells were returned to, and maintained in standard culture (no TPA).

Fluorescence-detected Antibody Binding

Pellets of 5 X 10^5 tumor cells were resuspended in 200 μl aliquots of whole or diluted syngeneic serum NAb or 10% FFBS. L5178Y-F9 cells were incubated in NAb for 1h at 37°C in 3% CO_2 while SL2-5 cells were incubated at 4°C in air for 1 h. Following this initial incubation, 0.1% sodium azide was added to all solutions. After washing, the cells were incubated in 100 μl of either 1/10 FITC anti-IgG + 1/10 FITC anti-IgM, 1/5 FITC anti-IgM, or 1/5 FITC anti-IgG containing 10% FFBS for 20 min at room temperature. The cells were washed in HEPES-buffered 10% FFBS and fixed for 5 min in 1% formaldehyde before being analyzed by flow cytometry at 4°C. Binding of anti-*H-2*^{d} major histocompatibility complex class I determinant monoclonal antibody HB101 (American Type Culture Collection) was similarly determined with tumor incubation in whole HB101 culture supernates for 20 min at 37°C for the L5178Y-F9 lines and 1h at 4°C or 30 min at 37°C for the SL2-5 followed by a 20 min incubation at room temperature with a 1/10 dilution of the FITC-labeled anti-IgG antibody. HB101 is a murine IgG2aκ monoclonal antibody which binds to both K^d and D^d (Ozato et al., 1983). The data are expressed in terms of the mean linear fluorescence channel number (MCF) minus the control MCF. The control cells treated with medium followed by the FITC-labeled second antibodies displayed virtually no fluorescence beyond that of untreated cells with typical mean channel values ranging from 3-15.

Cytolysis Assays

Complement-mediated Cytotoxicity. A two-step assay was used which has been described (Chow et al., 1981a). Briefly, ^{51}Cr-labeled tumor target

cells were incubated with whole or diluted LPS-induced mouse sera as sources of NAb(5 X 10^5 cells/200 μl serum). The cells were washed with Hank's balanced salt solution (HBSS) (Gibco) and exposed to specifically absorbed rabbit serum (Buxted Rabbit Co., Buxted, England) as a source of complement.

Natural Killer Cells. This measurement of lysis by splenic NK cells from syngeneic polyinosinic:polycytidylic acid-stimulated DBA/2 mice (100 μg i.p., 20h previously) was carried out in an 18 h assay using ^{51}Cr-labeled tumor target cells at effector-to-target ratios (E/T)of 150/1, 75/1 and 37.5/1 as described previously (Chow et al., 1981a).

Hypotonic Lysis. This assay was a modification of the hypotonic shock technique described by Russel *et. al.* (1980). Briefly, ^{51}Cr-labeled tumor cells were incubated in 15 or 25% isotonic medium (10% FFBS) for either 45 or 90 min in a 37°C, 3% CO_2 incubator.

Macrophage Cytotoxicity Assay. This assay was a modification of the procedure described by Keller (1977). Peritoneal exudate cells (PEC) were obtained by three, 5 ml peritoneal washes of mice given 2.5 mg *Corynebacterium parvum* (CP) (Burroughs Wellcome Co.) i.p. 4-5 days previously. The PEC were pelleted, treated with 0.83% NH_4Cl to lyse erythrocytes, washed twice with HBSS, and resuspended in a volume of 100-200 μl to wells of flat-bottomed Nunclon microwell plates and incubated for 75 min at 37°C in a 6% CO_2 atmosphere. The medium was removed and the wells were washed three times. Then 1 X 10^4 ^{51}Cr-labeled tumor target cells were added to each well in a total volume of 200 μl and the microtiter plates were incubated for 18 h in a 37°C, 6% CO_2 incubator. The plates were centifuged for 10 min at 200 X g and 100 μl aliquots were removed from the

supernate for γ counting.

Results from all of the cytotoxicity assays were expressed as:

% specific lysis =

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

where the spontaneous release was determined by incubating target cells alone in 10% FFBS for the hypotonic lysis, NK-cell, and macrophage assessment. For the complement cytolysis assay, lysis in the presence of complement alone was added to the spontaneous release in the presence of antibody alone.

In Vitro Growth Rate Determination

The *in vitro* growth rates were obtained from cell counts performed at various times after seeding fresh cultures according to the equation:

$$T_d = \frac{T \times 0.693}{\ln (N_T/N_0)}$$

where: T_d = doubling time; T = time of growth; N_T = final cell number; and N_0 = initial cell number (Dyson, 1978).

Tumorigenicity Assay

Small s.c. tumor inocula were used to simulate a spontaneously arising tumor. It has been shown that the rejection of threshold tumor inocula is mediated by thymus-independent mechanisms, because the frequency of tumors following such a challenge was not increased in adult thymectomized irradiated and bone marrow reconstituted mice (Greenberg and Greene, 1976). 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 middle of the lower back for each mouse. Tumors were detected as palpable lumps at the injection site and the tumor frequency was assessed 2 weeks after the appearance of the last tumor. All tumors appeared within 39 days.

Statistics

Student's *t* test was used to determine statistical significance for the differences in antibody binding and for the differences between the percentages of cytolysis in the presence of antibody and complement, CP-activated macrophages or hypotonic media for the different L5178Y-F9 and SL2-5 populations. *P* values greater than 0.05 were not considered significant. The *t*-dependent test was utilized in all determinations except where the *t*-independent test is indicated. The χ^2 test (Bourke et al., 1985) was used to determine the statistical significance for differences in tumor frequency.

RESULTS

Characterization of Cells Selected for Differential Binding of IgM NAb

An extensively cultured murine lymphoma cell line, L5178Y-F9 was found to contain two distinct populations of cells based on fluorescence-detected IgM NAb binding (Fig. 2.1). Fluorescence activated cell sorting based on IgM NAb binding was utilyzed to select high binding IgM NAb^+ and low binding IgM NAb^- populations. These selected populations maintained their respective IgM NAb binding phenotype (Table 2.1) for at least 13

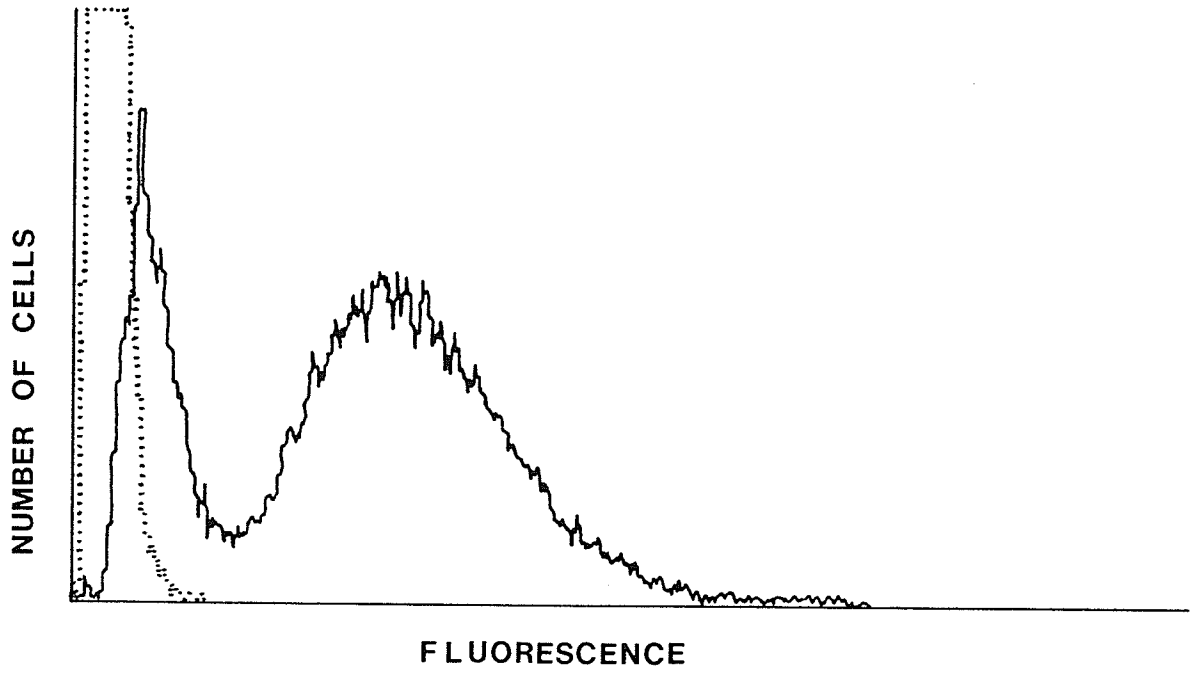

Fig. 2.1. Fluorescence profile of IgM NAb binding by extensively cultured L5178Y-F9 cells. Solid line represents fluorescence of cells exposed to whole serum NAb followed by FITC-anti-IgM while broken line shows fluorescence of cells exposed to FITC-anti-IgM alone.

Table 2.1

NAb binding of IgM NAb sorted L5178Y-F9

Tumor line	NAb binding[a] (MCF ± SE[b])			NAb + complement[a] (% cytolysis ± SE) (4)
	anti-IgM + anti-IgG (5)	anti-IgM (6)	anti-IgG (6)	
L5178Y-F9 IgM NAb$^+$	96.8 ± 12.2	73.4 ± 18.3	89.9 ± 22.1	56.1 ± 6.4
L5178Y-F9	74.9 ± 13.5	ND[i]	ND	17.2 ± 1.3[e,g,h]
L5178Y-F9 IgM NAb$^-$	73.9 ± 13.4[c]	44.2 ± 12.3[c]	40.4 ± 8.5[d]	12.8 ± 1.2[f,h]

[a] Number of experiments is indicated in parentheses.
[b] Since background fluorescence was the same for all three cell lines, in some experiments the control fluorescence was assessed for one line and subtracted from the total MCF of all three lines.
[c,d,f] $P_{td} < 0.02$, 0.03, 0.001 respectively as compared to L5178Y-F9 IgM NAb$^+$.
[e] $P_{ti} < 0.002$ as compared to L5178Y-F9 IgM NAb$^+$.
[g] $P_{ti} < 0.05$ as compared to L5178Y-F9 IgM NAb$^-$.
[h] The t-independent Student's t test was used.
[i] ND is not determined.

weeks in culture. L5178Y-F9 IgM NAb$^+$ cells were found to be significantly higher binding than IgM NAb$^-$ cells for IgM, IgG and combined IgM + IgG NAb binding, with mean channel fluorescence (MCF) increases of 66, 122 and 31% respectively. The starting L5178Y-F9 line was intermediate for combined IgM + IgG NAb binding.

The same ranking of NAb binding was observed when detected by incubation with NAb followed by exposure to complement (Table 2.1). L5178Y-F9 IgM NAb$^+$ was over three times more sensitive to NAb-dependent complement lysis than the starting line, which was 34% more sensitive than IgM NAb$^-$. All differences were significant.

The observation that the differences in fluorescence-detected NAb binding were reflected also in sensitivity to complement-dependent lysis suggested that the differences in NAb binding may be related to Fab reactivity and may therefore have biological significance. This possibility was examined by testing the ability of these cells to produce tumors from threshold s.c. inocula in syngeneic mice. An inverse relationship between NAb binding and tumorigenicity was observed as the IgM NAb$^+$ cells exhibited the lowest tumor frequency (52.2%) and IgM NAb$^-$ the highest (75%) after the s.c. inoculation of 50 cells (Table 2.2), although the difference in tumor frequency was not statistically significant ($P = 0.052$).

Characterization of TPA-treated, NAb-sorted cells

NAb binding. In order to generate cells with increased binding of NAb, cloned SL2-5 and L5178Y-F9 cell lines were treated with TPA followed by fluorescence-activated cell sorting for high NAb binding. TPA-treatment

Table 2.2

Tumorigenicity of IgM NAb sorted L5178Y-F9

Tumor line	Tumor frequency[a]		
	Inoculum	Number	Percent (3)
L5178Y-F9 IgM NAb$^+$	50	12/23	52.2
L5178Y-F9	50	14/24	58.3
L5178Y-F9 IgM NAb$^-$	50	18/24	75.0

[a] Number of experiments is indicated in parentheses.

of clonal cell lines has been shown to produce heterogeneity in NAb binding (Chow, 1984b). L5178Y-F9 TPA/NAb^{+}3 and SL2-5 TPA/NAb^{+}3 were obtained after three successive selections based on total IgM plus IgG NAb binding.

The TPA-treated, NAb-selected cells exhibited a significantly higher binding than their respective starting clones for IgM, IgG, and combined IgG plus IgM NAb binding (Fig. 2.2 and Table 2.3). L5178Y-F9 TPA/NAb^{+}3 showed increases of 147, 336 and 134% over its starting cell line, while SL2-5 TPA/NAb^{+}3 exhibited increases of 150, 99 and 91% over SL2-5 for IgM, IgG, and combined NAb binding respectively. This increased NAb binding was also demonstrated through a rise in complement-dependent NAb lysis of the selected cells, amounting to a 4-fold increase for the L5178Y-F9 variant and a 3-fold increase for the SL2-5 over the starting clones. Compared with their parental lines the L5178Y-F9 TPA/NAb^{+}3 bound slightly more anti-*H-2*^{d}, 7%, while the selected SL2-5 variant bound 15% less and both differences were significant (Table 2.4) The high NAb binding phenotypes of the cells were observed to be stable in culture for at least 10 weeks for SL2-5 TPA/NAb^{+}3 and at least 7 weeks for L5178Y-F9 TPA/NAb^{+}3.

NK cytolysis. The high NAb binding SL2-5 line exhibited a significant increase in sensitivity to NK lysis which amounted to approximately a 30% increase above the NK^{s} starting clone while the high NAb binding variant of the NK^{r} L5178Y-F9 was still NK^{r} (Table 2.5).

Macrophage cytolysis. Compared to the L5178Y-F9 the L5178Y-F9 TPA/NAb^{+}3 showed a small decrease, less than 10%, in sensitivity to lysis

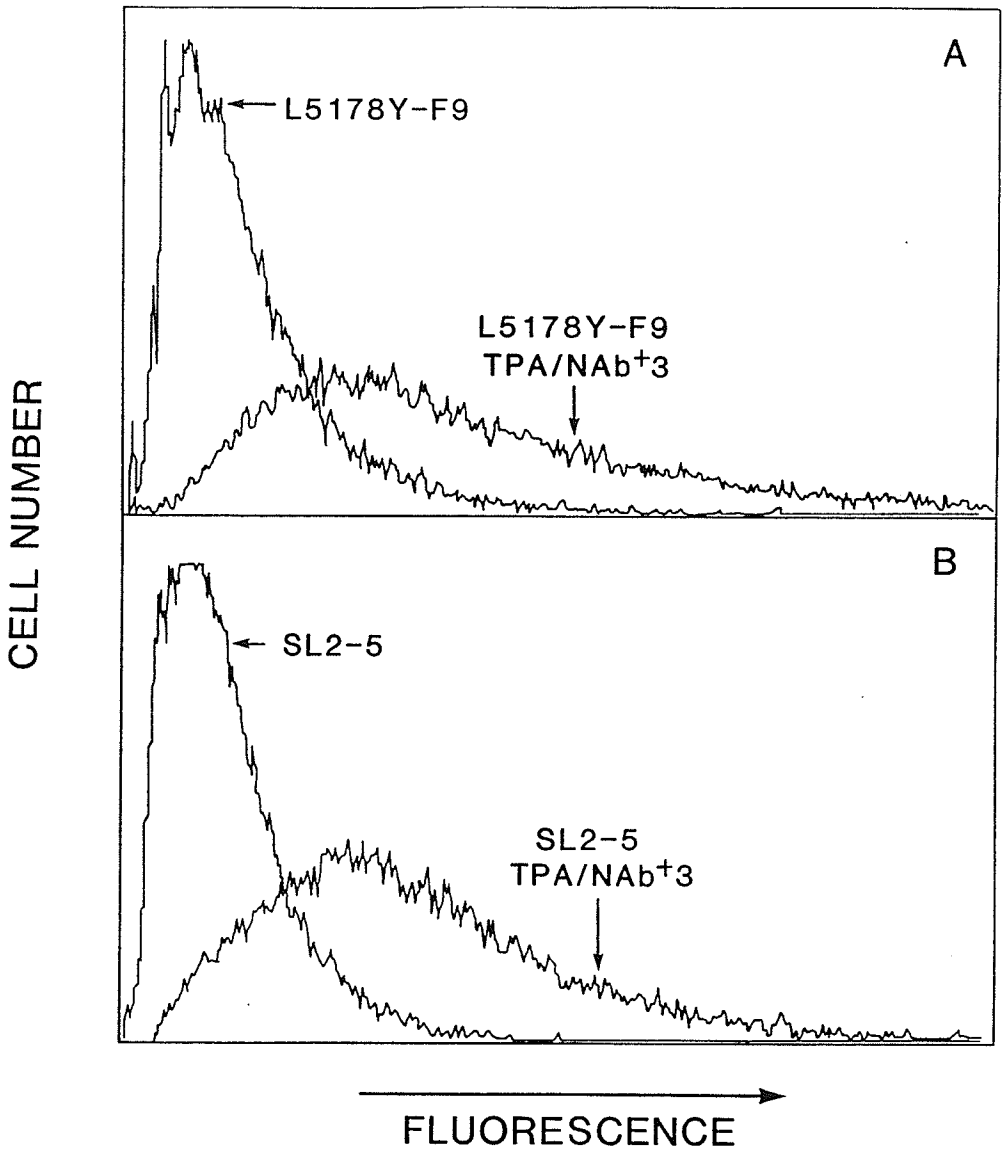

Fig. 2.2. Fluorescence profiles of NAb binding by TPA-treated, NAb-selected tumors. Three cycles of TPA treatment and fluorescence selection for high NAb binding cells (TPA/NAb^{+}3) yielded A, L5178Y-F9 and B, SL2-5 variants that bound markedly more IgG plus IgM NAb than their respective starting clones.

Table 2.3

NAb binding and sensitivity to syngeneic NAb and complement of the TPA-treated, NAb-sorted cells

Tumor target	NAb binding (MCF ± SE)			NAb + complement (% cytolysis ±SE)
	Anti-IgM + anti-IgG	anti-IgM	anti-IgG	
L5178Y-F9	39.7 ± 7.6	35.1 ± 3.4	15.5 ± 1.9	9.7 ± 2.3
L5178Y-F9 TPA/NAb$^+$3	92.7 ± 7.1	86.8 ± 10.0	67.1 ± 4.2	40.9 ± 5.5
P	<0.03 (4)[a]	<0.03 (4)	<0.002 (4)	<0.003 (6)
SL2-5	58.9 ± 8.6	31.1 ± 6.3	48.2 ± 9.7	19.9 ± 5.8
SL2-5 TPA/NAb$^+$3	112.4 ± 11.3	77.6 ± 7.7	96.0 ± 11.1	54.2 ± 3.2
P	<0.0005 (8)	<0.02 (3)	<0.05 (3)	<0.0006 (6)

[a] Number of experiments is indicated in parentheses.

Table 2.4

MHC class I expression of the TPA-treated, NAb-sorted cells

Tumor target	Anti-H-2^d binding (MCF ± SE)
L5178Y-F9	85.1 ± 1.4
L5178Y-F9 TPA/NAb$^+$3	91.3 ± 1.9
P	<0.03 (4)[a]
SL2-5	81.9 ± 3.0
SL2-5 TPA/NAb$^+$3	70.3 ± 5.4
P	<0.02 (7)

[a] Number of experiments is indicated in parentheses

Table 2.5

NK-cell cytolysis of TPA-treated, NAb-sorted cells

Tumor target	% NK-cytolysis ± SE (E/T ratios)		
	150/1	75/1	37.5/1
SL2-5	32.5 ± 2.8	27.5 ± 2.2	21.8 ± 2.4
SL2-5 TPA/NAb^{+}3	43.4 ± 1.6	32.6 ± 2.0	27.0 ± 1.9
P	<0.02 (6)[a]	<0.02 (6)	<0.02 (6)
L5178Y-F9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
L5178Y-F9 TPA/NAb^{+}3	0.0 ± 0.0	1.9 ± 2.2	0.9 ± 1.3
P	NS[b] (7)	NS (7)	NS (7)

[a] Number of experiments is indicated in parentheses.
[b] NS, not significant.

by CP-activated macrophages which was significant only at a 25:1 ratio (Table 2.6). Conversely, SL2-5 TPA/NAb^{+}3 was slightly more sensitive than its starting clone, less than 10%, although the differences were not significant.

Hypotonic lysis. In a previous study, tumor cells derived from the injection site of threshold s.c. inocula exhibited a unified natural defense-resistant phenotype including reduced sensitivity to hypotonic lysis, and this correlated with an increased tumorigenicity *in vivo* (Brown et al., 1986a). This result, along with a report of correlating sensitivities of tumor cells to NK cells, macrophages and hypotonic shock (Brooks et al., 1981), suggested that sensitivity to hypotonic stresses may be an important factor in determining susceptibility to antitumor natural resistance. Therefore, the ability of the selected cells to withstand hypotonic stress was also determined (Table 2.7). SL2-5 TPA/NAb^{+}3 was unchanged compared to SL2-5 while L5178Y-F9 TPA/NAb^{+}3 exhibited a slight but significant decrease in sensitivity to hypotonic stress which amounted to 15% of the control cytolysis when the control level was at least 30%.

Tumor Frequency. In order to assess the biological relevance of the changes in NAb binding, the tumorigenicity of the selected cells was examined in comparison with that of the starting clones. The ability of tumors to grow out from threshold s.c. inocula in syngeneic mice was used as a measure of their malignant potential and conversely their susceptibility to NR defense mechanisms *in vivo*. While the tumor latency

Table 2.6

Sensitivity to CP-activated macrophages of the TPA-treated, NAb-sorted cells

Tumor target	% PEC cytolysis ± SE (E/T ratios)		
	10/1	25/1	50/1
L5178Y-F9	47.1 ± 4.7	65.9 ± 1.2	ND[a]
L5178Y-F9 TPA/NAb$^+$3	44.0 ± 4.1	59.2 ± 2.5	ND
P	NS[b] (7)[c]	<0.03 (7)	
SL2-5	ND	24.9 ± 2.2	33.6 ± 2.6
SL2-5 TPA/NAb$^+$3	ND	27.1 ± 3.1	36.0 ± 3.1
P		NS (9)	NS (9)

[a] ND, not determined.
[b] NS, not significant.
[c] Number of experiments is indicated in parentheses.

Table 2.7

Sensitivity to hypotonic lysis of the TPA-treated, NAb-sorted cells

Tumor target	% Hypotonic lysis ± SE			
	45 min		90 min	
	25	15	25	15
L5178Y-F9	7.4 ± 0.7	22.4 ± 4.0	14.3 ± 1.6	39.4 ± 3.2
L5178Y-F9 TPA/NAb$^+$3	3.9 ± 0.6	17.6 ± 2.2	9.2 ± 1.1	33.4 ± 2.5
P	<0.05 (5)[a]	NS[b] (5)	<0.03 (5)	<0.01 (5)
SL2-5	11.1 ± 1.9	26.3 ± 4.7	15.8 ± 1.6	30.0 ± 2.4
SL2-5 TPA/NAb$^+$3	11.3 ± 2.6	24.5 ± 3.5	16.6 ± 2.6	28.4 ± 2.8
P	NS (3)	NS (3)	NS (3)	NS (3)

[a] Number of experiments is indicated in parentheses.
[b] NS, not significant.

of the selected and starting clones was similar, 21 days for the L5178Y-F9 lines and 12 days for the SL2-5 lines, in both cases the tumor frequencies of the high NAb binding cells were approximately one half of that of their respective starting clones, and these differences were statistically significant (Table 2.8).

DISCUSSION

A consistent inverse correspondence between NAb binding and tumorigenicity of threshold s.c. inocula was seen for the high and low NAb binding variants selected through cell sorting from the heterogeneous L5178Y-F9 line and for the TPA-treated, NAb-selected cells compared with their starting clones. This data supports the hypothesis that NAb contributes to the elimination of nascent tumors in syngeneic animals.

Threshold inocula of L5178Y-F9 and SL2-5 tumor cells surviving at a s.c. site *in vivo* displayed a unified natural defense-resistant phenotype which included reduced NAb binding and increased tumor frequencies (Chow et al., 1983; Chow, 1984a; Brown and Chow, 1985; Brown et al., 1986). SL2-5 and L5178Y-F9 cells exposed to TPA in culture and selected for resistance to NAb and complement lysis were reduced in sensitivity to complement-dependent NAb lysis and had decreased rates of i.p. clearance in the radiolabeled tumor elimination assay. These same cells, nevertheless, bound more NAb, as detected by indirect fluorescence and were less tumorigenic s.c. (Chow and Chan, 1988). The previous evidence of a relationship between NAb binding and tumorigenicity suggested that NAb may play a role in eliminating tumor cells *in vivo*. However, the tumors had been selected on the basis of other parameters in addition to NAb

Table 2.8

Tumor frequency of threshold inocula of the TPA-treated, NAb-sorted cells

Tumor line	Inoculum s.c.	Tumor frequency	
		Number	Percentage
L5178Y-F9	10^2	10/14 (2)[a]	71.4
L5178Y-F9 TPA/NAb$^+$3	10^2	5/14 (2)	35.7[b]
SL2-5	10^5	14/24 (3)	58.3
SL2-5 TPA/NAb$^+$3	10^5	6/24 (3)	25.0[c]

[a] Number of experiments is indicated in parentheses
[b] $P < 0.03$ compared to L5178Y-F9
[c] $P < 0.01$ compared to SL2-5

binding and exhibited phenotypes altered in other aspects of NR, so that the contribution and significance of changes concomitant with NAb binding were uncertain.

While TPA-treated, NAb-selected L5178Y-F9 and SL2-5 cells exhibited stable alterations in the high NAb binding phenotype for which they were selected, no consistent parallel changes in *in vitro* sensitivity to other NR effectors was observed. SL2-5 TPA/NAb^{+}3 maintained the same sensitivity as SL2-5 to CP-activated macrophages and hypotonic lysis while L5178Y-F9 TPA/NAb^{+}3 was slightly more resistant than its starting clone to both of these parameters. The NK^{r} L5178Y-F9 remained NK^{r} after selection and the NK^{s} SL2-5 became slightly more sensitive to *in vitro* NK lysis after TPA treatment and NAb selection. Since the high NAb binding SL2-5 cells were approximately three times as susceptible to NAb plus complement as the SL2-5 and only one-third more sensitive to NK lysis there may be some relationship between NAb binding and NK cytolysis but NAb-mediated mechanisms are more likely to contribute to the reduced tumor frequency of the high NAb binding SL2-5 cells *in vivo*. The lack of consistent changes in susceptibility to putative NR effectors other than NAb accompanied by no loss in tumor growth rate for the selected cells *in vitro* (data not shown) supports the hypothesis that the observed differences in tumorigenicity are related to the differences in NAb binding. The fact that the tumors which did form following injection of the high NAb binding selected cells did so with similar latencies to those of their parental cell lines indicated that the reduced tumorigenicity of the selected cell lines was not the consequence of reduced growth rates *in vivo*. Previous work in our laboratory has shown that TPA-treatment of the L5178Y-F9 and

SL2-5 cell lines without NAb selection results in a transient increase in their tumorigenicity which declines to near that of the parental cell lines within 8 days following the return of the cells to standard culture conditions (no TPA) (Sandstrom and Chow, 1988). Therefore, the reduced tumorigenicities of the L5178Y-F9 TPA/NAb^{+}3 and SL2-5 TPA/NAb^{+}3 cell lines were not due to direct negative effects of TPA. Thus the present analysis of tumors selected directly for altered NAb binding confirm the earlier findings and extend the support for a role for NAb in NR against tumors. The consistency of the inverse NAb binding/tumorigenicity relationship emphasizes the probable relevance of NAb reactivity in neoplastic development.

Although the selected cells clearly bound more fluorescence-detected NAb on their surfaces than their respective starting clones, the nature of the increased binding, whether specific or nonspecific, is not known. Specific binding involving Fab/antigen interaction was indicated by the parallel increases in sensitivity to lysis by NAb and complement coupled with no general increased susceptibility to lysis assayed as osmotic shock. Increased nonspecific binding of immunoglobulins to the surface of the selected cells, which may occur through Fc receptors or lectin-like carbohydrate interactions, does not appear to be responsible for the observed increases in NAb binding, since the binding of a murine IgG2aκ monoclonal anti-*H-2*^{K} antibody (Ozato et al., 1980), and more importantly the FITC 7S rabbit second antibody, were similarly low on both the starting and selected cells (data not shown). Therefore Fc receptors for IgG class antibodies were not increased on the high NAb binding selected cells. An increase in cell size cannot account for the augmented NAb

binding since no accompanying increases in cell volume were detected through Coulter counter analysis. Thus, the increased binding of NAb appears to be due to an increased density of antibody-accessible antigen. Increased cell surface antigen expression, however, was not a generalized phenomenon for the selected cells. While the L5178Y-F9 TPA/NAb^{+}3 showed a slightly higher *H-2*^{d} class I antigen expression than the L5178Y-F9, easily an order of magnitude less than the increase in NAb binding, the SL2-5 TPA/NAb^{+}3 exhibited a similarly small reduction in the same characteristic. Regardless of the nature of the increased NAb binding it has altered the fate of tumor foci *in vivo* suggesting that the bound NAb contributes to the tumor elimination. The mechanisms of NAb-mediated elimination which are increased against the high NAb binding tumor cells *in vivo* are unknown, but because we have selected for high IgG and IgM NAb binding, any mechanisms involving IgG and/or IgM NAb which can function at the s.c. site may contribute to the better elimination of the high binding cells.

In vitro selection of tumor cells has focussed mainly on obtaining cells resistant to some cytopathic agent, although concomitant increases in susceptibility to collateral effectors have been seen. Tumor cells with increased sensitivity to NK were obtained after selection for resistance to concanavalin A (Pohajdak et al., 1984; Pohajdak et al., 1986), wheat germ agglutinin (Nestel et al., 1984), and anti-*H-2*^{d} and complement (Pointek et al., 1985; Karre et al., 1986). We selected directly for increased sensitivity to a mediator of NR. The reduced tumorigenicity of the high NAb binding lines suggests that resistance to NAb would be an essential component of tumor escape from surveillance. Since the efficacy of NR is dependent on the tumor dose (Greenberg and Greene, 1976), the

NAb-mediated mechanisms would most likely contribute to antitumor surveillance in the early stages of neoplastic development when the tumor load is small. The high NAb binding, low tumorigenicity cells may therefore represent the phenotype of the initial stages of tumor development possibly associated with preneoplastic or early neoplastic alterations. The high NAb binding cells should facilitate the characterization of NAb-mediated mechanisms and of the biochemistry and function of NAb target antigens that may be central to early tumor development.

CHAPTER 3

NATURAL ANTIBODY RECOGNITION OF H-*RAS*-INDUCED 10T½ TRANSFORMATION

ABSTRACT

It has been shown that selection of transplantable tumor cell lines *in vitro* for increased NAb binding produced cells with a reduced ability to grow when transplanted s.c. into syngeneic mice, consistent with a role for NAb in the defense against tumors (see Chapter 2; Tough and Chow, 1988). While the generation of such NAb-sensitive cells *in vitro* suggests the possibility of a phase of tumor development which is susceptible to NAb-mediated antitumor defense, a role for NAb in tumor surveillance would imply that sensitivity to NAb should increase following events associated with cellular transformation. To test this prediction we examined by flow cytometry the effect on serum NAb binding of v-H-*ras* integration and expression in 10T½. The co-introduction of v-H-*ras* and the neomycin resistance (neo^r) gene into 10T½ followed by G418 selection resulted in a marked and heterogeneous increase in NAb binding. Clonal analysis of this population demonstrated that the increased NAb binding was associated with tumorigenic conversion and oncogenic *ras* p21 expression. Culture of 2H1, 10T½ cells expressing the activated human H-*ras* oncogene under the transcriptional regulation of the zinc-inducible mouse metallothionein-I promoter, in 50 μM $ZnSO_4$ produced increases in both *ras* protein p21 and NAb binding. The expression of p21 was maximal within 1 day, and NAb binding, which was significantly increased following 2 days of cell culture in $ZnSO_4$ continued to increase up to 4 days. Following the removal of added zinc ions from the culture medium, p21 decreased to near basal levels within one day and the increased NAb binding returned to uninduced levels within 2 days. The cells also demonstrated a significant increase in NK cell sensitivity following 2 days in $ZnSO_4$. This was maintained as long as the

zinc was in the medium, but returned to uninduced levels within 1 day following its removal. Repeated i.v. administration of whole serum NAb prior to tumor inoculation reduced the early appearance of tumors following the s.c. injection of Zn^{++}-induced 2H1 cells into Zn^{++}-treated syngeneic C3H/HeN mice, consistent with an *in vivo* role for NAb in the defense against *ras* transformed cells. The results provide the first evidence for an increase in sensitivity to NAb following *ras*-induced transformation and argue for an NAb and NK cell susceptible phase of *ras*-induced tumor development which is a prerequisiste for these mediators to contribute to a first line of defense against incipient neoplasia.

INTRODUCTION

For an antitumor immune surveillance system to be effective, it is necessary that sensitivity to the effectors of such a system arises during the course of tumor development. While numerous studies utilizing transplantable tumors have provided strong evidence for the participation of NAb in antitumor resistance, such established tumor lines which originated *in vivo* evolved in the presence of host selection factors and are likely not representative of cells in the early stages of tumorigenesis. Selection of transplantable tumor lines *in vitro* for increased NAb binding produced cells which were less tumorigenic than their starting clones (Chapter 2; Tough and Chow, 1988) and which may therefore represent tumor cells at an NAb susceptible stage of tumor development. In the present study, examination of an *in vitro* model of transformation was undertaken to assess the potential of NAb to recognize early changes associated with tumor nascence.

We employed a sensitive *in vitro* assay (Brown et al., 1986a) to determine the NAb binding capacity of C3H 10T½ cells before and after transformation with the v-H-*ras* oncogene. This assay has consistently shown an inverse correlation with the tumorigenicity of threshold inocula of two long established transplantable T lymphomas (Chow and Bennet, 1989; Brown et al., 1986; Tough and Chow, 1988). 10T½ is an immortalized but nontumorigenic cell line which has previously been used in *in vitro* models of multistep carcinogenesis, in which activated *ras* oncogenes cooperated with tumor promoters (Hsiao et al., 1984), additional oncogenes (Taparowski et al., 1987) or serum factors (Hsiao et al., 1987) in cellular transformation. NAb has been linked to the defense against *ras*-induced tumors by the demonstration that an L5178Y-reactive monoclonal NAb was found to have a better ability to bind to low rather than high tumorigenic clones of c-H-*ras* transformed NIH 3T3 cells (Agassy-Cahalon et al., 1988). Increased sensitivity to NK cell lysis has been observed for 10T½ cells constitutively expressing the *ras* oncogene (Greenberg et al., 1987a) and for 10T½ cells conditionally expressing the *ras* oncogene under the transcriptional regulation of the mouse metallothionein-I promoter (Trimble et al., 1986). The results from the most recently published study however, suggest that it is the changes in the steady-state p21 expression level in the conditionally *ras*-expressing cells, either increases or decreases, that provide the stimulus for their increased sensitivity to NK cells (Anderson et al., 1989).

In the present report we show that *ras* oncogene-induced transformation of 10T½ cells led to increased NAb binding, consistent with a natural immune susceptible phase of tumorigenesis. We have also utilized

a 10T½ clone carrying a Zn^{++}-inducible *ras* oncogene to more directly examine the association between NAb binding and *ras* p21 expression in 10T½ and to compare the induction of NAb sensitivity to that of NK cells.

MATERIALS AND METHODS

Mice and Sera

C3H/HeN and CBA/J mice were obtained from the University of Manitoba Vivarium (Winnipeg, Man.). Whole serum NAb was obtained through bleeding per axilla normal adult male mice anesthetized with ether and allowing the blood to clot at 4°C.

Cells

The continuous fibroblast line 10T½ was originally derived from C3H mouse embryos (Reznikoff et al., 1973). 2H1 was derived from 10T½ following transfection with the pMtEJ plasmid bearing the activated human c-H-*ras* oncogene from the EJ bladder carcinoma cell line under the transcriptional control of the mouse metallothionein-I promoter (Haliotis et al., 1990), and was provided by T. Haliotis (Queens University). The ψ-2 retrovirus packaging cell line was derived by transfection of NIH 3T3 with a mutant construct of the Moloney murine leukemia virus which has a cis-active deficiency for packaging of genomic RNA (Mann et al, 1983). This cell line can be used to produce stocks of defective retroviruses which are helper-free, since it provides the packaging proteins in trans. ψ-2 cells infected with the raszip 6 or SVX vectors (see below) producing helper-free virus were provided by A.H. Greenberg (University of

Manitoba). All cell lines were maintained in D-MEM/F12 (Gibco) containing 10% FBS (Flow, Mississauga, Ont.). For all experiments, cells grown to subconfluence, at which point the cells covered approximately 75% of the growing surface in 100 mm tissue culture dishes, were removed by light trypsinization with 0.05% trypsin-EDTA (Gibco) for 5 min. We have observed similar results in NAb binding assays when cells were removed from the tissue culture dishes in buffer containing EDTA alone, or when harvested mechanically by flushing cells with medium alone (data not shown). However, non-transformed 10T½ cells were very difficult to harvest in this manner, and hence a much longer harvesting time was required for these cells than for their transformed counterparts. In addition, there was a higher percentage of non-viable cells observed following mechanical or EDTA-mediated cell removal than following trypsin-mediated harvest. Therefore, light trypsinization was used to harvest cells for all experiments.

Recombinant Retroviruses and Infections

ZipneoSV(X) (SVX) is a Maloney murine leukemia virus-derived, recombinant retrovirus containing the bacterial neomycin phosphoryltransferase gene (neo^r) (Cepko et al., 1984). The raszip6 vector (Dotto et al., 1985) was derived from SVX by the insertion of the v-H-*ras* gene into the BamH1 site. Helper-free virus stocks obtained from ψ-2 cells which had been infected with the defective retroviral vectors were titered on 10T½ cells with their activity measured as G418-resistant colony-forming units produced per ml. Helper-free virus stocks were added to 10T½ cells in D-MEM/F12 containg 10% FBS and 8 μg polybrene/ml. For

experiments, a multiplicity of infection (MOI) of 0.02 was used for raszip6 while SVX virus was employed over a range of MOI from 0.01-0.04. Following incubation with virus for 24 h, the cells were selected in medium containing 400 μg G418/ml for 10-14 days. Supernatants from virally-infected, G418-selected 10T½ cells were tested for the presence of virus to ensure that the viral stocks had in fact been helper-free and were found to be negative for G418-resistant colony-forming units.

Fluorescence-Detected NAb Binding

Total IgM + IgG syngeneic NAb binding was determined by flow cytometry of fluorescein-labeled cells and expressed as MCF. In a modification of previous procedures (see Chapter 2), aliquots of 2 X 10^5 cells were incubated for 1 h at 4°C in 100 μl of whole serum or either a 0.5 or 0.25 dilution of whole serum NAb. Titrations of individual pools of serum NAb were initially performed to determine the optimal dilution. Total IgM + IgG NAb binding was then detected and quantitated as described in Chapter 2.

Fluorescence-Activated Cell Sorting and Cloning

A subpopulation was obtained from v-H-*ras*/neo^r-infected, G418-selected 10T½ by sorting the upper 3% NAb binding cells using flow cytometry of aliquots prepared asceptically for fluorescence analysis without formaldehyde fixation (see Chapter 2). Clones were obtained either by limiting dilution in multiwell plates (experiment 1) or by picking isolated colonies growing on 100 mm tissue culture dishes (experiment 2). When cloning by limiting dilution, cells were picked from positive wells

occurring at dilutions judged to contain less than one cell per well according to the Poisson distribution (>37% negative wells) (Lefkovits, 1972), and these wells were examined microscopically to ensure that only a single colony was present. In both cases, the picked colonies were transferred to 100 mm tissue culture dishes and subcloned by picking isolated colonies.

Western Blotting

Aliquots of 1-4 X 10^6 cells were washed 3 times in phosphate-buffered saline and frozen as pellets overnight at -20°C. They were thawed and lysed at a concentration of 1.33 X 10^7 cells /ml in a buffer consisting of 0.2 M LiCl, 0.02 M Tris, 10 mM EDTA, 0.5% NP-40 + 50 μg aprotinin at pH 8.0. Lysates were diluted in sample buffer (0.1 M Tris, 0.1 M dithiothreitol, 2% SDS, 10% glycerol, 0.2% bromophenol blue, pH 6.8) to a final concentration equivalent to 10^7 cells/ml. Samples were boiled for 5 min, passed through a 23-gauge needle 5-10 times to shear DNA and centrifuged to pellet debris. Following SDS-PAGE of equal volumes of cell lysates, along with 25 μl of biotinylated SDS-PAGE standards (low range) (Bio-Rad Laboratories, Ltd., Mississauga, Ont), on a 12.5% gel, proteins were transferred electrophoretically to nitrocellulose paper and probed with a pan-reactive monoclonal anti-*ras* p21 antibody (Cat. no. 8203317, Cetus Corporation, Emeryville, CA, or RAS-10, (Carney et al., 1988), Cat. no. NEI 725, Du Pont Company, Boston, MA). *Ras* p21 bands were then detected by staining with the Vectastain-AP kit (Vector Laboratories, Burlingame, CA), which involved sequential exposure of the blot to biotin-conjugated anti-mouse IgG, avidin-alkaline phosphatase and substrate

(Vector). Model 620 Video Densitometer (Bio-Rad) determinations of all Vectastain ABC-AP stained p21 bands were compared to obtain the ratios of p21 expression relative to 10T½ cells.

Natural Killer Cell Cytolysis

The measurment of lysis by splenic NK cells from polyinosinic-polycytidylic acid-stimulated syngeneic C3H/HeN and allogeneic CBA/J mice (100 μg i.p. 20 h previously) was carried out in an 18 h assay using ^{51}Cr-labelled target cells at effector-to-target ratios of 150:1, 75:1 and 37.5:1 as described previously (Chow et al., 1981a). Percent cytolysis was calculated as described in Chapter 2. Following linear regression analysis, the results were expressed as the number of lytic units (LU) per 10^6 cells where 1 LU is the number of effectors per target cell required to produce 30% cytolysis.

Tumorigenicity Assay

The cell lines listed in Tables 3.1 and 3.2 were washed 3 times and resuspended to a concentration of 5 X 10^6 cells /ml in HBSS. For each cell line, an aliquot of 10^6 cells in 200 μl HBSS was injected s.c into the middle of a shaved area on the back of 2 or more C3H/HeN mice as indicated, and tumor appearance was assessed as in Chapter 2. In addition, groups of 6 C3H/HeN mice, given $ZnSO_4$ in their drinking water (Trimble et al., 1987), were pretreated i.v. with serum or saline prior to the s.c. inoculation of aliquots of 10^5 Zn-induced 2H1 as indicated in Fig. 3.5.

Statistics

The Student's *t*-test, dependent (t_d) or independent (t_i), was used to determine statistical significance for the differences in MCF NAb binding and in NK cytolysis.

RESULTS

Analysis of v-H-*ras*-infected 10T½ populations

The retroviral vector raszip 6 has v-H-*ras* inserted into the neomycin resistance (neo^r)-bearing SVX virus. This provided an efficient vehicle for the introduction of the *ras* oncogene and a drug-selectable marker into NIH 3T3 cells leading to their morphological transformation (Dotto et al., 1985). In order to assess cell sensitivity to syngeneic NAb during transformation, we initially examined through flow cytometry the complete serum NAb binding profile of the G418-resistant population of C3H 10T½ cells obtained following their infection with the v-H-*ras*/neo^r bearing genes of raszip 6. Helper-free, raszip6 virus stock from infected ψ-2 cells was added to 10T½ at a MOI of 0.02 for 24 h and the cells were then selected for 10-14 days in medium containing 400 μg G418/ml. The surviving cells exhibited a range of *in vitro* growth morphologies, from flat and organized to rounded and highly disorganized. This *ras*/neo^r-infected, $G418^r$-resistant population showed a marked and heterogeneous increase in normal syngeneic C3H/HeN serum NAb binding compared to 10T½ (Fig. 3.1a). The increase was observed consistently in independent infections (Table

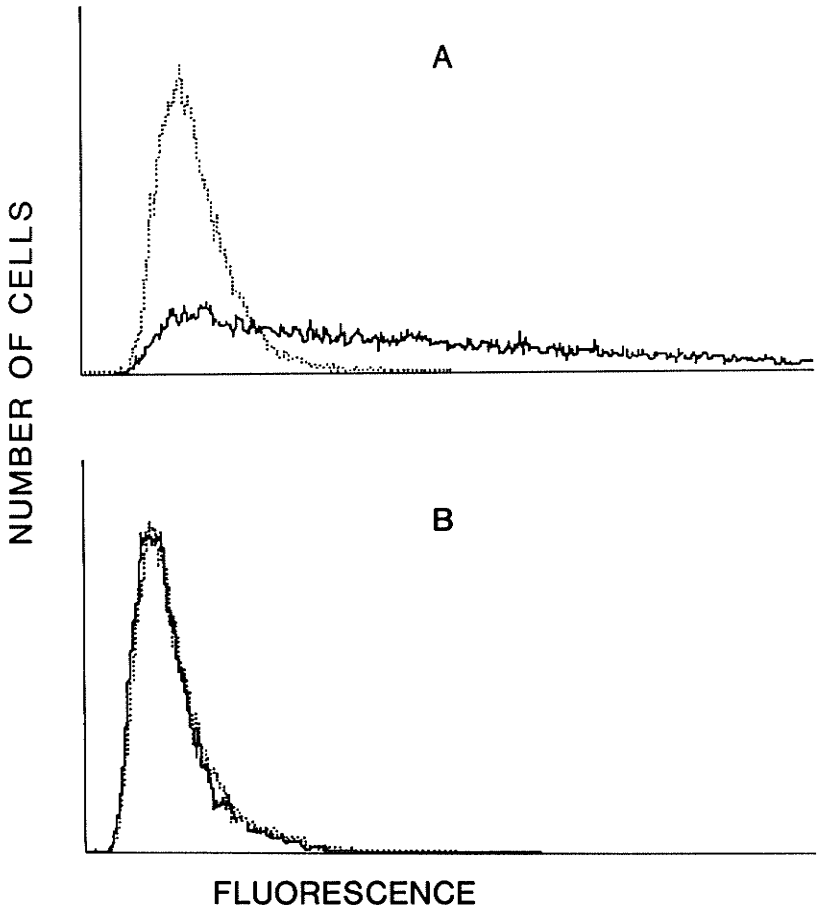

Fig. 3.1. NAb binding profiles of G418-selected, v-H-*ras*/neo^r- and neo^r-infected 10T½ cells. NAb acquisition *in vitro* was assayed for v-H-*ras*/neo^r-infected 10T½ (MOI 0.02; solid line) (A) and for neo^r-infected 10T½ (MOI 0.02; solid line) (B) both vs. 10T½ cells (broken line).

3.1) and the mean ± SE of the MCF for NAb binding by three independent *ras*/neo^r-infected populations was 107.7 ± 21.6 compared with 55.0 ± 14.2 for 10T½ with the difference being significant ($P_{td} < 0.04$). Similar retroviral infections of 10T½ with the neo^r-bearing SVX virus employed over a range of MOI from 0.01 to 0.04 followed by selective growth for 13 days in G418 produced cells which were morphologically indistinguishable from the 10T½ in tissue culture and exhibited no change in NAb binding (Fig. 3.1b, Table 3.1).

Expression of the introduced *ras* oncogene was examined by Western blotting. The *ras*-infected, G418-selected 10T½ cells expressed much more *ras* p21 than the parental 10T½, while the neo^r-infected, G418-selected cells exhibited p21 levels comparable to 10T½ (Fig. 3.2, Table 3.1). The more slowly migrating band observed in the lane corresponding to v-H-*ras*/neo^r-infected 10T½ appears similar to a phosphorylated form of the virally encoded p21 protein previously reported (Shih et al., 1979). Since modification of the murine p21 homolog to the phosphorylated form is not observed (Langbeheim et al., 1980), the presence of the phosphorylated species is diagnostic for expression of the viral-encoded gene product. The doublet which appears in both lane 1 (neo^r-infected 10T½) and lane 3 (parental 10T½) is due to the greater mobility of the processed form of proto-oncogenic p21 compared to its unprocessed precursor (Shih et al., 1982). The lysate of the v-H-*ras*/neo^r-infected 10T½ (lane 2) in fact also includes this doublet, plus a doublet corresponding to processed and unprocessed forms of unphosphorylated v-H-*ras* p21 which migrate with slightly lower mobilities than their respective proto-oncogneic counterparts (Shih et al., 1982).

Table 3.1

NAb binding, tumorigenicity and p21 expression of v-H-*ras*/neo^r-infected and G418-selected 10T½

Cell line	MCF NAb binding[a] ratio experimental / 10T½	Tumorigenicity number	p21[a] ratio experimental / 10T½
10T½	1	0/2	1
10T½ + v-H-*ras*/neo^r	1.96 (3)[b]	2/2	7.21 (2)
10T½ + neo^r	0.90 (3)	0/5	1.01 (1)

[a] The number of independent infections is indicated in parentheses.
[b] $P_{td} < 0.04$ compared with 10T½

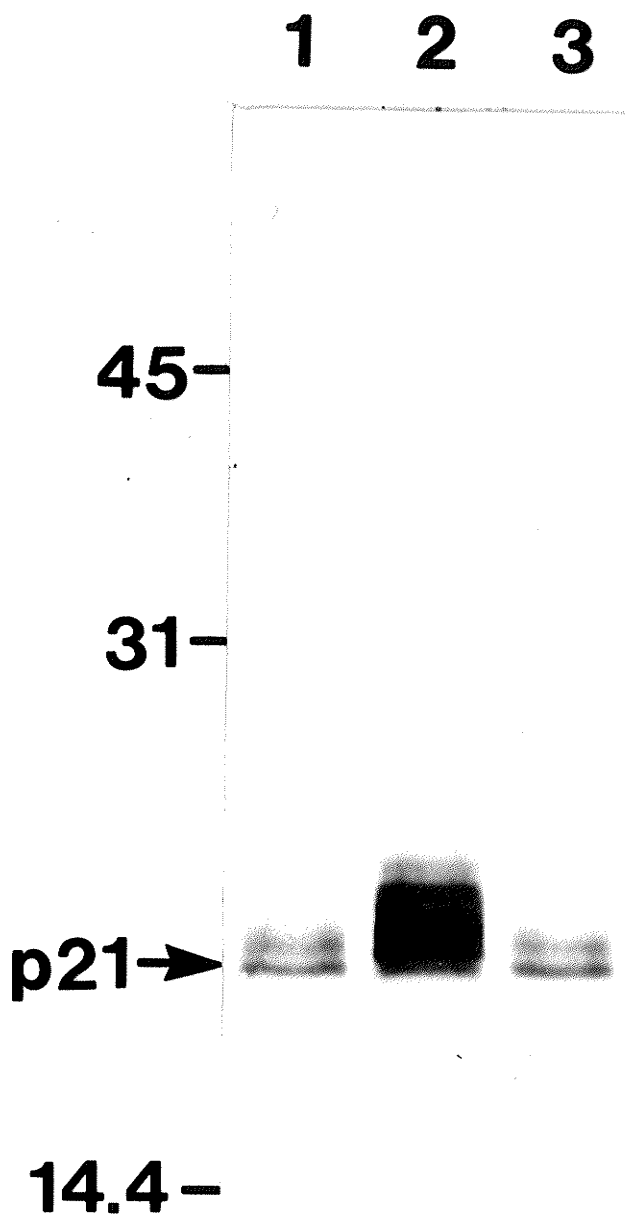

Fig. 3.2. p21 expression of v-H-*ras*/neo^r-infected, G418-selected 10T½. Lanes are as follows: 1 = neo^r-infected 10T½; 2 = v-H-*ras*/neo^r-infected 10T½; 3 = 10T½. Mr for standard markers are shown to the left (X 10^{-3}).

To test whether the v-H-*ras*-infected 10T½ cells were in fact neoplastically transformed, their tumorigenicity was assayed in syngeneic C3H/HeN mice. The v-H-*ras*/neo^r-infected 10T½ were tumorigenic, producing tumors in 2/2 mice inoculated s.c. with 10^6 cells, while neither the parental 10T½ nor the neo^r-infected cells produced tumors (Table 3.1). Taken together the results of the population studies suggested a correspondence between *ras*-induced transformation of 10T½ cells and a prominent increase in their ability to acquire NAb.

Clonal analysis of v-H-*ras*/neo^r-infected 10T½

Since the population of v-H-*ras*/neo^r-infected 10T½ was heterogeneous for NAb binding (Fig. 3.1a), and also likely heterogeneous for *ras* p21 expression and for tumorigenicity, it was uncertain from the population studies if the same cells were in fact positive for all three characteristics. To more directly examine this question, clones were selected based on one of these characteristics, increased NAb binding, and analyzed for the expression of the other two.

The upper 3% of NAb binding cells were selected from the v-H-*ras*/neo^r-infected 10T½ population by fluorescence activated cell sorting. Clones were subsequently generated from this sorted population either by limiting dilution in 96-well plates (Expt. 1, Table 3.2) or by picking isolated colonies from 100 mm tissue culture dishes (Expt. 2, Table 3.2). In both cases the picked clones were transferred to 100 mm tissue culture dishes and subcloned by picking isolated colonies.

Among the 21 clones generated, 10 grew with a flat, organized appearance *in vitro* which was indistinguishable from that of the parental

Table 3.2

Phenotypes of clones isolated following v-H-*ras*/neo^r-infection and G418-selection of 10T½

Expt.	Clone	Morphology	MCF NAb binding ratio[a] expt. / 10T½	Tumorigenicity number	p21 ratio[a] expt. / 10T½
1.	hE5.2	flat, organized	0.73 (1)	0/2	0.50 (3)
	hA9.2	flat, organized	0.82 (1)	0/2	0.53 (3)
	hH10.2	flat, organized	0.82 (1)	0/2	0.54 (3)
	hE12.2	flat, organized	0.85 (1)	0/2	0.58 (3)
	hG9.2	flat, organized	0.96 (1)	ND[b]	ND
	hC5.1	flat, organized	1.01 (1)	ND	ND
	hG6.2	flat, organized	1.11 (1)	0/2	0.52 (3)
	hC3.2	flat, organized	1.12 (1)	0/2	0.44 (3)
	hF1.2	flat, organized	1.17 (1)	0/2	0.68 (3)
	HF2.1	flat, disorganized	2.54 (13)[c]	2/2	9.8 (3)
	HE9.1.2	focus-forming	1.77 (9)[c]	2/2	4.2 (3)
	HF1.1	focus-forming	1.86 (10)[c]	2/2	6.0 (3)
	HC8.1	focus-forming	2.00 (9)[c]	2/2	17.6 (3)
2.	I8.1.1	flat, organized	0.79 (1)	0/2	0.46 (3)
	DT2	focus-forming	1.78 (5)[c]	2/2	8.0 (3)
	I3T2.1	focus-forming	3.24 (4)[c]	2/2	7.6 (3)
	T2	focus-forming	3.35 (1)	ND	ND
	I8T2.1	focus-forming	3.40 (4)[c]	2/2	12.8 (3)
	DT1	focus-forming	3.55 (1)	ND	ND
	I4.1.1.T	focus-forming[d]	2.30 (4)	2/2	10.2 (3)
	I6.1.1.T	focus-forming[d]	2.66 (4)[c]	2/2	8.3 (3)

[a] Number of experiments is indicated in parentheses.
[b] ND, not determined
[c] The differences between the MCF for 10T½ and 8/9 of the tumors tested repeatedly were statistically significant with $P_{ti} < 0.02$ to 0.00001 and $P_{td} < 0.06$ for the remaining clone I4.1.1.T.
[d] These two clones had a very rounded morphology.

10T½. When these flat clones were assayed for NAb binding, all bound levels of NAb which were comparable to that of 10T½ (Table 3.2). Eleven of the clones, however, exhibited *in vitro* growth morphologies characteristic of transformed cells in culture. All of these morphologically transformed clones were increased in NAb binding compared to the parental 10T½, showing increases which ranged from 77% to 255% (Table 3.2). For 8 of the 9 clones analyzed repeatedly, the difference in NAb binding compared to 10T½ was statistically significant, with p_{t1} values ranging from < 0.02 to < 0.00001 and $p< 0.06$ for the remaining clone. The clones produced in both experiments maintained their respective morphological and NAb binding phenotypes in culture for at least 2 months.

p21 expression and tumorigenicity of clones from v-H-*ras*/neo^r-infected 10T½

Nine clones exhibiting morphologically transformed phenotypes and eight flat clones were analyzed for *ras* p21 expression by Western blotting. The nine clones which appeared morphologically transformed and demonstrated increased NAb binding all expressed high levels of p21, ranging from 4-17 times that expressed by 10T½ (Fig. 3.3a, Table 3.2), while all clones which exhibited flat morphologies and were not increased in NAb binding did not express more p21 than 10T½ (Fig. 3.3b, Table 3.2). The phosphorylated form of the viral p21 protein was prominent among the morphologically transformed clones but was not detectable in any of the flat clones, providing evidence that these morphologically normal clones were not expressing the protein product of the introduced oncogene.

When assayed for their ability to form tumors in syngeneic mice, the nine morphologically transformed clones were all tumorigenic at an

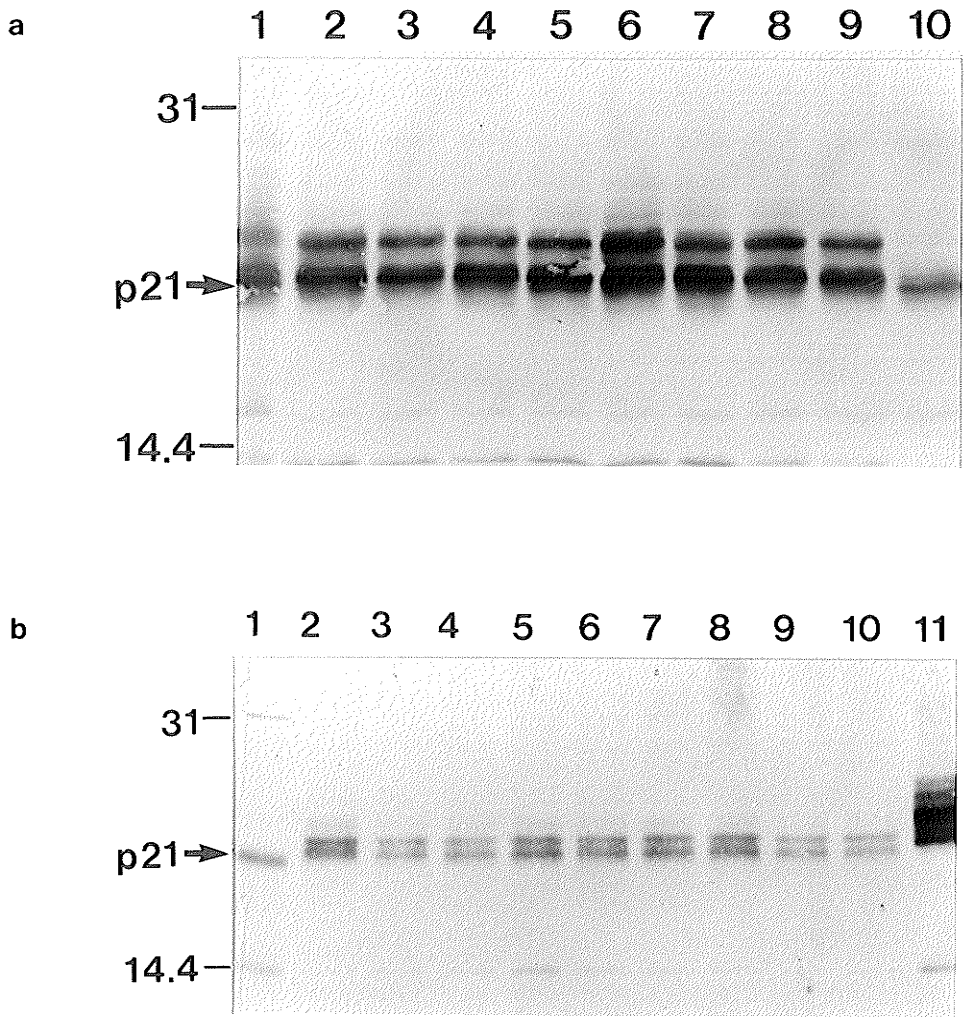

Fig. 3.3. p21 expression of clones from v-H-*ras*/neo^r-infected 10T½. a. Upper panel lanes are as follows: morphologically-transformed clones: 1 = HE9.1.2; 2 = I4.1.1.T; 3 = I6.1.1.T; 4 = DT2; 5 = I8T2.1; 6 = HC8.1; 7 = I3T2.1; 8 = HF1.1; 9 = HF2.1; 10 = the 10T½ parental line. b. Lower panel lanes are as follows: 1 = standard Mr markers; 2 = the 10T½ parental line and flat clones; 3 = hA9.2; 4 = hC3.2; 5 = hE5.2; 6 = hE12.2; 7 = hF1.2; 8 = hG6.2; 9 = hH10.2; 11 = HC8.1, a morphologically-transformed clone.

inoculum of 10^6 cells s.c. while none of the flat clones were able to produce tumors (Table 3.2). Therefore, the clonal analysis of v-H-*ras*/neo^r-infected 10T½ cells revealed a consistent correspondence between 10T½ cells which had undergone v-H-*ras*-induced neoplastic transformation and those expressing an increased NAb binding phenotype.

Relationship between *ras* oncogene expression and NAb binding

The relationship between *ras* oncogene expression in 10T½ and changes in NAb binding was further examined with the use of the 2H1, a 10T½ clone carrying an activated human H-*ras* oncogene under the transcriptional regulation of the mouse metallothionein-I promoter. 2H1 cells express a low basal level of human *ras*-specific mRNA and have an *in vitro* growth morphology similar to 10T½ in the absence of $ZnSO_4$, but show increased *ras* mRNA and exhibit the typical morphological characteristics of neoplastically transformed cells when grown in the presence of 50 μM $ZnSO_4$ (Haliotis et al., 1990). The cells exhibit no changes in morphology in response to 1.5 μM $ZnSO_4$, which is present in the DMEM/F12 medium used to culture the cells in the following experiments.

The effect of the addition of 50 μM $ZnSO_4$ to the culture medium on *ras* p21 protein expression in 2H1 was examined by Western blotting. Within 24 hours following the addition of $ZnSO_4$, p21 reached levels which were about 60% higher than that found in uninduced cells (Fig. 3.4, upper). This level of expression was maintained for at least 4 days of culture in the presence of 50 μM $ZnSO_4$. Conversely, addition of 50 μM $ZnSO_4$ to untransfected parental 10T½ cells did not increase *ras* protein expression (Fig. 3.4, upper).

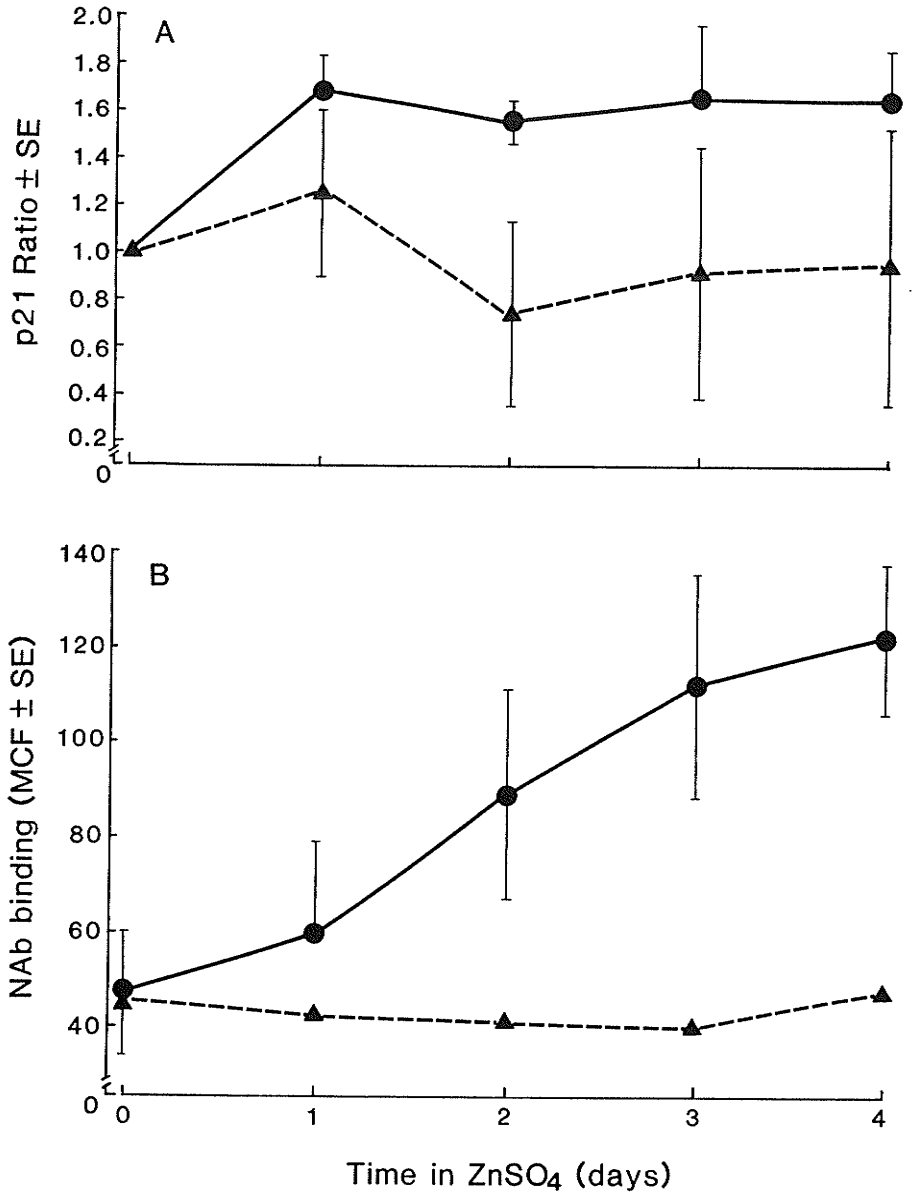

Fig. 3.4 Effect of growth in 50 μM $ZnSO_4$ on A) p21 expression and B) NAb binding of 2H1 (●) and 10T½ (▲). Each point represents the mean ± SE of: 3 independent experiments for NAb binding and p21 expression of 2H1; 2 independent experiments for p21 expression of 10T½; and 1 experiment for NAb binding of 10T½.

The NAb binding of these cells was simultaneously assayed (Fig. 3.4, lower). Following the addition of 50 μM $ZnSO_4$ to the culture medium, the NAb binding of 2H1 was increased within one day compared to uninduced cells and continued to increase for at least 4 days of culture in the presence of Zn. The 2H1 cultured in Zn for 2, 3 or 4 days exhibited significantly higher NAb binding than that of uninduced cells, with increases of 90, 139 and 160% respectively. Conversely, the addition of 50 μM $ZnSO_4$ to either untransfected 10T½ (Fig. 3.4, lower) or the I8T2.1, a 10T½ clone constitutively expressing v-H-*ras* (Table 3.3), did not increase their NAb binding. This result therefore demonstrates directly that increased expression of an activated *ras* oncogene in 10T½ cells results in increased NAb binding. The fact that p21 expression was maximal within 1 day of the addition of $ZnSO_4$, while NAb binding continued to increase for at least 4 days, suggests that the cell surface changes induced following *ras* expression which are responsible for the increased NAb binding are manifested as a late consequence of *ras* protein expression.

We next determined whether the high NAb binding phenotype induced in 2H1 cells upon p21 oncogene expression was dependent upon continued expression of the oncogene protein or was permanently imprinted following transient p21 expression. 2H1 cells were grown for 3 days in medium containing 50 μM $ZnSO_4$ and then transferred to normal culture medium for 1, 2, 3 or 4 days (Table 3.4). The cells cultured in the presence of added Zn^{++} for 3 days expressed more than twice the level of *ras* p21 expressed by the uninduced cells and bound significantly more NAb. p21 expression returned rapidly to the basal level in uninduced cells within 1 day following removal of $ZnSO_4$ from the culture. The NAb binding of the same

Table 3.3

NAb binding by I8T2.1 upon $ZnSO_4$ addition

Growth Conditions	NAb binding[a] MCF ± SE
no $ZnSO_4$	80.42 ± 11.4
+ $ZnSO_4$ 1 day[b]	72.54 ± 10.4
+ $ZnSO_4$ 2 days	73.51 ± 15.7
+ $ZnSO_4$ 3 days	65.79 ± 9.3
+ $ZnSO_4$ 4 days	71.24 ± 16.0

[a] The values are the mean of three experiments.
[b] 50 μM $ZnSO_4$ was added to the culture medium.

Table 3.4

NAb binding and p21 expression by 2H1 upon removal of $ZnSO_4$ from culture

Growth Conditions	NAb binding (MCF ± SE) (4)[a]	p21 Ratio ± SE[b](3)[a]
no $ZnSO_4$	33.4 ± 1.9	1.0
+ $ZnSO_4$ 3 days	69.7 ± 13.1[c]	2.31 ± 0.36[e]
+ $ZnSO_4$ 3 days - $ZnSO_4$ 1 day	84.9 ± 14.6[d]	1.12 ± 0.56
+ $ZnSO_4$ 3 days - $ZnSO_4$ 2 days	41.0 ± 9.7	1.08 ± 0.33
+ $ZnSO_4$ 3 days - $ZnSO_4$ 3 days	32.0 ± 2.8	0.61 ± 0.24
+ $ZnSO_4$ 3 days - $ZnSO_4$ 4 days	27.8 ± 2.8	0.71 ± 0.25

[a] Number of experiments is indicated in parentheses.
[b] Ratio $\frac{\text{expt. treatment}}{\text{no } ZnSO_4}$ ± SE.
[c] $P_{ti} < 0.04$ compared with no $ZnSO_4$.
[d] $P_{ti} < 0.02$ and $P_{td} < 0.03$ compared with no $ZnSO_4$ and + $ZnSO_4$ 3 days, respectively.
[e] $P_{ti} < 0.02$ compared with no $ZnSO_4$.

cells continued to increase for 1 day after removal of Zn^{++} from the culture medium with the cells binding significantly more NAb than those cultured in $ZnSO_4$ for 3 days ($P_{td} < 0.03$). After 2-3 days without Zn^{++} the NAb binding returned to uninduced levels. The magnitude of the increase in NAb binding for cells grown in the absence of $ZnSO_4$ for 1 day following 3 days in $ZnSO_4$ was similar to that observed on day 4 of continuous culture in the presence of $ZnSO_4$, with the MCF NAb binding values being 2.5 and 2.6 times that of ininduced cells respectively. The results demonstrate that the continued expression of the Zn^{++}-inducible H-*ras* oncogene protein is necessary to maintain the high NAb binding phenotype. The cell surface changes which are recognized by NAb therefore, are actively produced through a pathway involving *ras* p21 expression. The more rapid return to basal levels of p21 compared to NAb binding (1 vs 2-3 days) and the observation that 2H1 cells removed from $ZnSO_4$ for 1 day bound significantly more NAb than cells cultured in $ZnSO_4$ for 3 days but equivalent to day 4 cultures, again suggest that the changes in cell phenotype associated with NAb binding are manifested as a late consequence of *ras* p21 expression.

The sensitivity to syngeneic C3H NK cell lysis was assayed for 2H1 cells cultured for increasing periods of time in the presence of 50 μM $ZnSO_4$ (Table 3.5). Sensitivity to NK cytolysis and thus the number of $LU/10^6$ effectors acting against the Zn^{++}-induced 2H1 cells increased rapidly for 2 days and remained at a plateau level on the third day. The differences between reactivity with the Zn^{++}-induced and uninduced 2H1 were statistically significant for days 2 and 3. A similar observation was made using splenic effectors from allogeneic CBA/J mice (data not shown). The increased sensitivity to NK cytolysis was maintained in 2H1 cells cultured

Table 3.5

2H1 sensitivity to NK cytolysis upon addition or removal of $ZnSO_4$ and after prolonged growth in $ZnSO_4$

Expt.#[a]	Growth Conditions	# Lytic Units[b]/10^6 cells ($x10^3$) ± SE
1. (3)	no $ZnSO_4$	3.4 ± 0.8
	+ $ZnSO_4$ 1 day	5.4 ± 1.6
	+ $ZnSO_4$ 2 days	11.2 ± 1.4[c]
	+ $ZnSO_4$ 3 days	10.1 ± 0.1[d]
	+ $ZnSO_4$ 3 days - $ZnSO_4$ 1 day	4.8 ± 0.2
2. (1)	no $ZnSO_4$	4.4
	+ $ZnSO_4$ 3 days	8.2
	+ $ZnSO_4$ 7 days	11.6
3. (2)	$10T_{\frac{1}{2}}$	3.5 ± 0.8
	$10T_{\frac{1}{2}}$ + $ZnSO_4$ 3 days	3.4 ± 0.1

[a] Number of experiments is indicated in parentheses.
[b] 1 LU kills 30% of targets.
[c] $P_{ti} < 0.01$ compared with no $ZnSO_4$.
[d] $P_{ti} < 0.002$ compared with no $ZnSO_4$

in the presence of 50 μM $ZnSO_4$ for at least 7 days (Table 3.5). Since culture of non-transfected 10T½ cells in the presence of 50 μM $ZnSO_4$ for 3 days did not change their sensitivity to NK cell lysis (Table 3.5), the increased sensitivity observed for 2H1 was not due to a direct effect of $ZnSO_4$ on the cells. However, while growth in added $ZnSO_4$ did not alter the spontaneous lysis of 10T½ cells, the 18 hour spontaneous release of ^{51}Cr label by the Zn^{++}-induced 2H1 was almost double that of the uninduced 2H1 cells (data not shown) so that a general increase in fragility may contribute to the increased NK susceptibility of the Zn^{++}-induced p21-expressing 2H1. Nevertheless, the results are consistent with previous reports of increased 10T½ sensitivity to NK cell lysis following introduction of a *ras* oncogene leading to constitutive p21 expression (Greenberg et al., 1987a).

The requirement for continued *ras* p21 oncogene expression for the maintenance of the NK-sensitive phenotype was also assayed (Table 3.5). The NK sensitivity of the 2H1 cells cultured for 3 days in the presence of 50 μM $ZnSO_4$ was 3 times that of uninduced cells, while the sensitivity of 2H1 cultured in the absence of Zn for 1 day following 3 days in 50 μM $ZnSO_4$ was in the uninduced range. Therefore, the increased 2H1 sensitivity to NK lysis was associated with an increased steady-state level of *ras* oncogene p21 expression, not with the rise or fall in p21 level as proposed previously by Anderson et al (1989) for a related conditionally *ras*-expressing 10T½ clone. The more rapid reversion of NK cell sensitivity compared to the high NAb binding phenotype seen upon removal of $ZnSO_4$ from the culture medium (Table 3.4) suggests that different mechanisms associated with the activity of the *ras* p21 protein are responsible for

the increases in 10T½ sensitivity to these two effectors of natural resistance.

Serum NAb reduction of tumor frequency *in vivo*

Following a protocol established to examine the impact of i.v. administered serum NAb on the tumor forming capacity of the NAb binding RI-28 lymphoma in syngeneic xid-bearing B cell deficient mice (Chow, D.A. unpubl. obs.), i.v. injections of 0.3 ml of normal serum from C3H/HeN mice were given on days -2, -1 and 0 prior to the s.c. injection of a threshold inoculum of the zinc-induced 2H1. Thus aliquots of 10^5 2H1 cells grown in tissue culture containing 50 μM $ZnSO_4$ for 4 days were injected s.c. into groups of 6 C3H/HeN mice given drinking water containing 25 mM $ZnSO_4$ for one week and pretreated with 3 injections of serum NAb or the same volume of saline. Zn was maintained in the drinking water throughout the experiment. The tumors began to appear by day 11 and the tumor frequency at 30 days was markedly reduced in the serum NAb-treated mice with 1/6 exhibiting a tumor at the site of injection compared with 3/6 for the animals given saline (Fig. 3.5).

DISCUSSION

The essence of any form of immune defense is the ability of the effectors to discriminate between self and nonself, being specifically responsive to the latter while remaining tolerant of the former. Analagously, for an antitumor surveillance system to be effective, it is necessary that the effector mechanisms recognize changes associated with cellular transformation. We have demonstrated that NAb meet this criterion

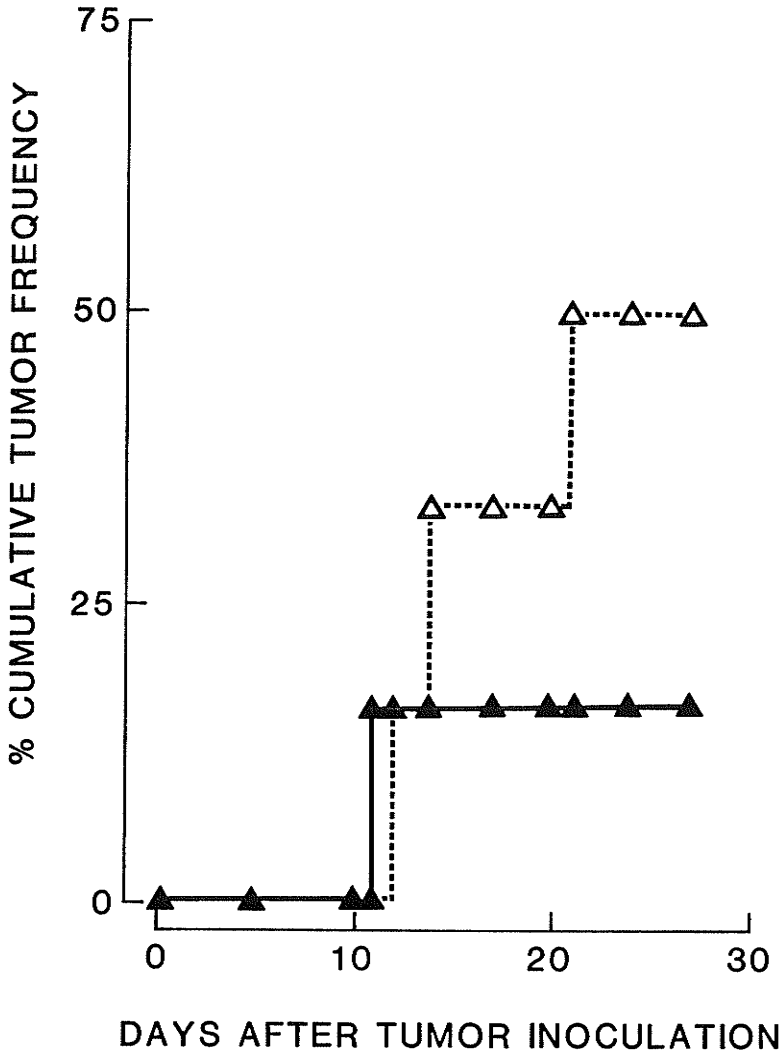

Fig. 3.5. Cumulative tumor frequency of Zn^{++}-induced 2H1 in serum NAb-treated mice. Aliquots of 10^5 Zn^{++}-induced 2H1 cells were injected s.c. into groups of 6 C3H/HeN mice maintained on 25 mM $ZnSO_4$ in their drinking water and repeatedly pretreated i.v. with either serum NAb (▲——▲) or saline (△---△).

of an effector of antitumor natural surveillance by utilizing an *in vitro* model of tumorigenesis in which cellular transformation induced by the *ras* oncogene was accompanied by an increased NAb binding capacity.

The introduction of the v-H-*ras* oncogene together with the neo^r gene into 10T½ followed by selection in G418 resulted in a marked and heterogeneous increase in NAb binding measured by flow cytometry. The *ras*-infected population expressed the oncogenic p21 protein and was tumorigenic. Clonal analysis of this population revealed a significant proportion of cells which despite their G418 resistance did not express the *ras* oncogene, were not tumorigenic and did not show increased NAb binding. In contrast, clones which expressed v-H-*ras* p21 were tumorigenic and showed increased NAb binding.

The effect on NAb binding of *ras* oncogene expression in 10T½ was further demonstrated with the use of 2H1 cells carrying a Zn-inducible human EJ-H-*ras* oncogene. The addition of 50 μM $ZnSO_4$ to 2H1 induced *ras* p21 expression and a subsequent increase in NAb binding. This result demonstrates that the cell surface changes associated with increased NAb binding are not due to a specific property of the viral form of the *ras* oncogene, but are associated with expression of an activated H-*ras* oncogene. The increase in NAb binding was dependent upon continued induction and expression of the *ras* oncogene, since removal of $ZnSO_4$ from the culture medium was followed by a return to baseline levels for both p21 and NAb binding.

The results also showed that the same Zn-inducible cells exhibited increased sensitivity to NK cell lysis upon Zn^{++}-induction of *ras* p21 expression. The NK sensitivity of the cells returned to uninduced levels

following removal of $ZnSO_4$ from the culture medium demonstrating the requirement for *ras* oncogene p21 expression in the maintenance of the NK sensitive state. The results, therefore, are consistent with a phase of tumor development which is susceptible to both NAb and NK cells, fulfilling a fundamental requirement for the effective participation of these mediators in the defense against incipient neoplasia.

A kinetic study revealed that p21 levels in 2H1 reached a maximal level within one day of Zn^{++}-induction and remained constant thereafter. However, increases in NAb binding occurred more slowly and continued up until at least 4 days. Assays carried out 7-28 days following exposure to zinc all showed increased NAb binding (data not shown). In addition, following culture of the cells in the presence of 50 μM $ZnSO_4$ for 3 days, removal of Zn^{++} from the medium resulted in p21 levels returning to near control levels within one day, while NAb binding continued to increase for 1 day in the absence of Zn^{++} before returning rapidly to the uninduced levels. Therefore, a comparison of the kinetics of zinc-induced *ras* p21 expression and NAb binding suggests that the accessibility of the NAb binding molecules occurs as a late consequence of *ras* p21 activity. Since the downstream components of the *ras* p21-inclusive signalling pathway(s) remain obscure, the biochemical events which link *ras* p21 activity to the cell surface changes responsible for the increased NAb binding are not known. The gradual increase in NAb binding compared to the more rapid rise in p21 expression suggests that there exists a slow, rate-limiting biochemical process in the pathway between *ras* activity and changes in NAb binding. The late appearance of increased NAb binding following p21 expression suggests that the cellular changes involved are likely not

related to the effects of *ras* on proliferation since they occur rapidly (Feramisco et al., 1984). The reversibility of the induction of NAb binding also suggests that the cellular changes are not associated with the induction of a differentiation pathway within the cells.

Interestingly, the kinetics of induction of sensitivity to NK cell cytolysis appear to differ from that of NAb susceptibility, since NK sensitivity returned to uninduced levels 1 day after removing the cells from $ZnSO_4$. This suggests that although sensitivity to both effectors arises following *ras* oncongene expression in 10T½, different cellular changes are likely responsible for the susceptibility to NAb vs NK cells. This result is consistent with previous studies in which differential changes in sensitivity to NAb vs NK cells have been observed (Gronberg et al., 1985; Tough and Chow, 1988).

The results relating to the relationship between p21 expression and NK cell sensitivity are not consistent with a previous report which utilized the conditionally *ras*-expressing 212 clone from which the 2H1 was subcloned (Anderson et al., 1989). In the earlier study, following an initial increase in NK sensitivity during the first 24 hours after Zn^{++} induction of p21, the NK sensitivity gradually decreased back to uninduced levels within the next 48 hours in Zn^{++} even though p21 levels remained constant. In addition, removal of Zn^{++} from the culture medium was associated with a decrease in p21 expression and an increase in NK susceptibility. Although the reason for the discrepancies is not known, a likely possibility is the fact that different clones were utilized. If, as is often proposed, *ras* protein functions as an intermediate in signal transduction, the manifestations of its activity would depend upon the

existing biochemical pathways present in the cell. Very likely for this reason, *ras* has been shown upon transfection to induce different effects depending upon the type of recipient employed. For example, the introduction of activated *ras* oncogenes into rat pheochromocytoma (PC12) cells or Schwann cells induces morphological differentiation (Bar-Sagi and Feramisco, 1985) or cell cycle arrest (Ridley et al., 1988) respectively. Since 10T½ is an aneuploid cell line (Reznikoff et al., 1973) which exhibits fluctuations in chromosome number (J.A. Wright, personal communication), it is possible that the clone used in our study may differ from that used in the previous paper with regard to the availability of biochemical pathways with which *ras* may interact. Alternatively, the differences between our results and the previous report may simply be one of kinetics such that had we maintained our cells in Zn^{++} for an even longer period of time (> 7 days), their NK sensitivity may have eventually returned to uninduced levels. Nevertheless, the present studies confirm the observation in 10T½ cells constitutively expressing *ras* p21 (Greenberg et al., 1987a) that increased sensitivity to NK cell lysis is associated with *ras* oncogene p21 expression in 10T½. The present study further shows that sensitivity to NAb is induced at the same time. The data therefore provide evidence that a basic requirement is fulfilled for a natural tumor surveillance system involving both NAb and NK cells.

The increases in NAb binding of the *ras*-transformed clones compared to 10T½ (approximately 80-250%), and Zn^{++}-induced versus uninduced 2H1 (160%), are comparable in magnitude to observed differences in NAb binding which have been shown to have relevance *in vivo* (Tough and Chow, 1988; Chow and Bennet, 1989). Tumor variants selected *in vitro* for a 100%

increase in NAb binding produced half as many tumors upon injection into syngeneic mice (Chapter 2; Tough and Chow, 1988), while normal (CBA/N X CBA/J) F_1 xid/+ female mice demonstrated twice the serum antitumor NAb reactivity and half the tumor susceptibility of the B cell-deficient (CBA/N X CBA/J) F_1 xid/- male mice (Chow and Bennet, 1989). These and other previous studies utilizing transplantable tumors consistently supported a role for tumor-reactive NAb in the defense against small tumor inocula (Greenberg et al., 1983; Menard et al., 1981; Chow et al., 1981a; Ehrlich et al., 1984; Chow and Bennet, 1989; Itaya et al., 1982; Chow et al., 1983; Chow, 1984a; Brown and Chow, 1985; Brown et al., 1986; Chow, 1984b; Chow and Chan, 1988; Tough and Chow, 1988) while the preferential *in vitro* reactivity of a monoclonal NAb against low rather than high tumorigenic clones of c-H-*ras*-transformed NIH 3T3 cells (Agassy-Cahalon et al., 1988) suggested the *in vivo* activity of NAb against *ras*-transformed cells. In the current study, the marked reduction in the number of early tumors which appeared following the s.c. inoculation of the high NAb binding Zn-induced 2H1 into normal syngeneic serum NAb-treated mice compared with saline-treated controls is consistent with a role for NAb in the *in vivo* defense against NAb-binding tumors, including *ras*-transformed cells. Although this difference in tumor incidence was not statistically significant due to the small sample size (n = 6), using the same approach, consistently lower tumor frequencies have been observed following the injection of the NAb-binding RI-28 lymphoma into normal syngeneic serum-injected, xid-bearing B cell-deficient mice compared to saline-treated controls in 4 separate experiments (data not shown). With the inclusion of this data the difference between the number of tumors appearing by day 30

in the serum-treated mice versus controls was statistically significant with $P_{td} < 0.007$. In addition, pre-treatment of xid mice with ammonium sulfate precipitated serum has also been shown to reduce the frequency of tumor formation following RI-28 injection (Chow, unpubl. obs.), providing further support for the idea that the tumor-protective effect of the serum injections was related to NAb activity. Therefore, the present results, taken in the context of the previous studies, provide strong evidence that NAb can recognize changes in cellular characteristics associated with *ras*-induced transformation of 10T½ and support the hypothesis that NAb would be effective in detecting the early events of tumorigenesis, consistent with a role in tumor surveillance.

While this is the first demonstration of increased NAb reactivity associated with oncogenic transformation, increased sensitivity to cytolysis by NK cells has been observed following *ras* transformation of rat-1 (Johnson et al., 1985) and 10T½ cells (Trimble et al., 1986; Greenberg et al., 1987a) but not NIH 3T3 cells (Greenberg et al., 1987a). Balb/c 3T3 fibroblasts have also been shown to acquire NK sensitivity following transformation with methylcholanthrene (Collins et al., 1981), an event which may be associated with mutation at the *ras* locus since methylcholanthrene-transformed 10T½ cells have been found to contain activated *ras* genes (Parada and Weinberg, 1983). A similar link between oncogenesis, chemical carcinogenesis and NAb reactivity may also exist. A natural monoclonal antibody which is cytotoxic for syngeneic and allogeneic lymphomas in the presence of complement decreased the number of carcinogen-induced tumor foci in the lungs of mice exposed to urethane (Agassy-Cahalon et al., 1988), while in a separate investigation both

benign papillomas and squamous cell carcinomas initiated in mouse skin by urethane and promoted with a phorbol ester were shown to contain activated cellular H-*ras* oncogenes (Bonham et al., 1989).

The increased NK sensitivity of *ras*-transformed 10T½ was shown to be relevant *in vivo* through the inverse correlation of tumor susceptibility to NK cells *in vitro* with the ability of tumor cells to arrest and survive in the lungs for the first 48 hours after intravenous inoculation (Greenberg et al., 1987b). The higher binding of a tumor-reactive natural monoclonal antibody to low rather than high tumorigenic clones of c-H-*ras*-transformed NIH 3T3 cells shown previously (Agassy-Cahalon et al., 1988) and the reduction of early-appearing *ras*-expressing tumors by i.v. injection of NAb shown in the present study are similarly consistent with an NAb-mediated defense against *ras*-transformed cells *in vivo* . Therefore, the evidence supports the concept of a natural immune surveillance system in which NAb and NK cells are both able to recognize and eliminate aberrant cells early in tumor development.

CHAPTER 4

MODIFICATION OF *RAS* ONCOGENE EFFECTS ON NATURAL ANTIBODY BINDING BY CELLULAR GENES

ABSTRACT

The extent to which *ras* oncogene-induced neoplastic transformation is associated with increased NAb binding was investigated by examining *ras* transformed 10T½ clones produced by two different transformation procedures, as well as *ras* transformed NIH 3T3 cells. Among 4 T24-H-*ras*-expressing 10T½ clones, a single clone selected as a G418^r colony following *ras* transfection, NR4, demonstrated a 76% increase in NAb binding compared to 10T½, while three clones selected as foci following *ras* transfection without G418 selection, CIRAS-1, -2 and -3, showed no increase in NAb binding. NR4 expressed the second highest *ras* p21 protein level but was the least tumorigenic, yielding a tumor frequency of 50%, while CIRAS-1, -2 and -3 had tumor frequencies of 92, 100 and 100% respectively following injection of 10^4 cells s.c. into syngeneic mice. Furthermore, the tumor incidence on day 9 following injection correlated inversely with the NAb binding of these 4 cell lines. Since our previous study of v-H-*ras*/neo^r-infected, G418-selected 10T½ had shown a consistent association between v-H-*ras*-induced 10T½ transformation and increased NAb binding (Chapter 3; Tough and Chow, 1991), it was hypothesized that the low NAb binding of CIRAS-1, -2 and -3 may have been related to the selection of these clones for focus formation rather than G418-resistance following *ras* oncogene intoduction. Nine cell lines selected as foci in the absence of G418 following v-H-*ras*/neo^r-infection of 10T½ all expressed high levels of v-H-*ras* p21 and were highly tumorigenic. Two of the nine were not increased in NAb binding compared to 10T½, while the remainder exhibited significant increases, ranging from 33-83%. On average, the nine focus-selected, v-H-*ras*/neo^r-infected 10T½ clones were both lower in NAb binding and more

tumorigenic than nine v-H-*ras*/neo^r-infected, G418-selected, FACS-sorted 10T½ clones produced in the previous study (Chapter 3; Tough and Chow, 1991). Comparisons of the NAb binding of somatic cell hybrids formed in individual fusions between 3 different high NAb binding *ras*-transformed 10T½ clones and either a low NAb binding *ras*-transformed 10T½ clone or a control drug-resistant clone suggested the presence, in both high and low NAb binding cells, of genes which can modify the effect of *ras* oncogene expression on the NAb binding phenotype. In addition, populations of G418^r cells obtained following v-H-*ras*/neo^r-infection and G418 selection of NIH 3T3 expressed v-H-*ras* p21 and were tumorigenic in Swiss mice but showed small decreases in NAb binding compared to parental cells. Overall, the results demonstrate that the ability of *ras* to induce increased NAb binding is dependent upon other genes in the recipient cell, and is not dependent solely on *ras* induction of neoplastic transformation.

INTRODUCTION

If NAb are to be effective in the defense against incipient neoplasia yet remain neutral with regard to the normal cells of the host, there must exist a rationale to explain how the newly arising aberrant cells may be selectively eliminated by NAb. One possibility is that NAb show increased binding to neoplastic cells compared to the normal cells from which they originated. Our previous study demonstrated that constitutive expression of the v-H-*ras* oncogene in v-H-*ras*/neo^r-infected and G418-selected 10T½ (Tough and Chow, 1991), or inducible expression of the activated human H-*ras* oncogene in 10T½ (Tough et al., 1992) was

associated with increased NAb binding (see Chapter 3). This result was consistent with a phase of tumor development which was sensitive to NAb-mediated defense by virtue of its increased NAb binding phenotype.

The mechanism by which *ras* oncogene expression in 10T½ led to increased NAb binding is not known. A comparison of the kinetics of changes in NAb binding to those in *ras* protein levels in a 10T½ clone carrying a Zn^{++}-inducible H-*ras* oncogene indicated that the cell surface changes associated with increased NAb binding were manifested as a late consequence of p21 expression and were thus likely far downstream of *ras* activity (Chapter 3; Tough et al., 1992).

Although the transforming properties of *ras* oncogenes have been well studied , the function of the normal *ras* protein in cells remains unknown. The structural and biochemical homology of *ras* to signal transducing G proteins has led to the suggestion that *ras* may similarly be involved in transducing signals from growth factor receptors. However, neither the upsteam nor downstream components of the signalling pathway with which *ras* may be involved are known. The phenotypic consequences of *ras* oncogene expression have been shown to be dependent upon the particular cell in which *ras* is expressed. While many cell lines become neoplastically transformed by the *ras* oncogene, expression of an activated *ras* oncogene is associated with growth arrest of both the rat embryo fibroblast cell line REF52 (Franza et al., 1986) and rat Schwann cells (Ridley et al., 1988), maturation of *Xenopus* oocytes (Sadler et al., 1990) and differentiation of both F9 embryonal carcinoma cells (Yamaguchi-Iwai et al., 1990) and the rat pheochromocytoma cell line PC12 (Bar-Sagi and Feramisco, 1985). These cell type-specific effects of *ras* have been shown

to include changes in susceptibility to proposed mediators of natural antitumor resistance. While expression of an activated *ras* oncogene in 10T½ (Trimble et al., 1986; Greenberg et al., 1987a), the rat-1 cell line (Johnson et al., 1985) or the human breast cancer cell line MCF-7 (Screpanti et al., 1991) led to increased sensitivity to NK cell lysis, similar expression in NIH 3T3 (Greenberg et al., 1987a) or a human colorectal carcinoma cell line (Bagli et al., 1990) resulted in either no change or reduced NK sensitivity respectively.

Since the consequences of *ras* oncogene expression may be specific to a particular cell, it was important to determine whether the observation of increased NAb binding following *ras*-induced 10T½ transformation can be extended to other cells. Presently we have examined the NAb binding of *ras* transformed 10T½ clones generated *in vitro* by two different methods and of v-H-*ras* transformed NIH 3T3 cells. In addition, we have examined the NAb binding of somatic cell hybrids produced by fusion of low and high NAb binding *ras* oncogene-expressing cells. The results suggest that *ras* induced changes in NAb binding can be modified by other genes present in the recipient cell and are not strictly associated with neoplastic transformation.

MATERIALS AND METHODS

Mice and Sera

C3H/HeN and Swiss mice were obtained from the University of Manitoba vivarium (Winnipeg, Man.). Whole serum NAb was obtained from normal male mice as described in Chapter 3.

Cell Lines

The continuous fibroblast cell lines 10T½ and NIH 3T3 were examined. NIH 3T3 was derived from outbred Swiss mouse embryos (Jainchill et al., 1969). NR3, NR4, CIRAS-1, CIRAS-2 and CIRAS-3, which were derived from 10T½ following transfection with a plasmid carrying the activated human T24-H-*ras* oncogene and the neo^r gene (Egan et al., 1987a), and 10T½RAS (Egan et al., 1989), which was derived from 10T½ following co-transfection with pEJ (Shih et al., 1982), a plasmid encoding the T24/EJ-H-*ras* allele from its own promoter and the hygromycin-resistance (hygr^r)-bearing PY3 (Blochlinger et al., 1984) were provided by A.H. Greenberg (University of Manitoba). SVX was cloned from a neo^r-infected 10T½ population (see Chapter 3) by picking an isolated colony from cells plated at low concentration on a 100 mm tissue culture dish (10 colonies per dish). All cells were maintained in D-MEM/F12 (Gibco) containing 10% FBS (Flow).

Recombinant Vectors and Gene Transfer

The defective retroviral vectors described in Chapter 3 were employed to introduce v-H-*ras*/neo^r or neo^r into the indicated cell line. Raszip 6- or SVX-infection and G418-selection of NIH 3T3 were carried out as described for 10T½ in Chapter 3, with SVX-infection done at an MOI of 0.02. For the generation of v-H-*ras*/neo^r-infected, focus-selected 10T½ cell lines, cells were grown in medium without G418 following v-H-*ras*/neo^r-infection at an MOI of 0.02. The transformed foci which arose in the resulting monolayer 16-23 days later were picked and subsequently shown to be G418-resistant. The hygr^r-bearing plasmid PY3 was transfected into 10T½ using the calcium phosphate method (Wigler et al., 1977). Following

transfection, cells were grown in medium containing 200 μg hygromycin B (Sigma)/ml for 13 days and an isolated $hygr^r$ colony was picked. The resulting cell line was called PY3.

Fluorescence-Detected NAb Binding

NAb binding was measured as described in Chapter 3. Normal male C3H/HeN or outbred Swiss mice were the source of NAb for 10T½- or NIH 3T3-derived cell lines respectively. Binding of C3H/HeN-derived NAb to NIH 3T3-derived cell lines was also assessed where indicated.

Western Blotting

Ras p21 protein expression was determined as described in Chapter 3.

Somatic Cell Hybridization

A modification of the procedure described by Fazekas De St. Groth and Scheidegger (1980) was employed. Following 3 washes in serum-free D-MEM/F12 (SFD), cell fusion partners were resuspended in SFD at equal cell numbers (2-2.5 X 10^6) and pelleted. A 0.5 ml aliquot of fusing solution, which consisted of 5 g polyethylene glycol (PEG 3350; Sigma), 0.5 ml dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ) and 5 ml of double distilled, deionized H_2O, was added dropwise to the pellet over 30 seconds with agitation. The mixture was incubated at 37° C for 90 seconds with agitation. Subsequently, SFD was added dropwise to the mixture as follows: 0.5 ml over the first 30 seconds, 1.5 ml over the next 30 seconds and 8 ml over the next 60 seconds. The mixture was then diluted to 25 ml with SFD and incubated for 5 min at room temperature. Cells were centifuged, washed

once in SFD and resuspended in D-MEM/F12 containing 10% FBS, 400 μg G418/ml and 200 μg hygromycin B/ml. Following growth in selective medium for 14 days, the entire population of drug resistant cells was examined.

Tumorigenicity Assay

Tumorigenicity was assessed as described in Chapter 3. For 10T½-derived cell lines, 10^4 cells in 100 μl HBSS were injected s.c. into syngeneic C3H/HeN mice. For NIH 3T3-derived cell lines, 10^6 cells in 200 μl HBSS were injected into outbred Swiss mice.

In Vitro Growth Rate Determination

The *in vitro* growth rates were obtained from cells grown to subconfluence from various initial plating densities as indicated in Chapter 2.

Statistics

Statistical significance of differences in NAb binding was determined by the Student's T test: td or ti.

RESULTS

Examination of T24-H-*ras*-transfected 10T½ clones

We initially examined five cell lines which were derived from 10T½ following transfection with the activated H-*ras* oncogene of the human bladder carcinoma cell line T24 (T24-H-*ras*), and which have been described previously (Egan et al., 1987a). Two of the clones, NR3 and NR4, were

derived as G418-resistant colonies following transfection with the pAL8A plasmid, which contains T24-H-*ras* and the neo^r gene, while the three remaining clones, CIRAS-1,-2 and -3, were cloned as transformed foci in the absence of G418 selection following transfection and subsequently shown to be G418-resistant (Egan et al., 1987a).

The expression of *ras* protein p21 in these cells was examined by Western blotting (Fig. 4.1, Table 4.1). NR3 exhibited little, if any increase in p21 expression compared to 10T½. CIRAS-3 expressed the most p21, 6.1 times that found in 10T½, followed by NR4 (3.3 times), CIRAS-2 (3 times) and CIRAS-1 (1.6 times). When the NAb binding of these 5 cell lines was examined, it was found that among the *ras* oncogene expressing clones, only NR4 showed increased NAb binding compared to 10T½ (Table 4.1). While NR4 bound significantly more NAb than 10T½ (76%), CIRAS-1 and -3 exhibited similar NAb binding to 10T½ while CIRAS-2 bound 30% less, although this difference was not significant. This result therefore demonstrated that not all *ras* transformed 10T½ cells were increased in NAb binding. Further, since CIRAS-1, -2 and -3 were tumorigenic (Egan et al., 1987a) but not increased in NAb binding, the data suggested that the effects of *ras* on the cell which are manifested as increased NAb binding are not strictly associated with the transformed state.

It has been previously shown that all five of the *ras* transfected clones were tumorigenic in syngenic C3H/HeN mice when injected s.c. at a dose of 3 X 10^5 cells. At this dose, NR4, CIRAS-1, -2 and -3 all produced tumors in 100% of recipient mice, while NR3 yielded a 75% tumor frequency, although the tumors produced by the latter cell line arose following a latency period which was 4-7 times longer than that of the other tumors

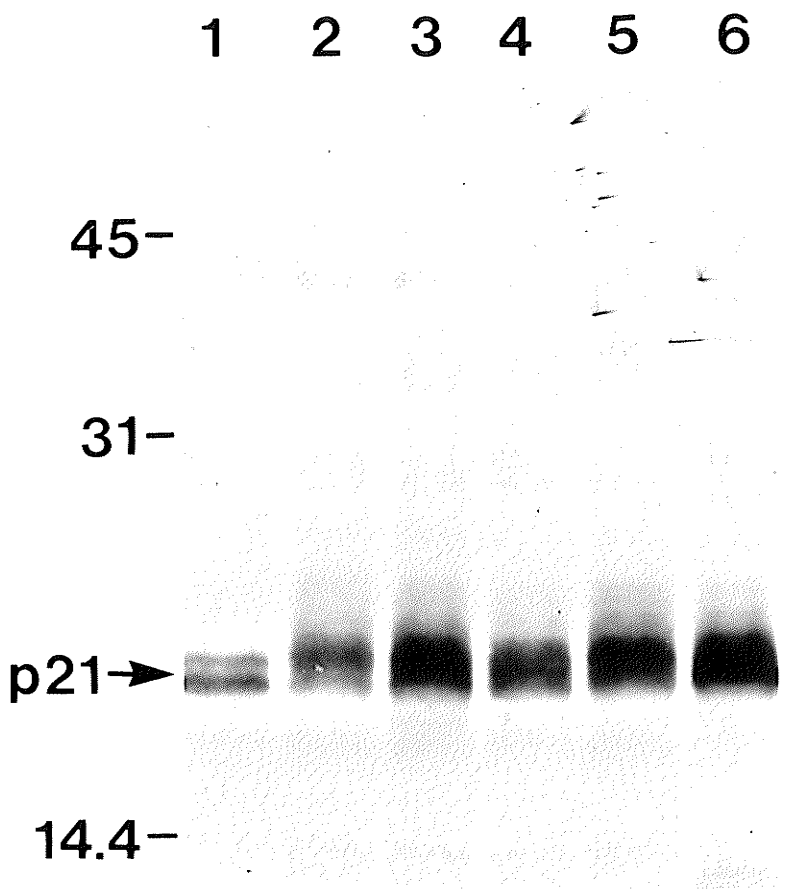

Fig. 4.1. p21 expression of T24-H-*ras*-transfected 10T½. Lanes are as follows: 1 = 10T½; 2 = NR3; 3 = NR4; 4 = CIRAS-1; 5 = CIRAS-2; 6 = CIRAS-3. Mr for standard markers are shown to the left (X 10^{-3}).

Table 4.1

NAb binding, Tumorigenicity and p21 expression of T24-H-*ras*-transfected 10T½ clones

Cell line	NAb binding MCF ± SE (3)[a]	Tumorigenicity number	Tumorigenicity latency (days ± SE)	p21 ratio (3)[a] *expt.* 10T½ ± SE
10T½	65.3 ± 20.1	0/11	-	1
NR3[b]	67.9 ± 14.7	1/12	75	1.2 ± 0.5
NR4[b]	115.1 ± 20.2[c]	7/14	19.9 ± 4.7	3.3 ± 1.3
CIRAS-1[d]	72.6 ± 12.7	13/14	11.8 ± 1.2	1.6 ± 0.6
CIRAS-2[d]	45.1 ± 7.0	14/14	10.6 ± 0.6	3.0 ± 1.0
CIRAS-3[d]	73.4 ± 9.4	14/14	21.1 ± 3.1	6.1 ± 3.1

[a] Number of experiments is indicated in parentheses.
[b] NR3 and NR4 were selected as G418-resistant colonies following T24-H-*ras*/neo^r-transfection and G418 selection (Egan et al., 1987a).
[c] $P_{td} < 0.01$ compared to 10T½.
[d] CIRAS-1, -2 and -3 were selected as foci at confluence following T24-H-*ras* + neo^r cotransfection without subsequent G418 selection (Egan et al., 1987a).

and frequently regressed (Egan et al., 1987a). In order to determine if differences existed between the tumorigenicity of the various clones, their tumor forming capacity was assessed at the lower inoculum of 10^4 cells s.c. (Table 4.1). CIRAS-1, -2 and -3 were all highly tumorigenic, producing tumors at frequencies of 13/14, 14/14 and 14/14 respectively. NR4 was moderately tumorigenic yielding a 50% tumor frequency, while NR3 was almost non-tumorigenic at this dose, producing a single tumor with an extremely long latency (75 days) in 1/12 mice. The long latency of NR3 suggests the presence of rare tumorigenic variants within a largely non-tumorigenic population. This observation, along with little or no increase in p21 expression compared to 10T½ as assessed by Western blotting, indicated that the NR3 cell line, with the possible exception of a minor subpopulation, was not expressing the *ras* oncogene. Comparison of p21 expression with tumorigenicity failed to show a correlation between these two parameters, since NR4 expressed the second highest level of *ras* p21 but exhibited the second lowest tumor forming capacity (Table 4.1). The higher NAb binding and lower tumorigenicity of NR4 compared to CIRAS-1, -2 and -3 was consistent with the inverse correlation between NAb binding and tumorigenicity observed for *in vitro* selected murine lymphomas (Chapter 2; Tough and Chow, 1988). A further examination of the early appearance of tumors following the s.c. inoculation of the four *ras* oncogene expressing cell lines into syngeneic mice showed a statistically significant inverse correlation between tumor incidence on day 9 and NAb binding, with the correlation coefficient $r = -0.9898$ and $P_t < 0.02$ (Fig. 4.2). This result, combined with the previous observation that prior injection of NAb reduced the early tumor appearance following injection of Zn-induced 2H1 cells

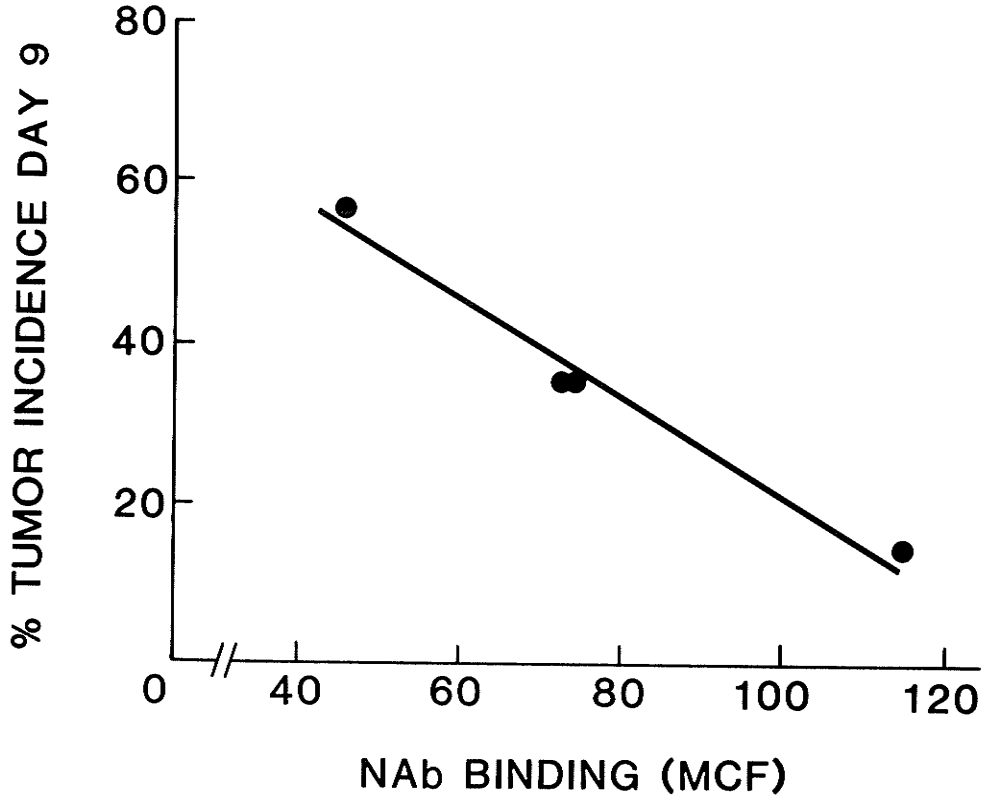

Fig. 4.2. Relationship between tumor incidence on day 9 and NAb binding. Aliquots of 10^4 cells were injected s.c. into C3H/HeN mice. The points from left to right represent CIRAS-2, CIRAS-1, CIRAS-3 and NR4 respectively.

(Chapter 3; Tough et al., 1992), provide support for a role for NAb in the defense against *ras*-transformed cells.

Examination of focus-selected v-H-*ras*/neo^r-infected 10T½

Among the T24-H-*ras*-transfected 10T½ clones, an association was observed between those cells which did not show increased NAb binding and those which were selected for focus formation in the absence of G418 following *ras* introduction. In our previous study, G418-selection following v-H-*ras*/neo^r-infection of 10T½ produced a drug resistant cell population which exhibited increased NAb binding and high NAb binding clones isolated from this population expressed v-H-*ras* p21 and were tumorigenic (Chapter 3; Tough and Chow, 1991). Therefore, it was possible that selection through focus formation in the presence of normal cells enriched for *ras*-transformed variants which were not increased in NAb binding.

In order to further examine the relationship between the selection for focus formation and changes in NAb binding, additional cell lines were picked as foci following raszip 6-infection of 10T½. Following the infection of 10T½ with the v-H-*ras*/neo^r-bearing defective retrovirus at an MOI of 0.02, the cells were allowed to grow to confluence, and the nine transformed foci which arose in the monolayer 16-23 days later were picked. The nine cell lines were subsequently shown to be G418-resistant.

All nine of the cell lines expressed high levels of the *ras* protein (Fig. 4.3, Table 4.2) and were highly tumorigenic when injected s.c. into syngeneic C3H/HeN mice at a dose of 10^4 cells, producing tumors in 100% of mice injected for all but 2 lines which produced 83% tumor frequencies

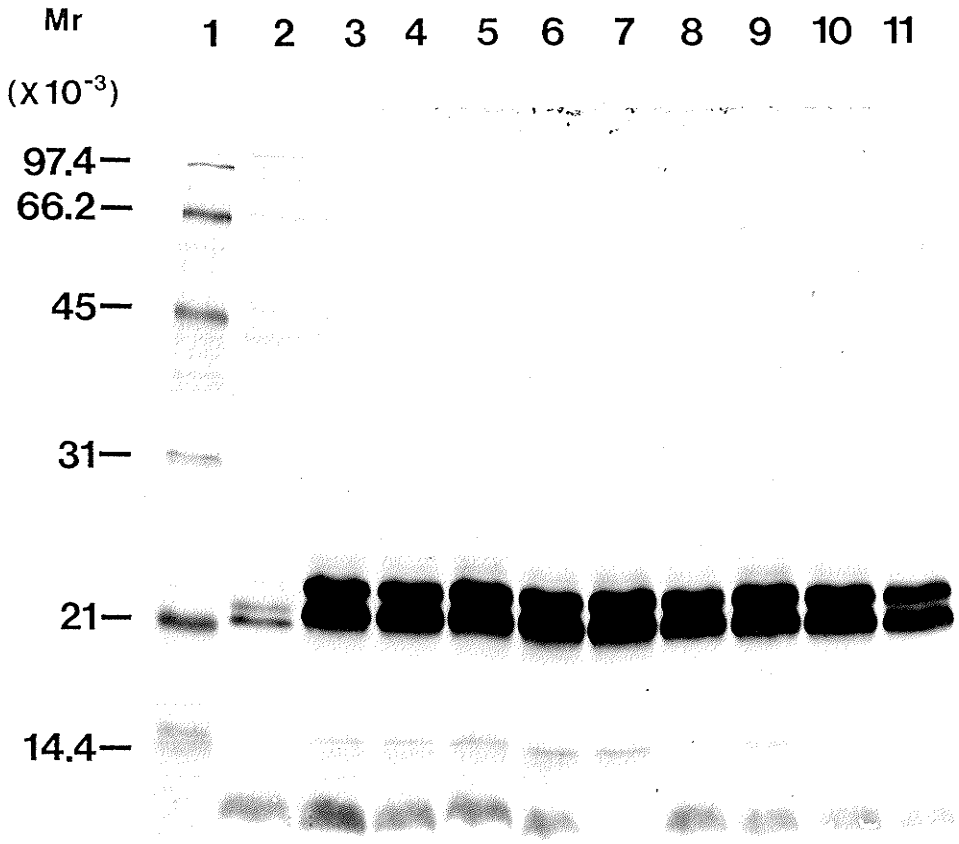

Fig. 4.3. p21 expression of v-H-*ras*/neo^r-infected, focus-selected 10T½. Lanes are as follows: 1 = standard Mr markers; 2 = the 10T½ parental line; 3 = NGRG1; 4 = NGRG2; 5 = NGRG3; 6 = NGRG4; 7 = NGRG5; 8 = NGRG6; 9 = NGRG7; 10 = NGRG8; 11 = NGRG9.

Table 4.2

NAb binding, tumorigenicity and p21 expression of focus-selected, v-H-ras/neo^r-infected 10T½ clones

Cell line	NAb binding MCF ± SE (6)[a]	Tumorigenicity number	Tumorigenicity latency (days ± SE)	p21 ratio expt. 10T½ ± SE (2)
10T½	36.8 ± 6.8	0/2	-	1
NGRG1	53.2 ± 8.9[b]	6/6	10.3 ± 1.1	11.4 ± 0.4
NGRG2	64.3 ± 7.1[c]	5/6	13.2 ± 0.8	11.2 ± 0.9
NGRG3	56.4 ± 5.2[c]	6/6	15.8 ± 0.7	9.7 ± 1.6
NGRG4	48.9 ± 7.1[b]	6/6	16.0 ± 0.9	13.2 ± 1.6
NGRG5	46.0 ± 9.7	5/6	19.0 ± 0.9	12.1 ± 1.9
NGRG6	57.0 ± 5.5[d]	5/5	11.2 ± 1.4	13.9 ± 0.6
NGRG7	40.2 ± 6.0	5/5	16.8 ± 1.4	10.0 ± 0.6
NGRG8	67.3 ± 10.6[e]	5/5	13.6 ± 0.7	13.1 ± 2.5
NGRG9	53.7 ± 6.9[f]	5/5	14.8 ± 0.8	12.0 ± 0.7

[a] Number of experiments is indicated in parentheses.

[b,c,d,e,f] P_{td} < 0.05, 0.007, 0.0008, 0.02, and 0.03 respectively as compared to 10T½.

(Table 4.2). Among the nine cell lines, two, NGRG7 and NGRG5, demonstrated no increases in NAb binding compared to 10T½ (Table 4.2). The remaining 7 clones exhibited small but statistically significant increases in NAb binding, ranging from 33% (NGRG4) to 83% (NGRG8). The results comfirm the previous study in showing that not all *ras*-transformed 10T½ cells were increased in NAb binding. Further, the fact that 2 of 9 clones in the present study, and 3 of 3 focus-selected clones in the previous study did not exhibit increases in NAb binding suggests that selecting cells in this manner may enrich for *ras*-transformed 10T½ cells which are not increased in NAb binding.

Although 7 of the 9 v-H-*ras*/neo^r-infected, focus-selected clones did exhibit significant increases in NAb binding compared to 10T½, the magnitude of the increases (33-83%) was on average lower than that observed previously for clones obtained from v-H-*ras*/neo^r-infected, G418-selected and FACS-sorted 10T½ (77-255%) (Chapter 3; Tough and Chow, 1991). Since our analysis of the T24-H-*ras*-transformed 10T½ clones revealed an inverse correlation between NAb binding and tumorigenicity, it was of interest to determine whether the same relationship existed between *ras* transformed clones expressing different levels of increased NAb binding. Nine of the G418-selected, FACS-sorted clones generated previously (Chapter 3; Tough and Chow, 1991) were injected s.c into syngeneic C3H/HeN mice at an inoculum of 10^4 cells to compare their tumorigenicity to that of the focus-selected *ras*-transformed cells of the current study (Table 4.3). These cells yielded tumor frequencies ranging from 59-100% with only 4 lines producing tumors in every mouse injected. In contrast to tumors which appeared following the inoculation of the v-H-*ras*/neo^r-infected

Table 4.3

Tumorigenicity of high NAb binding v-H-*ras*/neo^r-infected, G418-selected clones

Cell line	MCF NAb binding[a,b] ratio expt. / 10T½	Tumorigenicity number	percent	latency (days ±SE)
I4.1.1.T	2.30 (4)	7/7	100	12.7 ± 1.3
I6.1.1.T	2.66 (4)	7/7	100	14.0 ± 1.1
DT2	1.78 (5)	5/7[c]	71.4	19.8 ± 2.8
I8T2.1	3.40 (4)	7/7[d]	100	11.8 ± 1.3
HC8.1	2.00 (9)	4/7[c]	57.1	32.2 ± 11.4
HE9.1.2	1.77 (9)	7/7	100	18.7 ± 3.2
I3T2.1	3.24 (4)	6/7[c]	85.7	30.2 ± 3.9
HF1.1	1.86 (10)	6/7[c]	85.7	15.0 ± 1.1
HF2.1	2.54 (13)	6/8	75	38.1 ± 4.8

[a] Number of experiments is indicated in parentheses.
[b] MCF NAb binding ratios are those listed in table 3.2
[c] One tumor subsequently regressed.
[d] Two tumors subsequently regressed.

focus-selected clones, for which no regressions were observed, several of the tumors produced by the v-H-*ras*/neo^r-infected, G418-selected, FACS-sorted 10T½ clones eventually regressed (Table 4.3). No correlation was observed between NAb binding and tumorigenicity or between p21 expression and tumorigenicity among the individual clones produced by either selection procedure. However, when the properties of the cell lines were examined as a group based on the selection procedure used to produce them, the focus-selected *ras*-transformed cells had both a higher tumor frequency and a shorter tumor latency than the G418-selected, FACS-sorted *ras*-transformed cells. The average tumor frequency and mean latency of the focus-selected *ras*-transformed 10T½ clones were 96.3% and 14.5 ± 0.9 days respectively compared to values of 86.1% and 21.4 ± 3.2 days for the G418-selected, FACS-sorted *ras*-transformed cells (Table 4.4). The higher average tumorigenicity of the focus-selected clones was associated with a lower mean increase in NAb binding over 10T½ of 47%, compared to 139% for the G418-selected, FACS-sorted *ras*-transformed 10T½. Average p21 expression levels and *in vitro* growth rates were similar between the two sets of clones and did not correlate with tumor frequency or latency (Table 4.4). Therefore, although an inverse correlation was not observed between NAb binding and tumorigenicity when investigated at the clonal level, when the clones were compared as two distinct populations on average a relationship between higher NAb binding and lower tumorigenicity was detected among the v-H-*ras*-transformed 10T½ cell lines, consistent with a role for NAb in the defense against *ras*-transformed cells.

Table 4.4

Influence of Selection Protocol on NAb Binding and Tumorigenicity of v-H-*ras*/neo^r-infected 10T½

Tumor clone selection following v-H-*ras*/neo^r-infection[a]	Mean ± SE MCF NAb binding ratio[b]	Mean tumorigenicity %	Mean latency (days ± SE)	Mean ± SE p21 ratio[b]	Mean ± SE *in vitro* doubling time[c] (hours ± SE)
G418, FACS	2.39 ± 0.2	86.1	21.4 ± 3.2	11.1 ± 1.8	17.0 ± 0.5
Focus-formation	1.47 ± 0.08	96.3	14.5 ± 0.9	11.8 ± 0.5	17.5 ± 0.4

[a] The G418, FACS-selected and focus-selected clones are those listed in tables 4.2 and 4.3 respectively.
[b] Ratios are the values obtained for the experimental lines divided by that for 10T½.
[c] 3 independent determinations of *in vitro* doubling times were conducted for each cell line.

v-H-*ras*/neo^r-infection of NIH 3T3

The effects of *ras* have been shown to be dependent upon the specific cell type in which *ras* is expressed. The recipient-cell dependency of *ras* extends to changes in sensitivity to NK lysis. Since the results above suggest that effects of *ras* on NAb binding may similarly vary depending upon the characteristics of the cell in which *ras* is expressed, it was of interest to determine if *ras* oncogene expression in another cell line would lead to increased NAb binding. Therefore the effect on NAb binding of v-H-*ras* expression in NIH 3T3 was examined.

v-H-*ras*/neo^r-containing *ras*zip 6 was added to NIH 3T3 at an MOI of 0.02, the cells grown for 13 days in 400 μg G418/ml, and the total population of drug resistent cells was analyzed. Three independent raszip 6 infections yielded populations of cells which exhibited high levels of *ras* p21, including the more slowly migrating phosphorylated viral protein (Fig. 4.4, Table 4.5). The three v-H-*ras*/neo^r-infected populations were neoplastically transformed, as shown by their abilities to form tumors when injected s.c. into outbred Swiss mice (Table 4.5). Although tumors formed initially with a latency of 4 days, they began to regress after approximately 20 days, likely due to major histocompatibility complex differences between the tumor cells and the recipient mice. Neither parental NIH 3T3 nor neo^r-infected NIH 3T3 cells produced tumors when injected into Swiss mice (Table 4.5).

When the tumor binding of NAb derived from normal Swiss mice was examined, it was found that the v-H-*ras*/neo^r-infected NIH 3T3 did not bind more NAb than parental NIH 3T3 (Table 4.5). In fact, 2 of the three v-H-*ras*/neo^r-infected populations exhibited small but statistically significant

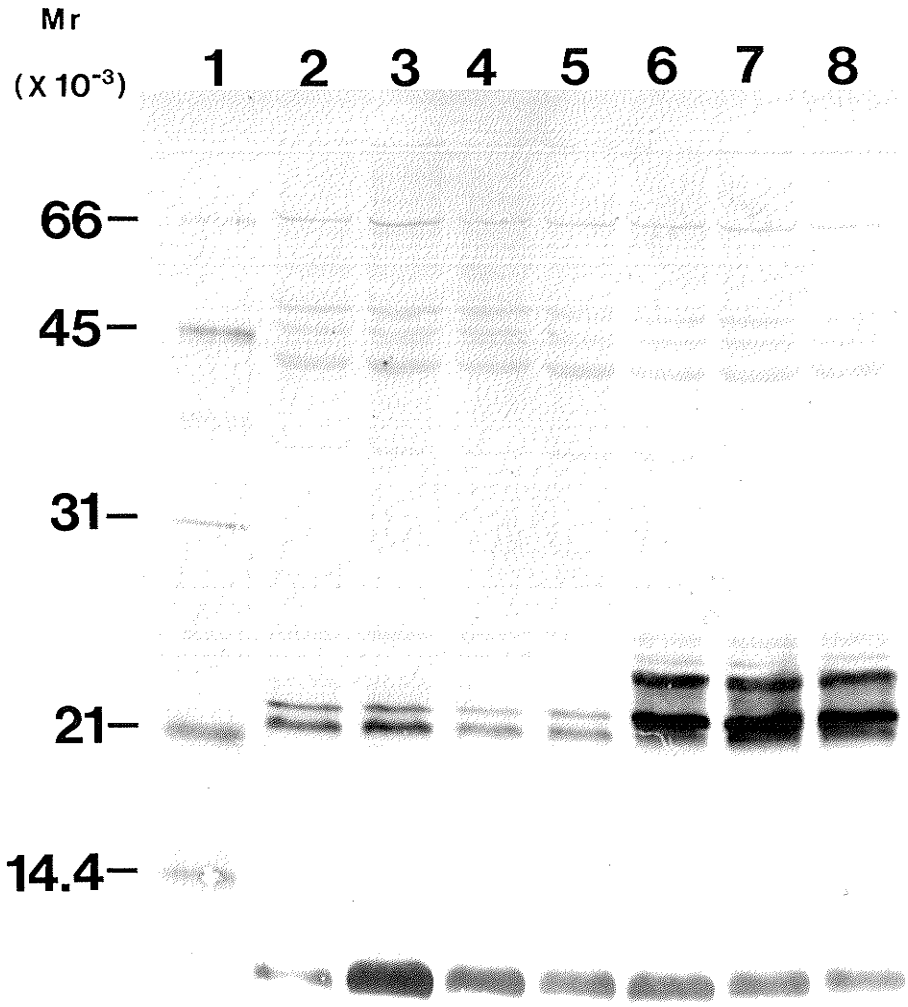

Fig. 4.4. p21 expression of v-H-*ras*/neo^r-infected, G418-selected NIH 3T3. Lanes are as follows: 1 = standard Mr markers; 2 = the NIH 3T3 parental line; 3-5 = independent neo^r-infected NIH 3T3 populations -1, 2, 3 respectively; 6-8 = independent v-H-*ras*/neo^r-infected NIH 3T3 populations -1, 2, 3 respectively.

Table 4.5

NAb binding, tumorigenicity and p21 expression of v-H-*ras*/neo^r-infected NIH 3T3 populations

Expt.#	Cell line	NAb binding (MCF ± SE) (4)[a]	Tumorigenicity number	p21 Ratio ± SE[b] (2)[a]
1.	NIH 3T3	52.9 ± 4.5	0/2	1
	NIH 3T3 + v-H-*ras*/neo^r-1	39.5 ± 2.0[c]	2/2	9.3 ± 4.9
	NIH 3T3 + v-H-*ras*/neo^r-2	40.4 ± 6.5	1/2	10.6 ± 5.4
	NIH 3T3 + v-H-*ras*/neo^r-3	40.6 ± 5.6[d]	2/2	10.4 ± 5.5
	NIH 3T3 + neo^r-1	52.7 ± 6.9	0/2	1.1 ± 0.1
	NIH 3T3 + neo^r-2	48.2 ± 4.2	0/2	0.4 ± 0.1
2.	NIH 3T3	58.7 ± 7.5[e]	0/2	1
	NIH 3T3 + neo^r-3	57.9 ± 4.8[e]	0/2	0.4 ± 0.1

[a] Number of experiments is given in parentheses unless indicated otherwise.
[b] Ratio $\frac{\text{expt.}}{\text{NIH 3T3}}$ ± SE
[c,d] $P_{td} < 0.03$ and 0.02 respectively compared with NIH 3T3.
[e] Only 2 experiments were performed on this population.

decreases in NAb binding. The third population also bound less NAb than NIH 3T3 but the difference was not significant. Therefore, the results with NIH 3T3 contrasted markedly with those observed following v-H-*ras*/neo^r-infection of 10T½ (Chapter 3; Tough and Chow, 1991) and suggested that the genetic characteristics of NIH 3T3 are non-permissive for the manifestation of the *ras*-induced high NAb binding phenotype. Further, the results once again demonstrate that neoplastic transformation and increased NAb binding are not strictly associated.

Two possible reasons could account for the absence of increased NAb binding detected on v-H-*ras*/neo^r-infected NIH 3T3. The first is that expression of an activated *ras* oncogene in NIH 3T3 does not result in the same cell surface changes as occur on 10T½. The second is that the same cell surface changes do occur, but the repetoire of NAb in Swiss mice differs from that in C3H/HeN mice so that these changes are not manifested as increased NAb binding. To assess whether this second possibility could account for the observed results, the binding of C3H/HeN-derived NAb to these cells was also examined. There was not an increase in C3H/HeN NAb binding to v-H-*ras*/neo^r-infected NIH 3T3 compared to neo^r-infected cells (Table 4.6). Therefore, expression of an activated *ras* oncogene in NIH 3T3 does not lead to the same cell surface changes as occur on 10T½.

Examination of somatic cell hybrids

The observation that *ras*-induction of increased NAb binding was not observed in NIH 3T3 or in 100% of 10T½ cells suggested that *ras* effects on NAb binding may be modified by additional genes present in the *ras*-expressing cell. This was further examined by performing somatic cell

Table 4.6

C3H/HeN serum NAb binding to v-H-*ras*/neo^r-infected NIH 3T3

Cell line	NAb binding MCF ± SE (6)[a]
NIH 3T3	48.4 ± 3.4
NIH 3T3 + v-H-*ras*/neo^r-1	59.4 ± 5.7
NIH 3T3 + v-H-*ras*/neo^r-2	59.4 ± 8.1
NIH 3T3 + v-H-*ras*/neo^r-3	61.2 ± 9.5
NIH 3T3 + neo^r-1	52.8 ± 5.6
NIH 3T3 + neo^r-2	67.1 ± 6.0
NIH 3T3 + neo^r-3	61.9 ± 7.2

[a] Number of experiments is indicated in parentheses.

hybridization between high and low NAb binding *ras*-transformed 10T½ cells. Three high NAb binding, v-H-*ras*/neo^r-infected 10T½ clones, I8T2.1, I3T2.1 and HF2.1, and a neo^r-infected 10T½ clone, SVX, were each hybridized to a low NAb binding, H-*ras* plus hygromycin resistance-transfected 10T½ clone, 10T½RAS, and a hygr^r-transfected 10T½ clone, PY3 (Table 4.7). The three high NAb binding *ras* transformed clones were obtained from a high NAb binding, FACS-sorted v-H-*ras*/neo^r-infected 10T½ population described previously (Chapter 3; Tough and Chow, 1991). The PY3 was cloned following transfection of 10T½ with the hygr^r-bearing plasmid and bound slightly less NAb than 10T½ (Table 4.7). 10T½RAS was cloned following transfection of 10T½ with EJ-H-*ras* and hygr^r (Egan et al., 1989) and bound similar amounts of NAb as 10T½ (Table 4.7). Following the hybridizations, the entire populations of G418-resistant and hygromycin-resistant cells were analyzed.

Hybrids formed between two of the high NAb binding clones, I8T2.1 or I3T2.1, and the low NAb binding, *ras*-transformed, hygr^r 10T½RAS were significantly lower in NAb binding than hybrids formed between the same high binding clones and the control untransformed hygr^r PY3 (Table 4.8). Hybrids formed between the remaining high NAb binding clone, HF2.1, and either 10T½RAS or PY3 showed levels of NAb binding which were similar to each other and also to those of the hybrids between the control PY3 and the I8T2.1 or I3T2.1. NAb binding profiles of each of the hybrid populations, as determined by FACS analysis, appeared as continuous smooth curves, suggesting that there was not selective loss of specific chromosomes among a large proportion of the cells of a given population. The tumorigenicity of 4 of the hybrid populations, SVX + PY3, SVX +

Table 4.7

NAb binding of cell clones used in hybridizations

Cell line	Introduced gene	MCF NAb binding[a] ratio expt. / 10T½
10T½	-	1
SVX	neo^r	1.03 (5)
PY3	hygr^r	0.85 (2)
10T½RAS	H-*ras*, hygr^r	0.92 (4)
I8T2.1	H-*ras*, neo^r	3.40 (4)
I3T2.1	H-*ras*, neo^r	3.24 (4)
HF2.1	H-*ras*, neo^r	2.54 (13)

[a] Number of experiments is indicated in parentheses.

Table 4.8

NAb binding, tumorigenicity and p21 expression of hybrid populations

Clones hybridized	Properties of neo^r, $hygr^r$ population		
	NAb binding[a] MCF ± SE	Tumorigenicity number	p21 ratio[b] expt. / 10T½
SVX + PY3	59.9 ± 3.6	0/2	1
SVX + 10T½RAS	59.5 ± 6.3	2/2	1.89
I8T2.1 + PY3	112.0 ± 10.4[c,d]	2/2	3.36
I8T2.1 + 10T½RAS	72.1 ± 8.2	2/2	2.59
I3T2.1 + PY3	103.7 ± 10.3[e,f]	ND[g]	3.33
I3T2.1 + 10T½RAS	71.9 ± 7.5	ND	2.87
HF2.1 + PY3	103.2 ± 7.8[c]	ND	3.98
HF2.1 + 10T½RAS	105.7 ± 10.4[e]	ND	3.48

[a] Values are the average of 3 experiments.
[b] Values were obtained from a single experiment.
[c] $P_{ti} < 0.01$ compared to SVX + PY3
[d] $P_{ti} < 0.04$ compared to I8T2.1 + 10T½RAS
[e] $P_{ti} < 0.02$ compared to SVX + PY3
[f] $P_{td} < 0.02$ compared to I3T2.1 + 10T½RAS
[g] ND means not determined

10T½RAS, I8T2.1 + PY3 and I8T2.1 + 10T½RAS was assayed (Table 4.8). While the hybrid population formed by fusion between the two drug-resistant control cells, SVX + PY3, was non-tumorigenic at an inoculum of 10^6 cells s.c., the other 3 hybrids tested each produced tumors in 2 of 2 syngeneic C3H/HeN mice. Also, compared to SVX + PY3, all hybrid populations expressed increased *ras* p21, and those hybrids in which one fusion partner was derived from a v-H-*ras*/neo^r-infected 10T½ clone also expressed the phosphorylated viral form of p21 (Table 4.8).

The presence in 10T½RAS of a gene or genes which may down-regulate *ras*-induced increases in NAb binding was suggested by the results from hybrids of this cell with the high binding I8T2.1 or I3T2.1. However, the hybrid produced following fusion of HF2.1 and 10T½RAS did not express the effect of this down-regulatory activity. Therefore the results suggest that genes other than *ras* can modulate the effect of *ras* on NAb binding and that interactions between multiple gene products may ultimately determine the effect of *ras* on NAb binding.

DISCUSSION

The results show that increased NAb binding is not universally observed following expression of the *ras* oncogene in all cells. Rather, the effect of *ras* on NAb binding is dependent upon other genetic characteristics of the cell in which *ras* is expressed. Although increased NAb binding was observed among the majority of *ras* oncogene-expressing 10T½ clones examined in this and a previous study (Chapter 3; Tough and Chow, 1991), certain T24-H-*ras*- or v-H-*ras*-transformed clones examined in the present study, which were selected based on their abilities to form

foci in the presence of untransformed cells following ras introduction, exhibited no increase in NAb binding compared to the parental 10T½. That genetic differences existed between the high and low NAb binding T24-H-*ras*-transfected clones was suggested by the observation that the high binding NR4, while expressing the second highest level of *ras* p21, produced tumors with approximately half the frequency of that of the three foci selected clones. Further, the v-H-*ras*/neo^r-infection and G418-selection of NIH 3T3, a procedure which has previously produced 10T½ populations with increased NAb binding (Chapter 3; Tough and Chow, 1991), yielded populations of cells which expressed v-H-*ras* p21 and were tumorigenic but were not increased in NAb binding compared to the initial NIH 3T3 cell line. Not only was there no increase in physiologically relevant Swiss mouse-derived NAb binding, there was also no increase in binding of NAb from C3H/HeN serum. Therefore, the cell surface changes responsible for the increased NAb binding of *ras* transformed 10T½ did not occur upon *ras* transformation of NIH 3T3. Thus, the genetic background found in the majority of 10T½ cells is permissive for the induction of these cell surface alterations while that of NIH 3T3 is not. The existence of tumorigenicity without increased NAb binding among both *ras* transformed 10T½ clones and *ras* transformed NIH 3T3 demonstrates that there is no strict association between these cell surface changes and neoplastic transformation.

Comparison of the NAb binding of somatic cell hybrid populations produced by fusion between high NAb binding *ras* transformed 10T½ clones and either low NAb binding *ras* transformed clones or control drug-resistant 10T½ clones provided more evidence for the ability of additional

genes to modify *ras*-induced increases in NAb binding. Two of the high NAb binding clones produced lower binding hybrid populations when fused to the low NAb binding *ras* transformed 10T½ clone than when fused to the control untransformed line. The hybridization between the third high binding clone and the control exhibited comparable binding to the fusion products of the control with the other high binders, but the hybrid with the low binding *ras* transformant showed no reduction in NAb binding. The results suggest: (1) that the three high NAb binding v-H-*ras* transformed 10T½ clones do not have identical genetic backgrounds, although the background of each is permissive for *ras*-induced increases in NAb binding and; (2) that the effect of *ras* on the NAb binding phenotype is likely determined by a combination of other genes in the cell, rather than the presence or absence of a single gene. The cell-type specific effects of *ras* oncogene expression observed in this and other studies may be explained if *ras* functions as a transducer of signals from growth factor receptors, since the availability of downstream effectors for *ras*, and the activation of other signalling pathways with which *ras* may interact, would determine the ultimate effect of *ras* activity. A different model for the function of *ras*, which could also account for the cell-type specific effects of *ras*, proposes that *ras* p21 may control assembly of cell surface receptors, and induce spontaneous activity in any receptors already present on the cell surface (Cantley et al., 1991). According to this hypothesis, the cell-type specific effects of *ras* would be due to the expression of cell-specific growth factor receptors.

While the nature of the difference in the genetic background between cells which become high NAb binding following *ras*-induced neoplastic

transformation and those that do not is not known, the results suggest that the failure of *ras* expression to produce increased NAb binding may be associated with cells which are more malignant. The focus-selected T24-H-*ras*-transfected clones, CIRAS-1, -2 and -3, were both lower in NAb binding and more tumorigenic than the G418-selected NR4. A previous study has also shown that CIRAS-1, -2 and -3 were more metastatic than NR4 (Egan et al., 1987a). Thus selecting cells following *ras* introduction based on their ability to form a focus in a monolayer of surrounding untransformed cells may enrich for cells with more autonomous growth characteristics and which are consequently more malignant when assayed *in vivo*. That the focus-forming ability was associated with a "more advanced" stage in the multistep process of tumorigenesis was suggested by studies which examined the ability of *ras* to transform either primary cells or established cell lines *in vitro*. Although the *ras* oncogene has been shown to be unable to induce focus formation when transfected into primary rodent cells without the simultaneous introduction of a cooperating oncogene, the cointroduction of *ras* and the neo^r gene into rat embryo fibroblasts followed by G418-selection yielded neoplastically transformed cells (Spandidos and Wilkie, 1984; Land et al., 1986). Therefore it appears that expression of an activated *ras* oncogene alone in normal cells is not sufficient to allow the cells to overcome some unknown growth inhibitory influence imposed by surrounding normal cells. In contrast, expression of a *ras* oncogene alone can induce focus-formation in established cell lines such as Rat-1 cells or NIH 3T3 cells (reviewed in Weinberg, 1989), suggesting that these immortalized cell lines differ from normal cells in a manner which mimics the effect of a cooperating oncogene. It has also

been reported that transfection of equal amounts of the *ras* oncogene into NIH 3T3 and 10T½ cells yielded 10-90 times more transformed foci in the NIH 3T3 (Hsiao et al., 1984; Taparowski et al., 1987), suggesting that a much smaller proportion of 10T½ than NIH 3T3 cells may have this property. The formation of foci following introduction of a *ras* oncogene has traditionally been regarded as an indication that the recipient cell contains genetic alterations relevant to the multistep process of tumorigenesis since primary cells do not form foci following *ras* transfection but can be induced to do so by cointroduction of a cooperating oncogene or treatment with a tumor promoter (reviewed in Weinberg, 1989). It has also been shown that phorbol ester tumor promoters (Hsaio et al., 1984), a factor present in fetal calf serum (Hsiao et al., 1987) and the *myc* oncogene (Taparowski et al., 1987) can all enhance the focus-forming ability of *ras* transformed 10T½. The results therefore suggest that the genetic background present in NIH 3T3 and a minor subpopulation of 10T½ cells can cooperate with the *ras* oncogene to produce cells with more autonomous growth characteristics and that this genetic background may further be non-permissive for *ras*-induced increases in NAb binding. However, since only 5 of the 12 focus selected *ras* transformed 10T½ clones failed to show increased NAb binding, there are likely different genetic characteristics which can cooperate with *ras* in producing the focus-forming ability, and only some of these are associated with a lack of a *ras*-induced increase in NAb binding.

An inverse correlation was observed between NAb binding and early tumor incidence among the four *ras* oncogene-expressing, T24-H-*ras*/neo^r-transfected 10T½ clones. A similar inverse relationship between NAb

binding and tumorigenicity was evident when the average properties of v-H-*ras*/neo^r-infected, focus-selected 10T½ clones were compared to those of v-H-*ras*/neo^r-infected, G418-selected, FACS-sorted 10T½ clones. These results are consistent with the inverse correlation between NAb binding and tumorigenicity observed previously for *in vitro*-selected murine lymphomas (Chapter 2; Tough and Chow, 1991), and extend support for a role for NAb in the defense against *ras* transformed cells. However, this inverse correlation was not evident when the v-H-*ras*/neo^r-infected 10T½ was analyzed at a clonal level. Clearly, numerous properties of the *ras*-transformed cells in addition to their NAb binding characteristics are important parameters in determining their ability to grow when transplanted s.c. into syngeneic mice. Greenberg et al (1987b) found that although the ability of *ras*-transformed 10T½ cells to arrest and survive in the lung during the first 48 hours after i.v. inoculation correlated inversely with their NK-sensitivity, the eventual outgrowth of lung tumors was independent of NK-sensitivity and instead correlated with *ras* mRNA expression. Although we observed no correlation between *ras* p21 expression and s.c. tumor frequency, our observations of both low and high NAb binding *ras*-transformed 10T½ cells, and of cell hybrids with different NAb binding phenotypes produced following the fusion of different *ras*-transformed 10T½ clones suggested the presence in individual clones of genes which modify *ras* effects on NAb binding. These genes, along with others which have no effect on NAb binding but which could also differ between clones, may affect other properties of the tumor cells, such as invasiveness or growth factor autonomy, which will influence their ability to grow *in vivo*. Since the increased tumorigenicity was associated not

only with reduced NAb binding but also with the focus-selection procedure it is possible that cell characteristics selected for by this process other than decreased NAb binding could account for the increased tumorigenicity. Nevertheless, it is significant that decreased NAb binding, whether a cause or a by-product, is associated with an enhanced malignant phenotype.

CHAPTER 5

ONCOGENE SPECIFICITY OF ALTERATIONS IN CELL SURFACE NATURAL ANTIBODY BINDING

ABSTRACT

It has been shown that expression of an activated H-*ras* oncogene in 10T½ cells is associated with increased NAb binding (Chapters 3 and 4; Tough and Chow, 1991; Tough et al., 1992). To determine whether the observed increase in NAb binding was specific to the *ras* oncogene, the NAb binding of 10T½ was examined following the expression of two additional oncogenes, v-*myc* and v-*src*. Infection of 10T½ with a defective retroviral vector containing the v-*myc* oncogene and the neo^r gene followed by G418-selection produced $G418^r$ populations which expressed the v-*myc* protein but were not increased in NAb binding. Introduction of a defective retroviral vector containing the v-*src* oncogene and the neo^r gene into 10T½, either by infection or transfection, followed by G418-selection produced cell populations which were neoplastically transformed but expressed slight decreases in NAb binding which were significant. The results demonstrate that increased NAb binding is not induced following the expression of all oncogenes in 10T½. Rather the high NAb binding phenotype appears to be specifically associated with properties of the *ras* oncogene.

INTRODUCTION

It has been shown that expression of an activated H-*ras* oncogene in 10T½ cells is associated with both neoplastic transformation and increased NAb binding (Tough and Chow, 1991). Subsequent studies demonstrated that *ras*-induced increases in NAb binding were modified by the expression of additional genes in the recipient cell, and were not obligately associated with neoplastic transformation (Tough et al., 1992). Neither the nature of the cell surface changes in 10T½ which results in increased NAb binding

nor the biochemical processes which follow *ras* oncogene expression and cause these changes are known. It would be of interest to determine whether the expression of other oncogenes in 10T½ can also lead to increased NAb binding. In the present study, we have examined the effect on NAb binding of the expression of two additional oncogenes, v-*myc* and v-*src*, in 10T½.

c-*myc* encodes a nuclear phosphoprotein which contains several structural motifs known to be involved in DNA-binding and protein-protein interactions, and is thought to function as a transcriptional regulator or as a controlling factor in DNA synthesis (reviewed in Luscher and Eisenman, 1990). The *myc* oncogene has been traditionally classified as a member of the "nuclear oncogene" category, which implies both its cellular location and its ability to cooperate with cytoplasmic oncogenes, such as *ras*, in the transformation of primary rodent cells *in vitro* (reviewed in Hunter, 1991). Nuclear oncogenes generally do not alter either cell morphology or growth factor requirements or induce anchorage-independent growth, but may immortalize cells, while cytoplasmic oncogenes tend to have the opposite characteristics (Weinberg, 1985). However, these generalizations are not absolute and likely depend upon the recipient cell, since an activated c-*myc* gene was shown to transform late but not early passage cultures of the FR3T3 cell line (Zerlin, et al. 1987), while inducible expression of a v-*myc* oncogene in the established cell line rat-1 resulted in morphological transformation (Bonham et al., 1991). NIH 3T3 cells were also transformed by v-*myc*, but these cells differed from *src*- or *ras*-transformed NIH 3T3 cells by lacking the ability to form foci in a monolayer of untransformed cells (Bignami et al., 1988). The effects of

myc oncogene expression have also been shown to differ from those of *ras* with respect to alterations in inositol phosholipid signaling pathways (Alonso et al., 1988) and a failure to activate the transcription factor PEA1 (Wasylyk et al., 1988). While the expression of the *myc* oncogene alone does not transform 10T½ cells, *myc* transfection into 10T½ has been shown to reduce cellular dependence for serum-derived growth factors (Leof et al., 1989), confer responsiveness to transforming growth factor β (Leof et al., 1987), and to cooperate with *ras* to produce a higher level of transformation than does expression of a *ras* oncogene alone (Taparowski et al.,1987). Thus, the *myc* and *ras* oncogenes appear be involved in the activation of different but complementary biochemical pathways in 10T½.

c-*src* encodes a protein, $pp60^{c\text{-}src}$, which is localized to the inner surface of the plasma membrane and is the prototype for a family of nonreceptor protein tyrosine kinases (reviewed in Hanks et al., 1988). The occurrence of common biochemical alterations, including alterations in inositol phospholipid signaling pathways (Alonso et al., 1988; Alonso et al., 1990), activation of the transcription factor PEA-1 (Wasylyk et al., 1988) and elevation of glucose transport and transporter messenger RNA (Flier et al., 1987) following cellular expression of activated *src* or *ras* oncogenes suggested that the two oncogenes may be located along a common cellular signalling pathway involved in cell transformation. Furthermore, the observation that microinjection of a neutralizing anti-*ras* antibody into NIH 3T3 cells transformed by the v-*src* oncogene reversed morphological transformation and blocked proliferation indicated that *ras* was located downstream of *src* along this transformation pathway (Smith et al., 1986). More evidence that *src* may act through c-*ras* in transformation

was provided by a study which showed that the ratio of *ras* p21 in the active GTP-bound form to that in the inactive GDP-bound form was increased 3-4 fold in transformed cells carrying v-*src* oncogenes (Satoh et al., 1990). The steps linking *src* and *ras* are not known, but a clue to the possible mechanism was provided by studies which showed that the GTPase activating protein (GAP) was both phosphorylated on tyrosine residues and associated in complexes with other tyrosine-phosphorylated proteins in v-*src* transformed cells (Ellis et al., 1990; Bouton et al., 1991; Moran et al., 1991). Since GAP regulates p21 GTPase activity (Trahey and McCormick, 1987), phosphorylation of GAP or GAP-associated proteins might modify p21 function. It has been shown that v-*src*-induced NIH 3T3 transformation was inhibited by overexpression of GAP (Nori et al., 1991), which also suppressed transformation of NIH 3T3 by c-*ras* but not v-*ras* (Zhang et al., 1990), further suggesting the importance of the link between *src* transformation and *src* modification of GAP. Thus, the evidence suggests that *src* transmits its mitogenic and transformation signals through a biochemical pathway involving *ras* p21.

Therefore, in order to provide insight into the mechanism by which *ras*-induced transformation of 10T½ results in increased NAb binding, we have examined the effect on NAb binding of expression in 10T½ of the *myc* oncogene thought to act along a distinct but complementary pathway to *ras*, and the *src* oncogene thought to act along a *ras*-inclusive pathway.

MATERIALS AND METHODS

Mice and Sera

Normal male C3H/HeN mice obtained from the University of Manitoba vivarium (Winnipeg, Man.) were utilyzed in all studies and were the source of whole serum NAb.

Cells

ψ-2 cells (Mann et al., 1983) and ψ-2 cells expressing the defective retrovirus VM (see below) were provided by A.H. Greenberg. These cells, along with 10T½ and derived lines were maintained in D-MEM/F12 (Gibco) containing 10% FBS.

Recombinant Retroviruses and Gene Transfer

The SVX virus carries the neo^r gene as described in Chapter 3. The VM virus carries the *myc* oncogene (p110 gag-*myc* fusion gene from pv-*myc* (Land et al., 1983a) in addition to the neo^r marker (Dotto et al., 1985). MX2122-B31/NEO is a defective retroviral vector containing v-*src* and neo^r genes (Egan et al., 1987). SVX was transfected into 10T½, and MX2122-B31/NEO transfected into 10T½ or ψ-2 using the calcium phosphate method as previously described (Wigler et al., 1977). Following transfection, cells were grown in medium containing 400 μg G418/ml for 13-14 days. Retroviral infections and G418-selections of 10T½ were carried out with helper-free virus stocks obtained from ψ-2 cells as described in Chapter 3, using VM at an MOI of 0.05 and MX2122-B31/NEO at an MOI of 0.02.

Fluorescence-Detected NAb Binding

Total IgM + IgG NAb binding was assessed as described in Chapter 3.

Western Blotting

Whole cell lysates were prepared as described in Chapter 3, except that cell pellets were not frozen prior to lysis. Following SDS-PAGE of 25 μl of cell lysates and 30 μl of SDS-PAGE molecular weight standards (high) (Bio-Rad) on a 7.5% gel, proteins were transferred to nitrocellulose. The p110 v-*myc*-gag fusion protein was detected by sequential incubations with a polyclonal sheep anti-v-*myc* antibody (Medac, Hamburg), biotinylated-rabbit-anti-sheep IgG (Dimension Labs, Mississauga, Ont.), avidin-alkaline phosphatase and the Vectastain ABC-AP substrate (Dimension Labs). Molecular weight standards were detected by india ink staining (Hancock and Tsang, 1983).

Tumorigenicity

Tumorigenicity of v-*src*/neo^r-transfected- or SVX-infected 10T½ was assessed at an inoculum of 10^6 cells s.c. as described in Chapter 3.

Statistics

Statistical significance was determined as described in Chapter 3.

RESULTS

Effect of v-*myc* oncogene expression in 10T½ on NAb binding

The defective retroviral vector VM (Dotto et al., 1985), which carries the v-*myc* oncogene (p110 gag-*myc* fusion gene from pv-*myc* (Land et al., 1983a) and the neo^r gene, was obtained from VM-infected ψ-2 cells. Virus was added to 10T½ at an MOI of 0.05 for 24 hours. The cells were grown in the presence of 400 μg G418/ml for 12-14 days, and the population of drug-resistant cells was analyzed. Three populations of cells expressing the gag*myc*-fusion protein of Mr 110 KD were obtained (Fig. 5.1). These cell lines had *in vitro* growth morphologies which were indistinguishable from 10T½. None of the v-*myc*/neo^r-infected cell populations were increased in NAb binding compared to the parental 10T½ (Table 5.1). The results demonstrate that not all oncogenes lead to increased NAb binding following their expression in 10T½. Rather, it suggests that a specific biochemical activity associated with the *ras* oncogene is necessary to produce the increased NAb binding.

Effect of v-*src* oncogene expression in 10T½ on NAb binding

The effect on NAb binding of the expression of the v-*src* oncogene in 10T½ was also examined. The defective retroviral vector MX2122-B31/NEO carries the v-*src* oncogene and the neo^r gene (Egan et al., 1987b). This vector was introduced into 10T½ either by direct transfection (Expt.1, Table 5.2), or by infection with helper-free virus obtained from MX2122-B31/NEO-transfected ψ-2 cells at an MOI of 0.02 (Expt.3 (ii), Table 5.2) or 0.04 (Expt. 3(i), Table 5.2). Following either procedure, the cells

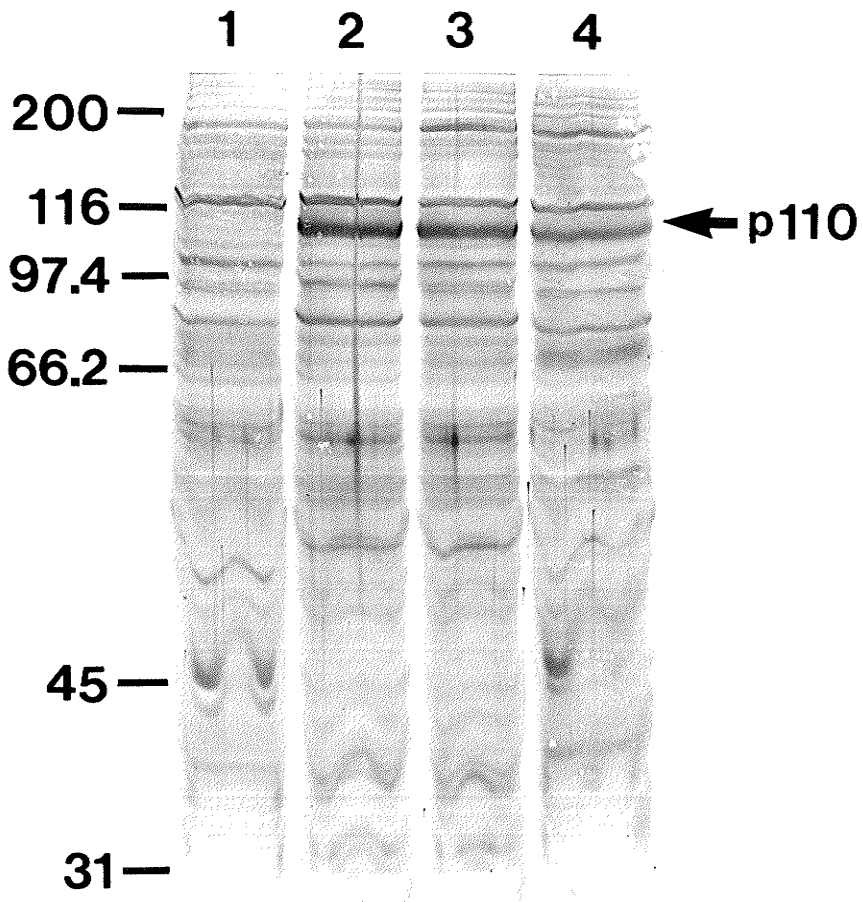

Fig. 5.1. $p110^{gag\text{-}myc}$ protein expression of v-*myc*/neo^r-infected 10T½. Lane 1 corresponds to the 10T½ parental line while lanes 2-4 are independent v-*myc*/neo^r-infected 10T½ populations -1, 2 and 3 respectively. Mr for standard markers are shown to the left (X 10^{-3}).

Table 5.1

NAb binding of v-*myc*/neo^r-infected 10T½ populations

Expt.[a]	Cell line	MCF NAb binding ± SE[b]
1.	10T½	38.5 ± 7.5 (3)
	10T½ + v-*myc*/neo^r-1	28.1 ± 5.7 (3)
2.	10T½	28.9 ± 6.8 (3)
	10T½ + v-*myc*/neo^r-2	27.5 ± 3.7 (3)
3.	10T½	33.4 ± 3.9 (3)
	10T½ + v-*myc*/neo^r-3	29.8 ± 3.0 (3)

[a] Individual experiments represent independent v-*myc*/neo^r-infections.
[b] Number of experiments is indicated in parentheses.

Table 5.2
NAb binding and tumorigenicity of v-src/neo^r-transfected and -infected 10T½ populations

Expt.#	Oncogene Introduction	Cell line	NAb binding[a] MCF ± SE	Tumorigenicity number[b]
1. v-src/neo^r transfection		10T½	66.2 ± 5.0 (4)	0/2
		10T½ + v-src/neo^r-1	55.8 ± 6.0 (4)[c]	2/2
		10T½ + v-src/neo^r-2	56.5 ± 3.9 (4)[d]	2/2
		10T½ + v-src/neo^r-3	49.0 ± 4.2 (4)[e]	2/2
2. neo^r transfection		10T½	53.3 ± 14.7 (3)	
		10T½ + neo^r-1	58.1 ± 14.1 (3)	ND
		10T½ + neo^r-2	53.5 ± 13.3 (3)	ND
		10T½ + neo^r-3	55.5 ± 15.6 (3)	ND
3. v-src/neo^r infection	(i)	10T½	52.5 ± 3.2 (4)	
		10T½ + v-src/neo^r-4	36.8 ± 3.8 (4)[f]	ND
		10T½ + v-src/neo^r-5	39.2 ± 3.4 (4)[e]	ND
	(ii)	10T½	54.5 ± 2.4 (6)	
		10T½ + v-src/neo^r-6	40.4 ± 3.9 (6)[f]	ND
4. neo^r infection		10T½	25.9 ± 2.0 (3)	
		10T½ + neo^r-4	21.4 ± 1.3 (3)	ND
		10T½ + neo^r-5	24.0 ± 0.6 (3)	0/5
		10T½ + neo^r-6	24.0 ± 1.3 (3)	ND

[a] Number of experiments is indicated in parentheses.
[b] ND means not determined.
[c,d,e,f] $P_{td} < 0.05, 0.01, 0.02, 0.03$ respectively compared with 10T½.

were grown for 13-14 days in the presence of 400 μg G418/ml and the populations of drug-resistant cells were examined.

The v-*src*/neo^r-transfected and -infected populations all exhibited markedly transformed morphologies *in vitro*. Each of the v-*src*/neo^r-transfected populations were tested for their abilities to produce tumors when injected s.c. into syngeneic mice. All of these populations produced rapidly growing tumors with short latencies upon injection of 10^6 cells, demonstrating that they had in fact been neoplastically transformed (Table 5.2). Neither the v-*src*/neo^r-transfected nor -infected populations, however demonstrated increases in NAb binding compared to 10T½. In fact, small but statistically significant decreases in NAb binding were observed in every case (Table 5.2).

The results demonstrate further the separation of the events leading to neoplastic transformation and increased NAb binding. The differential ability of *ras* but not *src* to induce increases in NAb binding has implications regarding the nature of the biochemical pathway activated by *ras* which results in increased NAb binding.

DISCUSSION

The results show that in contrast to the effect of *ras* oncogene expression in 10T½ (Chapter 3; Tough and Chow, 1991; Tough et al., 1992), neither v-*myc* nor v-*src* expression in 10T½ results in increased NAb binding. Expression of v-*myc* had no significant effect on NAb binding, while v-*src*-induced 10T½ transformation was associated with a small, but statistically significant decrease in NAb binding. Therefore, the cell surface changes responsible for increased NAb binding which occur

following *ras* oncogene expression in 10T½ are not associated with the expression of any oncogene per se, but rather are related to a specific activity of the *ras* oncogene. The results with *src* support and extend the results from the previous study (Chapter 4; Tough and Chow, manuscript in preparation) showing that morphological transformation and tumorigenic conversion of 10T½ are not strictly associated with increased NAb binding.

The failure of v-*myc* expression to produce increased NAb binding is not surprising considering that *myc* and *ras* likely act along distinct pathways in the disregulation of cell growth. Previous studies have also shown a discordance between the effect of *myc* and *ras* oncogenes on cell sensitivity to other parameters of natural resistance. Expression of the *ras* oncogene in 10T½ was associated with increased sensitivity to NK lysis while transfection of the same cells with the c-*myc* oncogene had no effect on sensitivity to this effector cell (Johnson et al., 1987). The *myc* oncogene, however has been shown to effect the NK sensitivity of some cells since high expression of the c-*myc* oncogene rendered human melanoma cells prone to lysis by NK cells, likely through the reduction of MHC class I expression (Versteeg et al., 1989).

The lack of increased NAb binding following *src* oncogene-induced 10T½ transformation is somewhat surprising, considering the evidence which suggests that *src* may act through a biochemical pathway involving *ras* in the induction of cell proliferation and transformation (Smith et al., 1986). However, revertants resistant to transformation by *ras* but sensitive to transformation by *src* suggest the existence of a *ras*-independent transformation pathway initiated by *src* (Yanagihara et al., 1990). In addition, the tyrosine kinase activity of *src* is likely involved

in initiating biochemical pathways which result in changes not observed following *ras* activation, such as tyrosine phosphorylation of the raf-1 protein (Morrison et al., 1988) and inositol tetrakisphosphate production (Johnson et al., 1989). These and other activities of *src* may be significant for the transforming activities of the gene, as shown by the ability of *src* but not *ras* to replace v-abl in lymphoid transformation (Engelman and Rosenberg, 1990). It has also been shown that *src* and *ras* block keratinocyte differentiation at distinct stages (Weissman and Aaronson, 1985). The differential effects of *ras* and *src* on cell sensitivity to a proposed effector of natural resistance was also previously shown (Anderson et al., 1989). In this study, expression of an activated *ras* oncogene but not a *src* oncogene in 10T½ resulted in increased sensitivity to NK cell cytolysis. Presently, we have demonstrated that the alteration of NAb binding is another cellular characteristic which differs following 10T½ transformation induced by the *ras* or *src* oncogenes.

One possible reason why *src* transformation does not result in increased NAb binding is that *src* may fail to stimulate the *ras*-inclusive pathway involved in increased NAb binding. Although *src* transformation has been shown to be dependent upon cellular *ras* activity, it is not known how the actions of *ras* which are necessary for *src* transformation compare to those of an oncogenically activated *ras*. There is evidence to suggest that *ras* may interact with more than one signalling pathway, and that the consequences of *ras* activity can be manifested through both protein kinase C-dependent and -independent pathways (Hiraki et al., 1989; Lloyd et al., 1989; Cai et al., 1990; Binetruy et al., 1991). Our previous observations

of a lack of increased NAb binding following *ras* transformation of NIH 3T3 cells (Chapter 4: Tough et al., 1992) and focus-selected *ras* transformed 10T½ clones which are not increased in NAb binding (Chapter 4; Tough and Chow, manuscript in preparation) indicates that the *ras*-induced pathways leading to increased NAb binding and cellular tranformation can be differentially regulated. It has been shown that v-*src* expression is associated with both an alteration in the subcellular distribution of GAP and with its association with other proteins (Moran et al., 1991). Since GAP may be either a regulator or an effector of *ras* activity (McCormick,1989), these altered properties of GAP may result in changes in *ras*-induced signalling through specific biochemical pathways. A proposed model for $pp60^{c\text{-}src}$ signalling involves the specific recruitment of enzymes, including the *ras* binding protein GAP, to critical locations in cell membranes (reviewed in Cantley et al., 1991). Therefore, it is possible that v-*src* may specifically recruit *ras* into a pathway involved in cell transformation without stimulating *ras* to activate the pathway involved in increasing NAb binding.

Another possibility for the failure of *src* to stimulate increased NAb binding is that, by virtue of its tyrosine kinase activity, *src* may stimulate a pathway which actively downregulates NAb binding. Our previous study of somatic cell hybrids has shown that additional cellular activities can modify the effects of *ras* on NAb binding. The observation that the NAb binding is slightly decreased following v-*src* transformation of 10T½ is consistent with either an active downregulation of NAb binding or alternatively a specific recruitment of *ras* into a transformation pathway since the latter process might reduce the basal *ras* activity in

the pathway leading to increased NAb binding.

CHAPTER 6

DISCUSSION

A considerable body of evidence has accumulated which supports a role for NAb in tumor defense as part of a natural immune surveillance system. In this thesis, the contribution of NAb to antitumor natural resistance has been further investigated by testing two predictions which arise from this hypothesis. The first prediction is that tumors with a relatively high sensitivity to NAb should have a reduced ability to grow *in vivo* compared to similar cells with lower NAb sensitivity. To approach this question, variants were selected from murine lymphoma cell lines *in vitro* directly on the basis of altered fluorescence-detected NAb binding and the *in vivo* behavior of the selected cells was assessed by s.c. transplantation of small tumor foci into syngeneic mice. A consistent inverse correspondence between NAb binding and tumorigenicity was observed both for the high and low NAb binding variants selected from a heterogeneous L5178Y-F9 cell line and for the TPA-treated, high NAb-sorted L5178Y-F9 and SL2-5 cells compared to their starting clones. Since no consistent changes in sensitivity to putative natural immune mediators tested other than NAb were observed for the TPA-treated, NAb-selected cells compared to their parental lines, the results strongly suggested that the reduced ability of the high NAb binding cells to form tumors in syngeneic mice was related to their enhanced NAb binding. The direct approach utilized to select NAb binding variants in the present work produced results which confirmed the inverse correlation that has been consistently observed between fluorescence-detected NAb binding and s.c. tumorigenicity in previous studies of low NAb binding tumor cells obtained following *in vivo* growth (Brown and Chow, 1985; Brown et al., 1986a) and of tumor cells which paradoxically expressed increased fluorescence-

detected NAb binding following TPA-treatment and selection for resistance to NAb and complement lysis *in vitro* (Chow and Chan, 1987). Although the mechanism(s) through which the bound NAb acts to inhibit tumor cell growth is not known, the results provide strong support for the hypothesis that sensitivity to NAb-mediated defense is an important parameter in determining the ability of transplanted tumor foci to grow and they further imply that nascent tumors will also be subject to the same constraints on their growth.

The extrapolation from the evidence supporting a role for NAb in defense against transplanted tumor cell lines to their proposed participation in surveillance against incipient neoplasia however is made based on the assumption that newly arising tumor cells will be sensitive to the effects of NAb. The observation that increased NAb binding *in vitro* was shown to correlate with sensitivity to antitumor natural resistance *in vivo* suggests that, for newly arising tumor cells to be susceptible to NAb-mediated surveillance, they should exhibit increased NAb binding compared to the normal cells from which they originate. Therefore, in order to test the validity of this proposal, we employed an *in vitro* model of cell transformation and examined changes in NAb binding following the oncogenic conversion of immortalized non-tumorigenic cell lines to tumor cells. Consistent with the hypothesis that increased sensitivity to NAb occurs in the early stages of tumor development, *ras* oncogene expression in 10T½ cells was associated with both neoplastic transformation and increased NAb binding. The increased NAb binding observed following v-H-*ras*/neo^r-infection and G418-selection of 10T½ was associated with v-H-*ras* p21 expression and tumorigenicity. Further, the 2H1 cell line, a 10T½

clone containing a Zn^{++}-inducible H-*ras* oncogene, exhibited increased NAb binding which was dependent upon Zn^{++}-induction of *ras* p21. The magnitude of the increases in NAb binding observed following *ras* oncogene expression in 10T½ was similar to that which has been shown to have an impact on the growth of transplanted tumors in this and previous studies. In addition, the pre-injection of whole serum NAb i.v. was shown to reduce the number of early tumors appearing following s.c. injection of Zn^{++}-induced 2H1 into syngeneic mice. Although the mechanism of the serum effect is currently unknown, the results are nevertheless consistent with NAb-mediated mechanisms being active against NAb-binding tumor cells *in vivo*. The Zn^{++}-inducible model also showed that expression of a *ras* oncogene in 10T½ was associated with both increased NAb binding and increased sensitivity to NK cell cytolysis, suggesting the existence of a stage of tumor development which was a target for antitumor natural resistance.

However, a further characterization of the *in vitro* tumorigenesis model revealed that the increased NAb binding exhibited by *ras* transformed 10T½ cells was not essential for tumorigenicity. Five of twelve *ras* transformed 10T½ cell lines selected following *ras* oncogene introduction based on their ability to form a focus in a monolayer of surrounding non-transformed 10T½ cells did not bind more NAb than 10T½ even though they were tumorigenic. In addition, neither v-H-*ras*-induced transformation of NIH 3T3 nor v-*src*-induced transformation of 10T½ was associated with an increase in NAb binding. The possible implications of these findings with regard to the proposed role of NAb in tumor surveillance must be considered. One interpretation of the data is that the apparently rare circumstance in which increased NAb binding was observed may suggest that

NAb have a limited potential to recognize cell changes associated with tumor development and therefore can be expected to play only a limited role in tumor surveillance. NAb recognition of cell transformation may be restricted to that induced by specific oncogenes (perhaps only *ras*?) in specific cell types and at specific stages of neoplastic development. Interestingly, reports of alterations in cell sensitivity to NK lysis following oncogene introduction have shown a pattern of specificity very similar to that observed in the present work for NAb binding. Increased NK sensitivity was observed following the expression of oncogenic *ras* (Trimble et al., 1986) but not *src* (Anderson et al., 1989) or *myc* (Johnson et al., 1987) oncogenes in 10T½, and was not observed following *ras* oncogene expression in NIH 3T3 (Greenberg et al., 1987a). The significiance of the observation that two proposed mediators of antitumor natural resistance show the same pattern of recognition is uncertain but may indicate either that *ras* oncogene transformation of 10T½ is a particularly relevant model with respect to tumorigenesis *in vivo* or that natural immune mediators in general show a limited potential to act in tumor surveillance. In order for the former hypothesis to be correct, one must propose that the expression of the particular combination of genes which results in increased sensitivity to natural resistance mediators also produces cell changes which are essential for tumor development *in vivo*. Currently, however, there is no basis for such a proposal, although the high frequency with which activated *ras* oncogenes have been detected in human and animal tumors does suggest an important role for this particular gene in tumor development.

Another possibility which could account for the rarity with which

increased sensitivity to NAb is observed in the current study relates to the nature of the target cells employed. Both 10T½ and NIH 3T3 are derived from mouse embryos, are aneuploid and exhibit non-senescent growth *in vitro*. Thus although these cells are not tumorigenic, clearly neither are they normal. The ability of the *ras* oncogene to transform immortalized but not primary cells *in vitro* without the requirement for a cooperating oncogene suggests that the former cells have already undergone alterations which are relevant to the multistep process of tumorigenesis (see Chapter 1). Therefore, these cells may already exhibit cellular characteristics related to tumor development which are recognized by NAb. In addition, the embryonic origin of these cell lines may result in substantial NAb binding, since tumor-reactive NAb have been shown to cross-react with antigens expressed on fetal tissue (see Chapter 1). For these reasons, the potential to induce further increases in NAb binding may be limited. Evidence that cell surface changes are associated with cell immortalization was provided by Rak et al (1991), who reported the 10 fold higher binding of two lectins, peanut agglutinin and soy bean agglutinin, to the surface of an immortalized human breast epithelial cell line than to the "mortal" cells from which they were derived. Support for the hypothesis that the immortalized mouse embryo cell lines may already be targets for natural resistance is provided by the fact that 10T½ and NIH 3T3 both exhibit considerable NK sensitivity before oncogene transformation (Greenberg et al., 1987a). Further, NK cytolysis of NIH 3T3 is higher than that of 10T½ suggesting that NIH 3T3 is "less normal" than 10T½, an idea which is supported by the greater susceptibility of NIH 3T3 to *ras*-induction of focus formation (Hsaio et al., 1984; Taparowski et

al., 1987). It might then be predicted that both 10T½ and NIH 3T3 may bind more NAb than their normal cell counterparts, and that NIH 3T3 may additionally bind more NAb than 10T½. Both 10T½ and NIH 3T3 do exhibit readily demonstrable NAb binding. However, because the 10T½ and NIH 3T3 cell lines were derived from whole mouse embryos following selection for immortalized growth (Todaro and Green, 1963; Reznikoff et al., 1973), the normal cell counterparts to which NAb binding should be compared are unknown and hence this hypothesis is untestable. It is similarly impossible to directly compare the NAb binding of 10T½ and NIH 3T3 since there is no syngeneic source of NAb for NIH 3T3, and levels of NAb in different strains of mice may well be different. Thus, while the experiments in this study were designed to examine changes in NAb binding which occur during an early stage of tumor development, the conversion of cells with an immortalized, non-tumorigenic phenotype to tumor cells, changes relevant to tumor surveillance may occur at an even earlier stage. In future experiments the effects on NAb binding of oncogene expression in primary cells derived from adult animals should be examined to assess this possibility.

The fact that increased NAb binding was only observed when the *ras* oncogene was expressed in certain cells indicated that the effects on NAb binding were dependent upon the genetic context in which the *ras* oncogene was expressed. This was further indicated by the observation that hybridization of two high NAb binding v-H-*ras* transformed 10T½ clones to a low NAb binding H-*ras* transformed 10T½ clone produced cells with different NAb binding than did hybridization between the same high NAb binding clones and untransformed 10T½. The nature of the genes which may

influence the effect of *ras* on NAb binding are not known, although the somatic cell hybridization studies suggested that interactions between multiple genes may determine the ultimate effect on NAb binding. The observation that focus-selected *ras* transformed 10T½ cells were enriched for cells which were not increased in NAb binding, and the failure to induce increased NAb binding following *ras* transformation of NIH 3T3 suggested that genes which confer increased cell growth autonomy may modulate *ras*-induced effects on NAb binding. Therefore, the expression of cooperating oncogenes or the loss of tumor suppressor gene expression would appear to be logical candidates for genetic alterations which can influence the effect of *ras* on NAb binding, and future studies should examine this possibility. As described earlier, *ras* induction of cellular characteristics such as tumorigenicity, metastatic potential and differentiation, have been shown to be recipient cell-specific. The present study has revealed that subtle differences exist at the cell surface between different *ras*-transformed cells and that these differences can be detected by NAb and may therefore be relevant to tumor development *in vivo*.

The *in vivo* relevancy of the differences in NAb binding among *ras* transformed 10T½ cells was suggested by the inverse correlation observed between NAb binding and early tumor incidence among four *ras* oncogene-expressing, T24-H-*ras*/neo^r-transfected 10T½ clones, and the similar inverse relationship between NAb binding and tumorigenicity which was evident when the average properties of v-H-*ras*/neo^r-infected, focus-selected 10T½ clones were compared to those of v-H-*ras*/neo^r-infected, G418-selected, FACS-sorted 10T½ clones. Since the higher tumorigenicity was associated with the

selection for focus-formation, it is possible that the genetic characteristics which allowed the cells to form foci were contributing to their increased tumorigenicity by mechanisms unrelated to NAb binding. Nevertheless, the results were consistent with the inverse correlation between NAb binding and tumorigenicity observed for the *in vitro*-selected murine lymphomas, and extend support for a role for NAb in the defense against *ras* transformed cells.

The basis of the increased NAb reactivity observed either following TPA treatment of the lymphoma cell lines or following H-*ras* transformation of 10T½ is not known. Because there is evidence that there exist multiple tumor-reactive NAb with distinct specificities, it is possible that the increased NAb reactivity may be due to NAb binding to a single antigen or to multiple antigens. At the level of the target cell, this may be due to either increased or modified expression of antigens present on the parental cell lines, which results in an increased number of binding sites or the expression of antigens to which NAb have an increased affinity respectively. Alternatively, NAb which did not bind to the parental cells may bind to the treated cells due to the exposure of novel antigens. Based on the data obtained in the current study it is impossible to differentiate between these possibilities.

The identities of the antigens which are targets for the increased NAb binding on H-*ras* transformed 10T½ are also not known. The increased NAb binding was not non-specifically associated with morphological transformation, since increased NAb binding was not observed on v-H-*ras* transformed NIH 3T3, v-*src* transformed 10T½ or a fraction of H-*ras* transformed 10T½, all of which exhibited transformed morphologies. Pronase

treatment of each of 10T½, a v-H-*ras*/neo^r-infected 10T½ population, a v-H-*ras*/neo^r-infected high NAb binding 10T½ clone (I8T2.1) and the 2H1 ± added $ZnSO_4$ has been shown to reduce subsequent NAb binding by 46-64%, demonstrating that a substantial proportion of the antigens are dependent upon protein (i.e. the antigens are either protein, glycoprotein or bound to one of these) (Chow, unpubl. obs.). Similar pronase-mediated reductions in NAb binding were observed for both the *ras* oncogene-expressing and non-*ras* oncogene-expressing cells, indicating that the exposure of both pronase-resistant and pronase-sensitive determinants were increased on the former cell type. *Ras* oncogene expression has been associated with altered expression of cell surface molecules in a number of cell systems. For example, *ras* oncogene expression was associated with increased expression of LFA-1 on EBV-immortalized B-lymphoblastoid cell lines (Endo et al., 1991) and unidentified, monoclonal antibody-detected molecules with molecular weights of 62,000, 86,000 and 101,000 on a rat fetus-derived fibroblast cell line (WFB) (Cho et al., 1991). However, because *ras*-induction of increased NAb binding was recipient cell specific, the relationship of cell surface alterations observed upon *ras* oncogene expression in cell types other than 10T½ to the antigens recognized by NAb is uncertain. It may be of interest that *ras* transformation of the WFB cell line was associated both with increased expression of the cell surface molecules mentioned above and with increased NK-sensitivity. Since we have observed similarities with regard to the effects of particular oncogenes on changes in both NAb binding and NK sensitivity of specific recipient cells, it is possible that *ras*-transformed WFB cells may also show increased NAb binding, in which case the increased expression of the

unidentified 62, 86 and 101 KD proteins may be relevant. It is a likely possibility that at least some of the cell surface alterations which occur following *ras* transformation of 10T½ and result in increased NAb binding are associated with changes in carbohydrate expression. Tumor-reactive NAb have been shown to frequently bind to carbohydrate antigens (see Chapter 1). In addition, it has been shown that H-*ras* expression in a number of cell lines results in changes in both glycolipid expression and in altered glycosylation of glycoproteins. An increased proportion of highly branched carbohydrate molecules was detected on the glycoproteins expressed on NIH 3T3 cells transformed by *ras* oncogenes (Santer et al., 1984; Collard et al., 1985). An alteration in cell surface carbohydrates following c-H-*ras* oncogene transformation of a human breast epithelial cell line was inferred from the increased binding of the lectin peanut agglutinin (Rak et al., 1991). Altered patterns of glycolipid expression have been observed following *ras* oncogene expression in various cell types including Balb/c 3T3 (Rosenfelder et al., 1977), NIH 3T3 (Matyas et al., 1987) and primary cultures of normal human renal proximal tubule cells (Nanus et al., 1989). Interestingly, the changes in glycolipid expression which occurred following *ras* expression were different in each of these cell types, suggesting that *ras*-induced alterations in glycolipids, like those in NAb binding, are dependent upon the genetic context of the cell in which *ras* is expressed. While these studies provide some information with regard to the types of cell surface changes induced by *ras* oncogene expression, the identification of the specific molecules bound by NAb awaits further study.

Also unknown is the mechanism through which the antigens to which

NAb bind are regulated by *ras* oncogene expression in 10T½. Although this question was not directly addressed in the current study, several observations may be pertinent to this matter. Firstly, the absence of increased NAb binding following v-*myc* expression in 10T½ or v-*src*-induced transformation of 10T½ suggested that the cell changes associated with increased NAb binding were regulated through a specific biochemical pathway activated by *ras* p21. In light of previous studies which have placed *ras* downstream from *src* along a common biochemical pathway in cell transformation, the failure of *src* expression to increase NAb binding may be interpreted as suggesting either that: (1) the activity of *ras* which is involved in producing increased NAb binding is not stimulated following *src* oncogene expression or; (2) in addition to stimulating *ras* activity, *src* may modify the outcome of *ras*-induced biochemical pathways. Future experiments designed to distinguish between these two possibilities should yield valuable information with regard to the elucidation of oncogene signalling pathways. Secondly, studies with 2H1 revealed that the changes in NAb binding occurred much more slowly than did changes in *ras* p21 expression, suggesting that the cellular alterations recognized by NAb are a result of activities far downstream of *ras* protein activity. Finally, the biochemical pathway which links *ras* p21 activity to increased NAb binding is separable at some point from that involved in neoplastic transfomation, since the two phenomena are not obligately associated.

The cell surface changes which result in the high NAb binding phenotype of the TPA-treated, NAb-sorted lymphomas have also not yet been characterized. The majority of the NAb binding sites present on the murine lymphomas are dependent upon protein, since pronase treatment of L5178Y-

F9, L5178Y-F9 TPA/NAb^{+}3 and SL2-5 inhibited the NAb binding of these cell lines by approximately 76, 71 and 80% respectively (Chow, unpubl. obs.). The similar percentages of pronase-resistant and pronase-sensitive NAb binding on L5178Y-F9 and L5178Y-F9 TPA/NAb^{+}3 suggests that both types of binding sites are increased on the selected cells. Carbohydrate determinants have been implicated as antigens, since NAb binding to the L5178Y-F9 and SL2-5 was partially inhibited by several monosaccharides (Reese and Chow, unpubl. obs.). Thus far, the saccharide-inhibition of NAb binding to the TPA-treated, NAb-sorted cells has not been tested. Alterations in cell surface saccharides have been observed following TPA-treatment of a number of cell types. For example, changes in glycolipid expression have been observed following TPA-induced differentiation of human leukemia cell lines (Momoi and Yokota, 1983, Kiguchi et al., 1986), and following TPA-treatment of mouse epidermal cells (Srinivas and Colburn, 1982), while TPA-treatment of 4 different human tumor cell lines was shown to decrease cell surface sialic acid (Nabi et al., 1989).

There are many molecules which could be regarded as candidates for NAb target antigens since the surface expression of numerous cell proteins has been shown to be upregulated following treatment of various cells with TPA, including both isoforms of the glucose transporter protein on rat adipose cells (Holman et al., 1990), CD13 on the human leukemia cell line HL60 (Kubota et al., 1991), CD5 on human T-leukemic cell lines (Chiron et al., 1990), fibronectin receptors, which also exhibited altered glycosylation on the K562 erythroleukemia cell line (Symington et al., 1989), class I and II major histocompatability antigens on human chronic lymphocytic leukemia cells (Guy et al., 1983), carcinoembryonic antigen

and the transferrin receptor on human breast carcinoma cell lines (Leon et al., 1989), and the T cell antigen receptor and the transferrin receptor on T cells (Vyth-Dreese and DeVries, 1984; Shackelford et al., 1987). The prediction of which molecules might be altered on the TPA-treated, NAb-sorted L5178Y-F9 and SL2-5 cell lines is difficult, since the pattern and extent of changes observed following TPA-treatment varies between different cell lines. In addition, TPA has been shown to produce such opposing effects as the inhibition of differentiation and the induction of terminal differentiation in different leukemic cell lines (reviewed in Delia et al., 1982). The fact that no change in *in vitro* growth rate was observed for the TPA-treated, NAb-sorted cells in the present study shows that these cells were not induced to terminally differentiate by TPA. The possibility exists, however, that the expression of some markers associated with T cell maturation may have been increased. Two such markers which have shown increased expression following TPA-treatment of T leukemia cell lines are the T cell receptor and CD8 (Ryffel et al., 1982; Delia et al., 1982; Shackelford et al., 1987). Antigens associated with T cell activation have also been induced on T cell leukemias following TPA-treatment. These include the transferrin receptor (Vyth-Dreese and DeVries, 1984) and the interleukin-2 receptor, which also exhibited an altered pattern of glycosylation (Shackelford and Trowbridge, 1984; Shackelford et al., 1987). The expression of these activation antigens following TPA-treatment is transient, so any relationship to the stable, irreversible increase in NAb binding exhibited by the TPA-treated, NAb-sorted cells is unknown. It has been shown that TPA-treatment of the L5178Y-F9 produces an early decrease in NAb binding followed by a

transient, PKC-dependent increase 4 days after initial exposure to TPA (Sandstrom and Chow, submitted). The kinetics of the alterations in NAb binding were similar to those observed for the T cell receptor and the transferrin receptor (May et al., 1984; Vyth-Dreese and DeVries, 1984; Minami et al., 1987; Shackelford et al., 1987) suggesting that these molecules may be targets for NAb binding. However, the antigens responsible for the TPA-induced, transient increase in NAb binding of L5178Y-F9 have not yet been identified and their relationship to the antigens which exhibit stable increases in expression on L5178Y-F9 TPA/NAb$^+$3 and SL2-5 TPA/NAb$^+$3 is unknown.

The identification of the antigens to which NAb bind, and the mechanisms of their regulation following TPA-treatment or *ras* oncogene expression should therefore be an important area for future research. The elucidation of the *ras*-induced effects on NAb binding will not only have important implications for the understanding of tumor cell/natural resistance interactions, but should also provide insight into the biochemical activity of the *ras* protein in the cell.

In conclusion, evidence is presented in support of a role for NAb in the rejection of transplanted syngeneic tumor foci, and the existence of a NAb-susceptible phase of tumor development was indicated, suggesting the potential for NAb to participate in surveillance against incipient neoplasia. In addition, numerous questions were raised by this study with regard to the regulation of NAb target antigens by genes associated with all stages of tumor development, which should be the focus of future studies.

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