PHYSIOLOGICAL MEDIATORS OF PSYCHOSOCIAL STRESSOR EFFECTS ON THE GROWTH OF A HORMONE-RESPONSIVE MOUSE MAMMARY CARCINOMA

by

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ABSTRACT

This thesis investigated the role of specific immune and endocrine variables in mediating the effects of social housing condition on the growth of the transplantable androgen-responsive Shionogi mouse mammary carcinoma (SC115). Mice were reared either individually (I) or group (G) housed, and then either remained in their rearing groups (II, GG) or were rehoused (IG, GI).

Splenic NK cell activity of tumor cell- and vehicle-injected mice from the 4 experimental housing groups was investigated at 1 d post tumor cell- or vehicle-injection. Splenic NK cell activity was suppressed in tumor cell-injected mice compared with vehicle-injected controls. Overall, mice of the GI group had significantly greater splenic NK cell activity than mice of the IG group. NK cell activity of tumor infiltrating lymphocytes in mice of the GI and IG groups was then investigated using a modification of the sponge allograft model. Overall, NK cell activity was greater in tumor cell-injected than in vehicle-injected sponges, and was greater in tumor cell-injected sponges of mice in the GI group than in those of mice in the IG group. Finally, the effects of modulating *in vivo* levels of NK cell activity on tumor growth rate were assessed. Injection ip of polyinosinic:polycytidylic acid (poly I:C, 100 µl 1mg/ml) or of anti-asialo GM1 ($ASGM_1$, 100 µl of a 1:5 dilution) every 5 d for 2 wk maintained stimulation or suppression (respectively) of splenic NK cell activity relative to that in saline-injected control mice, and had no effect on tumor growth rate in mice of the IG group. In mice of the GI group, stimulation of NK cell activity by poly I:C was accompanied by a significant stimulation of tumor growth rate compared with that of $ASGM_1$-injected or control mice. These studies suggest that NK cells may play an important role in mediating the stimulation of SC115 tumor growth rate in mice of the GI group.

In addition, the possibility that 1) selection for a slow growing, hormone-independent phenotype, or 2) alterations in plasma hormone levels may mediate the differential tumor growth rates was examined. Slow growing tumors from mice of the IG group had a morphological appearance similar to that of mice from the other experimental groups and dissimilar to that of slow growing androgen-independent tumors grown in females. Further, tumor cells from mice of the IG group showed greater proliferation in response to in vitro stimulation with dihydrotestosterone or hydrocortisone than tumor cells from mice of the GI group. In addition, mice of the GG and II groups had elevated basal levels of plasma testosterone at 1 d which declined significantly by 3d. Mice of the IG group had low basal plasma testosterone levels, whereas mice of the GG group had elevated basal plasma testosterone levels at all time points. In contrast, basal plasma corticosterone levels were significantly greater in mice of the IG group than in mice of all other groups at 1, 3 and 7 d. These data suggest that altered plasma levels of steroid hormones may also, in part, mediate the effects of psychosocial stressors on the differential tumor growth rate observed in this model, whereas selection for a subpopulation of SC115 cells with altered hormone responsiveness is likely not involved.

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Forward

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For all portions of these papers that are reported in this thesis, Gerry Rowse was the major contributor involved in conducting the research, analyzing the data and writing the papers.

CHAPTER 1: INTRODUCTION :

A) Human Studies of Stress and Cancer

The concept that psychological variables may alter the susceptibility of humans to disease processes, including cancer, is not new. For example, Galen observed that "melancholy women" were more prone to breast cancer than were "sanguine women" (LeShan, 1959). In the eighteenth century a number of noted British physicians recorded empirical observations of an association between breast cancer and personality amongst their patients (Le Shan, 1959). However, observations such as those presented above are anecdotal in nature and are subject to considerable observer bias. It was not until the middle of the 20th century that this question began to be examined by controlled scientific studies (Levenson and Bemis, 1991). Since the 1950's epidemiological studies have examined the association of such psychological attributes as affective state, coping style and stressful life events with the occurrence, progression and/or prognosis of cancer.

A.1) Definition of Stress and Psychosocial stressors

To investigate scientifically the possible role of stress in the initiation, promotion or progression of cancer, it is important to understand the current concepts of stress and to utilize a standardized terminology. The term stress has been much used and abused in both popular and scientific literature. The word has been used to refer interchangeably to a mental state, an external cue in the environment which is perceived as threatening or as the physiological changes which occur in response to external cues. For the purposes of this dissertation, the conventions established by Selye will be used. Stress will be defined as "an alteration in the body's hormonal and neuronal secretions caused by the central nervous system in response to a perceived threat".

Further, a stressor will be defined as "a change in an organism's internal or external environment which is perceived by the organism as threatening".

The modern concepts of stress arose from the pioneering works of Cannon and of Selye in the early part of the century (Mason, 1975a, 1975b, Selye, 1975). Cannon viewed stress as any event which disturbed the state of homeostasis within the organism. He considered stressors to include such events as exposure to cold, lack of oxygen, low blood sugar, loss of blood and emotional stimuli. Cannon emphasized the role of the autonomic nervous system (both sympathetic and parasympathetic) in responding to a stressor so as to restore homeostasis within the organism. In contrast, Selye emphasized the role of the pituitary-adrenal system in responding to noxious stimuli. He proposed, as a part of his theory of the general adaptation syndrome, that glucocorticoid release from the adrenal gland was a general and non-specific response by the organism to all forms of noxious stimuli, and equated "biologic stress" with the release of glucocorticoids (Mason, 1975a, 1975b, Selye, 1975). He suggested that unchecked or chronic stress resulted in exhaustion of the coping mechanism and then disease. Cannon and Selye each focused primarily on a specific hormone system as being the critical component of a general response to noxious stimuli. However, subsequent research has demonstrated that during the stress response, the brain is capable of modulating most if not all endocrine functions and that changes in the function of many endocrine systems may be important components of the stress response.

Although both Cannon and Selye primarily focused on the effects of noxious physical stimuli on the organism, more recently, it has been demonstrated that psychological stimuli, such as exposure to a novel environment, may be as effective as noxious physical stimuli in eliciting a stress response (Mason, 1975b, Weinberg and Levine, 1980). In fact, the response to both physical and psychological stimuli appears to depend on the cognitive processing of the stimuli by the organism. Following exposure to a stimulus, a number of factors, such as past experience,

emotional state or other environmental factors, will influence whether or not the organism perceives the stimulus as threatening. Mason suggests that the common mechanism underlying the nonspecific response of the organism to diverse noxious stimuli, as reported by Selye, is in fact the organism's perception of the stimulus as threatening (Mason, 1975b). Thus, it has been suggested that both noxious physical stimuli and psychological stimuli act through a common mechanism of increasing the organisms level of emotional arousal (i.e. level of fear or anxiety) (Mason, 1975b). The level of activity of the hypothalamic-pituitary-adrenal axis is a good indicator of an animal's level of emotional arousal and is accepted as a central feature of the stress response (Hennessy and Levine, 1979).

Moreover, it has been demonstrated that following exposure to an aversive stimulus, cognitive or psychological processes may act to reduce the impact (i.e. level of emotional arousal) of a stressor on the animal (Levine et al, 1978, Weinberg and Levine, 1980). Examples of such cognitive processes include habituation and coping responses. Habituation has been defined as the process whereby a novel stimulus that initially increases emotional arousal, but which is not intrinsically aversive, loses it's ability to induce a stress response as the animal learns to associate the stimulus with a nonthreatening outcome (Levine et al, 1978). In contrast, coping may be defined as the process that enables an animal to decrease its physiological response to a noxious or aversive stimulus (Levine et al., 1978). Coping may involve the performance of a behavioral response (such as fighting) or it may rely on purely psychological mechanisms (such as predictability of the stressor), but ultimately, coping occurs via cognitive processes that reduce the impact of the stressor on the emotional response of the animal (Levine et al., 1979).

An example of a purely psychological coping process is the effect that predictability of a stressor has on the response of the organism to that stressor. It is known that the physiological and hormonal responses to predictable or signaled shock are significantly less than those elicited by unsignaled shock, and that animals will choose signaled over unsignaled shock, even though

the signaled shock may be of greater intensity and of longer duration (Weinberg and Levine, 1980). An example of a behavioral coping response is the fighting observed when male rats receive electric shocks in pairs (Weinberg et al., 1980). Such animals exhibit a stereotyped fighting response to electric shock, that is, rearing, boxing and biting at each other. This response significantly decreases the physiological consequences (e.g. ACTH level and degree of gastric ulceration) of the electric shock compared with that seen in animals shocked individually. The protective effect of this coping response does not rely on the physical effects of fighting per se, as the reduced physiological responses are still observed if the rats are separated by a clear Plexiglas barrier and thus are only able to rear and posture but not to make physical contact (Weiss et al., 1976). Thus, this coping response appears to rely on the opportunity for the animal to perform a stereotyped or organized behavioral response which, through cognitive processes, somehow reduces emotional arousal. It has been suggested that this behavior may be equivalent to displacement behavior in humans (Weinberg and Wong, 1983). The concept that fighting represents a type of coping response is discussed further in section B of the introduction.

In summary, many types of aversive stimuli, both physical and psychological, affect the organism through the common mechanism of altering emotional arousal. Further, the ability of animals to cope with a stressor is known to decrease the physiological response of the animal to the stressor (Levine et al., 1978). As stated previously, coping, whether involving purely psychological processes or involving a behavioral response, acts to decrease the animal's level of emotional arousal in response to the stressor (Levine et al., 1978).

A.2) Affective Disorders and Cancer

Affective or emotional state has been strongly implicated as playing an important role in the etiology of cancer. A number of studies have demonstrated that depression and traits associated with depression may be associated with increased cancer incidence/and or poorer prognosis. For example, one study looked prospectively at the relationship between personality

and the incidence of cancer in 2,020 male workers at Western Electric using the Minnesota Multiphasic Personality Inventory (MMPI) to assess personality (Shekelle *et al.*, 1981). In this study, men working at the Chicago Western Electric plant in 1957-58, who were between the ages of 40 and 55, were given a medical exam to insure that they were healthy and were asked to fill out the MMPI forms. Seventeen years later the medical status of the subjects was examined. The overall cancer incidence was 10.5% (212 men) and it was found that men who scored high on a depression scale (380 men) had a significantly greater cancer mortality rate, exhibiting twice the incidence observed in non-depressed subjects. The authors note that the stronger association of depression with cancer mortality than with cancer incidence suggests that depression may affect tumor progression rather than tumor initiation. Similarly, Whitlock & Siskind (1979) found a statistically significant increase in the cancer mortality rate during a 4 year follow-up of 126 patients (initially cancer free) hospitalized for depression when compared with the normal population.

Studies also suggest the existence of a link between depression and length of survival in patients with advanced cancer. Blumberg *et al.* (1954) noted that patients with rapidly progressing tumors had a characteristic pattern of scores on the MMPI test, including a high rating on the depression subscale. This was in contrast to patients with slowly progressing or arrested tumors. In another study, 23 patients with various forms of malignant disease were asked to fill out a self report symptom inventory of psychological status (SCL-90) (Derogatis *et al.*, 1976). It was found that depression was significantly linked with a poor prognosis, but not with various demographic characteristics including sex, religion, social class and marital status. Finally, a study by Levy *et al.* (1988) indicated an association between depression in 34 women being treated for a first recurrence of breast cancer and poor survival (less than 2 years,$p<0.08$). Further, a patient's level of joy (as measured by SCL-90) was a significant predictor of length of survival in these women ($p<0.01$), being better than number of metastatic sites or the physicians

rating of prognosis. Thus, this body of experimental evidence supports the early observation of Galen (Le Shan, 1959) that depression is linked with cancer incidence and prognosis.

The role of depression in the development or prognosis of cancer remains controversial for several reasons. First, several large prospective studies have found no association between depression and cancer incidence or mortality. A prospective study of 6,848 adult residents of Alameda County assessed the link between depressive tendencies and cancer using the Human Population Laboratory questionnaire in 1965 (Kaplan and Reynolds, 1988). After a 17 year follow-up, no significant association between depression and either cancer incidence or cancer mortality was found. In another study, 8,932 females undergoing routine breast exams between 1968 and 1972 were asked to complete the MMPI form. This study failed to demonstrate a link between depression at the start of the study and the 117 cases of breast cancer observed during a 10 year follow up (Hahn and Petitti, 1988). Second, many studies in this area have been criticized for methodological flaws (Bieliauskas and Garron, 1982; Levenson and Bemis, 1991). For example the studies of Blumberg *et al.* (1954) and Derogatis *et al.* (1976) have been criticized for failing to control for stage of disease. In addition studies have been criticized for small, biased samples, inclusion of patients with different types of cancer, failure to control for stage of disease and treatment of cancer, retrospective subject bias and failure to standardize measures of psychosocial factors (Levenson & Bemis, 1991). Further, it has been suggested that differentiating among various depressive disorders and examining the chronicity of the depression is important but that many studies failed to consider these issues (Levenson & Bemis, 1991).

Another affective or emotional state suggested to be associated with cancer incidence is the feeling of hopelessness and/or helplessness. Interestingly, reported feelings of hopelessness or helplessness have also been considered as an indicator of depression (Bieliauskas and Garron, 1982). Here again, studies of the association of this trait with cancer have produced conflicting

results, demonstrating positive associations (Schmale and Iker, 1966; Wirsching, 1982) or no association (Casselith, 1988).

A.3) Personality / Coping Style and Cancer

A second psychosocial factor that has been suggested to be important in the incidence or the progression of cancer is personality and/or coping style. It has been convincingly demonstrated that personality is a factor in influencing the outcome of certain disease states. The best example of this is the association of heart disease with the type A personality (Rosenman *et al.*, 1964). Similarly, based on her observations of melanoma patients, Temoshok has proposed that a new personality type, the type C personality, may be associated with an increased risk of cancer (Temoshok and Fox, 1984). The type C personality is described as being intermediate between the type A and B personalities: an individual who strives to maintain a sense of pleasant interpersonal atmosphere with control over angry expressions, and a sense of themselves as well liked. According to this view, the type C personality differs from the type B personality in that the former represses negative emotions while the latter does not. Temoshok investigated 106 patients with malignant melanoma and found that significantly more of the patients who died or relapsed during an 18 month follow-up were of the type C personality (Temoshock and Fox, 1984). An important component of the type C personality is the repression of negative emotions. This characteristic has also been linked to cancer incidence in a number of studies (Grossarth-Maticek, 1985, Kissen and Eysenck, 1962, van der Ploeg, 1989, Wirsching *et al*, 1982). For example, a 10 year (1966 to 1976) prospective study of 1353 residents of a Yugoslavian village demonstrated that suppression of emotions was significantly correlated with increased risk of cancer (Grossarth-Maticek *et al.*, 1985). Also, using suppression of emotions or poor expression of emotions as the criteria, Wirsching *et al.* (1982) correctly predicted the diagnosis of 75% of 58 patients undergoing breast biopsy. Similarly, Greer and Morris (1975) found that patients exhibiting a "fighting spirit" had significantly increased 5 year survival rates.

It should be recognized that affect is an important component of personality and that suppression of emotion, a key factor in Temoshok's cancer prone personality, is considered by some to be a reflection of depression. Thus it is possible that all of the studies mentioned are measuring various facets of the same personality variable or personality type.

A.4) Childhood Stress/Loss and Cancer

Cancer is a disease which is believed in general to have a long development period. For example, colon cancer is thought to involve mutations in up to 4 discrete genes (oncogenes and suppressor genes, Vogelstein, 1989). Thus, some researchers feel that if psychosocial stressors really do play a role in the initiation or progression of cancer, it is important to look at early times in the patient's history for evidence of psychosocial perturbations. This approach is exemplified by a prospective study conducted by Thomas *et al.* (1979). In this study, psychological and medical data were collected from 1,337 Caucasian male medical students attending John Hopkins Medical School in 1946. The researcher used this information to investigate prospectively the association of quality of early family life with subsequent cancer incidence. Of 913 students who filled out a family attitudes questionnaire (FAQ), 48 subsequently developed cancer during the 30 year follow-up. It was found that the cancer patients had previously reported significantly less closeness with their parents ($p<0.01$) than had the control subjects. This finding has recently been replicated in a retrospective study in which it was found that women with breast and gynecological cancers reported significantly less closeness with their parents using Thomas's FAQ score, than did matched controls (Gehde and Baltrusch, 1990).

A.5) Life Stress/Psychosocial Stressors and Cancer.

There have long been anecdotal reports of stressful life events (or psychosocial stressors) preceding the onset or recurrence of cancer. Recently, a number of studies have demonstrated links between life stressors and cancer. For example, retrospective studies have demonstrated that severely stressful life events, such as the death of a close friend or a family member, are more common in a 1 to 6 year period preceding the diagnosis of breast cancer (Cooper *et al.*, 1989, Forsen, 1991, Geyer, 1992) or other types of cancer (Taylor *et al.*, 1988) than in control populations. Also, severely stressful life events were reported more frequently in women experiencing relapse of breast cancer than in women who did not experience a relapse (Forsen, 1991, Ramirez *et al.*, 1989). Retrospective studies of the link between life stressors and cancer have been criticized because cancer patients, knowing their diagnosis, may exhibit bias to negative interpretations of life events (Levenson and Bemis, 1991). However, it has recently been demonstrated that recall bias is not a significant factor in these studies (Geyer, 1992).

In contrast, a number of studies have failed to demonstrate an association between negative life events and cancer incidence. For example, cancer incidence in prisoners of war following World War II and the Korean war did not differ significantly from that in the normal population (Keehn, R.J. *et al.*, 1974,1980). Ewertz (1986) found no association between the assumed bereavement caused by widowhood or divorce and the diagnosis of breast cancer in 1782 women diagnosed with breast cancer and 1738 randomly selected control subjects. However, there is no indication of how well matched the two populations were with respect to age, socioeconomic status or ethnic origin. Further, studies of loss such as this have been criticized for failing to take into account the individual's perspective of a supposedly stressful event. For example, to some, divorce may be viewed as a release and as such a joyous occasion (Eysenck, 1988).

A.6) Social Contacts and Cancer

There is evidence that social relationships have a protective effect on general health and well being (House *et al.*, 1988). This effect is especially observed after traumatic events such as automobile accidents and heart attacks (Frasure and Prince, 1985, Porritt, 1979). There is also evidence that social relationships may have beneficial effects on cancer patients. One large prospective study found that low levels of social contact and social isolation increased the risk of cancer mortality in both men and women (Kaplan and Reynolds, 1990). The study also found that decreased social contact in women increased the risk of developing cancer (Kaplan and Reynolds, 1990). Further, several prospective studies have suggested that a correlation exists between perceived levels of social support or social contact and survival in women newly diagnosed with breast cancer (Ell *et al.*, 1992, Waxler-Morrison *et al.*, 1991). Perhaps the most convincing evidence for the effects of social support on cancer survival come from two studies which independently examined the effects of psychological counseling on survival in patients with advanced breast cancer. These studies demonstrated that psychotherapy can significantly prolong the survival of women with advanced breast cancer (Grossarth-Maticek *et al.*, 1989, Spiegel *et al.*, 1989). These findings are particularly impressive because Spiegel set out to disprove the theory that psychological counseling could affect the course of breast cancer. Further, one of these studies demonstrated that psychotherapy was as effective as chemotherapy in prolonging the life of women with advanced stage metastatic breast cancer and can exhibit synergistic effects with chemotherapy in prolonging life (Grossarth-Maticek *et al.*, 1989).

Thus, although there are many scientific studies that strongly suggest an association between cancer and psychological or emotional variables, the topic remains controversial. This controversy arises from two sources. First, it is probable that our attempts to study the effects of discrete psychological variables such as personality type or stressful life events are too simplistic. It is likely that affective state, personality, coping styles and social interactions

combine to affect how people view life events and ultimately how any of these factors will impact on cancer. Thus it is probably somewhat simplistic to look at one characteristic in isolation without examining all other variables. Further it is possible that subtle variations in several variables could have an impact on cancer initiation or progression and yet be beyond our current ability to measure or accurately control. Second, the lack of precise knowledge of the events and timing involved in the genesis and progression of cancer likely hamper studies of the effects of stressors on cancer.

B) Animal Models Of Stressor Effects on Cancer.

As noted above, research on the role of stressors in modulating tumor growth is complex and some studies have yielded ambiguous or even negative results. It is likely that the confusion in this field of research arises, at least in part, from an inability to control the frequency or severity of stressors experienced by the subjects and difficulty defining the temporal association of the stressors with the induction of the tumor. Thus, a number of researchers have turned to animal models to study the possible link between stressors and cancer. Animal models provide the ability to control all study variables including the type of stressor, the severity of the stressor and the timing of application of the stressor relative to tumor injection or induction. Studies using animal models have demonstrated that stressors can affect tumor growth. However, despite the increased control over study variables, the results of animal studies are also often inconsistent and contradictory. Data have demonstrated that stressors may cause an increase, a decrease, or have no effect on tumor growth. A number of factors have been suggested to be important in determining the effect that a stressor will have on tumor growth. These factors include the type of tumor used, the type of stressor used, the timing of application of the stressor (i.e. before or after tumor induction), whether the stressor is chronic or acute and whether or not the animal has a coping response available to help it deal with the stressor (Justice, 1985, Sklar and Anisman, 1981, Solomon and Amkraut, 1981).

Type of Tumor and Timing of Application of the Stressor. It is well known that stressors affect different types of tumors differently. For example, stressors are known to decrease the growth of most tumors induced by the chemical carcinogen 7,12 dimethylbenz[a]anthracene (DMBA) (Newberry, 1978, Ray and Pradhan, 1974), whereas stressors generally increase the growth of virally-induced tumors (Amkraut and Solomon, 1972; Riley, 1981). Justice (1985) suggests that stressor effects on viral tumors (immunoresponsive due to the expression of viral antigens) are due to the ability of stressors to affect immune functioning. Stressors applied during the growth of virally induced tumors suppress the immune system, allowing increased growth of the tumor. However, if the stressor is applied before or in the early phases of tumor induction, rebound enhancement of immune functioning occurs following cessation of the stressor and results in decreased growth of the virally induced tumor (Amkraut and Solomon, 1972; Sklar and Anisman, 1981). Justice (1985) further predicts that, with non virally-induced tumors, the immune system does not play a role in mediating the effects of stressors on tumor growth. Instead, stressors applied after tumor induction or injection appear to inhibit the growth of these tumors by other, possibly hormonal, mechanisms. In contrast, stressors applied prior to tumor induction or growth stimulate tumor growth by a rebound enhancement mechanism which would occur following the cessation of the stressor. Justice claims that these two factors, the type of tumor and the timing of application of the stressor relative to the tumor induction or injection, are sufficient to explain the variable results observed in this field.

Although Justice's theory explains many apparent discrepancies in this field of study, his model is not completely satisfactory for several reasons. First, although Justice's theory explains the growth of virally-induced tumors, it does not completely explain tumor growth in non virally-induced tumors such as carcinogen-induced tumors. Many studies support Justice's claim that stressors applied during tumor induction (using the chemical carcinogen DMBA) suppress or delay the growth of tumors. However, a number of studies have been published which have the

opposite finding: that chronic stressor administration during tumor induction by DMBA actually decreases tumor latency and increases tumor growth (Steplewski, 1985, Steplewski *et al.*, 1987, Tejwani *et al.*, 1991). Second, Justice does not consider studies which use the psychosocial stressor of differential housing. Interestingly, these studies show that individual housing as well as a change in housing condition typically increase the growth of various non viral tumors (Dechambre and Gosse, 1973, Sklar and Anisman, 1980, Steplewski *et al.*, 1987). Finally, studies have shown that stressors increase both the number and the size of metastases. Thus the type of tumor and the timing of stressor application are likely not the only factors responsible for altering the effect that stressors have on the growth of tumors.

Environmental Variables. Environmental variables are known to have an important impact on tumor growth. For example, mice switched from group to individual housing condition show increased tumor growth rates compared with that of mice remaining in their group (Sklar and Anisman, 1980, Weinberg and Emerman, 1989). Further, it has been demonstrated that housing condition can affect the impact of physiological stressors on tumor growth. Sklar and Anisman (1980) demonstrated that group housed mice exhibited increased growth of a transplanted syngeneic tumor following exposure to an acute electric shock, whereas mice moved from group to individual housing did not have an increase in tumor growth following shock exposure.

In addition to housing condition, the general environment in which the animals live (i.e. the intensity and timing of lighting in the animal room, the levels of noise and disturbance in the animal room, etc.) can affect their tumor growth. Riley *et al.* (1981) showed that in virgin female C3H mice, mammary tumor incidence was much greater in mice housed in "conventional" animal housing facilities than if mice were housed in a "low stress" environment. In "conventional" animal housing facilities, mice were housed in stainless steel cages on open racks in a communal animal room, exposing the mice to the daily activities of cage cleaning, bleeding procedures and

other stress-inducing experimental manipulations of other animals in the room. In the "low stress environment", mice were housed in plastic cages with bedding in specially designed racks which minimized the spread of pheromones between cages. Further, the "low stress" environment controlled the temperature variation, the light/dark cycle and the degree of disturbance induced by experimental and maintenance procedures (Riley *et al.*, 1981). Data demonstrated that basal plasma corticosterone levels, one indicator of arousal or stress, were 10 to 20 times lower in mice housed in the "low stress" environment compared with those in "conventional" housing (Riley *et al.*, 1981). Similarly, studies have demonstrated that, in animals housed in "low stress" conditions, some tumors which had previously been considered to be stress-nonresponsive could be shown to be modulated by stressors. Thus, some of the variability in the literature could be a result of failure to control adequately for such variables as housing condition, noise, disturbances and other stressors in communal animal housing facilities and pheromones released by stressed animals.

Acute Vs Chronic Stressors. It has also been demonstrated that the chronic vs acute nature of the stressor can alter the effect of that stressor on tumor growth. For example, exposure to a single (acute) session of electric shock at the time of injection of a syngeneic mastocytoma in mice reliably increased tumor growth. If, however, the animals were given 4 or 9 daily shock sessions before the tumor injection and acute shock session, tumor growth was not enhanced (Sklar and Anisman, 1981). In other experimental models, chronic stress has been shown to decrease tumor growth (Gershben *et al.*, 1974, Marsh *et al.*, 1959, Molomut *et al.*, 1963, Ray and Pradhan, 1974).

Coping Ability. The ability of animals to cope with a stressor is known to decrease the physiological response of the animal to the stressor (Levine et al., 1978). As stated previously, coping, whether involving purely psychological processes or involving a behavioral response, acts to decrease the animal's level of emotional arousal in response to the stressor (Levine et al.,

1978). For example, as described previously, it has been demonstrated that rats subjected to electric shock in pairs exhibit a stereotyped fighting behavior during the electric shock which decreases the number and severity of gastric lesions, the elevation in blood pressure and the plasma levels of ACTH compared with that in rats receiving the same amount of shock as individuals (Conner et al., 1971, Weiss et al., 1976, Williams and Eichelman, 1971). Coping processes may also effect stressor-induced changes in tumor growth. For instance, it has been demonstrated that the growth of a syngeneic mastocytoma is slower in mice exposed to escapable shock than in mice receiving an identical amount of inescapable shock (yoked paradigm) (Sklar and Anisman, 1981). Similarly, male mice that have experienced a change in housing condition from group to individual housing exhibit increased tumor growth, whereas mice transferred from individual to group housing do not show an increase in tumor growth (Dechambre and Gosse, 1973, Sklar and Anisman, 1980, Weinberg and Emerman, 1989). It has been suggested that the protective effect of being moved from individual to group housing may be due to the fighting that occurs in the group (Weinberg and Emerman, 1989). That is, fighting may serve as a kind of behavioural coping response that helps the mice deal with the stressor of changing housing condition. It is not intuitively obvious why fighting, which would appear to be a stressor in itself, may act as a coping response. However, as mentioned previously, it has been suggested that fighting acts as a coping response in rats receiving electric shock. Fighting is suggested to belong to a class of behaviors referred to as consummatory behaviors (Levine et al., 1979). Consummatory behaviors are stereotyped sequences of goal oriented activities such as eating, drinking and mating (Hennessy and Levine, 1979, Weinberg and Wong, 1983). Consummatory responses are known to decrease both the emotional arousal and the activation of the hypothalamic-pituitary-adrenal axis induced by stressors (Levine and Coover, 1976, Levine *et al.*, 1979). Thus, fighting, like other consummatory behaviors, may act to decrease an organisms' level of emotional arousal.

C) Psychoneuroendocrinology and Cancer

C.1) Hormones and Stress

Hypothalamic-Pituitary Axis: It has long been known that stressors may influence endocrine function. As previously mentioned, pioneering work by Cannon and Selye in the early part of the twentieth century demonstrated that stressors could alter the secretions of adrenaline, noradrenaline and glucocorticoids. More recently, it has been demonstrated that the central nervous system is involved in the control of almost all endocrine functions, either directly or indirectly. One method whereby the central nervous system controls endocrine function is via the hypothalamic-pituitary axis. The pituitary, a small neuroendocrine gland located at the base of the brain, is divided into three lobes- anterior, middle and posterior.

The posterior lobe or neurohypophysis is derived from neural ectoderm. Cells located in the hypothalamus synthesize and secrete the peptide hormones oxytocin and antidiuretic hormone. Axons of these cells project to the posterior pituitary. Oxytocin and antidiuretic hormone are stored in axon terminals and secreted from the posterior pituitary following the appropriate stimulation. The middle lobe is functionally unimportant in man. The anterior lobe or pars distailis is derived from oral ectoderm and consists of a number of distinct cell types which are responsible for the production of various peptide hormones. Some of these peptides have direct effects on target tissues (i.e. prolactin, growth hormone, somatostatin and β-endorphin), whereas others function as trophic hormones to stimulate the release of hormones by other endocrine glands (i.e. leutinizing hormone/follicle stimulating hormone, adenocorticotrophic hormone [ACTH], thyroid stimulating hormone). The activity of the anterior pituitary is modulated by the hypothalamus which produces releasing hormones that induce secretion of pituitary peptides. The releasing hormones are delivered to the pituitary by the hypothalamo-hypophyseal portal system, a specialized system of vessels. As an example of how this system functions, one can examine the

control of glucocorticoid secretion. Glucocorticoids are secreted by steroid producing cells of the adrenal cortex which are stimulated to synthesize and release glucocorticoids by the peptide ACTH (Axelrod and Reisine, 1984). ACTH is produced by corticotroph cells of the anterior pituitary and travels in the bloodstream to the adrenal glands. The primary mechanism of stimulating ACTH synthesis and secretion is via the peptide corticotrophin releasing factor (CRF) (Axelrod and Reisine, 1984, Jacobson and Sapolsky, 1991), which is produced by cells of the hypothalamus and released into the hypothalamic-hypophyseal portal system (Axelrod and Reisine, 1984, Reisine *et al.*, 1986). Studies have revealed that vasopressin and cholecystokinin may also be produced by CRF-containing hypothalamic neurons under certain conditions and that these substances also stimulate ACTH production by pituitary corticotrophs, possibly acting synergistically with CRF (Jacobson and Sapolsky, 1991, Reisine *et al.*, 1986). Finally, the catecholamines adrenaline and noradrenaline are known to stimulate pituitary corticotrophs to produce ACTH via β_2 adrenoreceptors (Jacobson and Sapolsky, 1991, Reisine *et al.*, 1986). The stimulation by catecholamines is not as great as that induced by CRF (Axelrod and Reisine, 1984). Plasma glucocorticoids in unstressed organisms follow a circadian rhythm, being lowest during sleep and highest just after waking. In response to stressors, CRF release from the hypothalamus is increased, resulting in increased ACTH and glucocorticoid production and secretion. The release of glucocorticoids is tightly controlled at several levels of the hypothalamic-pituitary-adrenal axis. First, high levels of plasma glucocorticoids act to inhibit the production of glucocorticoids by cells of the adrenal cortex. Elevated plasma glucocorticoid levels also act to decrease the sensitivity of pituitary corticotrophs to CRF. Further, glucocorticoids inhibit the release of CRF from the hypothalamus. The inhibition of CRF release caused by elevated plasma glucocorticoid levels has been demonstrated to be due, in part, to direct actions on the hypothalamus and in part to action on other regions of the brain such as the hippocampus.

Activation of the hypothalamic-pituitary-adrenal axis is a central feature of the stress response. In addition, many stress-induced changes in hormone secretion are modulated, at least

in part, by the action of the hypothalamic-pituitary-adrenal axis. For example, hormonal secretion by the gonads is affected at several levels by glucocorticoids. The hypothalamic release of gonadotropin-releasing hormone (GnRH) is decreased by increased CRF levels and by chronically elevated plasma glucocorticoid levels. Evidence suggests that this may be due to a direct effect on a corticosteroid regulatory element in the GnRH gene (Brann and Mahesh, 1991). However, corticosterone- and CRF-stimulated increases in opioid and catecholamine levels in the hypothalamus also appear important in blocking GnRH release and both naloxone and α-adrenergic antagonists block the effects of chronically elevated glucocorticoids on LH secretion (Brann and Mahesh, 1991, Rivier and Rivest, 1991). Chronically elevated corticosterone decreases the response of anterior pituitary gonadotroph cells to GnRH. Further, the gonads contain functional glucocorticoid receptors and elevated glucocorticoid or ACTH levels have been demonstrated to inhibit the response of Leydig cells to LH (Brann and Mahesh, 1991, Rivier and Rivest, 1991). Thus, the stress-induced alterations in the secretion of sex hormones involve multiple mechanisms acting at multiple levels. Similarly, stress-induced elevations of CRF, ACTH and glucocorticoids have been shown to be involved in the altered secretion of many hormones other than those of the hypothalamic-pituitary-gonadal axis, including insulin and glucagon.

Autonomic Nervous System: A second mechanism by which the central nervous system may control hormonal secretion is through modulation of autonomic nervous function. For example, stimulation of sympathetic nerves has been demonstrated to decrease the release of insulin and stimulate the release of glucagon from islet of Langerhans cells in the pancreas. It has further been demonstrated that this effect is reproducible by the direct administration of adrenergic agonists in the isolated pancreas (Yamaguchi, 1992). During stress responses, increased activity of sympathetic nerves to target organs such as the anterior pituitary, the adrenal medulla and the endocrine pancreas increases the local concentrations of noradrenaline (Axelrod and Reisine, 1984, Yamaguchi, 1992). Further, both increased activity of sympathetic nerves

and increased concentrations of plasma glucocorticoids act to increase the synthesis and secretion of adrenaline from chromaffin cells of the adrenal medulla (Axelrod and Reisine, 1984, Yamaguchi, 1992). Thus, stressor-induced alterations in the secretion of hormones such as glucocorticoids and catecholamines can interact both with each other and with other hormone-producing cells to cause wide spread changes in endocrine function following stressors.

C.2) Hormones and Cancer

Hormones play an important role in the initiation and/or progression of some tumors. For example, it has been reported that 40 - 60% of human cancers are etiologically associated with sex hormone exposure, either endogenously or exogenously (Li *et al.*, 1991). Cancers are often divided into two broad classes, hormone-responsive and hormone-nonresponsive, based on their hormone responsiveness. Hormone-responsive tumors typically arise from the endometrium, breast, prostate, immune system (lymphomas and leukemia) and endocrine system (Lippman *et al.*, 1985). In these tissues, hormones play a crucial role in modulating cell proliferation. Although the precise role of hormones in the carcinogenesis of these hormone-responsive tissues is not known, it is clear that hormones are required for the transformations to occur (Welsh, 1985). Further, most of the tumors that develop are initially dependent on the presence of the hormone for proliferation to occur (Dickson *et al.*, 1992, Miller *et al.*, 1990). Thus, modulation of hormone levels could drastically affect the growth of hormone-responsive tumors.

Steroid Hormones and Growth Factors: It is known that both during development and in many adult tissues, hormones and growth factors are involved in regulating the proliferation and differentiation of cells (Miller and O'Neill, 1990). This is exemplified by the role of steroids, especially estrogens and androgens, in regulating the growth, differentiation and function of a variety of normal tissues including the gonads, pituitary and secondary sex organs (Miller and O'Neill, 1990).

Steroids readily enter the cell, diffusing through the plasma membrane due to their lipophilic properties. In the cell, steroids bind to intracellular receptors specific for the individual class of steroid. Steroid receptors have been demonstrated to be a family of related proteins with molecular weights ranging from approximately 50 to 100 kDa in humans. (Jensen, 1992). The receptors contain a steroid binding domain, a hinge region and a DNA binding domain of approximately 66 - 68 amino acids (Jensen, 1992, Liao, 1992). The receptors bind to the DNA by a pair of zinc finger domains each of which consists of a peptide loop stabilized by zinc ions (Jensen, 1992). Binding of a steroid by the receptor is thought to cause a conformational change in the receptor, releasing a 90 kDa heat shock protein dimer from the DNA binding region of the receptor, thus activating the hormone-receptor complex and allowing it to bind DNA (Jensen, 1992, Liao, 1992). The steroid receptors bind to certain DNA sequences termed hormone-responsive elements (HREs) and there is apparently overlap in the ability of different steroid receptors to bind to different HREs (Jensen, 1992, Liao, 1992). The HREs are located upstream of various cellular genes and affect the transcription of the gene, causing either promotion or inhibition of transcription depending on the cell type and the genes involved (Liao, 1992). Administration of estrogen (17 β-estradiol) to cells of human breast cancer cell lines in culture results in increased transcription of a number of cellular genes and ultimately increases proliferation of the cells (Dickson *et al.*, 1990). However, the genes which are critical to estrogen's ability to stimulate cell proliferation have not yet been identified (Dickson *et al.*, 1990). Both estrogens and androgens have been shown to stimulate the transcription of a number of proto-oncogenes including peptide growth factors, growth factor receptors and transcription factors. Estrogen treatment of human breast cancer cells in culture induces increased expression of the growth factors transforming growth factor-α (TGF-α), insulin like growth factor I and II (IGF-I and -II), as well as the epidermal growth factor receptor (EGFR) and the nuclear transcription factors c-fos, c-jun and c-myc (Dickson *et al.*, 1992). In the prostate of castrated rats, testosterone administration rapidly up regulates the expressions of the proto-oncogenes c-

fos, c-myc and c-ki-ras (Thompson, 1992). The relative importance of hormone-induced stimulation of growth factors and their receptors versus nuclear proto-oncogenes in carcinogenesis is controversial but it is likely that both play a role (Lippman and Dickson, 1987, Vander Burg *et al.*, 1992). Further, it has been suggested that the effects of estrogens on carcinogenesis may be due to the ability of the hormone both to promote tumor development by stimulating cell growth and to initiate transformation in dividing cells by interfering with microtubule assembly, thereby inducing aneuploidy (Barrett, 1992, Metzler *et al.*, 1992).

Hormonal Control of Normal Breast Cells: In most hormone-responsive normal tissues, complex interactions of several hormones and growth factors are involved in mediating the balance of proliferation and differentiation of cells. Further it has been suggested that imbalances in the hormones could contribute to carcinogenesis or promotion of tumor growth (Ho *et al.*, 1992, Kuttenn *et al.*, 1992). As breast cancer growth in an animal model is a focus of this thesis, the hormonal control of breast cells (both normal and malignant) will be considered in greater detail. The breast is a good example of a hormone-responsive tissue.

Both the growth and the functional activity of the breast epithelium are tightly controlled by hormones. Unlike most other organs in the body, the breast is not fully formed at birth. The breast of female rodents consists of an epithelial rudiment and an underlying mammary fat pad (Imagawa *et al.*, 1990, Sakukura, 1991). At this stage, the epithelial rudiment consists of short ducts that extend into the underlying fat pad. The gland remains in this form until puberty, at which time there is a significant outgrowth of the ducts to form branched ductal trees that occupy most of the mammary fat pad. The mammary gland then undergoes cyclic expansion and involution of the ductal epithelium under the influence of the female sex steroids. The increased growth of the mammary gland involves further branching of the ductal structure and the development of lobuloalveolar sacs at the termination of the branches. During pregnancy, the development of lobuloalveolar sacs is greatly stimulated and secretory alveoli fill the gland,

displacing the fat cells. Following the cessation of lactation, the mammary gland undergoes involution and returns to the state seen in the cycling gland.

Studies have revealed that the hormonal control of the breast involves a network of interacting hormones and growth factors. *In vivo* experiments in rodents have revealed that a number of hormones are involved in the different stages of the development of the breast. It has been shown that either gonadectomy or hypophysectomy of female mice prevents the development of the breast epithelium at all stages of development (Haslam, 1987, Imagawa *et al.*, 1990). In gonadectomized, hypophysectomized and adrenalectomized prepubertal female mice, the addition of exogenous estrogen, growth hormone and either progesterone or corticosterone is required for the induction of normal development of the ductal tree (Haslam, 1987, Imagawa *et al.*, 1990), whereas in post pubertal virgin female mice, the addition of estrogen, progesterone and prolactin is required to induce the formation of the lobuloalveolar structures (Haslam, 1987, Imagawa *et al.*, 1990). It has been shown that the elevated serum levels of progesterone in the female during gestation block the premature expression of differentiated functions by the mammary epithelial cells (Haslam, 1987).

In an attempt to define more accurately the relative contributions of different hormones and growth factors to the development of the mammary epithelium, a number of researchers have investigated the growth of breast epithelium *in vitro*. *In vitro* studies have primarily utilized 1 of 2 different methods; early studies used whole gland or explant cultures whereas more recent studies have examined the growth of relatively pure populations of breast epithelial cells in cell culture (Imagawa *et al.*, 1990). These studies have revealed several important findings regarding the stimulation of breast epithelium. Organ cultures generally have demonstrated that the proliferation of breast epithelium at different stages of mammary gland development have similar hormonal requirements to those observed in *in vivo* studies (Imagawa *et al.*, 1990). A notable exception is the finding that unlike the *in vivo* situation, explants of breast tissue do not proliferate

in response to estrogen in defined medium (Haslam, 1987, Topper and Freeman, 1980). It has been speculated that estrogen may exert it's mammogenic effect in an indirect manner (Haslam, 1987), that is, by affecting the ability of cells to respond to other hormones and by stimulating the production of other hormones (see below).

Cell culture experiments have revealed that the stroma of the mammary gland is important in modulating the ability of the breast epithelium to respond to hormones and growth factors. For example, the growth of breast epithelial cells from rodents is dramatically affected by the substratum on which they are grown (Emerman and Pitelka, 1977). In contrast to cells grown on plastic substrata, cells grown on collagen gels exhibit normal morphology and produce breast milk components, considered to be an expression of the differentiated mammary phenotype (Emerman and Pitelka, 1977, Emerman *et al.*, 1977). In addition growing breast cells within a collagen gel matrix produced significantly greater proliferative responses to lactogenic hormones than growing cells on plastic (Imagawa *et al.*, 1990). Cell culture experiments using defined media have allowed the investigation of the minimal hormonal requirements for the proliferation of breast epithelial cells. The minimal medium which is capable of maintaining breast epithelial cells in culture contains insulin and either bovine serum albumin or phospholipids (Imagawa *et al.*, 1990). Using this medium, it has been demonstrated that progesterone and/or prolactin are capable of stimulating the growth of cultured mammary cells (Imagawa *et al.*, 1990). Interestingly, although estrogen does not increase cell proliferation in this system, it was demonstrated that the cells do possess estrogen receptors and that estrogen stimulates upregulation of the progesterone receptors in these cells (Haslam, 1987). It has been suggested that the *in vivo* effects of estrogen result from it's effect on progesterone receptors and it's ability to stimulate prolactin secretion (Imagawa *et al.*, 1985). As well, a number of growth factors have been demonstrated to affect the growth of mammary epithelial cells *in vitro* (Dembinski and Shiu, 1987). Epithelial growth factor (EGF) and transforming growth factor-α (TGF-α) have been shown to stimulate the proliferation of breast cells and to inhibit the differentiated functions of these cells (Dembinski and

Shiu, 1987, Imagawa *et al.*, 1990). Further, the combination of EGF and bFGF can substitute for prolactin in the induction of alveolar growth *in vitro* (Imagawa *et al.*, 1990). Transforming growth factor-β (TGF-β) has been demonstrated to have an inhibitory effect on the growth of mammary cells (Dembinski and Shiu, 1987, Imagawa *et al.*, 1990).

Implanting small pellets containing hormones or growth factors into the mammary glands of mice has demonstrated the functional activity of these compounds in the organism. It was demonstrated that in prepubertal mice (3-4 wk old), estrogen has a local or direct stimulatory effect on the development of the ductal epithelium but does not exert a systemic effect on mammary growth as the nonimplanted contralateral mammary glands are not affected (Haslam, 1987). Interestingly, in postpubertal mice (10 wk old), the estrogen pellet exerts a systemic effect (Haslam, 1987). Implants of pellets containing EGF result in an initial increase in the growth of the breast epithelium in virgin mice, followed by a subsequent decline in growth by 3 d post implantation (Imagawa *et al.*, 1990). It has been suggested that this bimodal effect of EGF may be due to the down-regulation of the EGF receptor in mice chronically exposed to EGF. Importantly estrogen, in addition to affecting serum prolactin levels and the expression of mammary epithelial cell progesterone receptors as discussed previously, acts to increase the production of EGF, up regulate the expression of the EGF receptor and decrease the secretion of TGF-β (discussed below). In contrast, it was demonstrated that implants of TGF-β appear to inhibit the growth of the ductal cells (Daniel and Robinson, 1992). TGF-β has been found to be localized in the stroma surrounding nondividing ductal epithelial cells, but not in the stroma surrounding the cells in the growing ends of the ducts (Daniel and Robinson, 1992).

In vitro studies of normal and malignant human breast epithelial cells, as well as cell lines derived from human breast cancers, have also greatly added to our understanding of the hormonal control of breast epithelial cells. Data indicate that estrogen in the presence of serum stimulates the growth of normal human mammary epithelial cells (Kutten *et al.*, 1986), human breast

carcinomas in primary culture (Emerman *et al.*, 1990), and human breast cancer cell lines (Dickson *et al.*, 1986). In the absence of serum, however, estrogen generally does not stimulate the growth of breast cells (Gableman and Emerman, 1992). Similar results have been observed in studies of rodent mammary epithelial cells in culture (Haslam, 1987). The mechanism whereby serum allows estrogen to stimulate breast epithelial cell growth is not currently known. However, studies have demonstrated that estrogen causes an up regulation of EGF receptors on normal human mammary epithelial cells (Colomb *et al.*, 1991) and in human breast-cancer derived cell lines (Berthois *et al.*, 1989), as well as increasing the synthesis of EGF- or TGF-α-like molecules (Dickson, 1986). Further, it has been demonstrated that estrogen down-regulates the production of TGF-β mRNA in human breast cancer cell lines (Jeng and Jordan 1991, Nutt *et al.*, 1991). If estrogen is able to up regulate EGF receptors and decrease the production of the inhibitory growth factor TGF-β, then the permissive effect of serum may be to supply an exogenous source of EGF for cell growth. This possibility is supported by the finding that in serum-free medium, estrogen has a synergistic effect on the ability of EGF to stimulate the growth of primary cultures of human breast epithelial cells (Gableman and Emerman, 1992) This summary clearly demonstrates that the control of growth and differentiated functions in mammary epithelial cells involves an interacting network of hormones and growth factors.

Hormonal Control of Breast Cancer: Breast cancers also appear to be regulated by complex interactions among a number of hormones and growth factors. Although the transformation of breast cells is dependent upon the presence of hormones and growth factors (Boot *et al.*, 1981), breast tumors exhibit considerable variability in the hormones and growth factors that are required for proliferation (Ethier and Cundiff, 1987, Ethier and Moorethy, 1991, Platica *et al.*, 1991). Furthermore, it has been demonstrated that many experimental breast tumors progressively lose their hormonal responsiveness (Sluyser, 1987). It has also been shown that preneoplastic mammary cells produced by *in vitro* transformation with chemical carcinogens have similar growth factor requirements for *in vitro* growth as normal mammary epithelial cells.

The most basic requirements are insulin and EGF (Kittrell *et al.*, 1992) or insulin and prolactin (Ganguly *et al.*, 1982). It has been demonstrated that approximately half of rat primary mammary tumors, generated *in vivo* by carcinogen treatment, exhibit similar *in vitro* growth factor requirements as normal mammary epithelial cells (Ethier and Cundiff, 1987). Interestingly, when these cultured tumor cells are injected into mammary fat pads, they are no longer tumorigenic and produce only normal appearing ductal structures. In contrast, tumor cells which were found to exhibit growth factor independence *in vitro* produced tumors when injected into mammary fat pads (Ethier and Cundiff, 1987, Ethier and Moorethy, 1991). The transition from hormone-dependence to hormone-independence has been studied in a pregnancy-dependent mammary tumor (Imagawa *et al.*, 1992, Matsuzawa, 1986). This tumor forms hyperplastic alveolar nodule-type preneoplastic lesions in virgin female mice. The development of the tumor is stimulated by the elevated levels of estrogen, progesterone and prolactin that occur during pregnancy, but the tumor subsequently regresses during lactation. Chronic stimulation with estrogen and progesterone or repeated pregnancies can induce the outgrowth of ovarian hormone-responsive tumors which do not regress during lactation and finally, ovary-independent tumors (Imagawa *et al.*, 1992, Matsuzawa, 1986). The transition from a pregnancy-dependent state to an ovary-independent state is observed to be accompanied by increased anaplasia and increased growth rate (Imagawa *et al.*, 1992). Further, data indicate that while pregnancy-dependent tumors are similar to normal mammary epithelial cells in their requirement for hormones (estrogen, progesterone and prolactin) or growth factors (EGF and bFGF) to proliferate in culture, ovary-independent tumors are not stimulated to proliferate by these factors (Imagawa *et al.*, 1992). A similar phenomena has been demonstrated in carcinogen-induced mammary tumors; cells that are tumorigenic in vivo have been shown to grow independently of EGF, insulin or cholera toxin *in vitro*, whereas cells that form preneoplastic lesions *in vivo* are dependent on these factors for growth *in vitro* (Ethier and Cundiff, 1987). Using this tumor model, the growth factor-independent cells have been demonstrated to achieve EGF autonomy by the autocrine production of EGF (Ethier and Moorethy, 1991). Thus, although hormones and growth factors are important in the

transformation of mammary tumors, with time the tumors seem to achieve autonomy from the effects of hormones and growth factors.

Breast Epithelial Cells and Androgens: Of particular interest to this thesis is the finding that breast epithelial cells respond to androgens. Treatment with androgenic compounds has been demonstrated to inhibit the growth of carcinogen-induced tumors in rats (Teller *et al.*, 1966) and 25 % of advanced human breast cancers regress in response to androgen treatment (The Cooperative Breast Cancer Group, 1961). The antiproliferative actions of these androgenic compounds were postulated to occur by decreasing the release of gonadotrophins by the pituitary (Blackburn and Albert, 1959). More recently, it has been demonstrated that some human breast cancer cell lines possess androgen receptors (Labrie *et al.*, 1992, Ormandy *et al.*, 1992). Normal human breast epithelial cells also possess androgen receptors (De Winter *et al.*, 1991, Wagner and Jungblut, 1976). Further, it has been estimated that as many as 78 % of human tumors possess androgen receptors and that up to 25 % of breast cancer patients whose tumors are progesterone-receptor negative still possess androgen receptors (Lea *et al.*, 1989). These authors suggest that the ability of high dose progesterone treatment to cause tumor remission in some breast cancer patients whose tumors are progesterone-receptor negative is due to the presence of androgen receptors, as progesterone is suggested to cross-react with the androgen-receptor (Bullock *et al.*, 1978).

Studies of androgen's effects on breast cancer cells have produced varied results. A number of studies using human breast cancer cell lines demonstrated that physiological concentrations of androgens have an antiproliferative effect on tumor cell growth (Labrie *et al.*, 1992). The specificity of androgen for the androgen receptor was insured by the ability of androgen antagonists to block the actions of androgenic compounds (Labrie *et al.*, 1992). Studies indicate that androgenic compounds completely block the growth stimulatory effects of estrogen on human breast cancer cell lines (Labrie *et al.*, 1992). However, several studies suggest

a possible role for androgens in breast epithelial cell carcinogenesis and the stimulation of the growth of breast cancer cells. First, alterations in testosterone metabolism in breast cancer patients is a consistent finding (Moore *et al.*, 1986, Secreto *et al.*, 1991). Further, studies have shown that lymph from vessels draining breast carcinomas have significantly higher levels of androgens than those observed in the patient's blood (Hamed *et al.*, 1991). It has also been observed that fluid in apocrine breast cysts contains high levels of both EGF and androgen metabolites (Lai *et al.*, 1989). The authors speculate that the androgens may stimulate the production of EGF in these abnormal breast cells (Lai *et al.*, 1989). Interestingly, apocrine breast cysts are associated with an increased risk of developing breast cancer (Secreto *et al.*, 1989). Thus, it has been suggested that altered androgen metabolism in the patient may be associated with the initiation or the progression of breast cancer (Secreto *et al.*, 1989). Finally, it has been demonstrated that treatment of some human breast cancer cells lines with androgen results in up regulation of progesterone-receptor expression (Ormandy *et al.*, 1992). Thus, it is clear that both normal and malignant breast epithelial cells possess functional androgen receptors which can play a role in altering the growth of these cells.

Hormone Nonresponsive Tumors: Altered hormone levels can also indirectly affect tumors that are considered hormone-nonresponsive. This effect can be mediated by several mechanisms. First, some hormones are involved in the general control of cellular metabolism. It has been demonstrated that all mammalian cells in culture require hormones and growth factors to proliferate (Carney *et al.*, 1981, Hayashi and Sato, 1976, Rizzino and Sato,1978). For instance, the basic requirements for growth of small cell lung carcinoma cells in culture are hydrocortisone, insulin, transferrin, estradiol and selenium (Carney *et al.*, 1981). However, *in vivo* other hormones or growth factors may substitute for some of these requirements. Second, it has been demonstrated experimentally that carcinogen-induced tumorigenesis of the skin, liver and lung are influenced by the hormonal environment of the host organism. For example, female mice normally exhibit higher basal levels of growth hormone than their male counterparts and this

increased level of growth hormone in female mice has been demonstrated to delay carcinogen-induced tumorigenesis in the liver (Blanck *et al.*, 1992). Third, altered hormone levels may affect host processes which have a critical impact on the growth of the tumor. For example, it has been demonstrated that elevated levels of glucocorticoids and heparin inhibit angiogenesis (Folkman and Haudenschild, 1980). Thus alterations in hormones can have very important effects on both hormone-responsive and hormone-nonresponsive tumors.

D) Tumors, Stress and the Immune System.

D.1) Tumor Immunology

The question of whether or not the immune system is capable of recognizing and responding to tumors has been the source of considerable controversy in the last 90 years. Studies of tumor transplantation conducted in the early 1900's by Erlich and others seemed to demonstrate that the immune system has the ability to reject tumors. However, subsequent studies demonstrated that the tumor rejection observed was in fact due to histoincompatabilities between the tumor cells and the recipient mice. This finding lead to the discovery of the major histocompatability complex (MHC), and an understanding of it's role in immune recognition of foreign cells and of the diversity of MHC molecules in allogeneic strains of animals of the same species. This discovery caused the concept of immune system-mediated rejection of tumors to fall into disfavor.

In the 1950's tumor rejection was demonstrated using tumors from syngeneic donor mice, here the donor and recipient were demonstrated to share the same MHC phenotype. Transplants of normal skin grafts from the donor mouse to the recipient mouse were not rejected and failed to immunize the recipient against a subsequent tumor graft from the donor mouse (Prehn and Main, 1957). Since that time, numerous studies have demonstrated immune mediated-rejection of

tumors in experimental animals due to recognition of tumor-specific antigens. The discovery of antibodies and humoral immunity caused further interest in the concept of antitumor immunity. However, there has been a general failure to demonstrate tumor-specific antigens that are recognizable by antibodies (Schreiber *et al.*, 1988). Most antitumor antibodies were found to react either with other normal tissues or with antigens expressed on fetal tissues. The overall failure to demonstrate the existence of tumor-specific antigens caused the concept of antitumor immunity once again to fall into disfavor by the late 1960's. The discovery of cytotoxic T lymphocyte (CTL)-mediated immunity to virally infected cells and bacteria lead to yet another resurgence of interest in antitumor immunity in the early 1970's (Burnet, 1970). Current research indicates that cell-mediated immunity and, to a lesser extent, humoral-immunity can play a role in controlling the growth of tumors.

Cytotoxic T lymphocytes (CTL): In contrast to the relative difficulty in demonstrating tumor-specific antigens with antibodies, CTL clones have been generated which specifically recognize tumor cells. CTL were the first cells which were found to be capable of cell-mediated cytotoxicity against cells of the organism with an altered surface antigen expression (i.e. virally infected cells and tumor cells); they recognize and directly act on the tumor cell to lyse it. CTL recognize antigen associated with class I molecules of the major histocompatability complex (MHC), which are expressed on the surface of all cells (Unanue and Cerrottini, 1989).

Studies of the mechanism by which T lymphocytes recognize target cells have given us insight into why T cells may be better suited to recognize transformed cells than are antibodies. It is well known that T lymphocytes recognize foreign antigen in conjunction with class I (CTL) or class II (Thelper cells) MHC through the T cell receptor (TcR) (Hedrick, 1988). It has been demonstrated that the association of antigen with MHC molecules involves the partial proteolytic digestion of the native peptide and the association of peptide fragments with the MHC molecule (Unanue and Cerottini, 1989). Class I MHC molecules associate with peptide fragments that are

produced by the digestion of the cell's own peptides, while class II MHC molecules associate with peptide fragments of molecules from outside the cell (Unanue and Cerottini, 1989). Recently the three dimensional structure of a class I MHC molecule has been elucidated using X ray crystallography (Hedrick, 1988). The upper surface of the molecule is in the form of a beta-pleated sheet topped by 2 alpha helixes. The alpha helixes form the sides of an elongated cleft and the beta-pleated sheet forms the base of the cleft. Comparison of the three dimensional structure of the MHC molecule with its amino acid composition reveals that the variable regions of the peptide would be those sequences involved in forming the top, sides and base of the cleft (Hedrick, 1988). Studies have shown that the smallest peptide fragment recognized by a T lymphocyte is 8 amino acids long, but that the length varies, depending on the peptide and the T lymphocyte clone involved (Hedrick, 1988, Unanue and Cerottini, 1989). It has been shown that the substitution of a single amino acid in a protein can dramatically increase the affinity of a CTL clone for that molecule (Hedrick, 1988). For example, studies looking at the reactivity of a mouse T lymphocyte clone specific for pigeon cytochrome c showed that it reacts with higher affinity to antigen-presenting cells which are presenting an insect cytochrome c peptide fragment than to the pigeon cytochrome c fragment. Analysis of the two peptide fragments showed that they differ only in that the insect peptide had an alanine molecule deleted (Hedrick, 1988). Thus it can be seen that T lymphocytes respond to small peptide fragments of proteins (which do not have to be membrane proteins) associated with MHC molecules. Small changes in the structure of a given peptide fragment are sufficient to alter the binding affinity of the TcR for the MHC antigen complex. This suggests that a point mutational change of an amino acid in the center of a globular protein, which may not significantly alter the three dimensional structure of the protein, could render the molecule antigenic for T lymphocytes while not changing its antibody affinity at all.

Although the previously mentioned studies suggest that tumor-specific antigens could theoretically be generated by a single point mutation in a cellular gene, experimental proof of such

an event occurring was lacking until several years ago. Several labs have used tumor-specific CTL clones to identify different tumor-specific antigens in both human and experimental animal tumors. One recent study generated antigenic variants of the non-immunogenic murine tumor P815 (a mastocytoma). The investigators subsequently generated CTL specific for one antigenic P815 subline (De Plain *et al.*, 1988). Following this, they constructed a genomic DNA library for the antigenic subline of P815. They used the CTL clone to screen non-antigenic P815 parental line cells transfected with the DNA library for the presence of the antigen. Following identification of the portion of the DNA library containing the antigenic gene, they sequenced the gene and compared it with the mRNA of the non-antigenic parental line. The results demonstrate that the gene, which is expressed in both antigenic and non-antigenic P815 cells, is made antigenic by a single point mutation. This strongly suggests that tumor-specific antigens do exist and can be generated by mutation of existing cellular genes. Thus, in addition to expressing normal cellular genes and fetal genes in an abnormal manner, tumor cells can also express antigenic, mutated cellular genes. The immune system probably can respond to both types of antigen.

The stimulation of CTL is thought to involve both CTL and helper T lymphocytes. The T helper (T_h) cells recognize tumor antigen fragments in association with class II MHC molecules on the surface of antigen-presenting cells, which include both macrophages and B lymphocytes (Unanue and Cerottini, 1989). Recognition of the antigen-MHC complex and simultaneous stimulation by cytokines produced by the antigen-presenting cell causes the T_h cell to secrete the lymphokines interleukin 2 (IL2) and interferon (IFN) and to proliferate (Street and Mosman, 1991). CTL precursor cells, referred to as virgin CTL, are not cytolytic. When the TcR of a virgin CTL recognizes a tumor-specific antigen fragment in association with class I MHC on the surface of the tumor cell, this causes an increase in the expression of IL2 receptors on the CTL's cell surface. The combination of binding the antigen-MHC complex and binding the IL2 produced by the T_h cell causes the CTL to proliferate and become cytotoxic. The generation of

effective CTL-mediated immunity in the organism following virus infection requires about 5 to 8 days to reach peak activity levels (Herberman and Ortaldo, 1981).

Natural Killer Cells: A second cell type found to be important in mediating antitumor immunity is the natural killer (NK) cell. NK cells were discovered in the early 1970's following the observation that nonimmunized human peripheral blood lymphocytes and mouse splenocytes consistently produce low levels of lysis of certain tumor cell lines (Greenberg et al., 1974, Kiessling et al., 1975). This activity was subsequently found to be due to a unique population of effector cells which were termed NK cells. NK cells have been defined as large granular lymphocytes which have the spontaneous ability to recognize and lyse a variety of normal, virally-infected and malignant cell types in an MHC-nonrestricted fashion (Herberman, 1985). These cells have been shown to lack some of the characteristic markers of T lymphocytes, B lymphocytes and macrophages (Herberman, 1985). Importantly, it has been shown that NK cells do not rearrange or express the genes for either the T cell receptor or immunoglobins, key markers for T and B lymphocytes respectively (Robertson et al., 1990, Trinchieri, 1989). Unlike the unique specificity of individual T lymphocytes for different antigens, NK cells are thought to possess a broad range of target cell specificities. However, there are indications that there is heterogeneity among NK cells with respect to target cell specificity (Dawson et al., 1992, Herberman, 1985). Despite more than 20 years of research into the phenomenon of NK cell activity, the NK cell receptor has yet to be characterized. Recently, however, several putative NK cell receptor molecules have been isolated for various species including mice, rats and humans (Anderson, 1992, Giorda et al., 1992, Harris et al., 1992). It is possible that NK cells may utilize more than one receptor molecule to increase the diversity of cell types that can be recognized (Anderson, 1992). As mentioned previously, NK cells are not MHC-restricted and thus do not require that their target cells express MHC class I or class II molecules for recognition and lysis. In fact, a number of studies have demonstrated that there may be an inverse relationship between the expression of class I MHC molecules and the ability of NK cells to lyse the target cell

(Dawson et al., 1992). Interestingly, studies using B lymphocytes as target structures have demonstrated that the expression of some class I MHC alleles protects the cells against NK cell lysis, whereas the expression of other class I MHC alleles does not (Dawson et al., 1992). Molecular analysis of the MHC alleles has revealed that the ability of class I MHC molecules to protect B lymphocytes from lysis by NK cells involves certain amino acid molecules which form a subpocket on the floor of the antigen-binding groove (Dawson et al., 1992). Substitution of a single amino acid in this region was shown to abrogate the ability of the class I MHC molecule to block NK cell lysis (Dawson et al., 1992). The exact mechanism by which MHC molecules regulate NK cell activity is controversial (Dawson et al., 1992, Ljunggren and Karre, 1990).

A variety of different molecules are capable of stimulating the activity and/or proliferation of NK cells. Treatment of NK cells with IL-2 or members of the interferon family (IFN-α, IFN-β, IFN-γ) has been demonstrated to increase dramatically the lytic ability of NK cells (Ellis et al., 1989, Ortaldo et al., 1984, Trinchieri et al., 1984), as well as to extend the spectrum of cells lysed, allowing NK cells to lyse target cells which are not normally sensitive to NK cell lysis. These activated NK cells are referred to as lymphokine-activated killer cells (LAK, Ellis et al., 1989; Trinchieri et al., 1984). IL-2 has been found to have the most potent and the most diverse effects on NK cells, stimulating increased NK cell activity, increased cell proliferation and the secretion of a number of different cytokines including IFN-γ and TNF-α (Robertson and Ritz, 1992). Research has demonstrated that there are many different subtypes of interferons α/β and that they differ in their ability to stimulate the cytotoxic activity of NK cells (Herberman, et al., 1983, Li et al., 1990). In contrast to IL-2, IFN only weakly stimulates the proliferation of NK cells. Studies indicate, however, that some interferons may synergize with IL-2 in stimulating the proliferation of NK cells (Robertson and Ritz, 1992). More recently, several other cytokines have been discovered that are capable of modulating NK cell activity, including IL-4 and IL-12. IL-4 alone has a weak stimulatory effect on NK cell cytolytic activity, but in combination with IL-2 it has been shown to inhibit the ability of IL-2 to induce LAK activity and proliferation in NK cells

(Nagler et al., 1988). IL-12 was originally discovered as a product of some malignant B lymphocyte cell lines which stimulated the cytolytic activity of both T lymphocytes and of NK cells (Naume et al., 1992, Robertson et al., 1992). Studies indicate that IL-12 is similar to IL-2 in its potent ability to stimulate the lytic activity of NK cells, but that IL-12 does not stimulate the proliferation of NK cells (Naume et al., 1992, Robertson et al., 1992). It has been demonstrated that, in the presence of the appropriate T helper lymphocyte population, IL-12 is a potent stimulator of the secretion of IFN-γ by NK cells (Naume et al., 1992). *In vitro* studies utilizing antibodies to IL-12 indicate that the production of this molecule by normal peripheral blood lymphocytes may be an important physiological mechanism for controlling NK cell activity *in vivo* (D'Andrea et al., 1992).

NK cells have been shown to constitutively express the intermediate affinity IL-2 receptor (p75) and approximately 10 % of human peripheral blood lymphocytes also express the p55 IL-2 receptor to form the high affinity IL-2 receptor heterodimer (Robertson and Ritz, 1992, Robertson et al., 1992). Stimulation of NK cells with IL-2, IL-12 or IFN has been demonstrated to cause a dramatic up regulation of the expression of both IL-2 receptor molecules on the surface of NK cells (Naume et al., 1992, Robertson et al., 1992). Thus, it is likely that all of these molecules would increase the response of NK cells to suboptimal doses of IL-2.

It has also been demonstrated that in vitro incubation of NK cells with target cells, including virally-infected cells, bacterial cells and tumor cells, stimulates NK cells (Biron and Welsh, 1982, Djeu et al., 1982, Rabinowich et al., 1992), inducing increased lytic activity, up regulation of IL-2 receptors and the secretion of the cytokines IFN-γ and TNF-α (Chong et al., 1989, Rabinowich et al., 1992). One study demonstrated that antibodies to IFN-α could block the stimulation of NK cells by target cells (Djeu et al., 1982). Thus NK cells are thought to be potent mediators of antitumor immunity against blood-borne metastases and primary tumors at early stages of tumor growth.

The ability of NK cells to recognize a broad range of transformed cells, and to respond immediately has prompted the suggestion that NK cells are the body's first line of defense against tumor cells (Herberman and Ortaldo, 1981). In experimental tumor models, NK cell have been shown to play an important role in the control of blood borne metastases (Talmadge et al., 1980). Studies of the kinetics of clearance of radioactively labeled tumor cells from the body following their injection into the bloodstream indicate that NK cells likely attack tumor emboli which have become arrested in the vascular beds of target organs (Aslakson et al., 1991, Johnson et al., 1990, Greenberg et al., 1987). Further, experimental models have demonstrated that tumor cell sublines with decreased sensitivity to NK cell lysis *in vitro* have increased metastatic ability *in vivo* (Talmadge et al., 1980). In humans, studies have indicated that the patient's level of NK cell activity is negatively correlated with the degree of lymph node involvement in breast cancer (Levy and Herberman, 1985). Studies in animals have also demonstrated that in some tumors, NK cell activity can affect growth of the primary tumor as well as the metastases (Talmadge et al., 1980). These studies suggest that NK cell activity may be important in controlling the early growth of the primary tumor when the tumor burden is small and that NK cells subsequently are immunosuppressed as the tumor grows in size (Talmadge et al., 1980).

Macrophages: A third effector cell which is active in cell-meditated antitumor immunity is the macrophage. Macrophages, previously mentioned as playing a role in the presentation of antigens to T_h lymphocytes, are also known to be capable of exerting a tumoricidal effect on tumors (Adams *et al.*, 1985, Adams and Johnson, 1982). Tumoricidal macrophages exert their effect either by phagocytosing and destroying the tumor cells or by the release of various cytotoxic and cytostatic cytokines (Adams and Johnson, 1982, Schwamberger *et al.*, 1991). The recognition of antigens by macrophages is poorly understood, but it has recently been suggested that they recognize negatively charged phospholipids such as phosphatidylserine (Fidler and Schroit, 1988). The abnormal distribution of phospholipids is postulated to allow macrophages to

recognize old cells, dead cells and tumor cells. Macrophages must be activated to exhibit tumoricidal activities (Fidler and Schroit, 1988), and this activation may be induced by lymphokines (of which γ-interferon is the most active) or certain bacterial cell wall products such as lipopolysaccaride (Fidler and Schroit, 1988).

Antibody Directed Cellular Cytotoxicity: A fourth mechanism whereby the immune system can recognize and lyse tumor cells is through antibody-directed cellular cytotoxicity (ADCC) (Kushner and Cheung, 1992). In ADCC, the recognition of the foreign cell occurs through the binding of an antibody to the tumor cell. The antibody (typically of the immunoglobin class gamma or IgG) is specific for and binds to a foreign antigen on the surface of the tumor cell. A number of cell types are then capable of recognizing the antibody-antigen complex and lysing the tumor cells. Cells involved in mediating ADCC include a special class of cytotoxic cells termed natural cytotoxic cells (NC), NK cells, macrophages and neutrophils (Kushner and Cheung, 1992, Liesveld *et al.*, 1991). NC cells and macrophages are the most potent mediators of ADCC and they possess receptors with the greatest affinity for the activated antibody-antigen complex (Liesveld *et al.*, 1991).

Thus, the antitumor activity of the immune system is a diverse and potent defense mechanism which allows the body to guard itself against the growth of abnormal or transformed cells. The broad specificity and spontaneous activity of NK cells, NC cells and macrophages allow them to recognize and destroy small foci of tumors. The specificity and potent cytotoxicity of CTL, although taking longer to develop, allows them to destroy larger tumors.

D.2) Psychoneuroimmunology

Classical immunologists have long considered the immune system as being largely separate from other systems in the body. The immune system has mechanisms which were thought to provide autonomous control of both cell division and cytolytic functions. However, in the past 15 to 20 years it has been demonstrated that other systems do indeed interact with the immune system and that these interactions are bi-directional. This field of study is termed psychoneuroimmunology. As the name implies, psychoneuroimmunology is the study of the interconnections of the immune system, the endocrine system and the central nervous system. I have previously discussed the interconnections of the endocrine system and the central nervous system; in this section I will discuss the interactions of these two systems with the immune system.

It has long been known that hormones can affect immune functioning. As early as 1936, Selye discovered that chronically elevated glucocorticoids can have a profound effect on the immune system, causing marked thymic involution (Selye, 1975). It was subsequently discovered that thymic involution occurs because chronically elevated glucocorticoids cause a marked suppression of immune function and lysis of developing T lymphocytes. A second key step in the development of the concept of psychoneuroimmunology was the finding that immune responses could be conditioned just like other physiological functions. In classical conditioning, an unconditioned stimulus (UCS), which elicits a desired physiological response, is administered in conjunction with a conditioned stimulus (CS) which is initially neutral and does not elicit the response. After many such pairings, the presentation of the conditioned stimulus alone will elicit the physiological response. In 1975, Ader and Cohen (Ader and Cohen, 1975) demonstrated that immune responses could be conditioned. They paired the injection of the immunosuppressive drug cyclophosphamide (UCS) with the taste of saccharin (CS), and demonstrated that subsequent presentation of saccharin alone suppressed the antibody response to the administration of sheep red blood cells compared to that seen in control animals. This indicated that the central

nervous system could act by some mechanism to suppress immune responses, since the association of the CS and the UCS is a central nervous system-mediated event. This finding has been repeated (Gorczynski, 1987, Rogers *et al.*, 1975; Wayner *et al.*, 1978) and extended to include conditioning of cell-mediated immune responses (Bovbjerg *et al.*, 1982) and of NK cell activity (Greenberg *et al.*,1986, Solvason *et al.* 1988).

These findings sparked interest in the ability of the central nervous system to control or at least modulate the actions of the immune system. It was discovered that various neuropeptides and hormones could affect immune responses *in vitro*. These various peptide molecules may affect lymphocyte functioning both *in vivo* and *in vitro*. However, the results have been conflicting due to methodological differences in the source of the hormones, the doses of hormones used, the presence of serum and the source and purity of white blood cells used (Dunn, 1989). Experiments have generally demonstrated that both β-endorphin and ACTH decrease antibody production, while thyrotropin, growth hormone and prolactin stimulate antibody production (Bernton, 1991, Blalock, 1992; Dunn, 1989; Johnson 1992; Kelley, 1991). T cell proliferation and functional activity has been reported to be stimulated by endorphins, growth hormone and prolactin, and inhibited by chorionic gonadotrophin (Bernton, 1991, Blalock, 1992; Dunn, 1989; Johnson 1992; Kelley, 1991). Researchers have subsequently discovered that not only do lymphocytes respond to neuropeptides but that lymphocytes can produce neuropeptide-like molecules during an immune response. It was first demonstrated that lymphocytes produce molecules which are similar to the pituitary hormones ACTH and β-endorphin during an immune response to viral infections or transformed cells (Blalock and Smith, 1980). The amino acid and mRNA nucleotide sequences of mouse splenic- and pituitary-derived ACTH have been demonstrated to be identical (Galin *et al.* 1990, Smith *et al.*, 1990). Other studies have demonstrated that lymphocytes may produce a number of different peptide hormone molecules including prolactin, thyrotropin, growth hormone, chorionic gonadotrophin, luteinizing hormone and follicle stimulating hormone (Blalock, 1992; Carr and Blalock 1991). It has been suggested

that different stimulating agents (e.g. concanavlin A vs allogeneic lymphocytes) result in the release of different peptide hormones (Blalock, 1992). Further, lymphocytes have been shown to possess specific receptors for a number of different neuropeptide and hormone molecules. The best characterized of these receptors is the ACTH receptor. Lymphocyte receptors for ACTH have been demonstrated which exhibit similar binding affinity to that reported for adrenal cortical cells (Clarke and Bost, 1989; Smith *et al.*, 1987). Interestingly, isolated rat B lymphocytes have been demonstrated to possess approximately 3 times the number of ACTH receptors that T lymphocytes possess (Clarke and Bost, 1989). Stimulation of the lymphocytes to proliferate causes an approximate doubling of ACTH receptor number (Clarke and Bost, 1989). The ACTH receptors are functional in that ACTH causes a dose-dependent increase in the cytoplasmic levels of the second messenger cyclic adenosine monophosphate (Clarke and Bost, 1989).

The central nervous system can also affect the functioning of the immune system directly through the actions of peripheral nerves. It is known that the lymphoid organs are innervated by the autonomic nervous system and recently it has been demonstrated by ultrastructural studies that these nerve processes make direct synaptic contacts with the lymphocytes (Felton, 1992). Cells of the immune system possess functional receptors for adrenaline (Madden, 1991), vasoactive intestinal peptide (Ottaway, 1991), and substance P (McGillis *et al.*, 1991) and neurons containing these neuropeptides have been demonstrated in most lymphoid organs (Felton and Felton, 1991). These neuropeptides have also been demonstrated to be capable of altering immune functioning *in vitro*.

Thus, the central nervous system is clearly able to influence the functioning of the immune system through the release of various hormones and neuropeptide molecules. The immune system is also capable of altering endocrine and central nervous system functioning. As mentioned previously, immune cells undergoing specific immune responses produce various peptide hormone molecules and it has been suggested that these molecules are capable of interacting with endocrine

tissues to influence endocrine functions (Blalock, 1992). This concept is controversial, however, and it has been questioned whether or not the peptides secreted by the immune system could reach sufficient concentrations in the blood to affect the endocrine system (Dunn, 1989). Finally, certain cytokines released by white blood cells during an immune response are potent stimulators of endocrine functions. For example, it has been demonstrated that interleukin 1 released by activated macrophages is a very potent stimulator of ACTH release by pituitary corticotrophs, but it is not clear if this is a direct effect or mediated by stimulation of CRF release from the hypothalamus (Dunn, 1989). Thus, there are bi-directional links between the central nervous system, the endocrine system and the immune system which allow coordination of the functional activities of these 3 systems.

E) Psychosocial Stressors

Animal models used to investigate the effects of stressors on the physiological functions of the animal have demonstrated that many different kinds of stressors may affect the organism. Stressors may be divided into two broad categories, physical and psychological. Physical stressors involve an insult to the tissues of the organism such as heat, cold or laparotomy, whereas psychological stressors induce the anticipation of threat or harm (Lazarus, 1971). Studies have demonstrated that a simple psychological stressor such as moving a rodent to a new cage can cause significant elevations in plasma glucocorticoid levels which may be as great as those observed with physical stressors such as electric shock. Psychosocial stressors, a type of psychological stressor, result from responses to intense social interactions, the lack of such interactions or even perhaps to other subtle social interrelationships (Asterita, 1985). Social interactions, or the lack of them, may have profound effects on both humans and animals. In rodents, psychosocial stressors may include individual housing, crowded housing conditions and changes in housing condition. In the research of this dissertation, we focused on psychological stressors as we feel that they are more relevant to the human condition than are physical stressors.

Individual Housing. Individual housing is known to cause marked changes in the behavior, endocrine function and immune competence of rodents. The most profound change is an increase in aggression of males seen in both mice and rats following even relatively brief periods of individual housing (Brain, 1975). Increases in inter-male aggression induced by individual housing occur more often in mice than in rats and are best documented in mice (Brain, 1975). Studies suggest that increases in the activity of neuronal circuits containing serotonin and noradrenaline in limbic regions of the brain may be involved in mediating this phenomenon (Frances *et al.*, 1990, Gentsch *et al.*, 1990, Olivier B. *et al.*, 1989). Individual housing has been demonstrated to decrease plasma glucocorticoid levels, increase plasma testosterone levels, and decrease adrenaline turnover in the adrenal glands of male mice (Brain, 1975). These hormonal changes are indicative of decreased stress levels and it has been suggested that individual housing is less stressful than group housing for mice (Brain, 1975). One basis for this argument is the observation that in the wild, male mice are territorial and will subordinate or kill all adult male mice in their territory (Crowcroft *et al.*, 1963, Mackintosh, 1970). Thus individual housing is suggested to induce territoriality in male mice (Brain, 1975). However, it has also been demonstrated that individually housed animals are hyperresponsive to stressors (Giralt and Armario, 1987, Hatch *et al.*, 1965). Thus, it is currently controversial whether or not individual housing is a stressor in rodents. Finally, individual housing is known to alter the immune competence of rodents. Individually housed mice have been demonstrated to have greater antibody responses to immunization with foreign proteins (Rabin *et al.*, 1987a, Salvin *et al.*, 1990, Vessey , 1964). Also, *in vitro* T lymphocyte responses to mitogenic stimulation (concanvalin A, phytohemagglutinin, pokeweed mitogen) (Raab *et al.*, 1986, Rabin *et al.*, 1987b) and macrophage stimulation by bacterial antigens (Salvin *et al.*, 1990) are greater in individually housed mice than in group-housed mice. However, differential housing does not appear to affect immune competence equally in all strains of mice (Rabin *et al.*, 1987b). Interestingly, individual housing has been shown to have variable effects on resistance of mice to different diseases. Individually

housed mice were shown to be more resistant to malaria (Plaut *et al.*, 1969), but less resistant to encephalomyocarditis virus (Freidman *et al.*, 1969) and to West Nile virus (Ben-Nathan and Feuerstein, 1990) than group housed mice.

Crowding. Crowding in mice can be defined by the number of mice that are able to interact with each other rather than the number of animals per square metre (Christian *et al.*, 1965). Some of the earliest studies on the effects of crowding on mice were conducted in the 1950's by Christian *et al.* (1965). These studies demonstrated that as the density of mice in a confined population increased, there was a significant increase in the weight of the adrenal gland and a corresponding decline in the weights of the thymus, testes and other sex organs (Christian *et al.*, 1965). These physiological changes had been previously described by Selye (1975) as hallmarks of a stress response. Subsequent studies have demonstrated that housing rodents in crowded conditions caused marked changes in basal plasma levels of several hormones including glucocorticoids (Brain and Nowell, 1970, Bronson, 1972, Peng *et al.*, 1989), testosterone (Koike and Noumura, 1989, Sayegh *et al.*, 1990), TSH (Restrepo and Armario, 1989), GH (Restrepo and Armario, 1989), insulin (Restrepo and Armario, 1989), LH (Bronson, 1972) and FSH (Bronson, 1972) in the first 2 wks post grouping . However, by 4 wks post grouping, there were no effects of crowded housing conditions on basal plasma hormone levels (Ortiz *et al.*, 1984,1985). Crowded housing conditions have also been demonstrated to affect immune function. Crowding has been shown to decrease antibody production in response to foreign antigens (Brayton and Brain, 1974, Edwards and Dean, 1977) and to decrease T lymphocyte proliferation in response to mitogens (Rabin *et al.*, 1987a). Generally, crowding has been found to have a depressive effect on resistance to disease, including Salmonella infection (Edwards and Dean, 1977), malarial infection (Plaut *et al.*, 1969) and viral leukemia (Ebbesen *et al.*, 1991). Thus crowding is probably a stressor although some adaptation may occur over time.

Dominance. It has been demonstrated that, in male animals, most effects of crowded housing conditions can be attributed to the establishment of a dominance hierarchy amongst the animals (Bronson, 1972, Christian, 1970). Mice are reported to form a non-linear dominance hierarchy, with one dominant male and several equal subordinate males (Christian *et al.*, 1965). Fighting is critical to the establishment and maintenance of the hierarchy, with the dominant male initiating and winning all encounters (Brain, 1972, Lyte *et al.*, 1990). Such fighting behavior is observed in most newly formed groups of adult male rodents, even when the group consists of only 2 animals (Brain and Nowell, 1970). As mentioned previously, prior individual housing increases the aggressiveness of rodents compared with that of continuously group housed animals. Subordinate rank in rodents and the defeat associated with such a position has been demonstrated to produce more dramatic alterations in endocrine and immune activity than are observed in the dominant male. Subordinate or defeated mice are known to have higher plasma levels of glucocorticoids and lower levels of androgens than their dominant cage mate (Brain and Nowell, 1970, 1971, Raab *et al.*, 1986). As well, subordinate mice exhibit higher activity of tyrosine hydroxylase (a key enzyme in the synthesis of catecholamines) in the adrenal medulla (Raab *et al.*, 1986) and increased release of opioid peptides (Miczek *et al.*, 1982). Subordinance or defeat has also been shown to affect the immune system. Defeated rodents exhibit decreased antibody production in response to bovine sera (Vessey, 1964), SRBC (Beden and Brain, 1982) and bacterial antigens (Ito *et al.*, 1983). In addition, defeated mice have increased susceptibility to viral leukemia (Ebbensen *et al.*, 1991), decreased responses to T lymphocyte mitogens (Raab *et al.*, 1986) and, in aged mice, decreases in NK cell activity (Ghoneun *et al.*, 1987). However, it has also been shown that defeated mice have increased levels of macrophage phagocytic activity which may be blocked by opioid antagonists (Lyte *et al.*, 1990). This study further demonstrated that defeat-induced enhancement of immune function is not opioid mediated in all strains of mice (Lyte *et al.*, 1990).

In summary, it is apparent that psychosocial stressors are important in modulating the activity of the central nervous system, the endocrine system and the immune system in animals. Thus, it is important to control for the effects of these potent stressors in all experiments.

F) Chronic vs Acute Stressors

Acute application of a stressor to an organism is known to cause alterations in the plasma levels of a number of hormones and neuropeptides including ACTH (Armario, 1985, Armario *et al.*, 1988), β-endorphin (Tejwani et al., 1991), prolactin (Kant *et al.*, 1983,1985), LH (Briski and Sylvester, 1988, 1987), growth hormone (Kant *et al.*, 1983, 1985), adrenaline and noradrenaline (Cox *et al.*, 1985, DeTurck and Vogel, 1980, Kvetnansky *et al.*, 1977, 1979), testosterone (Demura *et al.*, Frankel and Ryan, 1981, 1989, Sapolsky, 1986), glucocorticoids (Armario, 1985, Armario *et al.*, 1988, Kant *et al.*, 1983,1985) and many other hormones. However, in contrast to acute stressors, chronic or repeated administration of a stressor has been shown to result in a decrease in the responsiveness of the organism to the stressor. This effect, termed habituation or adaptation, has been demonstrated to occur for both physical manifestations of the stress response (for example, stress induced analgesia and body core hypothermia) and endocrine responses (Levine *et al.*, 1978). It is proposed that the phenomenon of habituation is a result of cognitive changes rather than physical alterations of the hormone secreting cells, since habituation to one stressor does not provide protection from a second stressor and therefore could not be acting at the level of hormone secretion (Kant *et al.*, 1985). Interestingly, not all hormones show habituation during exposure to chronic stressors and there are contradictory findings about the ability of stressors to cause habituation of other hormones. ACTH, prolactin and catecholamine secretion in response to chronic stressors have generally been demonstrated to consistently exhibit habituation (Armario, 1985, Armario *et al.*, 1988, Cox *et al.*, 1985, DeTurck and Vogel, 1980, Kant *et al.*, 1983, 1985, Kvetnansky *et al.*, 1977, 1979). Surprisingly, glucocorticoid levels have often been demonstrated not to decline with repeated presentation of the same stressor (Armario

et al., 1984a, 1988, 1990, Hennessey and Levine, 1977, Irwin *et al.*, 1986, Kant, 1983). This is likely due to the ability of chronic stressors to induce hyper-responsiveness of the adrenal corticotroph cells for ACTH (Armario *et al.*, 1988). It has been shown that plasma hormone levels may return to basal levels but if a subsequent stressor is applied, there is hyper-responsiveness to this stimulus. Other hormones such as growth hormone and TSH are reported not to alter their secretion patterns in response to repeated presentations of a stressor (Armario *et al.*, 1984b, Kant *et al.*, 1983). Different strains of inbred laboratory rodents have been demonstrated to exhibit differences in the magnitude of their hormone response to a given stressor and in the ability of chronic application of that stressor to induce adaptation of the hormone response (Kvetnansky *et al.*, 1979). Thus, when considering the effects of a stressor on an organism, one must take into account the chronicity of the stressor and the potential of the hormones being considered to undergo adaptation to chronic stress.

G) Animal-Tumor Model for Studying Effects of Psychosocial Stressors

An animal-tumor model which examines the effects of psychosocial stressors on the growth of a hormone-responsive mouse mammary tumor has been developed in our laboratory. In this model, the psychosocial stressors of differential housing has been demonstrated to induce both increases and decreases in tumor growth rate.

The Tumor. The tumor used in this model is the Shionogi carcinoma 115 (SC115). This tumor arose spontaneously in the breast epithelium of a female mouse of the DD/S strain. Following 19 generations of transplantation, a subline arose which grew more rapidly in male mice than in female mice. When 2×10^6 SC115 cells are injected subcutaneously in the interscapular region of a male mouse, a palpable tumor arises in approximately 7 d and grows to a mass of 2 to 3 g by 21 d. When a similar tumor inoculum is injected into a female mouse, tumor growth rate is considerably slower. In females, a tumor may require 40 d to reach a mass of approximately 1 g.

The SC115 tumor, similar to many human breast tumors , is hormone-responsive and a number of different hormones have been demonstrated to modulate its growth rate. Cells of the SC115 tumor have been demonstrated to possess functional androgen receptors and physiological concentrations of androgens stimulate the growth rate of the tumor (Bruchovsky and Rennie, 1978, Emerman and Siemiatkowski, 1984, Kitamura *et al.*, 1979). However, the SC115 tumor is heterogeneous and contains both androgen-responsive and androgen-nonresponsive cells. Removal of androgens from the environment results in the outgrowth of a slower growing androgen-independent cell population (Bruchovsky and Rennie, 1978, Emerman and Worth, 1985, Kitamura *et al.*, 1979). Pharmacological doses of glucocorticoids have been shown to stimulate the growth of the SC115 tumor, both *in vivo* (Watenabe *et al.*, 1982) and *in vitro* (Darbe and King, 1987). The SC115 tumor has been shown to possess estrogen receptors and to respond to supraphysiological doses of estrogen (Nohno *et al.*, 1982). However, the stimulatory effects noted with pharmacological doses of estrogen are apparently mediated by cross-reactivity with the androgen receptor and thus are only apparent at suboptimal androgen levels (Luthy *et al.*, 1988, Noguchi *et al.*, 1987, Nono *et al.*, 1982). More recently, it has been demonstrated that SC115 cells are stimulated to grow by basic fibroblast growth factor (bFGF) and that anti-bFGF antibodies partially inhibit the growth stimulatory actions of androgens and glucocorticoids *in vitro* (Furuya *et al.*, 1990, Tanaka *et al.*, 1990). It has also been demonstrated that TGF-β may suppress the ability of testosterone to stimulate the growth of SC115 cells (Yamanishi *et al.*, 1990). Thus, a number of hormones and growth factors are known to affect the growth of the SC 115 tumor, but androgens are the primary stimulator of cell growth. Further it has been demonstrated that morphological differences exist between tumors grown in male and female DD/S mice (Emerman and Worth, 1985, Kitamura *et al.*, 1979). Tumors grown in male mice exhibit a typical epithelial morphology, with cells appearing round to cuboidal and arranged in clumps or sheets with very little connective tissue separating the cells (Emerman and Worth, 1985). In contrast, tumors grown in female mice display a fibroblast-like morphology with

spindle-shaped cells arranged in loose sheets or irregular cords, separated by large amounts of connective tissue.

The Model. The model is based on a study by Sklar and Anisman (1980) which demonstrated that individual housing as well as a change in housing group could markedly affect the growth of a syngeneic mastocytoma tumor. Thus, Drs. Emerman and Weinberg designed a series of studies to investigate the effects of different social housing groups on the growth of the SC 115 tumor. The first study (Weinberg and Emerman, 1989) investigated the effects of housing group on the growth of the SC115 tumor . The design of the study was as follows (figure 1): Male mice of the DD/S strain were housed either individually [I] or in groups of 3 [G], at time of weaning (3 wk of age). When the mice were 2 to 4 months of age, they were injected with tumor cells and housed as follows. Mice raised as individuals were either rehoused as a male/female pair [IP] or were rehoused in groups of 5 males [IG]. Mice raised in groups either remained in their groups [GG] or were rehoused as individuals [GI]. Half of the mice in each housing group were subjected to an acute daily stressor consisting of exposure to 1 of 5 novel environments, 15 min/d, 5 d/wk. This daily stressor was designed to produce an acute rise in levels of plasma glucocorticoids. By 23 d post tumor injection there were significant differences in tumor growth among the different groups (Figure 2). Overall, mice experiencing acute daily novelty stress had significantly larger tumors than mice which did not experience this stressor ($p<0.05$). This effect was most prominent in mice of the GI and IP groups, and in fact, tumor growth was not significantly altered by novelty stress in mice of the GG group. In addition, collapsed across the novelty stress condition, there were significant differences in tumor growth among mice in the 4 housing groups ($p<0.05$). Mice of the GG group had a tumor growth rate which was similar to that previously reported for this tumor (approximately 2 g at 3 wk). In contrast, tumor growth rate was significantly greater in mice of the GI group than that of mice in the GG group. Whereas, mice in the IG group exhibited a significant retardation of tumor growth rate compared with tumors grown in mice of all the other housing groups ($p<0.01$). Plasma corticosterone levels were also

Figure 1. *Experimental Design of Model I.*

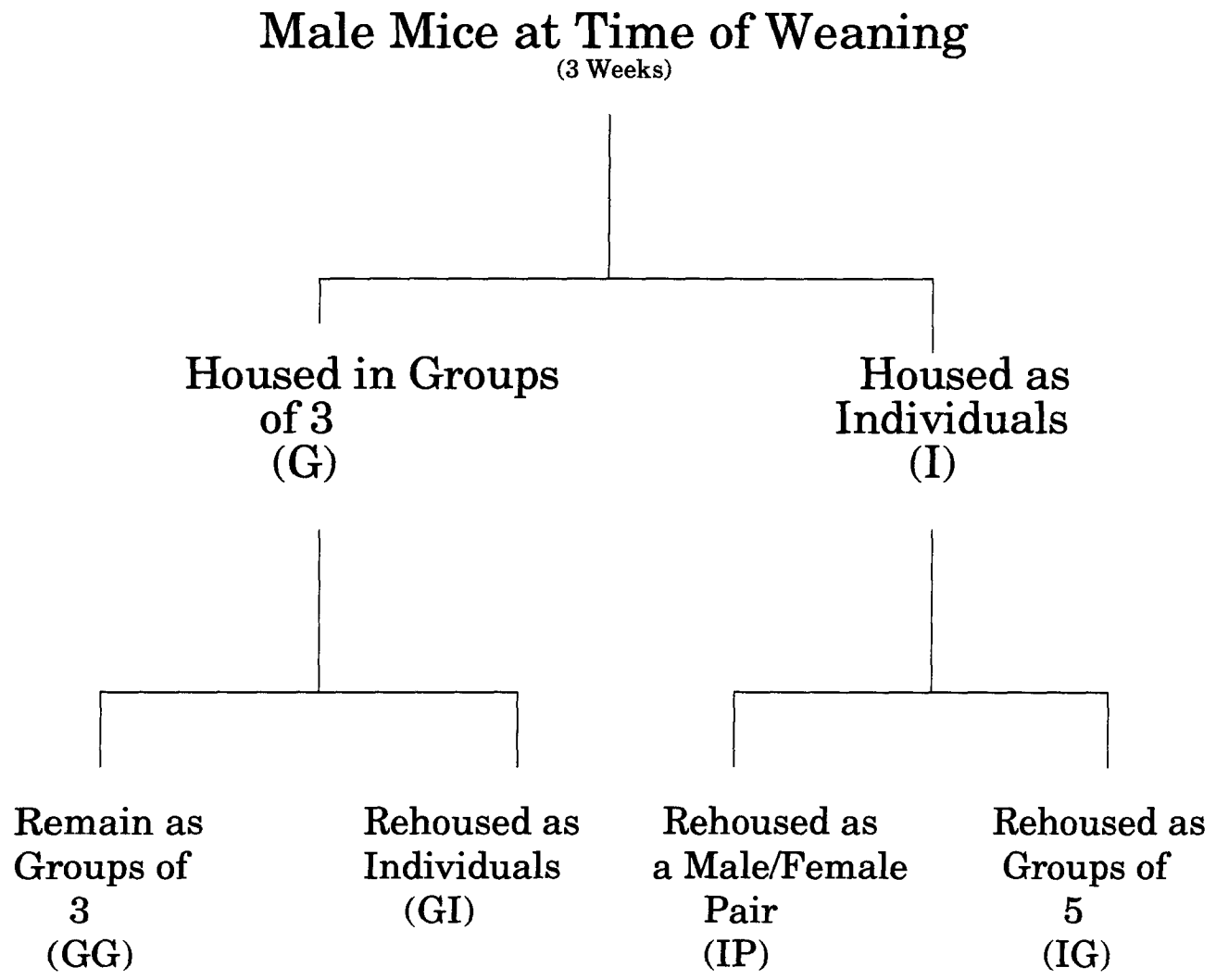
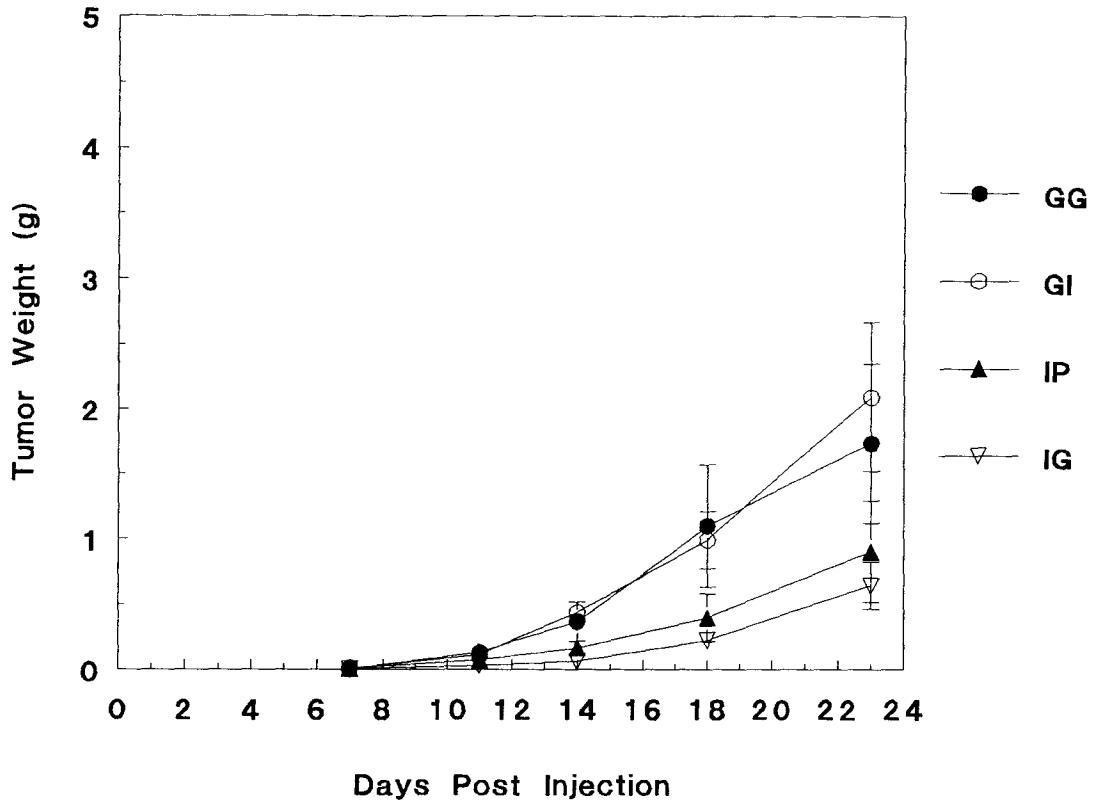
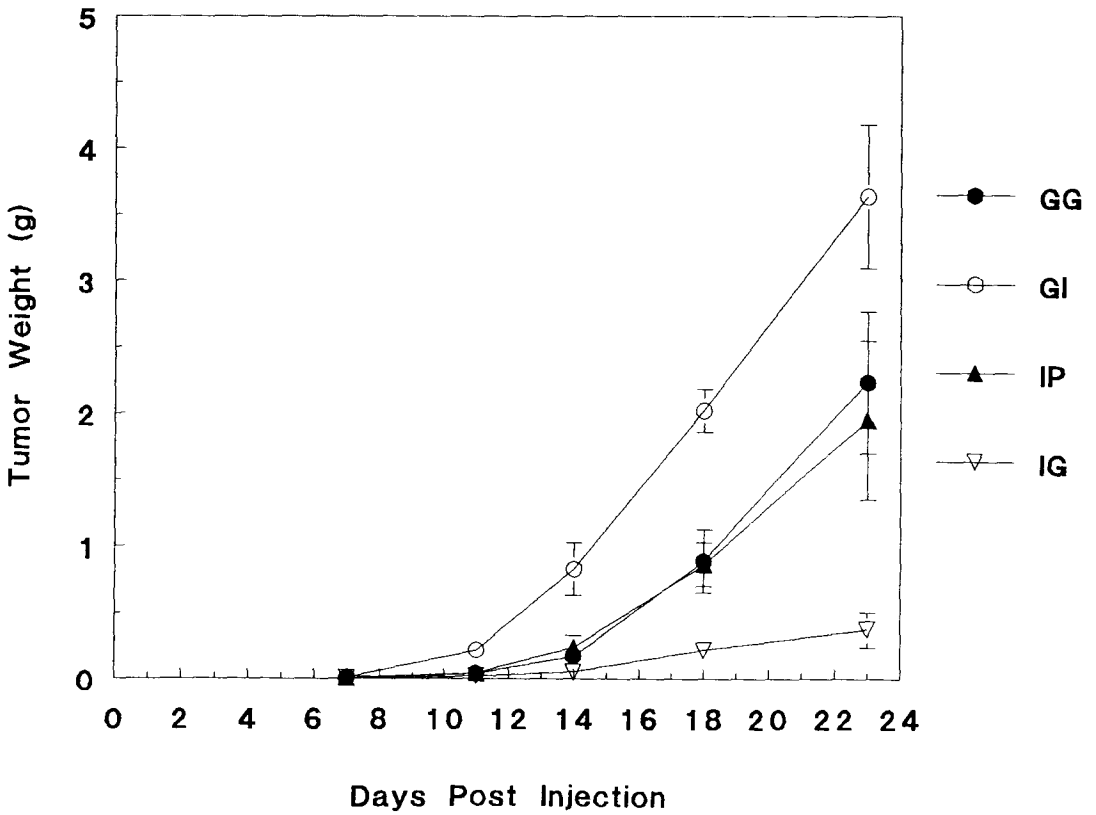

Male Mice at Time of Weaning
(3 Weeks)
Housed in Groups of 3 (G)
Housed as Individuals (I)
Remain as Groups of 3 (GG)
Rehoused as Individuals (GI)
Rehoused as a Male/Female Pair (IP)
Rehoused as Groups of 5 (IG)

Figure 2. *Tumor Growth in Male Mice in the Four Housing Groups.* Points represent mean ± SEM. GG, raised and maintained in sibling groups of three; GI, raised in sibling groups of three, then separated and housed singly; IG, raised singly housed, then rehoused in groups of 5 males; IP, raised singly housed, then rehoused with a female. All mice were injected with 2×10^6 SC115 tumor cells. At 23 d post tumor cell-injection, tumor growth collapsed across group, was greater in the presence of acute daily novelty stress than in its absense, $p<0.05$. In addition, collapsed across novelty stress, all groups differed significantly from each other in tumor size by 23 d, GI>GG>IP>IG, p's<0.05. (Weinberg and Emerman, 1989).

No Acute Novelty Stress



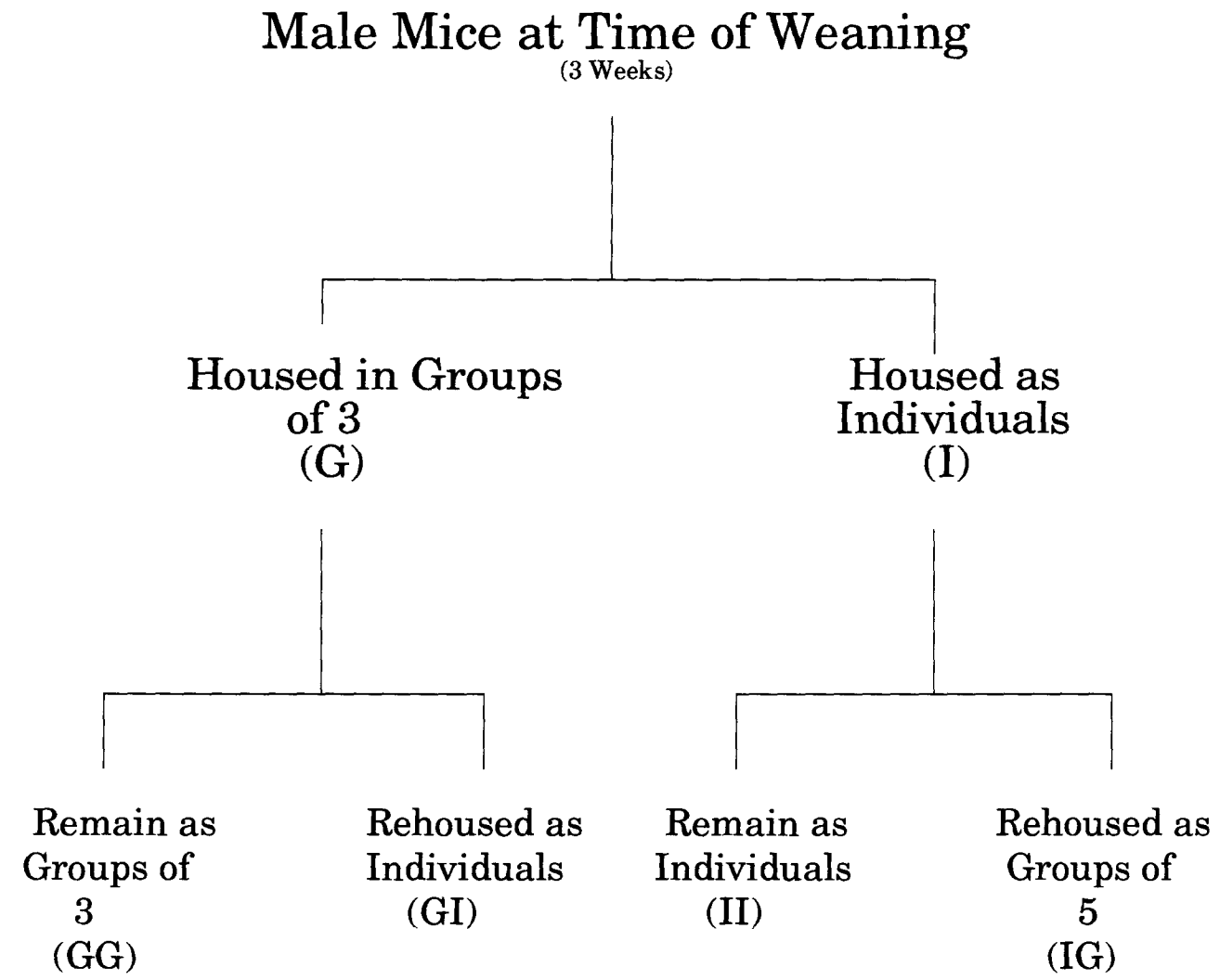
Acute Novelty Stress



measured in these mice at 3 wks post tumor injection and group formation. In mice not exposed to the acute daily novelty stressor, all mice exhibited low basal plasma corticosterone levels and there were no significant difference among mice from the 4 housing groups. Mice which had previously been exposed to the novelty stress were given a final exposure to novelty stress immediately before termination. It was found that there was still a significant corticosterone response to the acute daily novelty stress and that there were no significant differences among mice of the 4 housing groups in their corticosterone response to this novelty stressor.

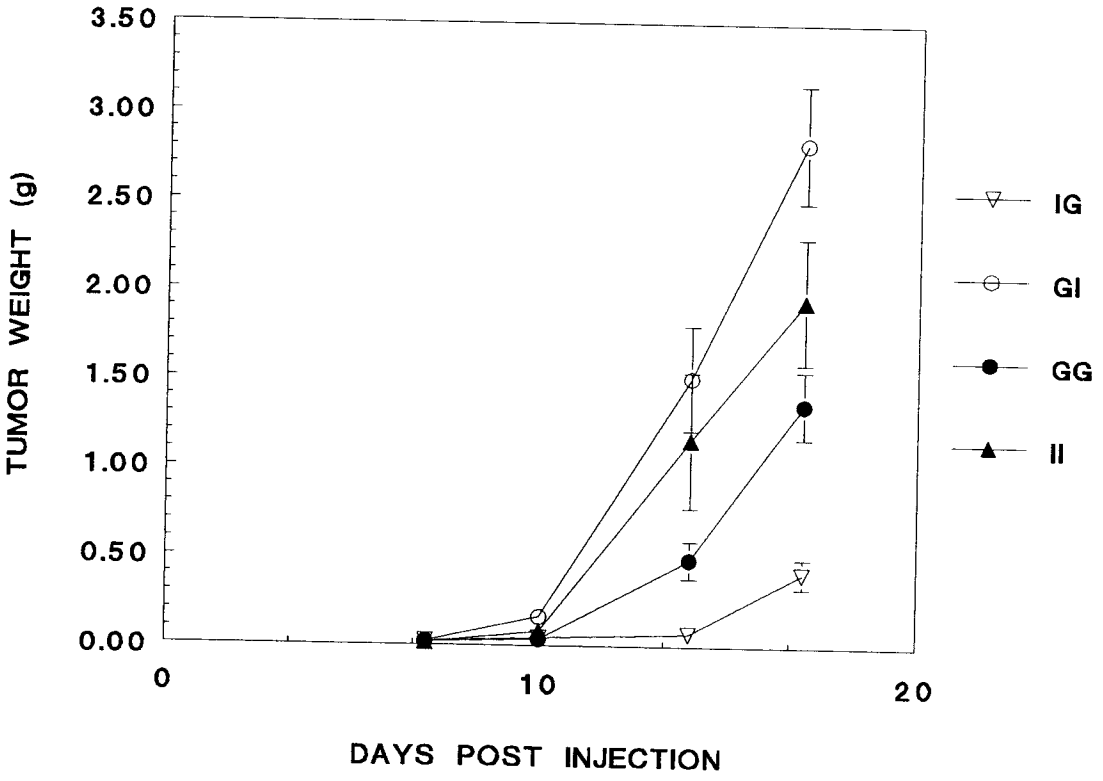
A second experiment was conducted to study the effects of group vs individual housing as well as a change in housing group on the growth of the SC 115 tumor. The design of the study was as follows (figure 3): Male mice of the DD/S strain were housed either individually [I] or in groups of 3 males [G], at time of weaning (3 wk of age). When the mice were 2 to 4 months of age, they were injected with tumor cells and housed as follows. Mice raised as individuals either remained as individuals [II] or were rehoused in groups of 5 [IG]. Mice raised in groups either remained in their groups [GG] or were rehoused as individuals [GI]. All mice were subjected to an acute daily stressor consisting of exposure to 1 of 5 novel environments, 15 min/d, 5 d/wk. This study replicated the tumor growth results of the previous study; in mice of the GG group (the standard colony housing group), tumors grew to a mass of approximately 2 g by 3 wk, whereas mice of the GI group had significantly increased tumor growth rates and mice of the IG group had significantly slower tumor growth rates compared with those of mice in the GG group (Figure 4). Interestingly, mice of the II group had a tumor growth rate that was intermediate between that of GG housed mice and that of GI housed mice. This study also examined the basal plasma levels of testosterone and dihydrotestosterone in mice from the 4 housing groups at 3 wk post tumor injection, as androgens are known to have an important stimulatory effect on SC115 growth. There were no significant differences in the plasma levels of testosterone or dihydrotestosterone among mice of the 4 housing groups. The mice were also studied for differences in humoral or cellular immunity as there are suggestions that the immune system may

Figure 3. *Experimental Design of Model II.*



All Mice Exposed to Acute Daily Novelty Stress
(15 min/d, 5 d/wk)

Figure 4. *Tumor Growth in Male Mice in the 4 Experimental Housing Groups.* Points represent mean ± SEM. GG, raised and maintained in sibling groups of three; GI, raised in sibling groups of three, then separated and housed singly; IG, raised singly housed, then rehoused in groups of 5 males; II, raised and maintained singly housed. All mice were injected with 2×10^6 SC115 tumor cells. At 18 d post tumor cell-injection,, GI=II>GG>IG, p's<0.05. (Weinberg and Emerman, 1989).


3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
TUMOR WEIGHT (g)
0
10
20
DAYS POST INJECTION
IG
GI
GG
II

play a role in modulating the growth of the SC115 tumor (Kitamura *et al.*, 1979, Nono *et al.*, 1986, Watenabe *et al.*, 1982). This study examined general measures of immune competence of B and T lymphocytes, looking at secondary antibody production in response to a foreign protein, bovine serum albumin (BSA) and the ability of T lymphocytes to proliferate in response to the mitogen concanavalin A (Con A) (Weinberg and Emerman, 1989). The presence of a tumor resulted in a general suppression of both B and T lymphocyte functions in all tumor-injected mice relative to their vehicle-injected counterparts at 3 wks post injection and group formation ($p<0.05$). Further, it was shown that, collapsed across the tumor/vehicle-injected condition, IG mice, who develop the smallest tumors, demonstrated a greater antibody response to BSA than did mice of the GI group, those mice who develop the largest tumors ($p<0.057$). T lymphocyte proliferation in response to Con A did not differ significantly among tumor-injected mice from the 4 housing groups. However, in vehicle-injected mice, mice of the GG group exhibited significantly greater T lymphocyte proliferation than mice in the IG group ($p<0.05$).

H) Thesis Objectives

In light of the ability of stressors to affect both the immune and the endocrine system, this thesis investigated the role of selected immune and endocrine variables in mediating the effects of psychosocial stressors on tumor growth rate as observed in our model.

Several studies suggest that the immune system may play a role in mediating the effects of psychosocial stressors on SC115 tumor growth rate. First, it has been demonstrated that, in DD/S mice, injection of heat killed Staphylococcus aureus prior to tumor cell-injection results in decreased growth of the SC115 tumor (Nohno *et al.*, 1986). As the injection of Staphylococcus aureus is known to stimulate the immune system, the authors conclude that the immune system may affect growth of the SC115 tumor. Thus, our laboratory is interested in the role of the immune system in mediating the differential tumor growth rates observed in our model. There are

several effector cells which could be involved in mediating the differential tumor growth including CTL and NK cells. Previous studies in our laboratory demonstrated that the presence of the SC115 tumor significantly stimulates splenic NK cell activity compared with that of vehicle injected mice at 3 and 7d post injection (Rowan, 1992). Furthermore, at 3 d post tumor cell-injection, when the greatest stimulation of NK cell activity was observed, mice of the GI group (largest tumors) had significantly greater levels of splenic NK cell activity than did mice in all other groups (Rowan, 1992). Thus, the role of NK cells in mediating the effects of psychosocial stressors on growth of the SC115 tumor in our model was examined further. The time course study of splenic NK cell activity was completed by examining NK cell activity of tumor cell- and vehicle-injected mice from the 4 experimental housing groups at 1 d post injection. As well, NK cell activity of tumor-infiltrating lymphocytes was studied in mice of the GI (largest tumors) and IG (smallest tumors) at 3 and 7 d post injection when NK cell activity of tumor-infiltrating lymphocytes was demonstrated to be maximal. Finally, the effect of direct in vivo modulation of NK cell on the differential tumor growth rates observed in our model was investigated in mice of the GI and IG groups.

The endocrine system is also likely to be involved in mediating the effects of psychosocial stressors on tumor growth in our model, as the SC115 tumor is hormone responsive and stressors are known to alter plasma hormone levels. Thus, to determine if alterations in plasma hormone levels may be involved in mediating the effects of psychosocial stressors on tumor growth rate in our model, plasma levels of testosterone and corticosterone were measured in mice of the 4 experimental housing groups in the first wk post tumor cell-/vehicle-injection. Furthermore, the SC115 tumor contains subpopulations of cells with different degrees of hormone responsiveness and selection for hormone-nonresponsive cells (i.e. tumors grown in female mice) results in a tumor with a slow growth rate. Hormone-nonresponsive tumors grown in female mice have been demonstrated to exhibit altered morphological characteristics compared with hormone-responsive tumors grown in male mice housed in our standard colony conditions. Thus, the morphological

characteristics of tumors grown in mice from the 4 experimental housing conditions were examined and compared with tumors grown in male and female mice housed in our standard colony conditions. As well, the hormone responsiveness of tumor cells was directly measured in an *in vitro* assay. The ability of tumor cells from mice of the GI (largest tumors) and IG (smallest tumors) groups to proliferate in response to in vitro stimulation with either dihydrotestosterone or hydrocortisone was examined.

CHAPTER 2: GENERAL METHODS :

A. Tumor Model.

The androgen-responsive SC115 mouse mammary carcinoma, subline class I (Bruchovsky & Rennie, 1978), was maintained by serial transplantation in male mice of the DD/S strain. For propagation, tumors were dissociated to a single cell suspension (as described below) and male mice (2-6 months old), housed in the standard colony condition, were injected with 2×10^6 cells/mouse. The standard colony condition consisted of male mice raised and housed in sibling groups of 3.

A.1) Dissociation.

Tumors weighing approximately 2 g were dissected free of extraneous tissue with sterile technique and finely minced with opposing scalpel blades. The tissue was transferred to a dissociation flask and approximately 15 ml Saline-Trypsin-Versine (STV) added. STV consisted of 0.05% trypsin (1:250) and 0.025% EDTA (Sigman Chemical Co., St. Louis, MO) in Ca^{2+} - Mg^{2+} - free Saline A, pH. 7.3. The flask was then gently swirled for 2 min; the contents transferred to a 50 ml conical centrifuge tube and spun at 80 x g for 1 min in a bench top clinical centrifuge (ICU). The supernatant was then transferred to a second 50 ml centrifuge tube with an equal volume of Dulbecco's Modified Eagle's Medium (DMEM; Terry Fox Laboratory, Vancouver, BC) and 5% Calf Serum (CS, to inactivate the trypsin). The tube was spun at 200 x g for 4 min to enrich for epithelial cells. The pellet was then resuspended in 5 ml DMEM and placed in a 37^{o} C waterbath.

The tissue in the original centrifuge tube was transferred to the dissociation flask and 15 ml STV was added. The flask was left shaking at 100 rpm on a gyrator shaker (Junior Orbit

Shaker, Lab-Line Instruments, Inc., Ill) in a 37° C incubator for 7 min. The contents of the flask were transferred to a 50 ml centrifuge tube and centrifuged at 80 x g for 1 min. The supernatant was collected and combined with an equal volume of DMEM and 5% CS. This was spun for 5 min at 400 x g, resuspended in 5 ml DMEM and placed in a 37° C waterbath. The remaining tissue was placed back in the flask for a third and final dissociation with STV for 7 min. The supernatant was collected as described above, combined with an equal volume of DMEM and 5% CS, and centrifuged. The resulting pellet was resuspended in 5 ml DMEM. All 3 cell suspensions were then pooled and passed through a 50 μm Nitex filter (Tetko, Inc., Elmsford, NY) to remove cell aggregates and debris. The resulting single cell suspension was centrifuged at 340 x g for 5 min and the pellet resuspeneded in 20 ml DMEM. An aliquot of this cell suspension was diluted 1:10 with DMEM and counted on a haemocytometer using trypan blue exclusion to determine the number of viable cells. The plasma membranes of dead cells are not able to prevent trypan blue (pH 7.2) from entering the cytoplasm, and therefore the dead cells stain blue. The suspension was then adjusted to the concentration desired for either freezing (see below) or injecting into mice (see below).

A.2) Freezing of Tumor Cells.

Although the SC115 carcinoma was maintained in vivo, SC115 cells were also stored in a liquid-nitrogen (LN2) storage tank. To freeze SC115 cells, dissociated SC115 cells were adjusted to a concentration of 1 to 1.5 x 10^7 cells/ml in freezing media (50% DMEM + 44% CS + 6% dimethylsulfoxide (DMSO)). Freezing vials were labeled with cell type and date of dissociation and 1 ml of cell suspension in freezing media was aliquoted to each vial. Vials were then slowly frozen according to a schedule provided by the manufacturer of the freezing tank (Handi-Freeze freezing tray, Union Carbide), before being transferred to a liquid nitrogen storage tank (MVE Cryogenics, TA).

A.3) Thawing of Tumor Cells.

Frozen vials of SC115 tumor cells were removed from the LN_2 storage tank and rapidly thawed in a 37° C water bath. The tumor cell suspension was transferred to a 15 ml conical centrifuge tube, diluted with an equal volume of warm (37° C) DMEM and spun for 5 min at 400 x g. The supernatant was discarded, the pellet resuspended in 10 ml DMEM and spun for 5 min at 400 x g (wash step). This wash step was repeated. The pellet was resuspended in 10 ml of DMEM and an aliquot counted as previously described. Cells were resuspended at 2×10^7 cells/ml for tumor injection.

A.4) Transplantation of Tumor Cells.

For tumor cell-injection, the cell suspension was adjusted to 2×10^7 cells/ml in DMEM. The total volume of tumor cell suspension required was 100 µl x the number of mice injected + 100 µl to allow for retention of fluid in the head of the syringe. The mice were lightly anesthetized with ether and then injected s.c. into the interscapular region with 100 µl of tumor cell suspension. Care was taken to lift the skin high up so as not to penetrate underlying tissue and to plunge the needle in deep enough to minimize the amount of cell suspension that leaked back out.

A.5) Monitoring Tumor Growth.

For tumor propagation, mice were palpated twice weekly after tumor injection, beginning on d 5 - 8 when a palpable tumor generally first appears and terminating at 18 - 21 days, when tumor size was between 2 and 3 g. When tumors were measurable, caliper measurements were taken and tumor weights calculated according to the formula (Simpson - Herron & Lloyd, 1970)

$$\frac{\text{length (cm) x [width (cm)]}^2}{2} = \text{g}$$

For experiments, mice were palpated twice a week, starting on day 6 or 7. Tumor weights were calculated when caliper measurements were able to be taken and the last measurement was taken on the day of termination.

B. Animal Model.

Mice were housed in polycarbonate cages (18 x 29 x 13 cm) with stainless steel lids, corn cobb bedding (Sanicel) and received food (Purina mouse chow pellets) and water ad libitum. Cages were placed on stainless steel racks in a room with a 12 h dark/light cycle (0700 h - 1900 h). The room was relatively free from extraneous building noise and remained at a constant temperature of 22^{o} C.

Following weaning (3 wk of age), male mice were housed individually [I] or in sibling groups of 3 [G]. When the mice were 2 to 4 months of age, they were injected s.c. in the interscapular region with a single cell suspension of 2 x 10^6 cells in 100 µl of DMEM (tumour groups) or with DMEM alone (Vehicle groups) and housed as follows:

1) II - males raised individually housed remained individually housed for the experiment.
2) IG - males raised individually housed were placed in groups of 5 animals for the experiment.
3) GG - males raised in sibling groups of 3 remained in their sibling groups for the experiment.
4) GI - males raised in sibling groups of 3 were rehoused as individuals for the experiment.

Animals in all groups were subjected to an acute daily stressor consisting of exposure, 15 min/d, 5 d/wk (prior to 1200 h), to 1 of 5 novel environments. The exposure to novel environments followed a set order of rotation as follows:

1) a round clear plastic jar 9 cm in diameter and 7 cm high with a white plastic screw top lid,
2) a polypropylene box 12 x 10 x 4 cm with a lid,
3) a covered cardboard box with cardboard divisions forming compartments 7 x 7 x 14 cm high,
4) a plastic cup (220 ml - 10 cm in height and top diameter of 6.5 cm, base diameter of 4.75 cm) with lid,
5) a clean cage (empty of bedding, food or water bottle) with a standard cage top.

All lids had hole punched in them for adequate ventilation.

For termination, the room was closed off the night before the experiment at 1800 h and no one entered the room prior to the start of the experiment. Previous data demonstrate that, under these conditions, animals are in a non-aroused state, as measured by plasma corticosterone levels (Weinberg & Bezio, 1987). For termination, each cage was quietly removed from the colony room and carried to an adjacent laboratory, the animals removed from their home cages, weighed and decapitated immediately. Where appropriate, trunk blood was collected and the required tissues removed from the animal.

CHAPTER 3 IMMUNE STUDIES :

A. Completion of Splenic NK Cell Activity Time Course Assay.

Introduction

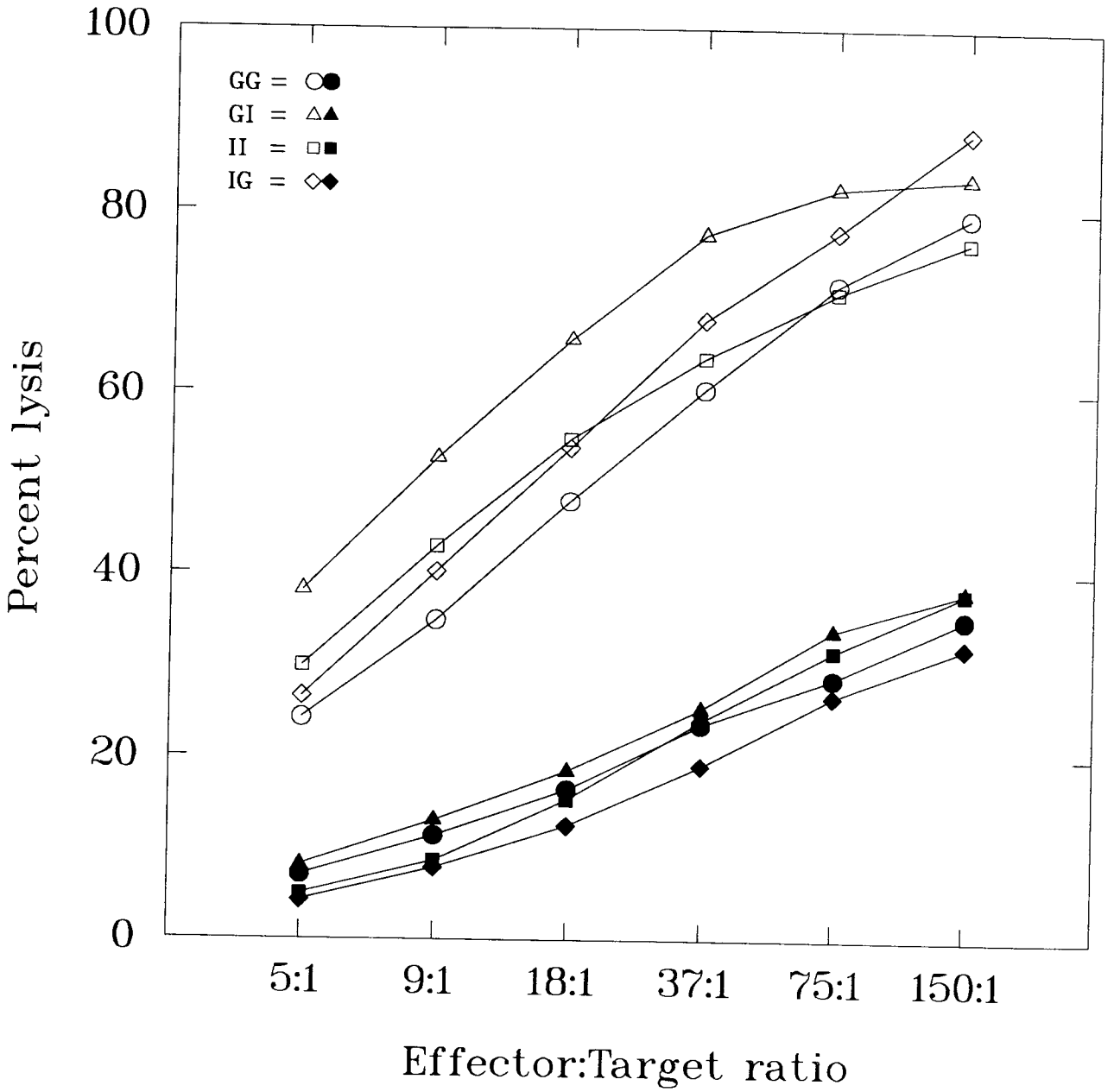
Tumor growth may be influenced by an animal's social housing condition (Riley, 1981, Riley *et al.*, 1981, Steplewski *et al.*, 1987). Group housed animals typically have smaller tumors and show increased rates of tumor regression compared with individually housed animals. We have demonstrated that the transplantable androgen-responsive Shionogi mouse mammary carcinoma (SC115), a typical adenocarcinoma with a sheet-like epithelial morphology (Emerman and Worth, 1985), also has differential growth rates when male mice are housed under different conditions (Weinberg and Emerman, 1989). Possible physiological mediators of this differential tumor growth were investigated in this thesis. The first set of studies focused on the immune system.

It has been demonstrated that the immune system is important in regulating the growth of some tumors (Cerottini and Brunner, 1974, Herberman and Ortaldo, 1981). Previous studies provide indirect evidence that the growth of the SC115 tumor is modulated by the immune system. First, it has been shown that the original androgen-dependent SC115 tumor, which does not grow in females or castrated males, can be induced to grow in DD/S mice by the administration of pharmacological doses of glucocorticoids (Watanabe *et al.*, 1982). The authors conclude that the growth-promoting effect of glucocorticoids is due in part to suppression of host immunity and in part to stimulation of a glucocorticoid receptor in the SC115 cells. Second, it was found that the development of the SC115 tumor is retarded by the injection of

Staphylococcus aureus on the day of tumor cell-injection (Nohno *et al*, 1986). This again is strongly suggestive of a role for the immune system in modulating the growth of the SC115 tumor. The immune cells which are responsible for this modulatory effect on SC115 tumor growth have not yet been determined. However, one possible candidate is the NK cell. NK cells are currently thought to be one of the body's first lines of defense against tumor cells (Haller *et al.*, 1977, Herberman and Ortaldo, 1981, Wei and Heppner, 1987). Data suggest that these cells are active very early in the antitumor immune response when the tumor burden is low and later are subject to immunosuppression as the tumor grows in size (Gerson *et al.*, 1981, Wei and Heppner, 1987). Studies have indicated that the degree of axillary lymph node involvement in breast cancer patients, a measure of the degree of metastasis of the tumor and thus an important prognostic indicator, exhibits a significant negative correlation with the patient's level of NK cell activity ($p<0.05$, Levy *et al*, 1985). It has also been shown that the patient's level of NK cell activity 15 months after removal of the primary tumor is negatively correlated with the chance of recurrence at 5 years (Levy *et al*, 1991).

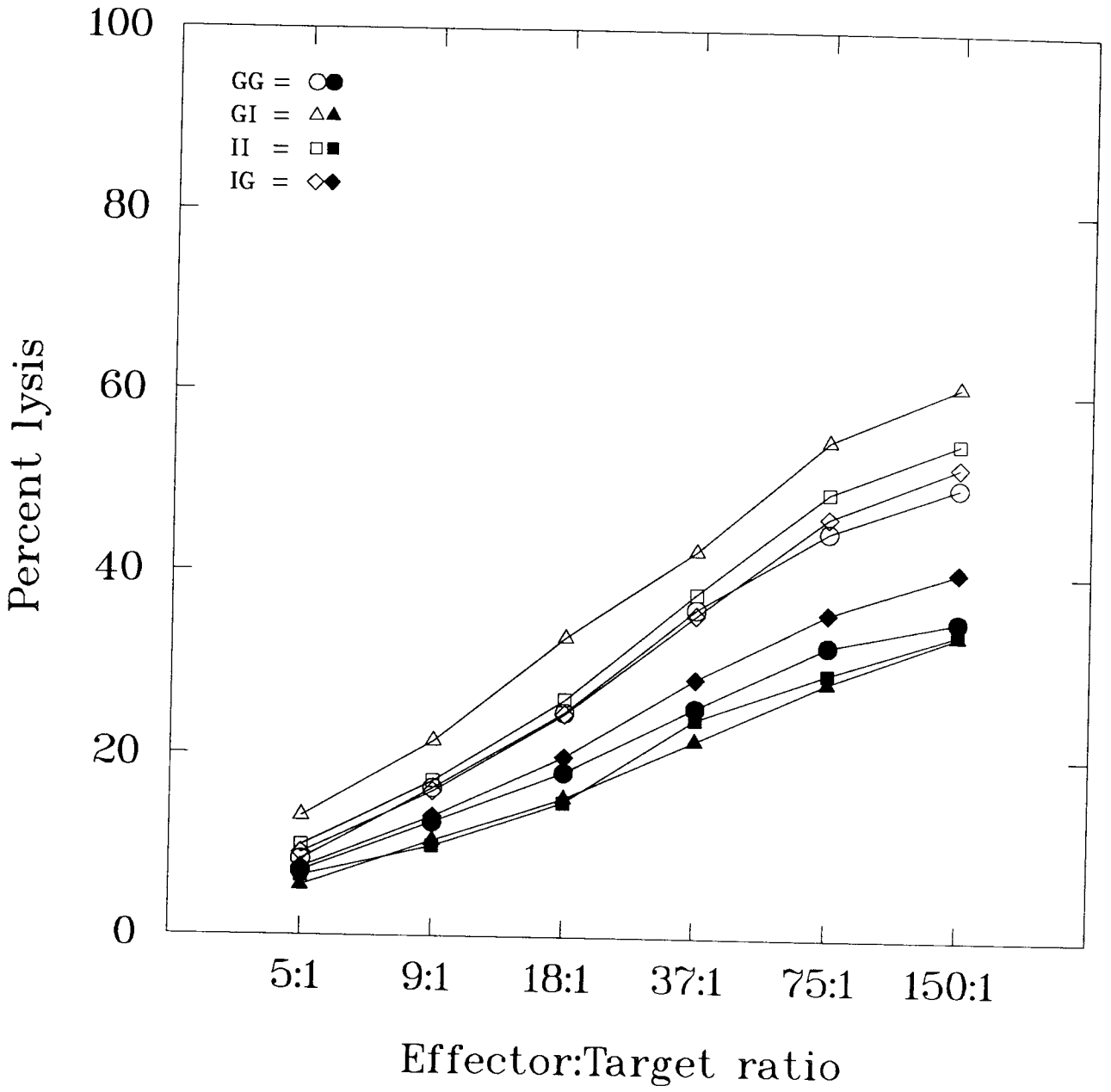
In light of the important antitumor role played by NK cells, a study was undertaken to investigate the role of NK cell activity in modulating the differential tumor growth rates observed in mice of the 4 housing groups of our experimental model. The initial part of this study, in which I played a significant role, investigated splenic NK cell activity in both tumor cell- and vehicle-injected mice from the 4 housing groups at 3 d and 7 d post injection and group formation (Rowan, 1992). Overall, data indicated a significant effect of both the presence of the SC115 tumor and differential housing group on splenic NK cell activity. At 3 d post injection and group formation, there was a significant ($p<0.001$) stimulation of NK cell activity in the spleens of tumor cell-injected mice compared with their vehicle-injected counterparts (Figure 5). In addition, there were significant differences among tumor cell-injected mice of the 4 housing groups in their level of splenic NK cell activity ($p<0.001$). Interestingly, post-hoc analysis revealed that mice of the GI

Figure 5. *Lytic Activity of Splenic NK Cells 3 d Post Injection.* Points represent mean ± SEM. Mice from the 4 experimental groups (as described in figure 2) were injected either with 2 x 10^6 SC115 cells (open symbols) or vehicle (solid symbols). Three days later, the lytic activity of splenic NK cells was measured. NK cell activity was significantly greater in tumor cell-injected than in vehicle-injected mice, $p<0.05$. Within the tumor injected condition, GI > GG = II, $p's<0.05$. (n's: GG-tumor (GG-T)(9), GG-vehicle (GG-V)(7); GI-tumor (GI-T)(6), GI-vehicle (GI-V)(5); II-tumor (II-T)(6), II-vehicle (II-V)(6); IG-tumor (IG-T)(11), IG-vehicle (IG-V)(15). Spontaneous release was usually less than 15% of the total release and always less than 20%.


100
80
60
40
20
0
Percent lysis
GG = ○●
GI = △▲
II = □■
IG = ◇◆
5:1
9:1
18:1
37:1
75:1
150:1
Effector:Target ratio

group (who develop the largest tumors) had significantly greater NK cell activity, at the lower effector to target cell ratios, than did mice of all other housing groups. No differences were found in NK cell activity among vehicle-injected mice from the 4 housing groups. At 7 d post injection and group formation (Figure 6), the spleen cell populations of tumor cell-injected mice once again had significantly greater levels of splenic NK cell activity than did those of vehicle-injected mice ($p<0.001$). However, at 7 d, there were no significant differences in splenic NK cell activity among mice from the 4 housing groups in either the tumor cell- or the vehicle-injected conditions. Furthermore, splenic NK cell activity of tumor cell-injected mice was significantly lower at 7 d than at 3 d post injection ($p<0.001$). Thus, this study demonstrated that the presence of the SC115 tumor significantly stimulated NK cell activity in mice from the 4 housing groups. However, at 3 d post injection, the differential levels of NK cell activity observed among tumor cell-injected mice of the 4 housing groups were not in the direction that we would hypothesize. That is, mice of the GI group, in which the largest tumors will develop (Weinberg and Emerman, 1989), also had the highest levels of splenic NK cell activity. Further, it was observed that the tumor-induced stimulation of splenic NK cell activity was clearly declining by 7 d post tumor cell-injection. Therefore it is possible that maximal stimulation of NK cell activity occurred earlier than 3 d post tumor cell-injection. This possibility is supported by the finding that injections of biological response-modifiers such as IL-2 or γ IFN causes stimulation of NK cell activity by 18 to 24 h post injection (Ortaldo *et al.*, 1989, Talmdge *et al.*, 1985). Thus, as part of this dissertation, a study was undertaken to examine splenic NK cell activity in tumor cell- and vehicle-injected mice from the 4 housing groups at 1 d (24 h) post injection and group formation. In addition, data on spleen weights and body weights of tumor cell- and vehicle-injected mice from the 4 housing groups at 1 d, 3 d and 7 d post injection were examined. Measurements of splenic NK cell activity at 3 and 7 d post group formation were reported in a previous thesis (Rowan, 1992) and at the same timepoints, spleen weight and body weight were measured. As these data were not reported in the previous thesis, they are reported and analyzed here. As the 1 d data described in this dissertation were part of the complete time course study (Rowse *et al.*,

Figure 6. *Lytic Activity of Splenic NK Cells 7 d Post Injection.* Points represent mean ± SEM. Mice from the 4 experimental groups (as described in figure 2) were either injected with 2 x 10^6 SC115 cells (open symbols) or vehicle (solid symbols). At 7 d, the lytic activity of splenic NK cells was measured. (n's: GG-T(9), GG-V(8); GI-T(5), GI-V(6); II-T(6), II-V(6); IG-T(15), IG-V(15)). Spontaneous release was usually less than 20% of the total release and always less than 30%.


100
80
60
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20
0
Percent lysis
GG = ○●
GI = △▲
II = □■
IG = ◇◆
5:1
9:1
18:1
37:1
75:1
150:1
Effector:Target ratio

1990), the discussion of this study will include data on spleen weight, body weight and NK cell activity from all 3 test days (1, 3 and 7 d).

METHODS AND MATERIALS

Animals: Seventy eight male DD/S mice were used in this experiment. Animals were reared and housed as described previously in the General Methods section.

Tumor Cells: SC115 tumor cells were prepared and injected as described previously in the General Methods section.

NK cell assay: NK cell activity was assayed as described by Greenberg (Kraut and Greenberg, 1986) with spleens from mice from the 4 housing groups injected with either tumor cells or vehicle. Briefly, animals were weighed, exposed to ether (10-15 sec) and rapidly terminated by decapitation. Spleens were then aseptically removed.

Dissociation of Spleens. In a laminar flow hood (Canadian Cabinets Co., Ottawa, Ont.) each spleen was placed in a sterile 60 mm petri dish (spleens were not pooled) and washed with approximately 10 ml warm (37°C) RPMI 1640 media (Terry Fox Laboratory). The spleens were then aseptically transferred to a second set of 60 mm petri dishes containing sterile stainless steel wire mesh screens and approximately 15 ml RPMI. The spleens were cut into several sections and gently pressed through the wire mesh using the flat end of a plunger from a plastic 10 ml syringe. The spleen cell suspensions were transferred to 50 ml polypropylene conical centrifuge tubes using 10 ml disposable pipettes. The screens were each washed with approximately 15 ml RPMI which was then added to the centrifuge tubes. The spleen cells were centrifuged for 2 min at 600 x g to pellet the cells. The supernatants were decanted and the cells resuspended in 3 ml trisma base-NH_4Cl solution and incubated at 25°C for 3 min to lyse the red blood cells. The cells were pelleted again by spinning at 600 x g for 2 min and the trisma base solution was decanted.

The cells were washed twice in approximately 10 ml RPMI containing 10 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO).

Removal of B Lymphocytes. Nylon wool columns were prepared by placing 0.7 g nylon wool (Fenwal, Deerfield, IL) into 10 ml pipettes and packing the wool to the 7 ml mark. The columns were then wrapped in aluminum foil and autoclaved. On the day of the experiment, the columns were conditioned by first wetting the column with 10 ml warm RPMI (tapping the side of the column with a 10 ml pipette until all of the nylon wool was wet with medium) and then pouring 20 mls of RPMI containing 10 mM Hepes and 10 % fetal bovine serum (Grand Island Biology Co., Burlington, Ontario) (RPMI + H + FBS) through the column and incubating it for 1 h in a humidified incubator at 95 % air, 5% CO_2. The spleen cells were resuspended in 1 ml of RPMI + H + FBS and placed on the column with a pastuer pipette one drop at a time. RPMI + H + FBS (0.5 ml) was then added to each column to ensure that the spleen cells entered the nylon wool. The columns were incubated for 1 h (as above). The T lymphocytes were eluted from the columns with 10 ml RPMI + H + FBS (added to the columns drop-wise) and collected in a 15 ml polystyrene centrifuge tube (Falcon). This procedure has been shown to eliminate B lymphocytes and macrophages, thus enriching the spleen cell preparation for T lymphocytes and NK cells (Julius *et al.*, 1973).

Target Cells. Yac-1 lymphoma cells (a generous gift of Dr. D. Chow, Manitoba Institute of Cell Biology), a Maloney lymphoma virus infected lymphocyte cell line which is highly susceptible to NK cell lysis, were stored in liquid nitrogen in 1 ml aliquots (10^7 cells/ml). At least 48 h before use in an assay, the cells were rapidly thawed as described for SC115 cells in General Methods, resuspended in 50 ml RPMI + H + FBS at a concentration of 5 x 10^4 cells/ml, placed in a 75 cm^2 tissue culture flask (Corning Glass Works, Corning, NY) and incubated at 37°C in 95 % air, 5% CO_2. The cells were resuspended in fresh medium at 5 x 10^4 cells/ml every 24 h to ensure that cells remained in an exponential growth phase. For the experiment, 1 x 10^7 Yac cells

were placed in a 15 ml round bottomed centrifuge tube (Falcon), spun for 2 min at 600 x g and the supernatant decanted. The cells were then resuspended in 10 ml fresh RPMI + H + FBS, centrifuged for 2 min at 600 x g and the supernatant decanted, leaving approximately 100 µl supernatant behind with the cell pellet. Sodium [^{51}Cr] chromate (500 µCi/ml, Amersham, Oakville, Ont.) (100 ul) was added to the tube and the cells resuspended by hand vortexing. The cells were then incubated in a water bath at 37°C for 80 min with frequent hand vortexing to maintain the cells in suspension. Following this, the cells were washed twice with 10 ml RPMI + 10mM hepes and resuspended at 10^5cells/ml of medium (RPMI + H + FBS).

Final Preparation and Plating of Cells. Viable effector cells were counted on a hemocytometer and resuspended in RPMI + H + FBS at a concentration of 1.5 x 10^7 cells/ml medium. For each spleen, cells were plated by adding 100 µl spleen cell suspension to 3 wells of rows 1 and 2 of a 96 well v-bottomed plate (Flow Laboratories Inc., McLean, VA) and adding 100 µl media to wells of rows 2 through 5; spleen cells were serially diluted by resuspending the cells in row 2 and placing 100 µl of this suspension into the wells of row 3. The cells in row 3 were then resuspended and 100 µl of this suspension placed in the wells of row 4. This process was repeated for rows 4, 5 and 6. This serial dilution produces effector to target cell ratios of 150:1, 75:1, 37.5:1, 17:1, 9:1, 4.5:1 (3 wells per ratio per spleen). Aliquots of 100 µl target cell suspension (10^5 cells/ml) were added to each well of effector cells. In addition, 6 wells were plated with 100 µl of target cells and 100 µl of media alone to measure the spontaneous lysis of the target cells and 6 wells were plated with 100 µl of target cells and 100 µl of 1 N HCl to measure the total radioactivity available to be released by the target cells. Plates were incubated for 6 h at 37°C in 95% air, 5% CO_2. The plates were centrifuged for 10 min at approximately 150 x g, 100 µl of supernatant was removed and the [^{51}Cr] release from lysed target cells was determined by gamma counting. The percent of specific lysis (chromium released) at each effector to target cell ratio was computed using the formula:

$$\% \text{ specific lysis} = \frac{\text{Test CPM - Spontaneous CPM}}{\text{Total CPM - Spontaneous CPM}} \times 100$$

where Test CPM = counts in experimental wells containing target cells and effector cells.
Spontaneous CPM = counts in wells containing only target cells.
Total CPM = counts obtained by adding 100 µl 1N HCl to target cells to lyse all cells.

To ensure that NK cells were in fact the primary cells responsible for the target cell lysis observed in these assays, 2 additional groups (GG, n=3, of and GI, n=3) of mice were injected with tumor cells. Three days later spleens were removed, dissociated and treated with anti-Asialo GM1 (Wako Chemical USA Inc., Dallas, Tx) and complement (Cedar Lane Laboratories Ltd., Hornby, Ontario), a procedure shown to suppress NK cell activity (Kasai *et al.*, 1980). The treated spleen cells were then assayed for NK cell activity.

Data Analysis: Statistical analyses were performed with appropriate analyses of variance for the factors of Group, Tumor and Ratio, with repeated measures on the last factor. Significant main effects were analyzed by Tukey's post-hoc tests.

Results

Body Weights: Body weights were analysed separately for tumor cell- and vehicle-injected conditions at 1 d, 3 d and 7 d post injection and group formation. The body weights of vehicle-injected animals from the 4 groups did not differ at 1 d or 3 d. At 7 d, vehicle-injected animals in the GI group were heavier than mice in the other 3 groups ($F(3,31) = 7.3$, $P<0.01$). There were no significant differences in body weights among the tumor cell-injected animals from the four experimental groups on any of the termination days (Table 1). Since body weights of the tumor cell-injected animals do not differ, we conclude that neither body weights nor nutritional variables affect tumor growth in this model.

Table 1. *Body Weights of Tumor Cell- and Vehicle-Injected Mice From the 4 Experimental Housing Groups 1, 3 and 7 d Post Injection.* Male mice were injected with either 2 x 10^6 SC115 cells or vehicle. Mice were weighed and immediately terminated at 1, 3 and 7 d post injection. The body weights of vehicle-injected animals from the 4 groups did not differ at 1 d or 3 d. At 7 d, vehicle-injected animals in the GI group were heavier than mice in the other 3 groups ($p<0.05$). There were no significant differences in body weights among the tumor cell-injected animals from the four experimental groups on any of the termination days.

Table 1.

	Body Weight (g)			
	GG	GI	II	IG
1 Day Tumor	36.1 [1] ± 0.98	37.2 ± 0.98	37.6 ± 0.95	35.9 ± 0.70
1 Day Vehicle	36.6 ± 1.35	36.5 ± 0.83	38.9 ± 1.63	37.4 ± 0.99
3 Day Tumor	33.2 ± 0.68	32.6 ± 1.96	31.8 ± 2.32	34.5 ± 0.96
3 Day Vehicle	34.5 ± 0.72	36.4 ± 0.88	34.8 ± 1.55	32.8 ± 0.72
7 Day Tumor	36.6 ± 0.88	34.1 ± 1.15	35.8 ± 1.62	34.2 ± 1.00
7 Day Vehicle	37.3 ± 1.24	42.8 ± 1.44	36.9 ± 0.77	35.0 ± 0.85

1. Mean ± sem.

Spleen Weights: Spleen weights were adjusted for the body weight of the animal (relative spleen weight). Overall, there were significant effects of Tumor, $F(1,195) = 19.86$, $P<0.001$, Group, $F(3,195) = 5.75$, $P<0.001$ and Day, $F(2,195) = 22.09$, $P<0.001$. Tumor cell-injected mice had significantly larger relative spleen weights than those of vehicle-injected mice ($p<0.001$) (Table 2). Separate analyses of relative spleen weights in tumor cell- and vehicle-injected animals revealed that, in tumor cell-injected mice, relative spleen weights increased significantly over days (1 d < 3 d < 7 d, $p<0.001$). In addition, there was a significant Day x Group interaction ($F(6,102) = 3.326$, $p<0.01$). At 1 d post injection, mice of the GI group had significantly larger relative spleen weights than mice of the IG group ($p<0.05$, Figure 7). At 3 d, there were no significant differences in relative spleen weights among groups, whereas by 7 d, mice of the IG group had significantly larger relative spleen weights than did mice of the II group ($p<0.05$) (Figure 7). In contrast, in vehicle-injected mice, there were significant effects of Group and no Group x Days interaction. Overall, relative spleen weights were greater at 3 d than at 1 d ($p<0.02$), but relative spleen weights at 7 d post injection were not significantly different from spleens of mice at either 1 d or 3 d post injection (Table 2).

Splenic NK Cell Activity at 1 d Post Injection: At 1 d post injection and group formation, the ANOVA revealed significant main effects of Group ($F(3,64) = 15.16$, $P<0.001$), Tumor ($F(1,64) = 12.05$, $P<0.001$), and Ratio ($F(5,320) = 821.98$, $P<0.001$), as well as a significant Group x Tumor x Ratio interaction ($F(15,320) = 3.94$, $P<0.001$) (Figure 8). Overall, tumor cell-injected animals showed decreased splenic NK cell activity compared with vehicle-injected controls. Importantly, post-hoc analysis of the Group x Tumor x Ratio interaction revealed that for both tumor- and vehicle-injected conditions, GI animals generally had the greatest and IG animals generally had the least NK cell activity ($p<0.05$). In addition, it is interesting that in the vehicle-injected condition, II animals were similar to GI animals in their high levels of NK cell activity, whereas in the tumor-injected condition II animals had significantly lower NK cell activity

Table 2. *Spleen Weights of Tumor Cell- and Vehicle-Injected Mice From the 4 Experimental Housing Groups 1, 3 and 7 d Post Injection.* Methods were as described in table 1. Spleens were removed immediately following termination of the mice. In tumor cell-injected mice, relative spleen weights increased significantly over days (1 d < 3 d < 7 d, $p<0.001$). In vehicle-injected mice, relative spleen weights were greater at 3 d than at 1 d ($p<0.02$), but relative spleen weights at 7 d post injection were not significantly different from spleens of mice at either 1 d or 3 d post injection.

Table 2

		Tumour Injected		Vehicle Injected	
		Spleen Wt. (g)	Spleen Wt. / Body Wt x100 (g)	Spleen Wt. (g)	Spleen Wt. / Body Wt x100 (g)
1 DAY	GG	0.133 [1] ± 0.008	0.371 ± 0.029	0.149 ± 0.014	0.392 ± 0.045
	GI	.0148 ± 0.009	0.396 ± 0.021	0.150 ± 0.018	0.364 ± 0.030
	II	0.117 ± 0.003	0.313 ± 0.010	0.125 ± 0.006	0.322 ± 0.005
	IG	0.108 ± 0.006	0.302 ± 0.017	0.122 ± 0.005	0.327 ± 0.013
3 DAY	GG	0.168 ± 0.010	0.506 ± 0.027	0.181 ± 0.021	0.526 ± 0.060
	GI	0.172 ± 0.017	0.536 ± 0.058	0.170 ± 0..041	0.476 ± 0.128
	II	0.140 ± 0.008	0.443 0.020	0.123 ± 0.012	0.353 ± 0.031
	IG	0.152 ± 0.006	0.442 ± 0.014	0.142 ± 0.007	0.432 ± 0.021
7 Day	GG	0.260 ± 0.019	0.711 ± 0.50	0.142 ± 0.014	0.385 ± 0.044
	GI	0.186 ± 0.023	0.540 ± 0.054	0.146 ± 0.020	0.344 ± 0.043
	II	0.170 ± 0.009	0.483 ± 0.041	0.108 ± 0.006	0.292 ± 0.014
	IG	0.238 ± 0.015	0.713 ± 0.062	0.183 ± 0.020	0.514 ± 0.047

1 Mean ± sem

Figure 7. *Relative Spleen Weights of Tumor Cell-Injected Mice From the 4 Experimental Groups in the First Wk Post Injection.* Points represent mean ± SEM. Mice from the 4 experimental groups (as described in figure 2) were injected with 2 x 10^6 SC115 cells, terminated 1, 3 or 7 d later. At 1 d post injection, mice of the GI group had significantly larger relative spleen weights (spleen weight/body weight) than mice of the IG group ($p<0.05$). At 3 d, there were no significant differences in relative spleen weights among groups, whereas by 7 d, mice of the IG group had significantly larger relative spleen weights than did mice of the II group ($p<0.05$). Points represent the mean of "n" mice ± sem. (n's: 1 d: GG(9); GI(6); II(6); IG(11); 3 d: GG(9); GI(5); II(6); IG(15); 7 d: GG(7); GI(7); II(6); IG(13)).

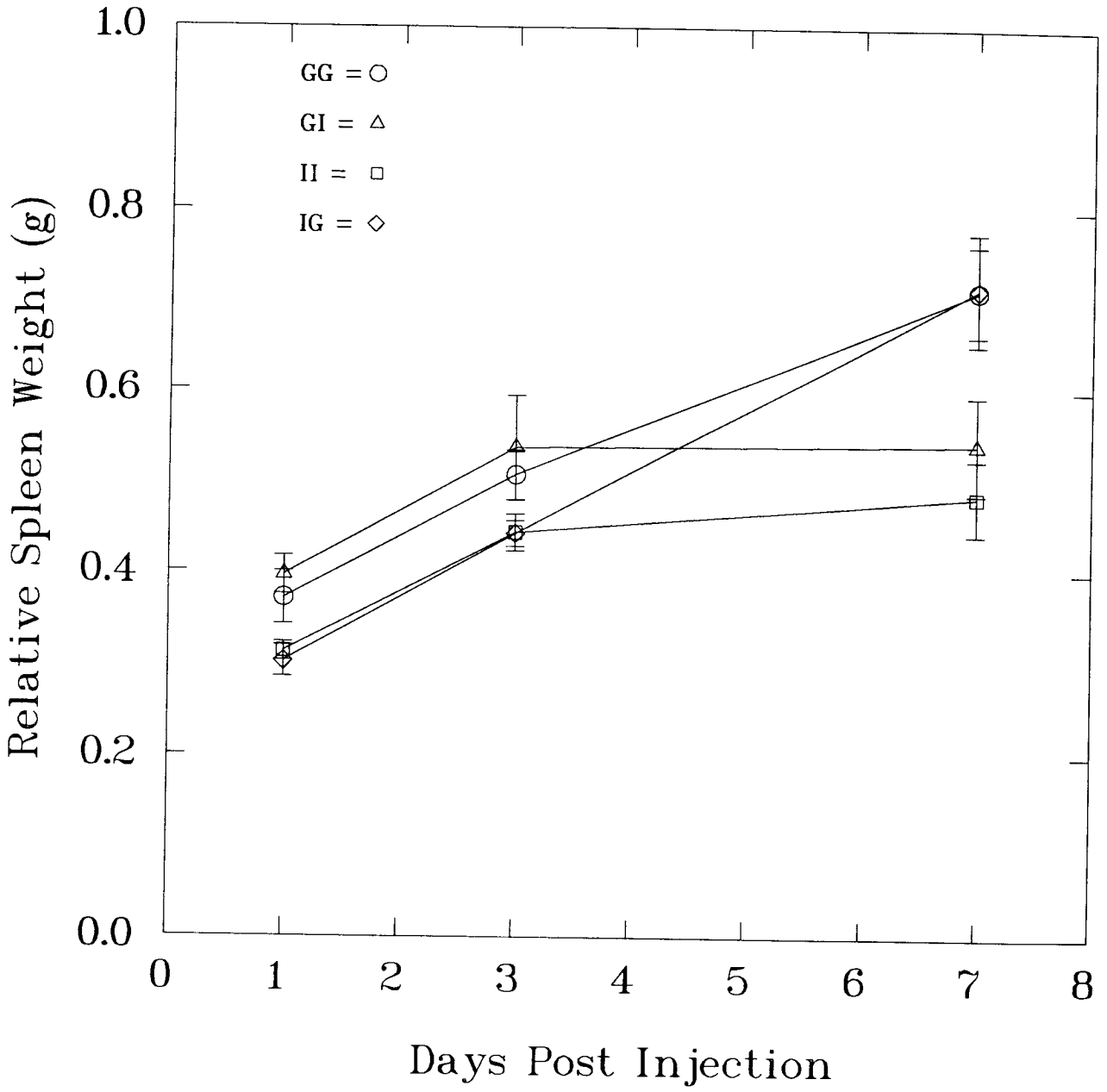
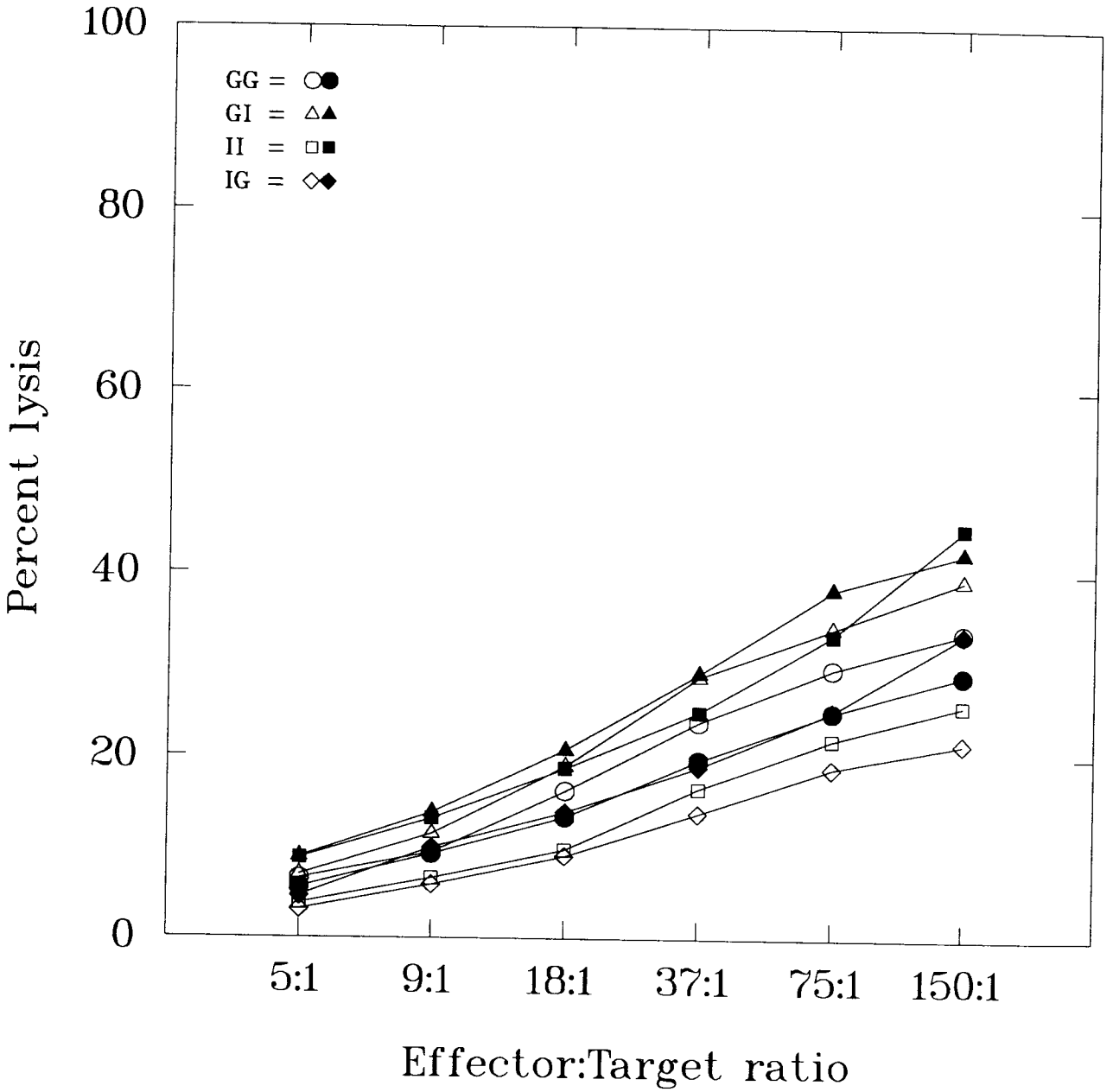

1.0
0.8
0.6
0.4
0.2
0.0
Relative Spleen Weight (g)
GG = ○
GI = △
II = □
IG = ◇
0
1
2
3
4
5
6
7
8
Days Post Injection

Figure 8. *Lytic Activity of Splenic Natural Killer (NK) Cells 1 d Post Injection.* Points represent mean ± SEM. Mice from the 4 experimental groups (as described in figure 2) were either injected with $2x10^6$ SC115 cells (open symbols) or vehicle (solid symbols). Twenty-four hours later, the lytic activity of splenic NK cells was measured. Within the tumor injected condition, GI and GG > IG and II, p's<0.05. Within the vehicle injected condition, GI = II > GG = IG, p's<0.05. (n's: GG-T(7), GG-V(11); GI-T(7), GI-V(9); II-T(6), II-V(7); IG-T(13), IG-V(12)). Spontaneous release was usually less than 20% of the total release and always less than 30%.


100
80
60
40
20
0
Percent lysis
GG = ○●
GI = △▲
II = □■
IG = ◇◆
5:1
9:1
18:1
37:1
75:1
150:1
Effector:Target ratio

than GI animals ($p<0.05$). Further, data on NK cell activity at 1 d were compared with the data previously obtained at 3 d and 7 d post injection. Collapsed across the group condition, vehicle-injected mice at 1 d post injection and group formation did not have significantly different levels of splenic NK cell activity than their vehicle-injected counterparts at 3 d and 7 d ($F(2,96) = 0.211$, $P>0.8$). In contrast, tumor cell-injected mice have significantly lower levels of splenic NK cell activity than did tumor cell-injected mice at 3 d and at 7 d ($F(2,95) = 322.558$, $P<0.001$).

Anti-asialo GM1 Treatment: When spleen cells from mice injected with tumor cells 3 d prior to termination were treated with anti-Asialo GM_1 and complement prior to the NK cell assay, there was a greater than 80 % decrease in NK cell activity in all cases compared to spleen cells from the same animals that were not treated with anti-Asialo GM_1 and complement (data not shown).

DISCUSSION

This is the first study to demonstrate that the SC115 tumor is capable of stimulating splenic NK cell activity. Stimulation of NK cell activity followed a definite time course. Activity was suppressed 1 d post tumor cell-injection, was maximally stimulated at 3 d and had begun to decline by 7 d. Anti-Asialo GM1 treatment of stimulated spleen cells resulted in a greater than 80 % decrease in activity. This indicated that the main effector cell in this assay was the NK cell, although it is possible that allospecific cytotoxic T lymphocytes were involved to a small degree.

Inducers of NK cell activity such as interferon maximally stimulate NK cell activity 18 -24 h following their injection (Ortaldo *et al.*, 1989, Talmadge *et al.*, 1985). In the present study, we found that maximal stimulation of NK cell activity occurred at 3 d post tumor cell-injection. This delay in NK cell stimulation would be expected since the immune system must first recognize the tumor and respond with the production of lymphokines before NK cell stimulation can occur.

This phenomenon has been demonstrated in other forms of immune stimulation. For example, it has been shown that augmentation of NK cell activity is maximal 3 d following an injection of bacterial cells (Klein and Kearns, 1989, Savary and Lotzova, 1987). The decline in NK cell activity seen in tumor cell-injected animals at one week may be due to tumor mediated-suppression of NK cell activity. The suppression of NK cells by tumors is a well documented phenomenon (Fulton, 1987, Savary and Lotzova, 1987, Wei and Heppner, 1987).

We have previously shown (Emerman and Siemiatkowski, 1984a) that SC115 tumor cells injected into male mice raised under our standard laboratory conditions (groups of 3, GG) produce a palpable tumor within 6-8 d, which grows to a mass of 2-3 g in approximately 3 wk. Moreover, growth rate of the SC115 tumor may be increased or decreased by housing mice under different conditions (Weinberg and Emerman, 1989). Tumor growth rate is increased in GI and II mice and decreased in IG mice compared to that seen in mice in the standard GG group. In the present study, we demonstrate an effect of differential housing on NK cell activity in mice in the 4 experimental groups. At 1 d post-injection, GI animals generally had the greatest NK cell activity whereas IG animals had the lowest NK cell activity in both tumor and vehicle-injected conditions. At 3 d, when NK cell activity was markedly stimulated in tumor cell-injected mice, GI animals in the tumor injected condition again had the greatest NK cell activity, whereas in the vehicle-injected condition, the groups did not differ from each other. Finally, at 7 d, although NK cell activity was still stimulated to some extent in tumor cell-injected mice, mice in the 4 experimental groups did not differ from each other.

We hypothesized that GI and II animals, which have the largest tumors, would have decreased NK cell activity, and that IG animals, which have the smallest tumors, would have increased NK cell activity. Surprisingly, we found that at 3 d post injection, when NK cell stimulation was the greatest, GI animals had greater NK cell activity than IG animals. Further GI and II animals did not have similar NK cell activity at this time.

One explanation for these data is that NK cell activity in the spleen may not accurately reflect the activity of NK cells at the tumor site. For example, experimental findings demonstrate that the recruitment of lymphocytes, including NK cells, from the circulation (Greenberg *et al.*, 1986, Migliori *et al.*, 1987, Zangemeister-Wittke *et al.*, 1989), and the ability of NK cells to infiltrate solid tumor targets (Jaaskelainen *et al.*, 1989, Wei and Heppner, 1987) may be critical factors in regulating tumor growth. Thus, it is possible that NK cells of mice in the IG group are better able to remain localized at the tumor site than are NK cells from mice of the GI group. This could result in mice of the IG group having higher levels of tumor-infiltrating NK cells and lower levels of splenic NK cells than mice in the GI group.

A second possibility is that NK cell activity may not be a significant regulator of SC115 tumor growth. This view is supported by the findings of Kitamura (1980), who demonstrated that the original SC115 tumor could grow in female nude mice (who lack T lymphocytes but have high levels of NK cell activity) but not in female DD/S mice. The authors concluded that T lymphocytes are an important mediator of SC115 cell growth. However, the class 1 subline of the SC115 tumor used in this experiment differs from the original SC115 cell line in that tumors do grow in female DD/S mice. It is possible that this change could be the result of a modification of the immunogenicity of the SC115 tumor, such as down regulation of class I MHC expression. Decreased expression of the class I MHC molecules by tumor cells has been demonstrated to decrease the ability of CTL to lyse some tumors while increasing the susceptibility of the cells to NK cell-mediated lysis (Ljunggren and Karre, 1985).

Finally, a third possibility is that NK cells may positively modulate the growth of the SC115 tumor. It is possible that the growth of the SC115 tumor is stimulated by hormones or cytokines released by the activated NK cells at the site of the tumor. This possibility is supported by a study which demonstrated that increased NK cell activity in preneoplastic alveolar nodules in

the breast of female mice stimulated the development of cancerous outgrowths from the nodules (Wei and Heppner, 1989). The difference in NK cell activity between the II and GI groups, both of which develop large tumors, may be due to the factors discussed above or could indicate that different mechanisms may be regulating tumor growth in these two groups of mice.

Previously, it was demonstrated that at 3 wk post injection, tumor cell-injected mice had significantly enlarged spleens compared with their vehicle-injected counterparts (Weinberg and Emerman, 1989). However, no significant differences in spleen weights among mice in the 4 experimental groups were observed at that time. The present study measured spleen weights at 1, 3 and 7 d following injection. Overall, spleen weight was lowest in both tumor cell- and vehicle-injected animals at 1 d post injection. At 3 d post injection, there was a significant increase in spleen weight in both tumor cell- and vehicle-injected animals. The spleen weights of tumor cell-injected animals continued to increase and were significantly greater at 7 d than at both 1 and 3 d. In the vehicle-injected condition on the other hand, spleen weights at 7 d were intermediate to those observed at 1 and 3 d. These results suggest that several different processes may be affecting spleen weight. First, the finding that both tumor cell- and vehicle-injected mice exhibit enlarged spleens at 3 d post injection and group formation suggests that a non-specific process is operating at this time. It is possible that exposure to the acute daily novelty stress regimen or the stress/trauma associated with the injection process may be responsible for the increase in spleen weights at 3 d. At 7 d post injection and group formation, spleen weights continue to increase in tumor cell-injected mice, but not in vehicle-injected mice. This suggests that the presence of a tumor may induce an increase in spleen weights which becomes apparent at this time. These data support the previous finding that the presence of a tumor increases spleen weights in mice at 3 wk post injection (Weinberg and Emerman, 1984).

The observed increase in spleen weights of tumor cell-injected mice at 7 d does not correlate with the changes in NK cell activity in these mice as splenic NK cell activity is declining

while spleen weights are increasing at this time. It is possible that the increased spleen weights at 7 d in tumor cell-injected mice reflects an increase in the number of cytotoxic T lymphocytes. Alternatively, the increase in spleen weights could reflect a tumor-induced increase in the number of erythoblasts in the spleen as, in mice, the spleen is capable of erythyropoiesis (Milas and Scott, 1978). An increase in erythropoiesis could correlate with the gradual suppression of splenic NK cell activity as erythroblasts have been shown to exert a suppressive effect on NK cell activity (Savary and Lotzova, 1987). Histological studies of spleen cell composition and perhaps a FACS (flourescence activated cell sorter) analysis of the relative composition of cell types in spleens of tumor cell- and vehicle-injected animals would distinguish between these possibilities.

In summary, this study demonstrates that the SC115 tumor is capable of stimulating NK cell activity. Further, differences in social housing condition alter the degree of NK cell activation produced by tumor cell-injection. However, it is not yet clear if modulation of NK cell activity has an effect on the growth rate of this tumor. An increase in the spleen weight in tumor cell-injected mice, but not in vehicle-injected mice was observed at 7 d post injection. It is not clear at this time if this increase in spleen weights reflects an increase in lymphoid cell numbers or an increase in erythropoeisis in these spleens. We conclude that either NK cell activity in the spleen does not reflect the activity at the tumor site or NK cells are not an important mediator of the differential tumor growth observed in this model, or that NK cells may positively modulate the growth rate of SC115 cells.

B. Natural Killer Cell Activity At The Tumor Site.

Introduction

In the previous study, it was demonstrated that the presence of the SC115 tumor caused a significant stimulation of splenic NK cell activity. However, the relevance of this finding to the modulation of the differential tumor growth rates observed in mice in the experimental housing groups of our model is unclear. Thus, the present study was undertaken to investigate the hypothesis that NK cell activity of tumor-infiltrating lymphocytes differs among animals of the experimental housing groups and that these differences are involved in mediating the differential tumor growth rates observed in this model.

The previous study demonstrated that tumor-induced stimulation of NK cell activity in the spleen was greatest at 3 d post tumor cell-injection and that, as early as 7 d post tumor cell-injection, the tumor-induced stimulation of NK cell activity had declined significantly from that seen at 3 d. These data suggested that the optimum time to investigate NK cell activity of tumor-infiltrating lymphocytes is in the first wk post injection. This presents a problem for studying tumor-infiltrating lymphocyte activity as, at 7 d, the tumor is barely palpable. It would be impossible to collect sufficient amounts of tumor tissue to isolate the required numbers of lymphocytes to conduct the study. To circumvent this problem, the sponge allograph model developed by Roberts and Hayry (1976) was adapted for use in the SC115 model. The sponge allograft model involves implanting a small polyurethane sponge in the peritoneal cavity of a mouse. After several days, the sponge becomes infiltrated with host connective tissue and immune cells. The sponge is then removed and implanted subcutaneously on the dorsal surface of an allogeneic mouse. The sponge can be removed at different time points and the immune cells infiltrating this allogeneic graft can be isolated by gently squeezing the sponge. Using this

procedure, Hoffman (1988) demonstrated that NK cells infiltrate the sponge matrix if the matrix contains allogeneic cells but not if it contains syngeneic cells. Roberts and Hayry (1976) also demonstrated that tumor cells are capable of growing in the sponge matrix of subcutaneously implanted sponges. Thus, the sponge is capable of supporting tumor growth and allowing the infiltration of immune cells, yet the sponge itself is not immunogenic. The present study was designed to investigate the NK cell activity of lymphocytes infiltrating sponges injected either with tumor cells or with vehicle alone. Mice from only GI (who develop the largest tumors) and IG (who develop the smallest tumors) were tested in this study.

Materials and Methods

Animals: One hundred fifty-four young adult DD/S mice (2-4 months of age) were used in this study. In experiments designed to develop and test the sponge model, mice were housed individually for the course of the experiments and were not exposed to the acute daily novelty stress regimen. In the experiment to investigate the effects of differential housing on NK cell activity of lymphocytes infiltrating tumor cell- and vehicle-injected sponges, mice from the two experimental housing groups (GI and IG) were reared and housed as described previously in the General Methods, were used and these mice were subjected to acute daily novelty stress.

Tumor Cells:

SC115 Cells: All SC115 cells used in this study were taken from frozen stocks as previously described in General Methods. For each experiment in the time course study of NK cell activity in mice from the GI and IG groups, 2 additional mice were injected with tumor cells s.c. as is typically done for tumor propagation, and monitored for 21 d to ensure that the cells were viable. All tumors grew as expected in these control mice.

Yac 1 Cells: These cells were used in the NK cell assays as previously described.

Polyurethane Sponges:

Preparation: Sponges (1 x 1 x 1.5 cm) were cut from a polyurethane foam block and sterilized as previously described (Hoffman *et al.*, 1988). Briefly, sponges were washed 3 times with distilled water (1 l/wash), taking care to remove the air from the sponges. The sponges were then boiled 3 times in distilled water (1 l/boiling), soaked for 30 min in approximately 100 ml of acetone, soaked for 30 min in 95% EtOH (500 ml), washed 3 times with distilled water (1 l/wash) and finally boiled 3 times in distilled water (1 l/boiling). The sponges were then carefully sealed in aluminum foil so as not to compress the sponge and autoclaved.

Implantation: The sponges were surgically implanted into mice anesthetized with halothane (Ayerst Laboratories Inc., Montreal, Que) - oxygen mixture (Medigas Inc., Vancouver, B.C.). Briefly, a mouse's back was swabbed well with 70% ethanol to sterilize it. A 1 cm long cut was made in the skin with sterile scissors and a pocket was made under the skin by blunt dissection using the scissors. The sponge was wet in sterile phosphate buffered saline, compressed using sterile forceps and inserted into the subcutaneous pocket. The wound was closed with three sutures (Ethicon Inc., Somerville, NJ). The animals were allowed 5 d to recover and for the sponge to vascularize before the next experimental procedure was initiated.

Tumor/Vehicle Injection: An animal was lightly anesthetized with ether for injection. The sponge was gently compressed and the needle inserted into the sponge. As the 0.1 ml of fluid (tumor cell suspension or vehicle) was injected, the sponge was gently released.

Histology of SC115 Tumor Growth in Polyurethane Sponge Matrices: Sponges were implanted into 2 male and 2 female DD/S mice. After 5 d, sponges in the male mice were injected with SC115 cells (2 x 10^6 cells); mice were terminated at 19 d post injection. Four additional male

mice were injected with tumor cells in the usual fashion as controls for tumor growth. The female mice were not injected and were terminated at 21 d post sponge-implantation. The sponges were removed, cut in half and fixed in 10% formalin for 1 wk. The sponges were then paraffin-embedded, sectioned and stained with hematoxylin and eosin for microscopy.

Time Course Study of White Blood Cells Infiltrating Polyurethane Sponges: Twelve male mice (raised in groups of 3) were implanted with sponges and individually housed. After 5 d, sponges were injected with either tumor cells (n=6) or vehicle (n=6). Mice were terminated at 1, 3 or 7 d post injection and the sponges removed. Sponge-infiltrating lymphocytes were isolated by cutting a sponge in half and gently squeezing the contents of the sponge into 3 changes of RPMI (approximately 35 mls in total) in a 60 mm petri dish. The lymphocytes were centrifuged (600 x g, 2 min), the supernatant decanted and the cells resuspended in 1 ml RMPI. Several 100 µl aliquots were spun onto glass slides using a cytospin apparatus (Shanndon) at 120 x g for 1 min. The slides were stained with a Wright's stain and differential counts were made by counting 200 cells from each of 2 slides for each animal. The relative proportions of the different white blood cells in the sponge infiltrates of each animal were thus determined.

Pilot Study of the Time Course of NK Cell Activity in Sponge-Infiltrating Lymphocytes: In an initial experiment, 4 male mice were implanted with sponges and 5 d later were injected with either tumor cells (n=2) or vehicle (n=2). All mice were terminated 3 d post injection and sponge-infiltrating cells isolated as previously described. This time point was chosen for termination as 3 d was when maximal stimulation of splenic NK cell activity was observed in a previous study. The cells isolated from the sponges were used in the NK cell assay previously described with the exception that, due to the low numbers of cells isolated from each sponge, effector to target cell ratios of 15:1 and lower were used in this study. The cells from each mouse were processed and used in the NK cell assay separately.

In a second experiment, 12 male mice were implanted with sponges and the sponges injected with SC115 tumor cells 5 d later. Due to the low cell yields observed in the first study, sponges from 2 mice were pooled for each data point. Mice were terminated at 1, 3 and 7 d post injection (4 mice/day) and the NK cell activity of their tumor-infiltrating cells was determined.

In a third experiment, 12 mice were implanted with sponges and 5 d later sponges were injected with either SC115 tumor cells (n=8) or vehicle alone (n=4). Half the mice in each condition were terminated at 10 d post injection and half at 17 d post injection. The lymphocytes from the sponges of 2 mice in the same condition were pooled for each data point and NK cell activity was measured.

NK Cell Activity of Sponge-Infiltrating Lymphocytes at 3 d and 7 d in Mice from the Experimental Groups: Ninty-six male mice were implanted with sponges and 5 d later sponges were injected with either tumor cells (n=26) or vehicle (19). The mice were then rehoused as either GI (T, n=10, V, n=7) or IG (T, n=16, V, n=12) groups and subjected to the acute daily novelty stress regimen as described previously in General Methods. The mice were terminated at 3 d or 7 d post injection and the sponges were removed. Sponge-infiltrating lymphocytes from 2 mice of the same housing group were combined for a single data point in assays of NK cell activity.

Results

Histology of SC115 Tumor Growth in Polyurethane Sponge Matrices.

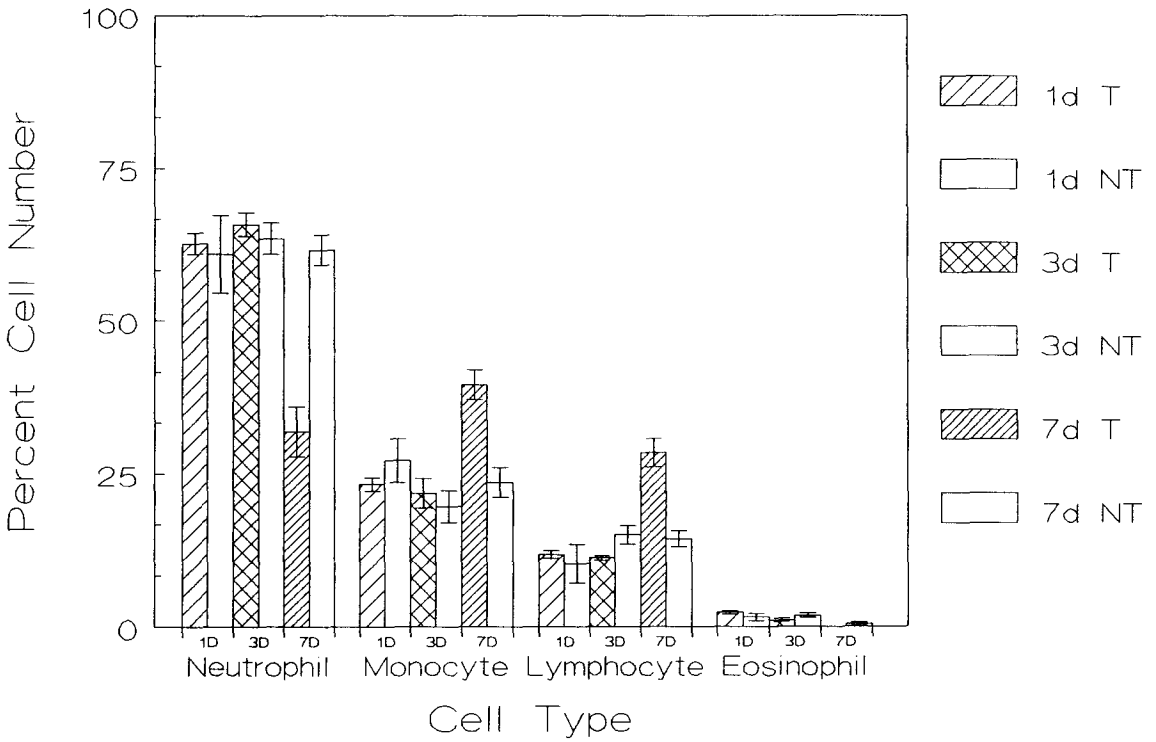
Both tumor-injected and noninjected sponges became invested with a thick connective tissue capsule, not unlike that observed surrounding the SC115 tumor, and were firmly attached

to the overlying skin. Microscopic examination of the sponges revealed that both tumor cell-injected and noninjected sponges contained many small blood vessels. In the tumor-injected sponges, colonies of tumor cells were observed growing within the matrix of the sponge. Such colonies were not observed in the noninjected control sponges. Although tumor growth was observed in the sponges, it appeared to be delayed compared to the growth of tumors in animals injected at the same time by the usual subcutaneous procedure. At 19 d, tumors growing in the sponges were small foci of cells whereas, in the s.c. injected control mice, tumors were all larger than 1 g. In addition, noninjected sponges exhibited a mild granulocytic invasion which was not observed in the tumor cell-injected sponges.

Time Course Study of White Blood Cells Infiltrating Polyurethane Sponges.

Analysis of cytospin slides of cells harvested from sponges at 1, 3 and 7 d revealed that on all 3 test days, both tumor cell- and vehicle-injected sponges contained notable numbers of neutrophils, monocytes, lymphocytes and eosinophils (Figure 9). However, there were marked differences across days in the relative percentages of these cell types in tumor cell-injected sponges. The relative proportions of different white blood cells were similar in vehicle-injected sponges at all time points. They consisted of approximately 60 % neutrophils, 25 % monocyte, 15 % lymphocytes and a small percentage of eosinophils . A similar distribution of white blood cells was observed in the infiltrate of tumor cell-injected sponges at 1 and 3 d post injection . Importantly, at 7 d post-injection, there was a marked change in the relative proportions of different white blood cells infiltrating tumor cell-injected sponges . There was a decline in the relative numbers of neutrophils in the infiltrate and a corresponding increase in the relative numbers of lymphocytes and monocytes compared with that seen at 1 and 3 d. As well, no eosinophils were observed in the cell infiltrates of tumor cell-injected sponges at 7 d, whereas they were present in low numbers in all other groups. Although this was a pilot study and the group size was small (2 mice/group, 2 slides/animal), T-tests were conducted on this data. Analysis

Figure 9. *Time Course Study of White Blood Cells Infiltrating Polyurethane Sponges.* Mice were implanted s.c. with polyurethane sponges (1 x 1 x 1.5 cm); 5 d later sponges were injected with either tumor cells (T)(2×10^6) or vehicle (V), and mice terminated at 1, 3 and 7 d post injection. White blood cells infiltrating the sponges were isolated, stained with Wright's stain and counted. In infiltrates from vehicle-injected sponges, no differences in the relative numbers of white blood cell types was observed on 1, 3 and 7 d post injection. In infiltrates from tumor cell-injected sponges, the relative numbers of white blood cell types on d 1 and 3 were similar to those in vehicle-injected sponges. At 7 d post-injection, there was a marked decline in the relative numbers of neutrophils in the infiltrate and a corresponding increase in the relative numbers of lymphocytes and monocytes compared with that at 1 d and 3 d ($p's<0.01$). No eosinophils were observed in the infiltrates of tumor cell-injected sponges at 7 d, whereas they were present in low numbers in all other sponges. (n = 2 mice/condition, 2 x 200 cells counted/mouse).

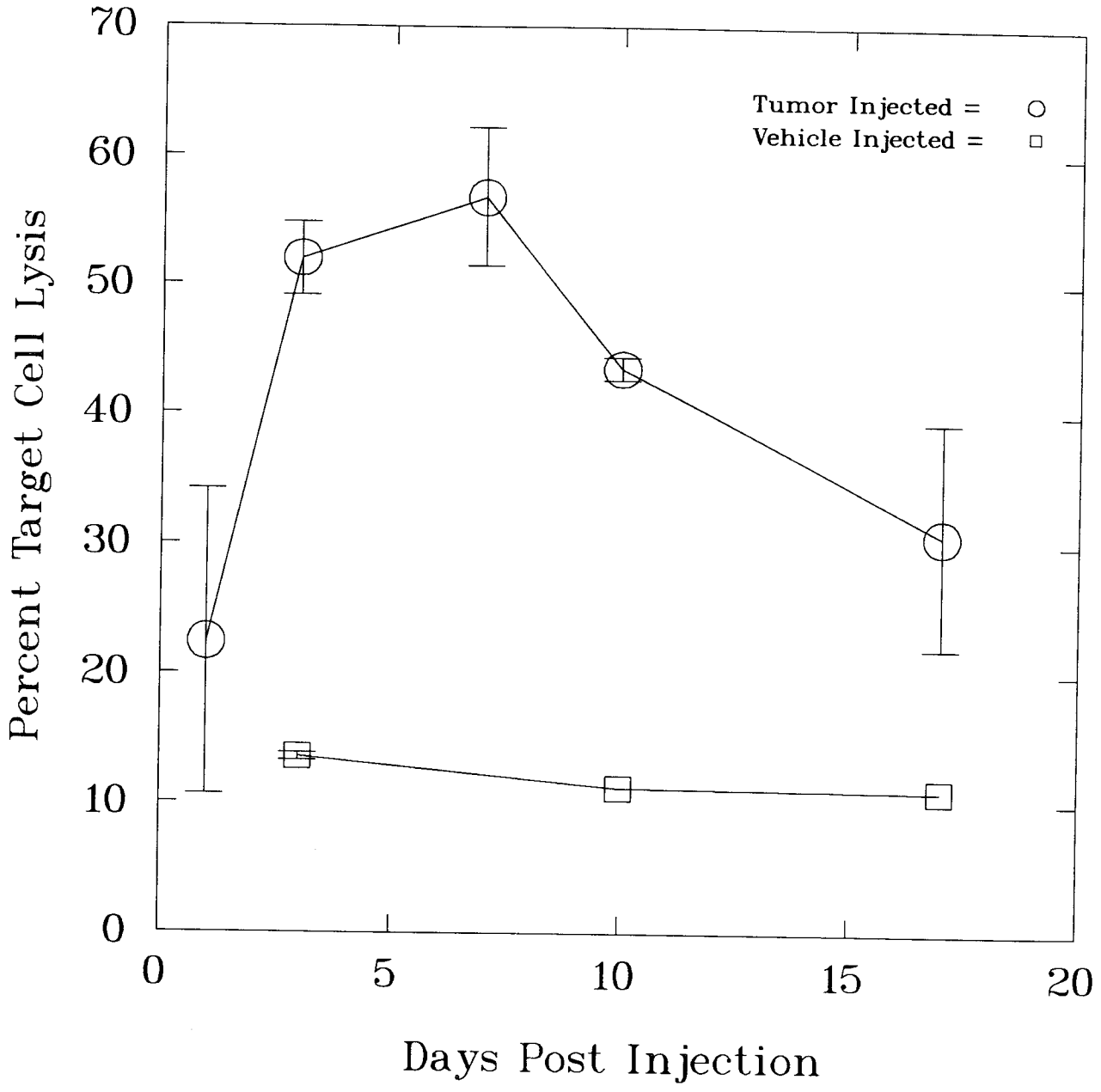

100
75
50
25
0
Percent Cell Number
1D
3D
7D
Neutrophil
Monocyte
Lymphocyte
Eosinophil
Cell Type
1d T
1d NT
3d T
3d NT
7d T
7d NT

revealed that the white blood cells infiltrating tumor cell-injected sponges at 7 d post injection differed significantly from both that of both tumor cell-injected sponges at 1 and 3 d post injection and from vehicle-injected sponges at all 3 d measured ($p<0.01$). It is understood that, due to the small group size, the power of this analysis is low. However, the results clearly support the conclusion that there is a significant change in the composition of the sponge-infiltrating white blood cells in tumor cell-injected sponges at 7 d post injection relative to those of tumor cell-injected sponges at 1 and 3 d and of vehicle-injected sponges at all 3 days.

Pilot Study of the Time Course of NK Cell Activity in Sponge-Infiltrating Lymphocytes.

There were marked differences in levels of NK cell activity of sponge-infiltrating lymphocytes of tumor cell- and vehicle-injected mice (Figure 10). However, due to the small numbers of mice per group in this pilot study, no statistical analysis of the data was possible. Consistent with NK cell activity observed in the spleen, NK cell activity of sponge-infiltrating lymphocytes did not differ in tumor cell- and vehicle-injected mice at 1 d post injection. However, by 3 d post injection, lymphocytes infiltrating tumor cell-injected sponges showed an increase in NK cell activity compared with that of vehicle-injected mice. This increase was maintained through 7 d post tumor cell-injection. Although NK cell activity began to decline after 7 d post tumor cell-injection, it was still elevated in lymphocytes infiltrating tumor cell-injected sponges compared with that in their vehicle-injected counterparts at 10 d and 17 d post injection. Interestingly, the tumor-induced stimulation of NK cell activity at 3 d post injection observed in sponge-infiltrating lymphocytes was similar to that observed previously in splenic lymphocytes.

Figure 10. *Pilot Study of the Time Course of NK Cell Activity in Sponge-Infiltrating Lymphocytes.* Lymphocytes infiltrating tumor cell- and vehicle-injected sponges were isolated as described in figure 9, mice were terminated at 1, 3, 7, 10 and 17 d post injection. NK cell activity of vehicle-injected mice did not differ across days. At 1 d post injection, NK cell activity (effector to taget cell (E:T) ratio of 15 to 1) of lymphocytes infiltrating tumor cell-injected sponges did not differ from that of vehicle-injected sponges. By 3 d, lymphocytes infiltrating tumor cell-injected sponges showed an increase in NK cell activity compared with that of vehicle-injected sponges and, this increase was maintained through 7 d. Although NK cell activity began to decline after 7 d, it was still elevated in lymphocytes infiltrating tumor cell-injected sponges compared with that in their vehicle-injected counterparts at 10 d and 17 d post injection. (N's: 1 d, T(2); 3 d, T(4), V(2); 7 d, T(2); 10 d, T(2), V(1); 17 d, T(2), V(1)).


Tumor Injected =
Vehicle Injected =
Percent Target Cell Lysis
70
60
50
40
30
20
10
0
0
5
10
15
20
Days Post Injection

NK Cell Activity in Sponge-Infiltrating Lymphocytes at 3 d and 7 d in Mice from the Experimental Groups.

This experiment investigated NK cell activity of sponge-infiltrating lymphocytes in mice from the GI group (who develop the largest tumors) and IG group (who develop the smallest tumors) following either tumor cell- or vehicle-injection. Based on the findings of the previous pilot study, NK cell activity of sponge-infiltrating lymphocytes was assayed at 3 d and 7 d post injection and group formation when NK cell stimulation in tumor cell-injected mice was likely to be the greatest. At 3 d post-injection (Figure 11), ANOVAs revealed that there was a significant effect of Group ($F(1,20) = 7.548$, $P<0.02$). Overall, NK cell activity in sponge-infiltrating lymphocytes was significantly greater in mice of the GI group than in mice of the IG group ($p<0.05$). However, it is clear from Figure 11 that the overall group effect was due primarily to the fact that Tumor cell-injected mice of the GI group showed greater NK cell stimulation than all other mice. However, the main effect of Tumor was not significant. By 7 d post-injection (Figure 12), the effect of Tumor was significant ($F(1,17) = 29.293$, $P<0.001$) and a Group x Tumor interaction was found ($F(1,17) = 5.852$, $P<0.03$). Post-hoc analysis revealed that, overall, levels of NK cell activity in sponge-infiltrating lymphocytes were significantly greater in tumor cell-injected mice than in vehicle-injected mice at 7 d ($p<0.005$). Also, at this time, tumor cell-injected mice of the GI group had significantly greater levels of NK cell activity than did mice in all other groups ($p<0.05$). In addition, tumor cell-injected mice of the IG group had significantly greater NK cell activity than vehicle-injected mice of both the GI and IG groups ($p<0.05$).

Discussion

This study demonstrates three important findings. First, the study established a procedure for utilizing the polyurethane sponge model to investigate tumor-infiltrating lymphocytes in the SC115 mouse mammary tumor. It was demonstrated that polyurethane sponges implanted s.c. in

Figure 11. *NK Cell Activity in Sponge-Infiltrating Lymphocytes at 3 d Post Injection in Mice from the Experimental Groups*. GI and IG mice (as described in figure 1) were implanted with polyurethane sponges and injected with either tumor cells (2×10^6) or vehicle as described in figure 9. Mice were terminated at 3 d post injection and NK cell activity of lymphocytes infiltrating tumor cell- and vehicle-injected sponges was determined. Points represent mean ± SEM. Overall, NK cell activity in sponge-infiltrating lymphocytes was significantly greater in mice of the GI group than in mice of the IG group ($p<0.05$). The overall group effect appears to be due primarily to the greater NK cell stimulation in GI-T mice than in mice of all groups ($p<0.05$). (N's: GI, T(5), V(4); IG, T(8), V(7)). Spontaneous release was usually less than 15% of the total release and always less than 25%.

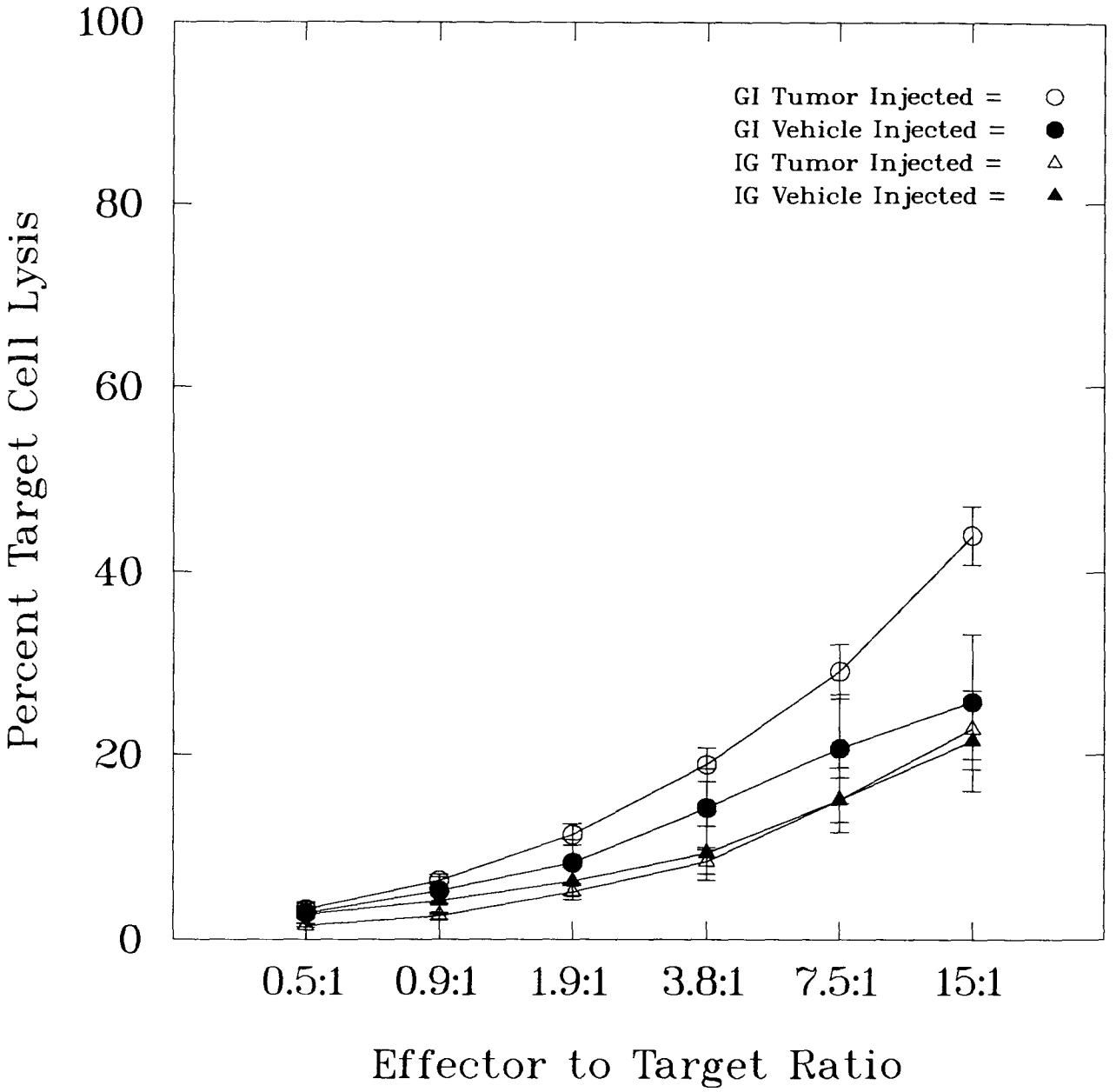
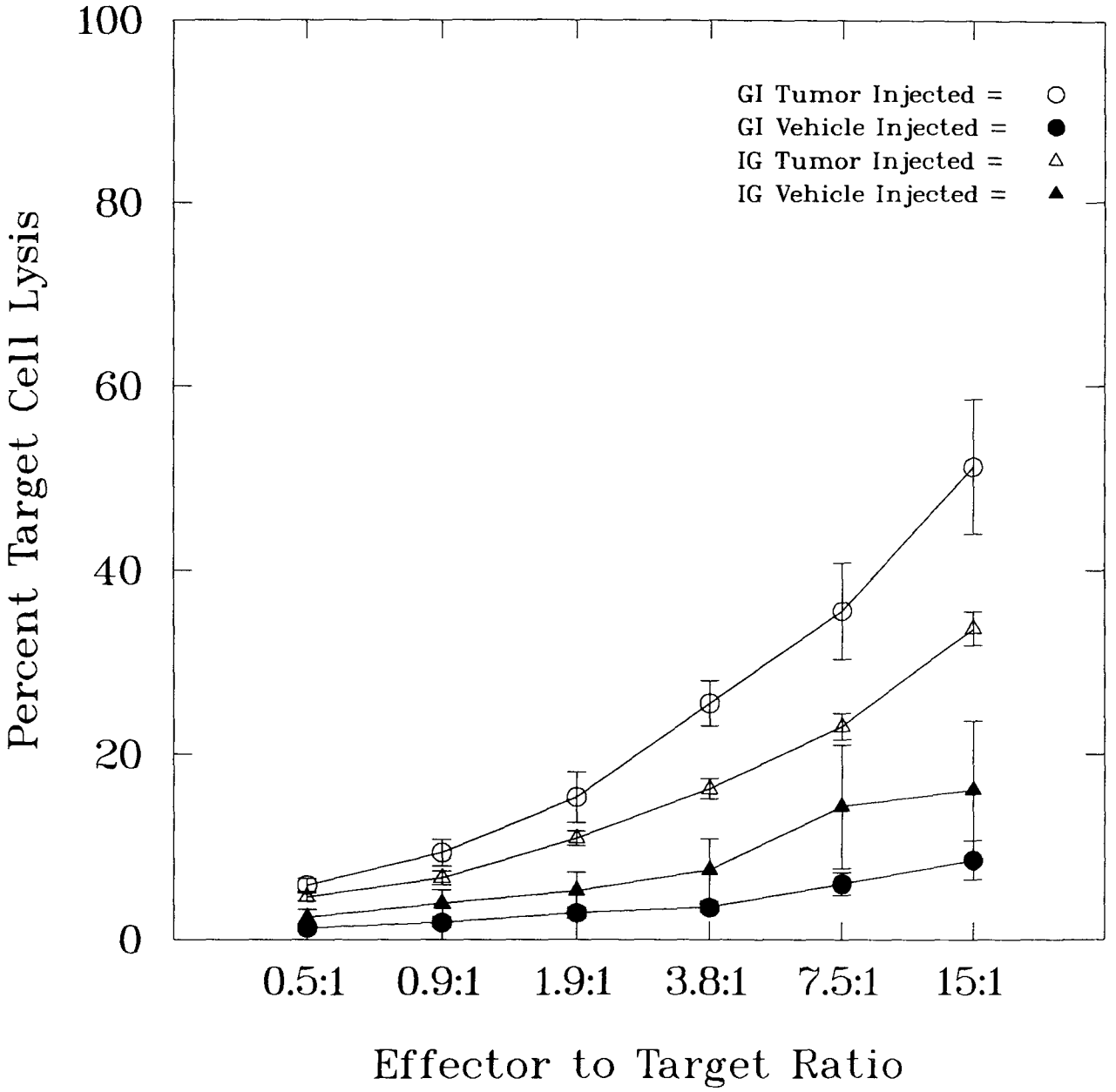

100
80
60
40
20
0
Percent Target Cell Lysis
GI Tumor Injected =
GI Vehicle Injected =
IG Tumor Injected =
IG Vehicle Injected =
0.5:1
0.9:1
1.9:1
3.8:1
7.5:1
15:1
Effector to Target Ratio

Figure 12. *NK Cell Activity in Sponge-Infiltrating Lymphocytes at 7 d Post Injection in Mice from the Experimental Groups.* GI and IG mice (as described in figure 1) were implanted with polyurethane sponges and injected with either tumor cells (2×10^6) or vehicle as described in figure 9. Mice were terminated at 7 d post injection and NK cell activity of lymphocytes infiltrating tumor cell- and vehicle-injected sponges was determined. Points represent mean ± SEM. At 7 d, NK cell activity in sponge-infiltrating lymphocytes was significantly greater in tumor cell-injected sponges than in vehicle-injected sponges ($p<0.005$). Also, at this time, tumor cell-injected mice of the GI group had significantly greater levels of NK cell activity than did mice in all other groups ($p's<0.05$). In addition, tumor cell-injected mice of the IG group had significantly greater NK cell activity than vehicle-injected mice of both the GI and IG groups ($p's<0.05$). (N's: GI, T(5), V(3); IG, T(8), V(5)). Spontaneous release was usually less than 15% of the total release and always less than 20%.


100
80
60
40
20
0
Percent Target Cell Lysis
GI Tumor Injected =
GI Vehicle Injected =
IG Tumor Injected =
IG Vehicle Injected =
0.5:1
0.9:1
1.9:1
3.8:1
7.5:1
15:1
Effector to Target Ratio

DD/S mice could support the growth of the SC115 tumor and that it was possible to isolate lymphocytes infiltrating the sponge. Secondly, we have shown that the SC115 tumor significantly stimulates the activity of NK cells at the tumor site. Finally, it was demonstrated that mice of the GI group had significantly greater levels of NK cell activity at the tumor site than did mice of the IG group.

This study utilized polyurethane sponges implanted s.c. in the mouse as a solid support matrix in which tumor cells could grow. This facilitated the isolation of immune cells infiltrating the tumor at early time points post tumor cell-injection. To our knowledge, this is the first study to utilize this model for the study of the SC115 tumor, although it has previously has been used in the study of several other tumor systems (Roberts and Hayry, 1976, Zangemeister-Wittke *et al.*, 1989). It was demonstrated that the SC115 tumor is capable of growing in the sponge matrix and that blood vessels infiltrate the sponge to nourish the developing tumor. Tumor growth in the sponge was delayed compared with the tumor growth observed in control mice injected s.c. (the standard injection protocol).

White blood cells were isolated from both tumor cell- and vehicle-injected sponges. Cells from vehicle-injected sponges demonstrated a white blood cell infiltrate indicative of a mild granulocytic response. This is similar to previous reports that the presence of the polyurethane sponge itself does not induce significant immune reactivity (Hoffman at al, 1988, Roberts and Hayry, 1976). However, in tumor cell-injected sponges, there was an increase in the relative numbers of monocytes and lymphocytes and a corresponding decrease in the number of neutrophils at 7 d post injection compared with those at 1 d and 3 d post injection as well as with all vehicle-injected sponges. This change in relative cell numbers suggests that a specific immune response might be occurring in the tumor cell-injected sponges at 7 d. A similar shift from a granulocytic infiltrate to a monocytic infiltrate has previously been reported to occur at 8-11 d post implantation of sponges impregnated with allogeneic cells (Hoffman *et al.*, 1988). The

conclusion that the SC115 tumor may be immunogenic is also supported by the finding that white blood cells from tumor cell-injected sponges at 7 d post-injection completely lacked the small but persistent component of eosinophils found in all other sponges. It has been suggested that eosinophils are stimulated by the cytokines IL-4 and IL-5, which are primarily associated with a humoral immune response, and that such a response may act to inhibit the stimulation of CTL and NK cells by T_{H1} cells (Street and Mosman, 1991). Although this finding that the SC115 tumor may be immunogenic is not conclusive, it is supported by the demonstration that prior immunopotentiation of mice by the injection of heat-killed Staphylococcus aureus virus decreases SC115 tumor growth (Nohno *et al.*, 1986) and that the presence of the SC115 tumor stimulates splenic NK cell activity (Rowse *et al.*, 1990).

Importantly, this study demonstrates that the presence of the SC115 tumor was capable of stimulating NK cell activity, not only in the spleen, but also in tumor/sponge-infiltrating lymphocytes. At 3 d post tumor cell-injection, the level of NK cell activity at the tumor site was similar to that observed in the spleen at 3 d. Interestingly, NK cell activity remained stimulated for a longer period of time at the tumor site than at the spleen. Whereas NK cell activity in the spleen had declined significantly by 7 d post tumor cell-injection, NK cell activity at the tumor site did not decrease at 7 d post tumor cell-injection and at, 17 d post injection, NK cell activity of tumor-infiltrating lymphocytes was still greater than that of lymphocytes infiltrating vehicle-injected sponges. These data are consistent with the report that in the allogeneic sponge model lymphocytes infiltrating a sponge impregnated with allogeneic peritoneal cells demonstrate a significant increase in NK cell activity at 5-11 d (Hoffman *et al.*, 1988).

There was a significant difference in the level of NK cell activity in tumor-infiltrating lymphocytes between mice in the GI and IG groups. At both 3 d and 7 d post injection, mice of the GI group, who would develop the largest tumors, had greater NK cell activity than mice of the IG group, who would develop the smallest tumors. A similar result was observed in the

spleens of tumor cell-injected mice at 3 d post injection. Thus, tumor-induced stimulation of NK cell activity is greater in mice of the GI group than in mice of the IG group both at the spleen and at the tumor site. This finding is contrary to what one would expect if NK cells are involved in decreasing the tumor growth observed in this model.

There are a number of possible explanation for this apparent contradiction. Firstly, it is possible that NK cells are not involved in mediating the differential tumor growth rates observed in this model. NK cells may be stimulated by cytokines released by another immune reaction (i.e. a CTL response to the tumor) and may not in fact be able to recognize and/or lyse the SC115 tumor cells. Alternately, NK cells may be able to recognize and lyse the SC115 tumor, but the level of lysis may not be sufficient to inhibit the growth of the tumor to any great extent. Secondly it is possible that NK cells may act to modulate SC115 tumor growth in a positive manner. This could occur if, as a result of activation, NK cells released a cytokine or hormone-like molecule (i.e. IL-2) which was capable of stimulating SC115 tumor growth. A study by Wei and Heppner (1989) supports this latter possibility. They demonstrate that the neoplastic transformation of preneoplastic hyperplastic alveolar nodule cells in the mammary glands of mice is stimulated by a factor released by activated NK cells which are attracted to the glands by the hyperplastic lesion. It has also been demonstrated that in early human breast cancer, levels of NK cell activity are higher than those found in normal control subjects (Brenner and Margolese, 1991, Pross *et al.*, 1984, Zielinski *et al.*, 1989). It is not clear if this increase in NK cell activity plays a role in inhibiting the spread of metastatic disease or if this activity is in some way facilitating the growth of these tumors. The subsequent study is designed to distinguish between the hypotheses that in DD/S mice of our model NK cells act to increase the growth rate of the SC115 tumor, or that NK cell activity does not affect the growth rate of the SC115 tumor. The effect of *in vivo* modulation of NK cell activity, both up-regulation and down-regulation, on the differential growth of the SC115 tumor as observed in this model is investigated.

C. *In Vivo* Modulation of NK Cell Activity and Its Effects on the Differential Growth of the SC115 Tumor Observed in the Model.

Introduction

Our previous studies demonstrated that the presence of the SC115 tumor markedly stimulated NK cell activity both at the spleen and at the tumor site. Further, there were significant differences in the levels of NK cell activity among mice from the different housing groups of our model. Interestingly, mice of the GI group, that develop the largest tumors, showed the greatest stimulation of NK cell activity at both sites, whereas mice of the IG group, that develop the smallest tumors, showed significantly lower levels of NK cell activity than mice of the GI group. It was concluded that although NK cell activity is clearly stimulated by the SC115 tumor, either NK cells are not involved in mediating the differential tumor growth observed in this model or NK cells may actually be facilitating the growth of the SC115 tumor in some manner. However, it has been suggested that immune functioning observed in *in vitro* studies does not necessarily reflect the situation *in vivo* due to loss of modulatory influences which are found in the *in vivo* environment (Ben-Eliyahu and Page, 1992). The present study was designed to investigate if NK cells are likely to play an important role in mediating the effects of psychosocial stressors on tumor growth rate in this model. This question was examined by directly modulating (stimulating or suppressing) *in vivo* NK cell activity of mice from the GI (largest tumors) and the IG (smallest tumors) groups and observing the effect of these manipulations on tumor growth rate.

It has been demonstrated that NK cell activity is stimulated by a variety of endogenous compounds, of which IL-2 and molecules of the IFN family are the most potent (Ben Arribia *et al.*, 1989, McGinnes *et al.*, 1988, Robertson and Ritz, 1992a). NK cells have been shown to possess specific receptors for IL-2 (Ben Arribia *et al.*, 1989, Robertson and Ritz, 1990) and for IFN (Welsh, 1984). A number of exogenous molecules also stimulate NK cell activity.

Polyinosinic-polycytidylic acid (poly I:C), a double stranded synthetic DNA polymer has been shown to be a potent stimulator of NK cell activity (Talmadge *et al.*, 1985a). An important physiological effect of poly I:C is that it stimulates the production of IFN (Fresa *et al.*, 1985, Korngold and Doherty, 1985), and the stimulation of NK cells by the injection of poly I:C is blocked by the injection of anti-IFN antibodies (Fresa *et al.*, 1985). Interestingly, although repeated injections of IL-2 or IFN induce hypo-responsiveness of splenic NK cells to further stimulation by these compounds, multiple injections of poly I:C do not induce hypo-responsiveness to further stimulation by poly I:C (Talmadge *et al.*, 1985a, Talmadge *et al.*, 1985b). Thus, poly I:C is a useful compound for inducing long lasting alterations in *in vivo* NK cell activity. It should be noted, however, that injections of poly I:C may have other effects on the animal in addition to stimulating NK cell activity (Talmadge *et al.*, 1985a). The induction of IFN in the animal (by poly I:C) can have many effects on the antitumor immunity of the animal, including stimulation of tumoricidal macrophages (Schwamberger *et al.*, 1991) and CTL (Chen *et al.*, 1986, Knop, 1990) and an increase in the expression of MHC class I antigens on the tumor cells (which could decrease NK cell lysis of the tumor, Dawson *et al.*, 1992). Thus, the *in vivo* effects of poly I:C on NK cell mediated immunity must be interpreted with caution.

Elimination or suppression of *in vivo* NK cell activity is generally accomplished by the injection into the animal of antibodies specific for NK cells. Several such antibodies have been developed. One of the earliest antibodies shown to react specifically with NK cells is the anti-asialo GM_1 antibody ($ASGM_1$). This polyclonal antibody is produced in rabbits by immunization with the ganglioside asialo GM_1 isolated from bovine brain (Kassai *et al.*, 1980), and has been demonstrated to recognize NK cells preferentially over T and B lymphocytes (Kasai *et al.*, 1980, Habu *et al.*, 1981). $ASGM_1$ is known to recognize NK cells in many strains of mice and rats (Habu *et al.*, 1981, Mason *et al.*, 1990, Pelletier *et al.*, 1987). More recently, it has been demonstrated that several other cell types also express the asialo GM_1 epitope, including thymocytes, macrophages and, at a lower density, CTL (Suttles *et al.*, 1987). Data generally

indicate that *in vitro* and *in vivo* treatment with low doses of $ASGM_1$ result in the selective depletion of NK cells over CTL, due to the higher expression of the asialo GM_1 epitope of NK cells than of CTL (Habu *et al.*, 1981). However, several studies have suggested that, under certain conditions, CTL are quite sensitive to lysis by $ASGM_1$ (Doherty and Allen, 1987, Stitz *et al.*, 1986, Stout *et al.*, 1987). Thus, caution should be exercised in attributing alteration in immune functioning induced by anti-asialo GM1 antibody solely to NK cells. A second antibody that has been produced against the NK cell is the monoclonal antibody NK 1.1 which was generated by immunizing (Balb/c x C3H)F_1 mice with NK cells from CE mice (Koo and Peppard, 1984, Seaman *et al.*, 1987). It has been demonstrated that in C57BL/6 mice, the NK1.1 antibody is expressed on most functional NK cells as defined by morphological and functional analysis (Lemieux *et al.*, 1991, Seaman *et al.*, 1987). However, in many common strains of mice other than the C57BL, the NK1.1 antigen is reported not to be present (Burton *et al.*, 1991, Lemieux *et al.*, 1991). Thus, a number of studies have continued to use the $ASGM_1$ antibody due to the lack of a more specific antibody which will universally recognize NK cells in different strains of mice. The $ASGM_1$ antibody was used in the present study as our preliminary experiments demonstrated that the anti-NK1.1 antibody did not recognize NK cells from DD/S mice.

The present study was designed to test the hypothesis that *in vivo* NK cell activity plays a role in modulating the differential tumor growth rates observed in our model. $ASGM_1$ and poly I:C were administered to mice from GI (largest tumors) and IG (smallest tumors) groups to increase (poly I:C) or decrease ($ASGM_1$) their *in vivo* NK cell activity. The tumor growth rates of these mice were monitored over 21 d post tumor cell injection.

Materials and Methods

Animals: Two hundred twenty-six male DD/S mice (2-4 months of age) were used in this study. In most experiments, mice were housed individually for the course of the experiment and

were not exposed to the acute daily novelty stress regimen. The notable exception was the study to investigate the effects of *in vivo* modulation of NK cell activity on the differential tumor growth of mice from the GI (largest tumors) and IG (smallest tumors) housing groups. For this study. mice were raised and housed as described previously in General Methods, and these mice were subjected to acute daily novelty stress during the experimental period.

Tumor cells:

SC 115: SC115 cells used in this study were fresh cells continuously propagated in male DD/S mice as described in General Methods. For experiments involving tumors, tumor cells were injected into unanesthetized male mice as it has been suggested that anesthesia may inhibit the ability of subsequent poly I:C injections to stimulate NK cell activity (Markovic and Murasko, 1990). As this injection protocol was different from that used in previous studies in which mice were briefly anesthetized for the injection procedure, 6 mice raised according to our standard protocol were injected in the usual fashion with anesthesia, as described in General Methods. All tumors grew as expected in these control mice and reached a mass of approximately 3 g by 21 d.

Yac 1 Cells: These cells were used in NK cell assays as previously described.

Modulation of NK Cell Activity: Initial experiments investigated $ASGM_1$-induced suppression and Poly I:C-induced stimulation of *in vivo* levels of NK cell activity to determine the dose, injection schedule and time course of suppression or stimulation of NK cell activity. For $ASGM_1$, doses of 20 and 50 µl of $ASGM_1$ (diluted to a final volume of 100 µl: 1:5 and 1:2 dilution) were tested as single doses, repeated doses and with or without prior (18 h) injection of poly I:C. Animals were terminated 1 to 15 d post injection. For poly I:C, a dose of 100 µg was used to investigate the time course of stimulation of NK cell activity induced by repeated doses of poly I:C. Anti-asialoGM_1 (Wako Chemicals Inc., Texas) was purchased in lyophilized form. Antibody titer was estimated at 1:1000 by immunoflocculation. Each vial was reconstituted with

1 ml distilled water and stored at 4°C. The $ASGM_1$ was used within one month of reconstitution. Poly I:C (Sigma) was obtained as a sodium salt, diluted to a concentration of 1 mg/ml and stored at -20°C in 1 ml aliquots.

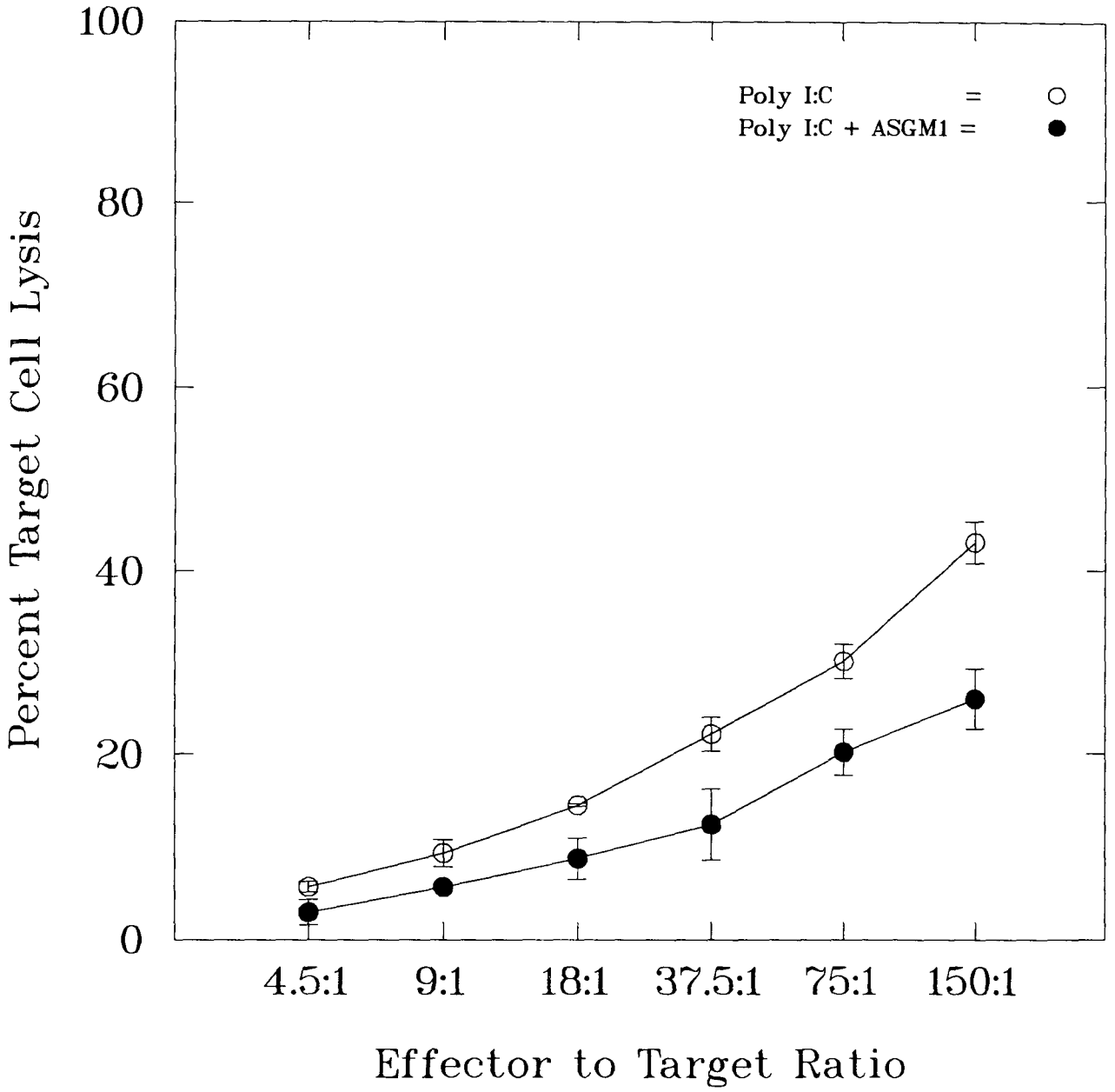
Methods for determining the effect of *in vivo* modulation of NK cell activity on the differential tumor growth rates of mice from GI and IG groups were based on the results of the initial experiments. To inhibit NK cell activity, mice were injected (ip) with 100 µl $ASGM_1$ (1:5 dilution). To stimulate NK cell activity, mice were injected (ip) with 100 µl poly I:C (1 mg/ml). Mice in each housing group were randomly assigned to one of 4 experimental treatment conditions: injected ip with either poly I:C, $ASGM_1$ or saline (to control for the stress of ip injection), or not ip injected at all. The ip injections were given every 5 d (-1, 4, 9 and 14 d). Mice were injected with tumor cells without anesthesia on day zero and tumor growth was monitored for 21 d.

Assays of Splenic NK Cell Activity: Splenic NK cell activity was measured as previously described.

Results

Anti-AsialoGM_1-Induced Decreases in <u>In Vivo</u> Levels of NK Cell Activity: In the first experiment, the ability of $ASGM_1$ to decrease NK cell activity *in vivo* following prior stimulation with poly I:C was measured. All mice were injected ip with poly I:C (100 µl, 1 mg/ml) and, 18 h later, half the mice were injected ip with $ASGM_1$ (100 µl, 1:5 dilution) and half the mice were ip injected with vehicle. Three days after the injection of $ASGM_1$, splenic NK cell activity was measured (Figure 13). At this time, stimulation of splenic NK cell activity by poly I:C was not

Figure 13. *Effect of a Single Injection of Anti-AsialoGM1 on In Vivo NK Cell Activity in Mice Previously Treated With Poly I:C.* All mice (individually housed) were injected ip with 100 µl of polyinosinic:polycytidylic acid (Poly I:C, 1mg/ml). Eighteen h later, mice were injected ip with 100 ul of anti-asialoGM$_1$ antibody (ASGM$_1$, 100 µl, 1:5 dilution) (n=3) or vehicle (n=3). Three days later, mice were terminated and splenic NK cell activity was assessed. Points represent mean ± SEM. In mice receiving ASGM$_1$, NK cell activity decreased to approximately 50 % of that observed in mice receiving poly I:C alone. In mice stimulated by poly I:C alone , NK cell activity was comparable to that previously observed in vehicle-injected mice (Rowse *et al.*, 1990). Spontaneous release was less than 10% of the total release.


Poly I:C =
Poly I:C + ASGM1 =
Percent Target Cell Lysis
100
80
60
40
20
0
4.5:1
9:1
18:1
37.5:1
75:1
150:1
Effector to Target Ratio

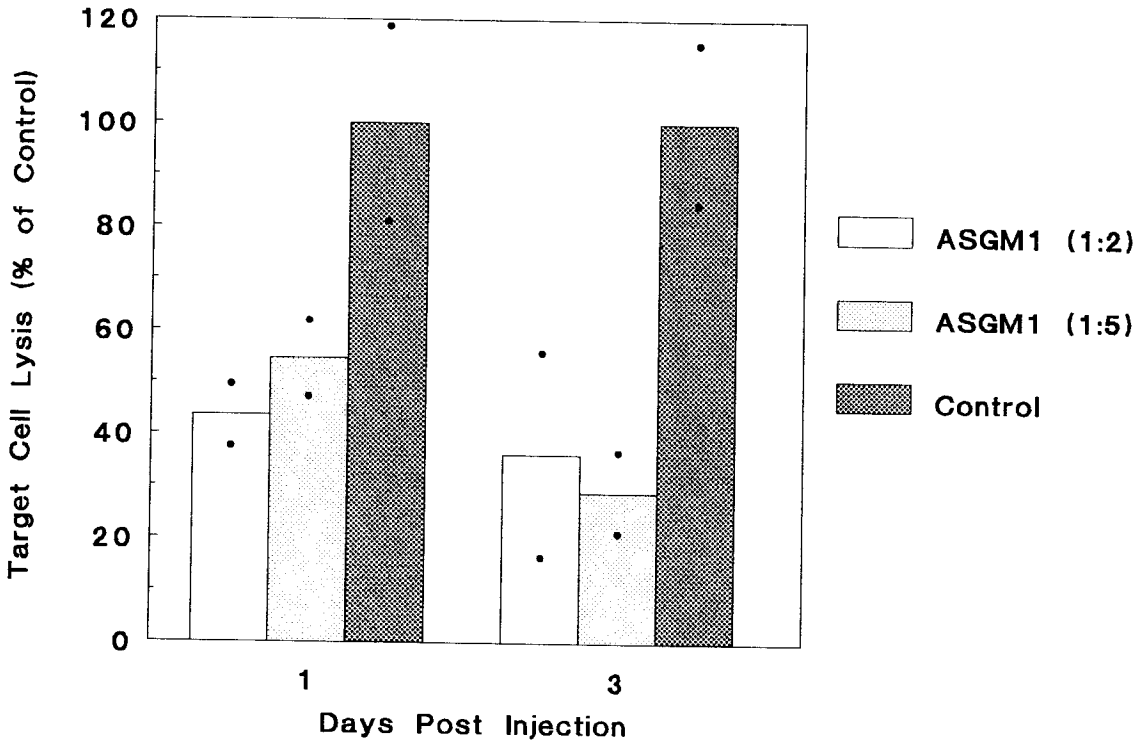
very remarkable. In mice stimulated by poly I:C alone, NK cell activity was comparable to that previously observed in vehicle-injected mice (Rowse *et al.*, 1990). Treatment with $ASGM_1$ decreased NK cell activity to approximately 50 % of that observed in mice receiving poly I:C alone. This decrease in activity was less than had previously been reported for $ASGM_1$ in other strains of mice (Habu *et al.*, 1981).

A second experiment was conducted to investigate the effects of different dosages of $ASGM_1$ (1:5 vs 1:2 dilutions) and terminating the mice at different times post injection (1 and 3 d) on the ability of a single injection of $ASGM_1$ (mice were not previously injected with poly I:C) to decrease splenic NK cell activity (Figure 14). Overall, there was approximately a 60 % suppression of splenic NK cell activity compared with that in vehicle-injected controls. Data indicate that the effect of $ASGM_1$ was greater at 3 d than at 1d, but that increasing the dose of $ASGM_1$ from 1:5 to 1:2 dilutions did not appreciably increase the suppression of NK cell activity observed.

In a third experiment, the effect of 2 injections of $ASGM_1$ (100 ul each, 1:2 dilution), 1 d apart, was investigated. This procedure has been reported to increase the effectiveness of the antibody (Ben-Eliyahu and Page, 1992). The data indicate that, at 3 d following the second injection of antibody, there was approximately a 60 % decrease in splenic NK cell activity compared with that in vehicle-injected controls. This was similar to the inhibition observed with a single injection of $ASGM_1$. Thus, in the DD/S strain of mice, there did not appear to be a significant advantage of multiple injections of $ASGM_1$.

A final experiment investigated the effects of repeated injections of $ASGM_1$, with doses of 1:5 and 1:2 dilutions (100 µl each), on NK cell activity over a 2 wk period. Antibody was injected every 5 d (the distributor's recommended optimum) for 2 wk and mice were terminated at varying times (2, 5, 7, 10, 12, 15 d). Data indicate that both doses of $ASGM_1$ caused a lasting

Figure 14. *Effect of Dosage of Anti-AsialoGM1 and Time Post Injection on In Vivo NK Cell Activity in Mice.* Mice were injected ip with 100 µl $ASGM_1$ (1:5 or 1:2 dilution), or saline. Mice were terminated at 1 d or 3 d post injection and splenic NK cell activity was assessed. Bars represent mean ± the range (dots). The suppression of NK cell activity (E:T ratio of 150:1) induced by $ASGM_1$ was greater at 3 d than at 1d. However, $ASGM_1$ doses of 1:5 and 1:2 dilutions appeared to be equally effective at inducing suppression of NK cell activity. (N's: 2 mice /treatment/day). Spontaneous release was less than 10% of the total release.


120
100
80
60
40
20
0
Target Cell Lysis (% of Control)
1
3
Days Post Injection
ASGM1 (1:2)
ASGM1 (1:5)
Control

suppression of NK cell activity over the 15 d time course when injected every 5 d (Figure 15). There appeared to be a small degree of recovery of NK cell activity by 5 d following each injection. However, suppression of NK cell activity was greater than 60 % in most cases. Once again, there did not appear to be a marked difference between the 200 μg and the 500 μg doses of $ASGM_1$ in the level of inhibition of splenic NK cell activity observed.

Poly I:C-Induced Stimulation of In Vivo Levels of NK Cell Activity: In the preceding pilot study, a single injection of poly I:C did not produce a large increase in splenic NK cell activity at 90 h (approximately 3 1/2 d) post injection (figure 16). Thus, an experiment was undertaken to investigate the effects of repeated doses of poly I:C in stimulating and maintaining the stimulation of NK cell activity over time. Animals were injected with poly I:C or saline on day 0, 5 and 10 and mice were terminated at day 1, 3, 5, 8, 10, 12 and 15.

Poly I:C injection induced a marked increase in splenic NK cell activity at 1 d post injection compared with that in vehicle-injected mice (approximately 300 %, Figure 16). However, by 3 d post injection, NK cell activity in poly I:C injected mice had declined noticeably (to approximately 210 %) and it declined further (to approximately 160 %) by 5 d post injection. Subsequent injections of poly I:C at 5 and 10 d did not increase splenic NK cell activity observed at 8 and 10 d and at 12 and 15 d, respectively, above the levels observed at 5 d.

In light of the apparent lack of effect of the 5 and 10 d injections of poly I:C, a second experiment was undertaken to investigate the possibility that anesthesia might block the stimulatory effects of poly I:C on NK cell activity. Mice were injected with either poly I:C or saline on day 0 and 5 and terminated at 1, 6 or 7 d following the first injection. Half of the poly I:C-injected mice were briefly exposed to anesthesia (ether) during the injection. It was found that poly I:C induced a strong stimulation of splenic NK cell activity at all 3 time points examined (Figure 17).

Figure 15. *Effect of Repeated Injections of Anti-AsialoGM1 on In Vivo NK Cell Activity in Mice Over Time.* Mice were injected ip with either 100 µl of $ASGM_1$ (1:2 and 1:5 dilutions) or saline on d 0, 5 and 10 and terminated either on d 2, 5, 7, 10, 12 or 15 (1:2 dilution $ASGM_1$ or saline), or on d 2, 5 or 10 (1:5 dilution $ASGM_1$). Splenic NK cell activity was assessed. Points represent mean ± SEM, the cross-hatched bar represents the mean of the controls ± 1 standard deviation. Repeated injection of both doses of $ASGM_1$ induced lasting suppression (greater than 60 %) of NK cell activity (E:T ratio of 150:1) over time, although there appeared to be a small degree of recovery of NK cell activity by 5 d following each injection. Once again, $ASGM_1$ doses of 1:2 and 1:5 dilutions appeared to be equally effective at inducing suppression of NK cell activity. (N's: $ASGM_1$=3 mice /dose/day, Vehicle= 2 mice /day). Spontaneous release was less than 15% of the total release.

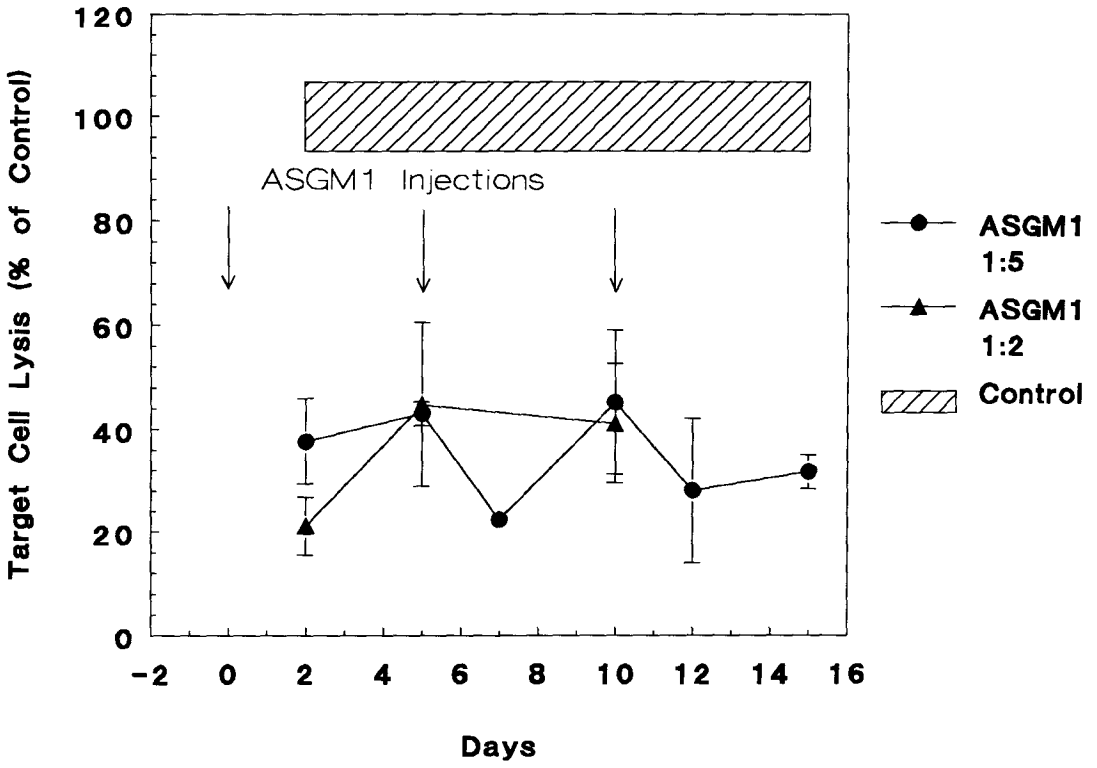

120
100
80
60
40
20
0
Target Cell Lysis (% of Control)
ASGM1 Injections
-2
0
2
4
6
8
10
12
14
16
Days
ASGM1 1:5
ASGM1 1:2
Control

Figure 16. *Effect of Repeated Injections of Poly I:C on In Vivo NK Cell Activity in Mice Over Time.* Mice were injected ip with either 100 µl poly I:C (1mg/ml) or saline on d 0, 5 and 10 and were terminated on days 1, 3 , 5, 8, 10, 12 and 15. Splenic NK cell activity was assessed. Points represent mean ± SEM, the cross-hatched bar represents the mean of all controls ± 1 standard deviation. Poly I:C injection induced a marked increase in splenic NK cell activity (E:T ratio of 150:1) at 1 d post injection compared with that in saline-injected mice. There was a progressive decrease in NK cell activity in poly I:C injected mice from d 3 to 5. Subsequent injections of poly I:C at 5 and 10 d did not increase splenic NK cell activity observed at 8 and 10 d and at 12 and 15 d, respectively, above the levels observed at 5 d. (N's: poly I:C, 2 mice /day (1, 3, 8, 12 d), 3 mice /day (5, 10, 15 d); Vehicle = 2 mice /day). Spontaneous release was usually less than 10% of the total release and always less than 15%.

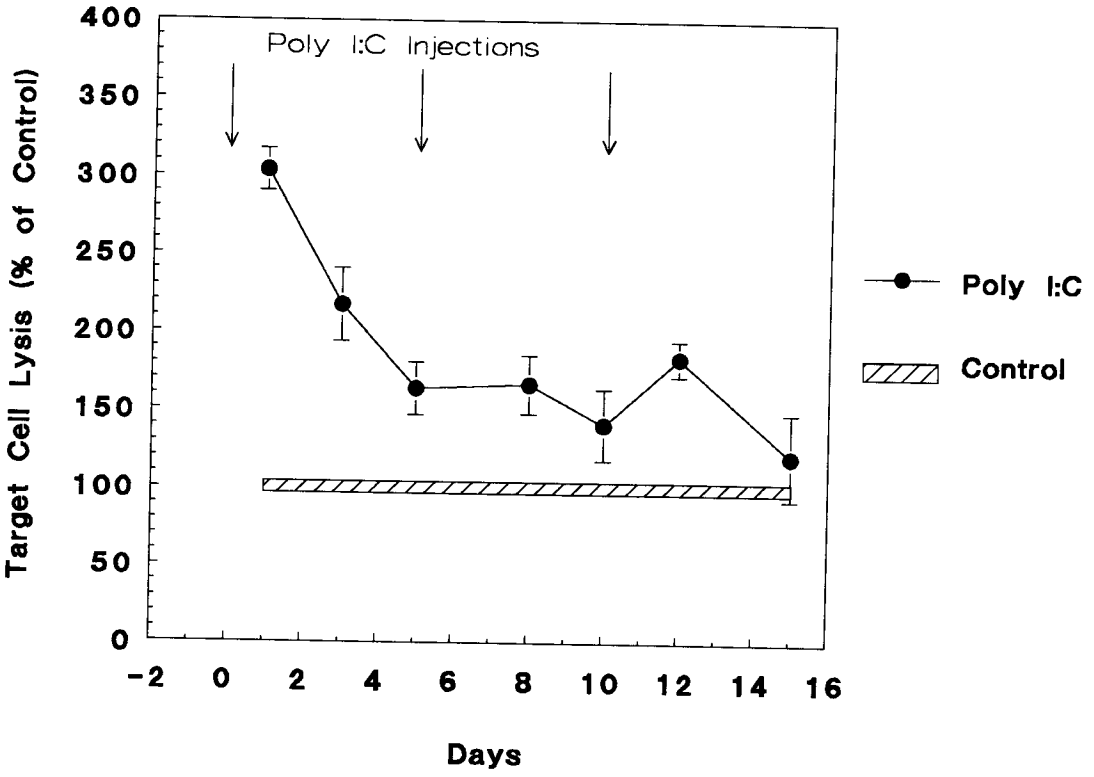
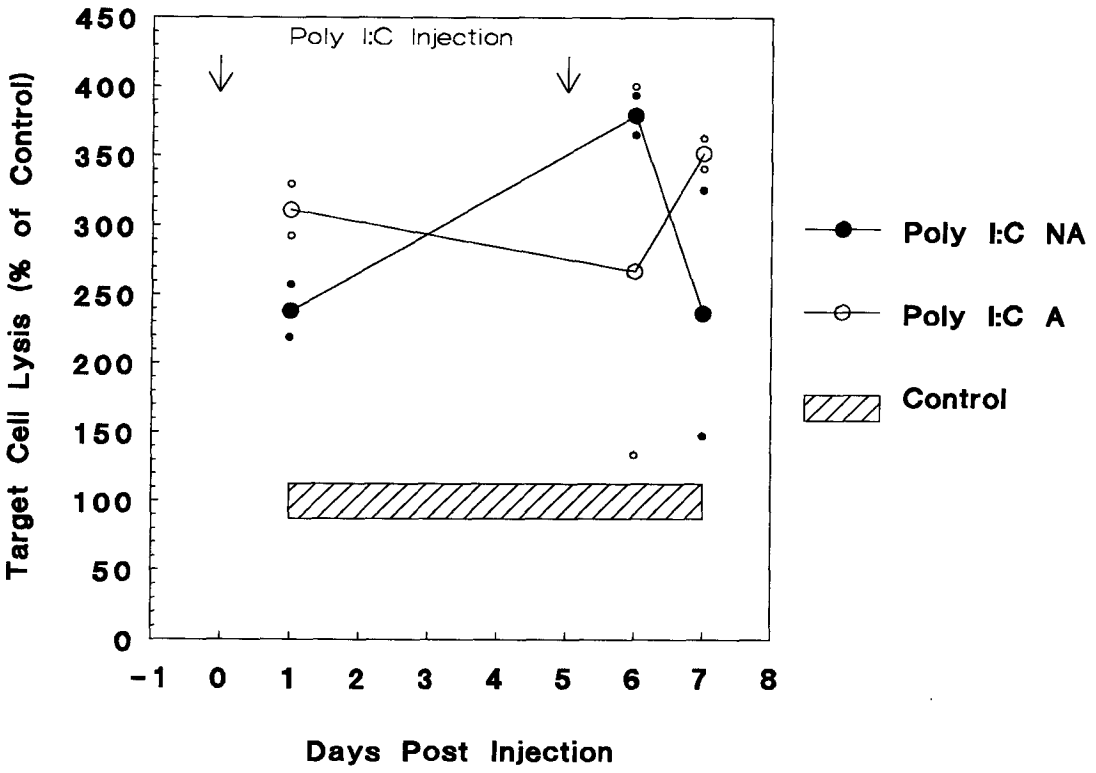

Poly I:C Injections
Target Cell Lysis (% of Control)
400
350
300
250
200
150
100
50
0
-2
0
2
4
6
8
10
12
14
16
Days
Poly I:C
Control

Figure 17. *Effect of Anesthesia on the Ability of Repeated Injections of Poly I:C to Stimulate In Vivo NK Cell Activity in Mice Over Time.* Mice were injected ip with either poly I:C (1mg/ml) or saline on day 0 and 5 and terminated at 1, 6 or 7 d following the first injection. Poly I:C-injected mice were either briefly exposed to ether anesthesia during the injection (n=2) or received no anesthesia (n=2). Points represent mean ± range (dots), the cross-hatched bar represents the mean of all controls ± 1 standard deviation. Poly I:C induced a strong stimulation of splenic NK cell activity at all 3 time points examined. There were no apparent differences in the level of NK cell stimulation observed in mice injected with or without anesthesia. (N's: 2 mice /treatment /day). Dots indicate the range. Spontaneous release was less than 10% of the total release.


Poly I:C Injection
Target Cell Lysis (% of Control)
450
400
350
300
250
200
150
100
50
0
-1
0
1
2
3
4
5
6
7
8
Days Post Injection
Poly I:C NA
Poly I:C A
Control

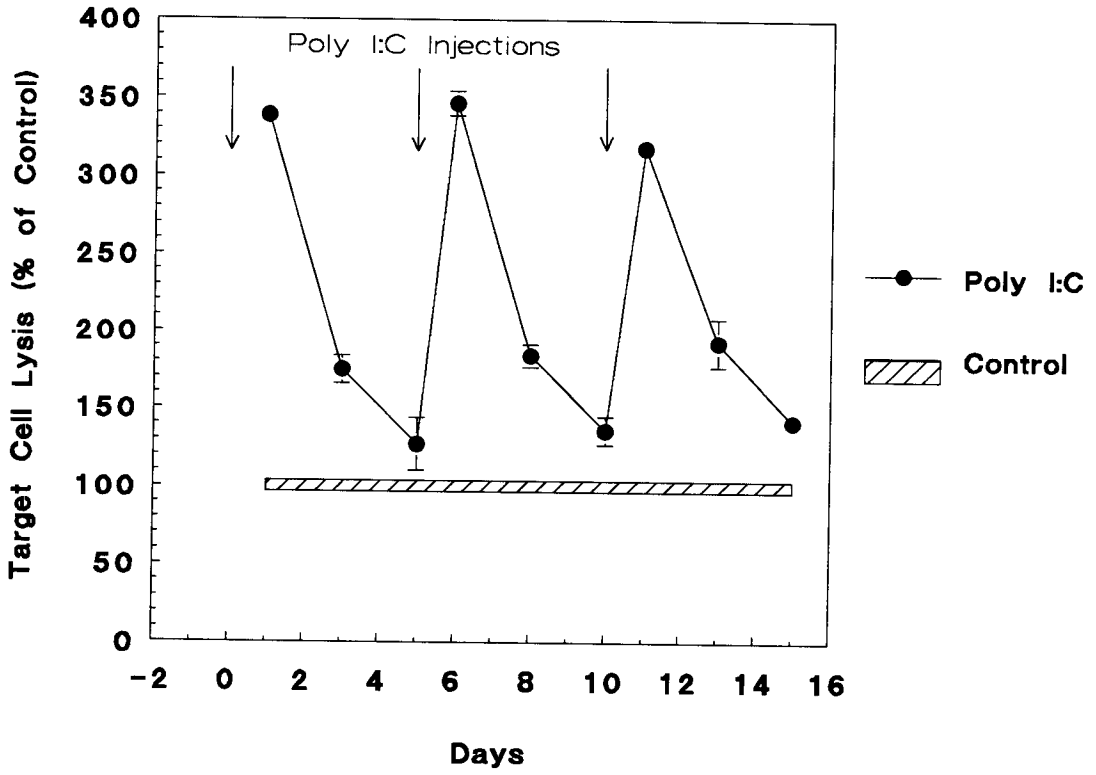
There were no apparent differences in the level of NK cell stimulation observed in mice injected with or without anesthesia.

The results of the first 2 experiments present an apparent contradiction concerning the ability of repeated injections of poly I:C to stimulate an increase in the levels of splenic NK cell activity. The first experiment demonstrated that the second and third injections, at 5 and 10 d respectively, failed to induce an increase in NK cell activity 2 d later. However, in the second experiment, a second injection of poly I:C at 5 d caused a stimulation of NK cell activity on both 6 and 7 d that was of equal magnitude to that observed 1 d following the initial injection. A third experiment was undertaken to resolve this apparent conflict in the results. Mice were injected without anesthesia, with either poly I:C or vehicle, on day 0, 5 and 10, and were terminated on days 1, 3, 5, 6, 8, 10, 11, 13 and 15 post injection. With this more complete time course, we found marked stimulation of splenic NK cell activity on days 1, 6 and 11, that is one day after each injection of poly I:C (Figure 18). NK cell activity subsequently declined back to control levels by 5 d after each injection.

Effect of In Vivo Modulation of NK Cell Activity on The Differential Tumor Growth Rates Observed in Our Model: The effects of *in vivo* modulation of NK cell activity on the differential tumor growth rates observed in our model were examined. Only mice from GI (largest tumors) and IG (smallest tumors) groups were included. Mice in each housing group were randomly assigned to one of 4 experimental treatment conditions, mice were injected ip with either poly I:C, $ASGM_1$ or saline (to control for the stress of ip injection), or were not injected at all. The ip injections were given every 5 d (-1, 4, 9 and 14 d). Mice were injected with tumor cells without anesthesia on day zero and tumor growth was monitored for 21 d.

An overall ANOVA indicated that control mice injected ip with saline and control mice who were not injected did not differ significantly from each other in tumor growth rate ($F(3,58) =$

Figure 18. *Effect of Repeated Injections of Poly I:C on In Vivo NK Cell Activity in Mice Over Time.* Mice were injected ip without anesthesia, with either 100 µl of poly I:C (1mg/ml) or saline on days 0, 5 and 10 and terminated on days 1, 3, 5, 6, 8, 10, 11, 13 and 15. Splenic NK cell activity was assessed. Points represent mean ± SEM, the cross-hatched bar represents the mean of all controls ± 1 standard deviation. There was marked stimulation of splenic NK cell activity on days 1, 6 and 11 (i.e. 1 d after each injection of poly I:C). NK cell activity decreased back to control levels by 5 d after each injection. (N's: poly I:C = 3 mice /day, Vehicle = 2 mice /day). Spontaneous release was less than 10% of the total release.


Poly I:C Injections
Target Cell Lysis (% of Control)
400
350
300
250
200
150
100
50
0
-2
0
2
4
6
8
10
12
14
16
Days
Poly I:C
Control

2.106, P = 0.109). Therefore data were collapsed across these 2 control conditions. The subsequent ANOVA revealed significant main effects of Group ($F(1,60) = 41.574$, $P<0.001$) and Day ($F(4,240) = 175.949$, $P<0.001$). Further, effects of Treatment ($F(2,60) = 2.953$, $P=0.060$) and the Day x Treatment interaction ($F(8,240) = 1.884$, $P=0.063$) approached significance. Post-hoc tests revealed that, overall, mice of the GI group had significantly larger tumors than mice of the IG group ($p<0.001$, Figure 19). Interestingly, simple main effects analysis of the Day x Treatment interaction revealed that on days 7 through 15, mice in the 3 treatment conditions ($ASGM_1$, poly I:C and control) were not significantly different from each other, whereas on day 18 and 21 significant treatment effects emerged ($p's<0.01$). Post-hoc analysis revealed that at 18 d, mice treated with poly I:C had significantly larger tumors than mice treated with $ASGM_1$ ($p<0.01$) or control mice ($p<0.05$, Figure 19). At 21 d post injection, both mice treated with poly I:C ($p<0.001$) and control mice ($p<0.05$) had significantly larger tumors than mice treated with $ASGM_1$.

The data were further analysed with the 21 d time point omitted. Our previous experiments demonstrated that the effects of $ASGM_1$ and poly I:C gradually decrease and are significantly reduced by 5 d following the last injection. We reasoned that because the effects of $ASGM_1$ and poly I:C decrease over time and because the last injection of these agents was at 14 d, it was possible that, by 21 d, tumor growth was no longer affected by these agents. With this subset of data, the ANOVA revealed significant effects of Group ($F(1,60) = 45.822$, $P<0.001$), Treatment ($F(2,60) = 4.166$, $P=0.020$) and Day ($F(3,180) = 129.506$, $P<0.001$). Further, the Group x Treatment x Day interaction was now highly significant ($F(3,180) = 2.726$, $P=0.015$). Post-hoc analysis revealed that at this time, mice treated with poly I:C had significantly larger tumors than mice treated with $ASGM_1$ and control mice ($p's<0.001$, Figure 20). In contrast, for mice in the IG group tumor growth did not differ among mice in the 3 treatment conditions at any time point ($p>0.50$).

Figure 19. *Effect of In Vivo Modulation of NK Cell Activity on The Differential Tumor Growth Rates Observed in GI and IG.* Mice from GI (open symbols) and IG (closed symbols) (as described in figure 4) were injected ip with 100 µl of either poly I:C (1mg /ml), $ASGM_1$ (1:5 dilution), saline or received no injection. Injections were given on d -1, 4, 9 and 14. Mice were injected with tumor cells without anesthesia on d 0 and tumor growth was monitored for 21 d. Points represent mean ± SEM. Overall, mice of the GI group had significantly larger tumors than mice of the IG group ($p<0.001$). At 18 d, mice treated with poly I:C had significantly larger tumors than mice treated with $ASGM_1$ ($p<0.01$) or control mice (combined data for saline and uninjected conditions, $p<0.05$). At 21 d post injection, both mice treated with poly I:C and control mice had significantly larger tumors than mice treated with $ASGM_1$ ($p<0.05$). (N's: GI, $ASGM_1$(8), poly I:C(9), control(12); IG, $ASGM_1$(9), poly I:C(9), control(19)).

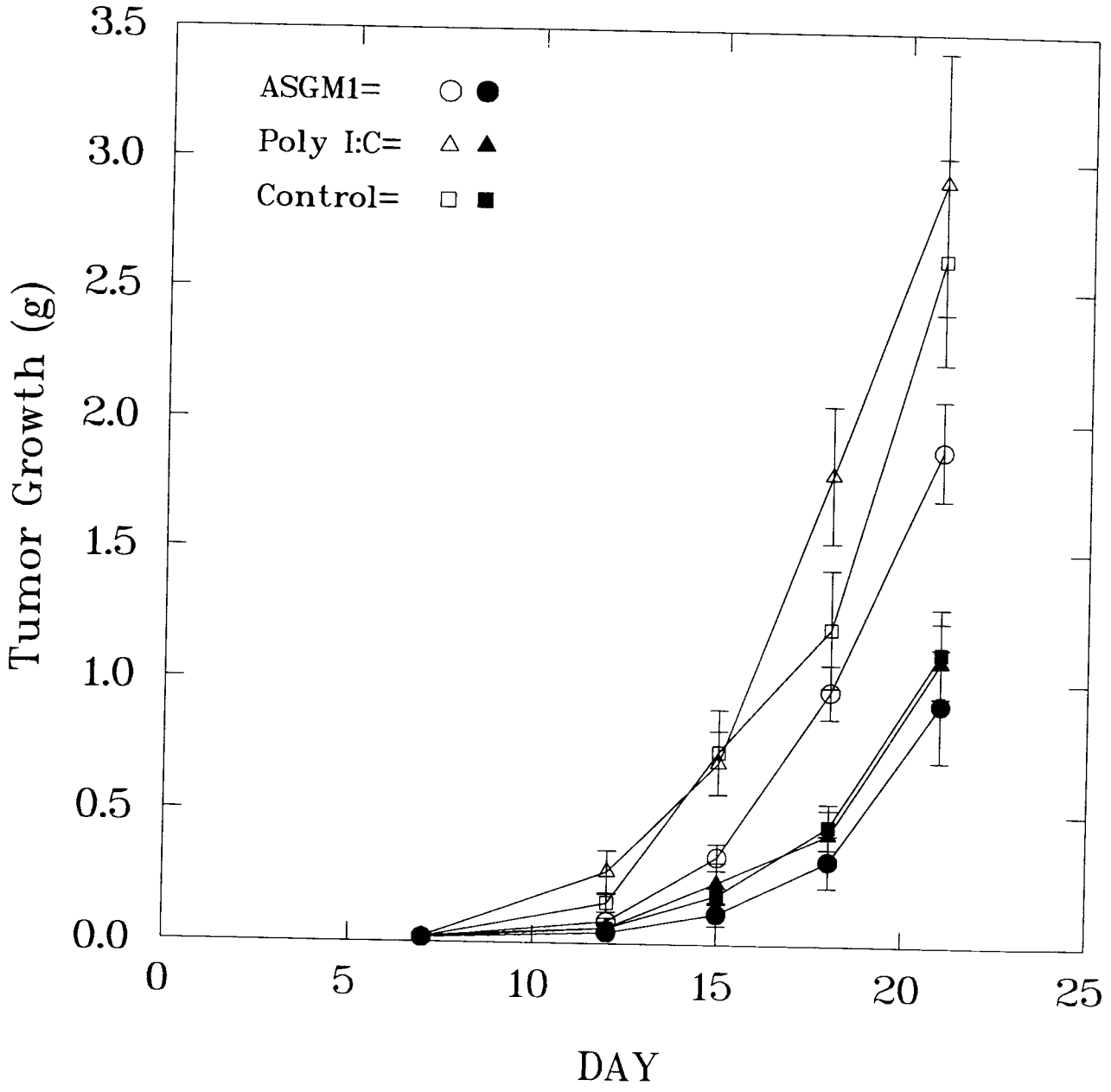
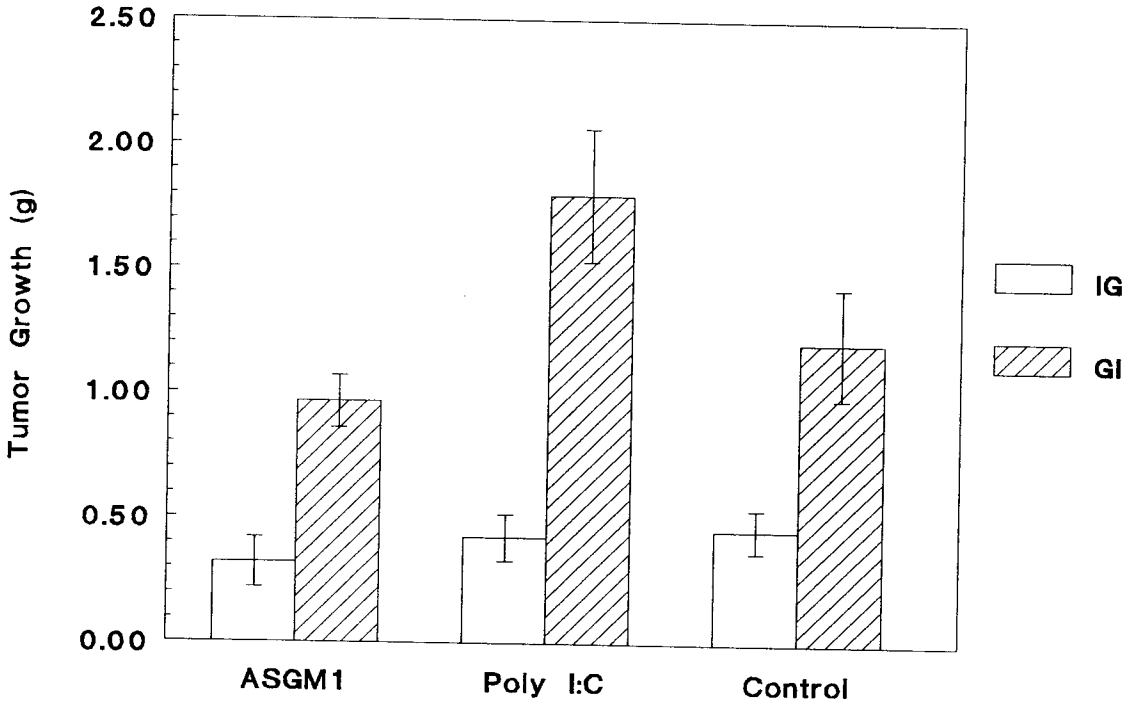

ASGM1=
Poly I:C=
Control=
Tumor Growth (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
DAY

Figure 20. *Effect of In Vivo Modulation of NK Cell Activity on The Differential Tumor Growth Rates Observed in GI and IG Mice at 18 Days Post Injection.* Mice from GI and IG (as described in figure 4) were injected ip with either poly I:C (1mg /ml), $ASGM_1$ (100 μ of a 1:5 dilution), saline or received no injection. Injections were given on d -1, 4, 9 and 14. Mice were injected with tumor cells without anesthesia on d 0 and tumor growth was monitored for 18 d. Bars represent mean ± SEM. Mice treated with poly I:C had significantly larger tumors than mice treated with $ASGM_1$ and control mice (combined data for saline and uninjected conditions) ($p's<0.001$). In contrast, for mice in the IG group tumor growth did not differ among mice in the 3 treatment conditions at any time point ($p>0.50$).


2.50
2.00
1.50
1.00
0.50
0.00
Tumor Growth (g)
ASGM1
Poly I:C
Control
IG
GI

Discussion

This study investigated the effect of *in vivo* modulation of NK cell activity on the differential growth rates of the SC115 tumor in mice from the experimental housing groups of our model. To accomplish this, we utilized agents that produce prolonged suppression or stimulation of *in vivo* NK cell activity. Suppression of NK cell activity was induced by ip injection of the antibody $ASGM_1$. It was found that injection of $ASGM_1$ resulted in an approximately 60 % reduction of *in vivo* splenic NK cell activity. Further, this reduction was maintained over time by the repeated administration of the antibody every 5 d. Interestingly, the reduction of NK cell activity observed in DD/S mice was less than that previously reported for this dose of antibody in other strains of mice (Habu *et al.*, 1981). It is possible that the route of administration might be responsible for the differences between our study and other studies. Most studies using $ASGM_1$ administer the antibody by tail vein injection. This route of injection was unacceptable for this study as it would have involved an increase in the level of stress experienced by the animals injected with $ASGM_1$ compared with animals injected with poly I:C. A second possibility is that NK cells of DD/S mice do not uniformly express the asialo GM_1 antigen, do not express high levels of the antigen or are for some reason less susceptible to antibody- and complement-mediated lysis using this antibody. A third possibility is that DD/S mice develop an immune response to the rabbit $ASGM_1$ antibody. However, this last possibility is unlikely as one would expect to see a decrease in the effectiveness of the antibody with repeated injections and no such decrease was observed. Despite the less than complete reduction of NK cell activity, this treatment was demonstrated reliably to cause a greater than 60 % reduction in NK cell activity for a period of up to 2 wk.

Stimulation of NK cell activity was accomplished by repeated injection of poly I:C. It was found that 1 d after each injection of poly I:C, there was a significant stimulation of splenic NK cell activity. This finding supports previous reports that the effects of poly I:C do not attenuate

with repeated injections (Talmadge *et al.*, 1985a, Talmadge *et al.*, 1985b) and is in contrast with the reports of the hyporesponsiveness of splenic NK cell activity following repeated injections of IL-2 (Talmadge *et al.*, 1985a, Talmadge *et al.*, 1985b). However, the stimulation of NK cell activity induced by an injection of poly I:C was observed to decline rapidly and, by 3 d post injection, was approximately half that observed at 1 d post injection. By 5 d post injection the levels of splenic NK cell activity were close to the levels observed in vehicle-injected mice. The apparently conflicting results on the effects of repeated injections of poly I:C observed in the first 2 poly I:C time course experiments were resolved by the third experiment which revealed that the discrepancy was caused by the differences in the time of sampling (8 d vs 6 and 7 d). This decline in the stimulation of NK cell activity with time following an injection of poly I:C is more rapid than has previously been reported in the literature. One study reported that, in C_3H mice, a single ip injection of 0.5 mg/kg poly I:C resulted in a significant stimulation of splenic NK cell activity for at least 7 d post injection (Talmadge *et al.*, 1985a). This dose of poly I:C is 5 times lower than that used in our study (approximately 2.5 mg/kg, a commonly used dosage), but comparable levels of NK cell stimulation were observed.

It is assumed that splenic NK cell activity is reflective of overall levels of NK cell activity. This assumption is supported by our previously finding (Chapter 1B, part 4) that the stimulation of NK cell activity by the SC115 tumor is of similar magnitude in the spleen and at the tumor site. In contrast, it has been reported that following repeated injections of IL-2, stimulation of NK cell activity declines in the spleen but is maintained in nonlymphoid target organs (Talmadge *et al.*, 1985a). However, as attenuation of the NK response to poly I:C following repeated stimulation was not observed, it appears that the attenuation of NK cell responses to poly I:C is not an issue in this study.

Finally, the effect of modulating *in vivo* NK cell activity on the differential growth of the SC115 tumor in mice housed in different experimental groups was examined. We postulated that

if NK cell activity played an important role in modulating the differential tumor growth rates observed in our model, then *in vivo* modulation of NK cell activity would result in an alteration of the differential tumor growth rates observed in mice from the IG and GI groups. That is, since poly I:C increases NK cell activity and $ASGM_1$ decreases NK cell activity, one would expect that these treatments should produce opposite effects on tumor growth relative to each other.

The data revealed that modulation of NK cell activity did result in a significant alteration in tumor growth rate. Importantly, poly I:C-induced stimulation of NK cell activity resulted in a significant increase in tumor growth rate at 18 d and 21 d post tumor cell-injection compared with that in $ASGM_1$-injected mice. As expected, the tumor growth rate of control mice was found to be intermediate to that of poly I:C- and $ASGM_1$-injected mice. The finding that poly I:C stimulates the growth rate of the SC115 tumor compared with that observed in $ASGM_1$-injected mice suggests that NK cells may actually facilitate the growth of the SC115 tumor. These data are consistent with our previous finding that mice of the GI group (who develop the largest tumors) have significantly increased levels of NK cell activity both in the spleen and at the tumor site compared with mice of the IG condition (who develop the smallest tumors).

It has been demonstrated that NK cells are active early in the immune response and that their activity declines at later stages. For example, NK cell activity of lymphocytes infiltrating highly immunogenic allografts declines by about 8 d (Hoffman *et al.*, 1988). We have demonstrated that NK cell activity of tumor-infiltrating lymphocytes has begun to decline by 10 d post tumor injection, although it is still elevated at 17 d post injection when compared with that in control mice. In our experiment, the last injections of poly I:C and $ASGM_1$ were given at 14 d post-tumor injection. As we have demonstrated that the stimulatory and inhibitory effects of these compounds decline markedly by 5 d following each injection, it is likely that the growth of the tumor observed at 21 d post injection was not influenced by the treatment conditions to the same extent as was observed at 18 d. When only the first 18 d of tumor growth were considered,

it was found that the effects of poly I:C and $ASGM_1$ were observed only in mice of the GI condition and that these effects were highly significant. The observation that the modulation of NK cell activity only affects the growth of tumors in mice of the GI group and not in mice of the IG group suggests that several different processes may be involved in controlling the growth of the SC115 tumor. This possibility is strengthened by the finding that modulation of *in vivo* NK cell activity, either stimulation of inhibition, does not result in similar tumor growth rates in mice of the GI and IG groups. Obviously, although NK cells appear to play a role in modulating the growth of the SC115 tumor, other mechanisms must also be involved in mediating the effects of psychosocial stressors on the growth of tumors in this model.

In conclusion, this study demonstrates that NK cells do play a role in modulating the differential tumor growth rates observed in our animal tumor model. Surprisingly, NK cells appear to be involved in increasing SC115 tumor growth rate through as yet undetermined mechanisms and appear to be involved selectively in mice of the GI group. Further, it is apparent that mechanisms other than those related to NK cell activity are also involved in mediating the effects of psychosocial stressors on the differential tumor growth rates observed in this model.

CHAPTER 4 ENDOCRINE STUDIES.

In addition to the immune system, the endocrine system may also play a role in mediating the effects of psychosocial stressors on the differential tumor growth observed in our model. The SC115 tumor is a hormone-responsive tumor and several hormones and growth factors are known to affect its growth (Bruchovski and Rennie, 1978, Emerman and Siemiatkowski, 1984, Furuya *et al.*, 1990, Kitamura *et al.*, 1979, Tanaka *et al.*, 1990). Further, the endocrine system has been shown to be exquisitely sensitive to stressors. Thus, the endocrine system may also be a mediator of the differential tumor growth rates observed in this model.

A) Morphological Studies of the SC115 Tumor Grown in Male and Female Mice.

Introduction

Breast cancers are composed of a heterogeneous population of cells including hormone-responsive and hormone-independent cells (Heppner *et al.*, 1981). This heterogeneity results in metabolic and functional variability within a tumor and subsequent variation in response to treatments such as chemotherapy and hormone therapy (Emerman and Siemiatkowski, 1984, Heppner *et al.*, 1981, Miller *et al.*, 1981). The environment of the tumor may modulate the composition of subpopulations in the tumor in several ways. There may be negative selection against some cell populations if required growth factors or nutrients are absent from the environment. Alternately, there may be positive selection of some cell populations if the growth of these cells is stimulated by a factor present in the environment to which other cell subpopulations do not respond. The SC115 tumor is an example of a tumor that is heterogeneous in its response to hormones (Bruchovski and Rennie, 1978, Emerman and Siemiatkowski, 1984,

Yates *et al.*, 1980). The predominant subpopulation of the SC115 tumor grown in intact male mice is androgen-responsive, whereas the predominant subpopulation is androgen-independent when SC115 cells are grown in female or castrated male mice (Bruchovski and Rennie, 1978, Emerman and Siemiatkowski, 1984, Yates *et al.*, 1980). It is possible that the development of the androgen-independent tumors in female and castrated male mice may result from the selective growth advantage of androgen-independent cell subpopulations in an environment where the concentrations of androgens are not sufficient to stimulate the proliferation of the androgen-responsive cell populations. Both androgen-responsive and androgen-independent cell clones have been isolated from androgen-responsive SC115 tumors (Yamaguchi *et al.*, 1992, Darbe and King, 1987). The androgen-responsive cell clones exhibit low rates of proliferation in the absence of testosterone and a 15 fold increase in the rate of proliferation in the presence of testosterone (Yamaguchi, 1992, Darbe and King, 1987). In contrast, androgen-independent cell clones do not respond to the presence of testosterone with increased cell proliferation rates (Yamaguchi *et al.*, 1992). Thus, when androgens are not present at the levels required to stimulate the growth of the androgen-responsive cell subpopulations, cells of the androgen-independent subpopulations have been shown to have a selective growth advantage and become the predominate subpopulation within the tumor.

The heterogeneity of tumors is often manifest as an alteration of the morphology of the cells. For example, hormones are known to alter the morphology of the SC115 tumor (Emerman and Worth, 1985, Yates *et al.*, 1980). Tumors grown in intact male mice (maintained in our standard laboratory conditions) have been shown to exhibit significant phenotypic differences from tumors grown in castrated male mice and female mice (Emerman and Worth, 1985). Androgen-responsive tumors grown in intact male mice have a sheet-like growth pattern. The tumors are highly vascularized but there are considerable areas of necrosis in the center of the tumor. Androgen-independent tumors grown in female and castrated male mice lose this cohesive

growth pattern. The cells form loose sheets and irregular cords dispersed within large amounts of loose connective tissue stroma.

In our experimental model, the slow growth rate of tumors observed in male mice moved from the individual to the group condition (IG) was of particular interest to us as it approximates the growth rate of the SC115 tumor maintained in female mice (Weinberg and Emerman, 1989). Tumors from female and castrated male mice develop in a low androgen environment where androgen is provided solely by the adrenal gland. It has been demonstrated that, in male mice, crowded housing conditions may induce the suppression of testosterone secretion at all levels of the hypothalamic-pituitary-gonadal axis. Thus, in mice of the IG group, crowding could induce a low androgen environment. It is possible that in environments which provide suboptimal levels of androgens, androgen-independent cells may have a growth advantage relative to the androgen-responsive cells such that there is selective outgrowth of the androgen-independent subpopulation. Considering the dramatic morphological differences between tumors grown in intact male and female mice raised in our standard housing conditions, the present study was designed to investigate: 1) if the slow-growing tumors seen in male mice moved from individual to group housing would display histological characteristics similar to those seen in the androgen-independent tumors grown in female mice and 2) to examine further the histological characteristics of the SC115 tumor grown in androgen-rich (male) and androgen-deprived (female) environments.

METHODS AND MATERIALS

Animals: Male (n=17) and female (n=2) mice of the DD/S strain, 2 to 4 months of age, were used in this study. In accordance with our model, fourteen males were used to form the 4 experimental housing groups (GG, GI, II, IG) and were subjected to acute daily novelty stress as

previously described in the General Methods. Two additional groups of animals were included for comparison; males (n=3) and females (n=2) raised in the standard sibling rearing groups were maintained in their groups following tumor cell injection and were not subjected to acute daily novelty stress.

Tumors: The SC115 tumor was used in this study as described in General Methods. Male mice were terminated 3 wk post tumor cell-injection and the tumors were excised. Female mice were terminated when the tumors reached a mass of 1.5 g (approximately 50 d).

Fixation and Staining:

Tissue blocks approximately 0.125 cm^3 were cut from the center and periphery of the tumors and placed immediately into 10% formalin (4% formaldehyde) containing 2% (w/v) calcium acetate. Blocks were routinely paraffin processed after one wk of fixation. Serial sections cut at a thickness of 5 μm were stained with the following techniques:

1) Carbohydrate Histochemistry

a) Selective periodate oxidation-Schiff (PA*/S). This permits the specific demonstration of sialic acids without side chain 0-acyl substituents or with an 0-acyl substituent at position C7 (Volz *et al.*, 1987).

b) Saponification selective periodate oxidation-Schiff (KOH/PA*/S). This permits the specific demonstration of all sialic acids (Volz *et al.*, 1987).

c) Saponification selective periodate oxidation-Alcian blue pH 1.0-Schiff (KOH/PA*/AB1.0/S). With this stain, all sialic acids stain magenta, o-sulphate esters stain aquamarine blue; mixtures stain in various shades of purple (Reid *et al.*, 1987).

d) KOH/AB2.5/PAS. This is the standard Alcian Blue pH 2.5 Periodic Acid Schiff of Mowry (Mowry, 1963) preceded by a saponification step to remove any 0-acyl esters blocking vicinal diols. This stain serves as a control, demonstrating the presence of carboxyl groups and o-sulphate esters in aqua marine blue and all sugars containing vicinal diols magenta; mixtures stain in various shades of purple (Mowry, 1963).

e) Allochrome (Lillie, 1954). This provides a rapid visualization of the connective tissue present in the tumor.

f) Haematoxylin and Eosin, H and E (Culling 1974). This is used for standard morphological analysis (Culling, 1974).

2) Immunohistochemistry

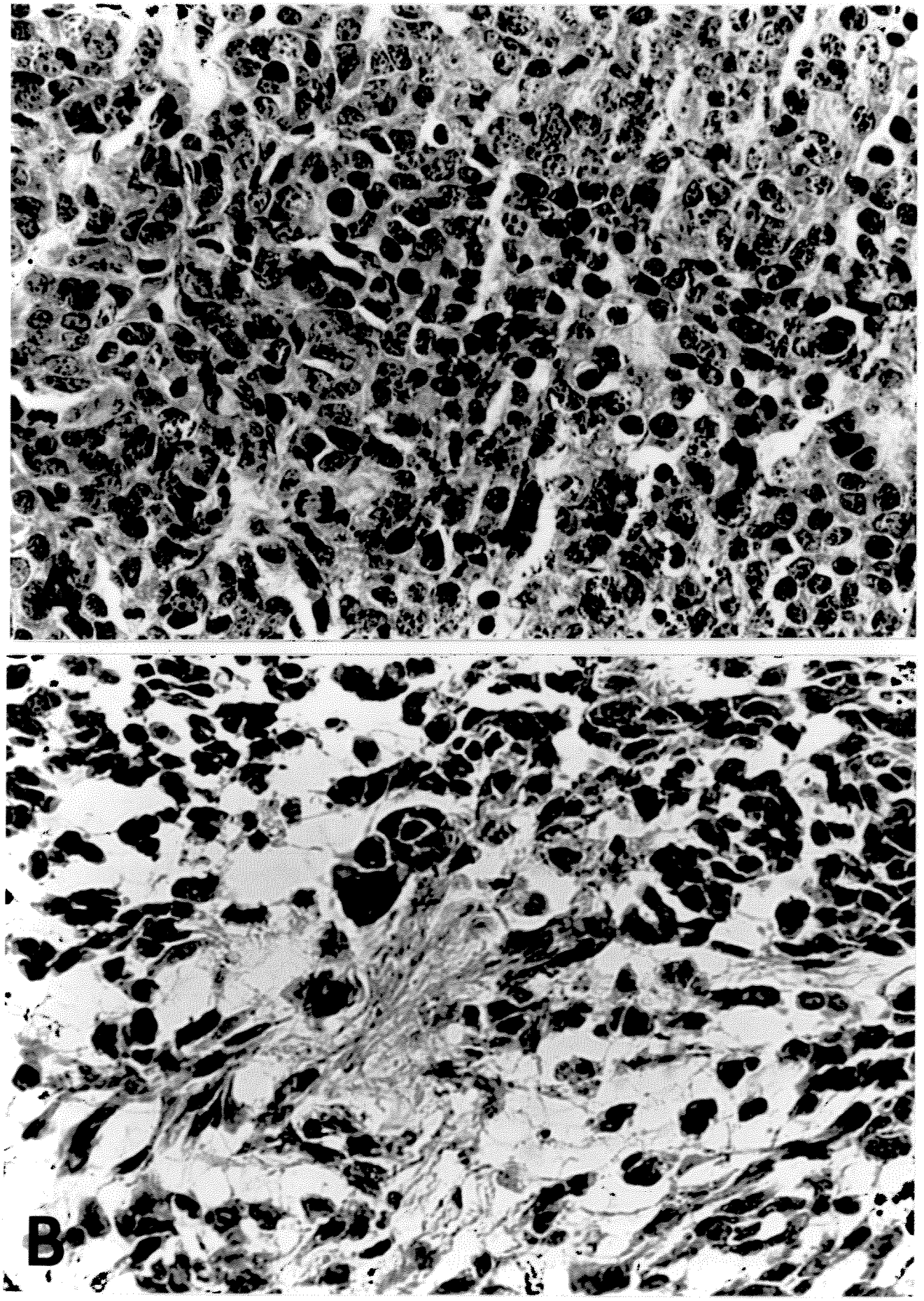
a) Representitive sections from tumors of animals in each condition were stained with muscle specific actin (MSA) and visualized by the immunoperoxidase technique (Papotti *et al.*, 1988) to determine if cells had characteristics of myoepithelial cells. Slides were counterstained with haematoxylin. Muscle tissue served as a positive control.

b) Representitive sections from tumors of animals in each condition were stained with S-100 protein antiserum and visualized by the immunoperoxidase technique (Dwarakanath *et al.*, 1987). S-100 has been shown to be associated with myoepithelial cells. Slides were counterstained with haematoxylin. Peripheral nerves, which also stain with S-100, served as a positive control.

RESULTS:

H & E stained sections of the tumors grown in females and males maintained under our standard laboratory conditions conformed to previous morphological descriptions of these tumors (Figure 21). The tumors from males had a cohesive epithelial-like growth pattern, high degree of vascularization and large areas of necrosis. The tumors from female mice contained cells dispersed into loose sheets and irregular strands growing in loose connective tissue. The morphology of tumors from the male mice exposed to the 4 experimental housing conditions were the same as that of the males housed in the standard conditions. Lillie's allochrome stain confirmed that the significant amounts of loose stromal connective tissue present throughout the female tumors were not present in any of the male tumors.

Figure 21. *Morphology of SC115 Tumors Grown in Male and Female Mice*. Male and female mice raised under standard colony conditions were injected with SC115 cells (2×10^6) and terminated at 21 d (males) or 50 d (females; when the tumors were approximately 1.5 g) post injection. Tissue blocks approximately 0.125 cm^3 were cut from the center and periphery of the tumors and placed immediately into 10% formalin containing 2% (w/v) calcium acetate. Blocks were routinely paraffin processed after one wk of fixation. Serial sections cut at a thickness of 5 μm were stained with H&E. The morphology of SC115 tumors grown in male (A) and female (B) mice conformed to previous descriptions. Tumors from males exhibited a cohesive epithelial-like growth pattern. Tumors from female mice contained cells dispersed into loose sheets and irregular strands growing in loose connective tissue. (x 812.8).


B

In addition, we observed that the tumors from female mice contained regions of extracellular material which appeared osteoid-like in H & E stained sections. As it has been shown that osteoid of cortical bone contains chondroitin sulphate and sialic acid-rich glycoproteins (Andrews *et al.*, 1969, Herring and Kent, 1963), all male and female tumors were investigated histochemically for the presence of these moieties.

The development of osteoid-like regions in female mice appears to proceed in several defined steps. First a cluster of cells lost their attachments with neighbouring cells forming a lobule (Figure 22A). The cell clusters then began to secrete an extracellular product, (Figure 22B), spreading away from each other as they did so until they achieved an osteoid-like appearance (Figure 22C). When these extracellular regions were investigated histochemically (Table 3), they stained moderately positive with the PA*/S stain, indicating the presence of sialic acids. There was a moderate increase in the staining intensity of these extracellular regions when the KOH/PA*/S technique was used indicating the presence of substituted sialic acids. Further, when stained with the KOH/PA*/AB1.0/S technique these regions stained purple, indicating the presence of sulphated moieties as well as sialic acid moieties in the osteoid-like regions. No such osteoid-like regions were observed in any of the tumors grown in male mice with any of the stains used.

It has been suggested that, under the influence of the endocrine environment, breast cancer develops from undifferentiated cells which have the ability to become either mammary secretory epithelial cells or mammary myoepithelial cells (Hayashi *et al.*, 1984). Thus, it is possible that the different hormonal milieu of the male and female mice induces different phenotypes in the breast tumor cells. Therefore, we hypothesized that the slow growing tumors of the IG male mice might have a phenotype similar to that of tumors from female mice. To investigate this possibility, near serial sections of tumors were stained with the myoepithelial cell-specific markers S-100 and MSA to display myoepithelial cells present in the tumors. All tumors stained for MSA to some

Figure 22. *Development of Osteoid-Like Regions in Tumors of Female Mice*. The development of osteoid-like regions in tumors of female mice proceeded in several defined steps. A. First a groups of cells (arrowheads) lost their attachments with the surrounding cells. B. The cell clusters then began to secrete an extracellular product (arrowheads). C. Secretion continued until an osteoid-like appearance was acheived. (x812.8)

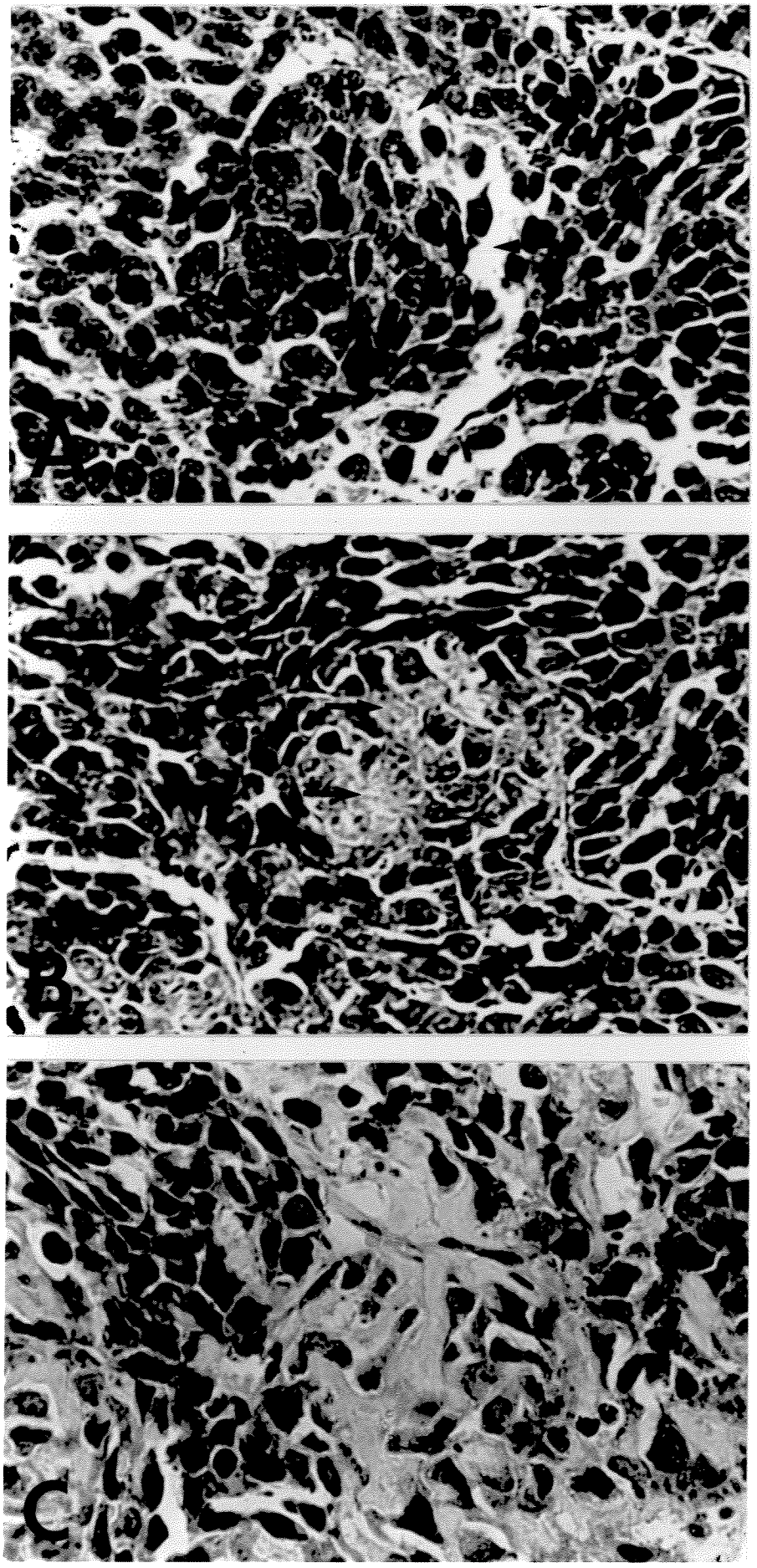

A
B
C

Table 3. *Histochemical Staining of the Osteoid-like Pools in the Female Mice*. Female mice (n=2) were injected with SC115 cells (2×10^6) and terminated when the tumors reached a mass of 1.5 g (approximately 50 d). Tissue blocks approximately 0.125 cm^3 were cut from the center and periphery of the tumors and placed immediately into 10% formalin containing 2% (w/v) calcium acetate. Blocks were routinely paraffin processed after one wk of fixation. Serial sections cut at a thickness of 5 μm were stained. Osteoid-like pools were examined.

TABLE 3. Histochemical Staining of the Osteoid-like Pools in the Female Mice.

Stain	Female Osteoid Pools
PA*S	++[1]
KOH/PA*/S	++
KOH/PA*/AB1.0/S	P,M,A[2]
KOH/PA*/AB2.5/S	P,M,A
PAS	+++
AB1.0	+
AB2.5	++
Lilles Allochrome	P,B[3]

1. +, weakly positive; ++, moderately positive; +++, strongly positive.
2. P, purple; M, magenta; A, aquamarine blue.
3. P, purple; B, blue.

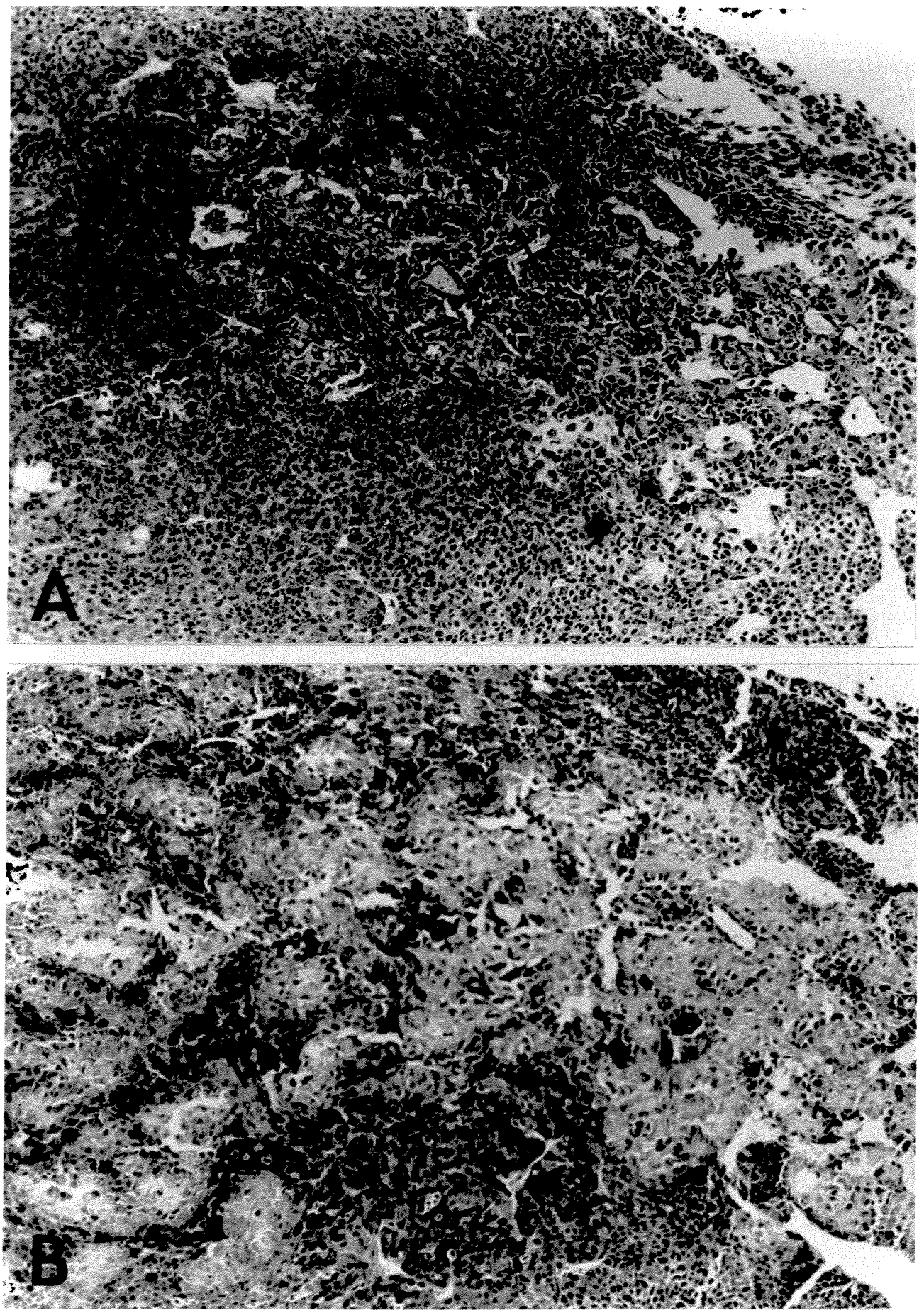
degree and staining was not observed in negative controls. Tumors from both males and females contained small clusters of strongly positive cells within the regions of viable cells. In addition, in tumors taken from female mice, a layer of MSA positive cells lined the osteoid-like regions (Figure 23A).

Specific S-100 staining was observed in all tumors examined and was not observed in negative controls. In tumors from male mice, the S-100 staining was less extensive than the MSA staining. In contrast, the tumors grown in female mice had large regions of viable cells which stained intensely S-100 positive. S-100 stained the cells in areas surrounding the osteoid-like regions, but did not stain the MSA positive cells immediately adjacent to the osteoid-like regions (Figure 23B). Further, in both males and females there appeared to be little overlap in the areas that were stained by these two techniques using near serial sections.

DISCUSSION:

It has been demonstrated that the SC115 mammary carcinoma consists of a heterogeneous population of androgen-responsive and androgen-independent cells (Bruchovsky and Rennie, 1978, Emerman and Siemiatkowski, 1984). The predominant subpopulation of the SC115 tumor grown in intact male mice is androgen-responsive, whereas an androgen-independent subpopulation is selected for when SC115 cells are grown in female or castrated male mice. These two subpopulations of cells have different growth rates and morphologies (Emerman and Siemiatkowski, 1984; Emerman and Worth, 1985). Based on previous data (Weinberg and Emerman, 1989) indicating that tumors grown in male mice moved from individual to group housing conditions (IG) and tumors grown in female mice have similar slow growth rates, it was hypothesized that the slow growth rate in mice of the IG group could be due to selection for a slow growing androgen-independent cell subpopulation similar to that in tumors of female mice,

Figure 23. *Comparison of MSA and S-100 Staining of Serial Sections From Osteoid-Like Regions of a Tumor Grown in a Female Mouse*. Comparison of MSA (**A**) and S-100 (**B**) staining of serial sections from osteoid-like regions of a tumor grown in a female mouse. The osteoid-like regions (arrows) are surrounded by MSA-positive cells. In contrast, cells positive for S-100 (arrows) are found in the areas outside the MSA-positive regions. (x 203.2)


A
B

and thus that tumors grown in IG males might also share other characteristics of the predominantly androgen-independent subpopulation of cells.

This study confirmed previous work (Emerman and Worth, 1985) showing morphological differences between tumors grown in female and intact male mice housed under our standard conditions. Importantly, tumors grown in IG males had a morphology similar to tumors of the male controls.

The presence of osteoid-like extracellular material was observed in tumors grown in female mice but not in male mice. It is notable that the slow growing tumors in IG males were again similar to tumors grown in males of the other housing groups rather than to the tumors grown in female mice.

The osteoid-like regions of the female tumors contained sulphate and sialic acid moieties similar to the osteoid of cortical bone (Andrews *et al.*, 1969, Herring and Kent, 1963). Although breast carcinomas are reported not to undergo differentiation to bone, calcification is a well documented occurrence in human breast tumors (Bouropoulou *et al.*, 1984, Frappart *et al.*, 1986; Hatter *et al.*, 1969, Sickles, 1980). Such regions of calcification have been reported to contain sialic acid moieties (Bouropoulou *et al.*, 1984). The production of extracellular material by the SC115 tumor grown in female mice may be similar to the production of regions of calcification reported in human breast cancers. Such osteoid-like regions have been previously reported in SC115 tumors grown in female mice (Kitamura *et al.*, 1979).

S-100 and MSA immunohistochemical staining patterns differed markedly from each other in both male and female tumors. Myoepithelial cells, as demonstrated by the MSA immunohistochemical stain, were found in small isolated clusters in all tumors investigated. These clusters were usually located in the center of sheets or cords of viable cells. Importantly, MSA

staining was more prominent in female tumors than in male tumors and it was associated with the osteiod-like regions in female tumors. It has been suggested that the differentiation of breast tumor stem cells into epithelial or myoepithelial cells is controlled by local environmental conditions (Hayashi *et al.*, 1984). It appears that the SC115 tumor is capable of myoepithelial cell differentiation and that this differentiation is promoted by the hormonal environment of the female. Furthermore, in tumors from female mice, myoepithelial cells appeared to be linked to the production of the osteoid-like extracellular material.

The S-100 staining pattern in males was less extensive than that seen with MSA,whereas in tumors of females the S-100 staining was at least as extensive as seen with the MSA stain. In both males and females there was no overlap between the regions stained by the two techniques. The large regions of S-100 positive cells in tumors from female mice are of particular interest. It has been shown that the S-100 protein belongs to a family of structurally related proteins which share a high degree of sequence homology and exhibit extensive cross-reactivity immunologically (Kligman and Hilt, 1988). The S-100 family consists of calcium-binding proteins which probably act as second messengers, similar to calmodulin (Kligman and Hilt, 1988), and may be involved in the promotion of cell division and the calcium-induced depolymerization of microtubles. One S-100 protein, p9ka, has been isolated from rat myoepithelial cells (Barraclough *et al.*, 1987). The lack of correlation between MSA and S-100 staining of tumors from female mice suggests that the S-100 protein being recognized is not the myoepithelial cell-associated p9ka, but rather, may be an S-100 protein involved in regulation of cell division or microtubule formation. This view is consistent with studies of primary human breast cancer, which find that both epithelial and myoepithelial cells stain positive with the S-100 technique (Dwarakanath *et al.*, 1987; Stroup and Pinkus, 1988).

In this study, it was demonstrated that although tumors grown in male mice moved from individual to group housing are similar to tumors grown in female mice with regard to their growth rate, they clearly do not resemble tumors grown in female mice in their histology. Rather, slow growing tumors of IG males are histologically similar to the fast growing tumors of control

males. These results suggest that the growth of tumors in IG mice may not be the result of selection for an androgen-independent tumor phenotype as observed in female mice.

B) Selected Studies of Endocrine Functioning in Mice from the 4 Experimental Housing Groups.

Introduction

One possible mediator of the differential tumor growth rates observed in this model is an alteration in endocrine functioning of the mice. Animals in our model experience a variety of potentially stressful stimuli, including brief anesthetization, tumor/vehicle-injection, and, for some animals, a change in housing condition and/or individual housing. It is known that androgens and glucocorticoids are responsive to stress (Amario and Castellanos, 1984, Christian and Davis, 1964, Frankel and Ryan, 1981) and also have significant effects on the growth rate of the SC115 tumor (Omukai *et al.*, 1987). Thus, it is possible that changes in plasma levels of these hormones may be responsible for the altered tumor growth rates observed in this model.

Previously we have demonstrated that, at 3 wk post injection and group formation, plasma levels of testosterone, dihydrotestosterone and corticosterone were not significantly different among mice from the 4 housing conditions (Weinberg and Emerman, 1989). This suggests that housing condition may not influence hormone levels. However, it is known that hormonal responses to stressors are dynamic and adapt to the chronic application of stressor with time. It has recently been demonstrated that when male mice are moved from individual housing to a large group, there is an immediate rise in basal corticosterone which returns to normal levels within 2 wk (Peng *et al.*, 1989) . Further, acute stressors such as ether anesthesia (used in our model during tumor/vehicle injection) have been shown to cause transient alterations in both corticosterone and testosterone levels (Armario and Lopez-Calderon, 1986, Frankel and Ryan,

1981). Thus, functionally important changes in the endocrine system may occur within the first wk post tumor cell-/vehicle-injection and group formation and have returned to normal by 3 wk.

Another possible mediator of differential tumor growth rates in our model could be a shift in the responsiveness of the tumor cells themselves to hormones. As noted, the SC115 tumor is heterogeneous, containing androgen-reponsive and androgen-independent cells (Emerman, 1988,Emerman and Worth, 1985). Growing SC115 tumor cells in an androgen-deprived environment (in a female mouse or *in vitro*) results in the selection of androgen-independent cells (Emerman, 1988, Emerman and Worth, 1985). Thus, selection for cells with greater or lesser hormone sensitivity may occur in animals in the 4 housing conditions resulting in differential tumor growth. In a previous study we demonstrated that the morphology of the slow growing tumors from mice of the IG group did not resemble morphologically the slow growing androgen-independent tumors from female mice. However, it was possible that morphology and hormone responsiveness of the tumor cells are not directly linked.

The present study was designed to test the hypothesis that the differential tumor growth rates observed in this model are the result of differences in testosterone and/or corticosterone secretion which occur in mice of the 4 experimental housing groups during the first wk post injection and group formation. Also, this study investigated the hypothesis that the differential tumor growth rates observed in this model result from the selection of a subpopulation of tumor cells which have altered hormonal responsiveness and associated changes in growth rate. To determine if the experimental conditions resulted in an early change in basal hormone levels, we examined basal levels of plasma testosterone and corticosterone early in the experimental period: 1, 3 and 7 d post tumor cell- /vehicle-injection and group formation. To determine if our experimental conditions resulted in a change in the hormone sensitivity of the tumor cells themselves, the *in vitro* hormone responsiveness of tumor cells from mice in the different housing

conditions was examined at 3 wk post tumor cell-injection (the time when the tumor mass was large enough for study).

METHODS AND MATERIALS

Animals. Two hundred and twenty two male DD/S mice (2-4 months of age) were used in this study. Animals were raised and housed as described in General Methods.

Tumors. SC115 cells used in this study were fresh cells continuously propagated in male DD/S mice as previously described in General Methods.

Plasma Hormone Levels. Two hundred and six animals were terminated 1 d (24 h), 3 d and 7 d following tumor cell- or vehicle-injection and group formation. Animals were not subjected to novelty stress on the termination day so that basal hormone levels could be measured. Cages were quickly and quietly carried from the colony room to an adjacent laboratory, animals were briefly anesthetized (15 sec) with ether and immediately decapitated. For cages with more than one mouse per cage, several experimenters were involved in the decapitation procedure to ensure than all animals were terminated within 1 min of first touching the cage. Trunk blood was collected in heparinized tubes. Following centrifugation at 2200 x g, plasma was separated and stored at -20^{o}C for subsequent assay.

Testosterone levels were measured by radioimmunoassay using a modification of the methods of Auletta *et al.* (Auletta *et al.*, 1974). Briefly, 200 µl of plasma was extracted twice with 4 mls of anhydrous diethyl ether. Samples were pooled and dried under nitrogen in a 50^{o}C water bath. Testosterone was then measured by standard radioimmunoassay (RIA). Antiserum and tracer were obtained from Radioassay Systems Laboratories, Inc. (Carson, C.A.). Samples

were counted in Formula 989 (New England Nuclear, Lachine, Quebec). Cross reactivity of the antiserum with dihydrotestosterone (DHT) was less than 20% and we have previously demonstrated that, in DD/S mice, DHT comprises less than 10% of total plasma androgen (Weinberg and Emerman, 1989).

Corticosterone was measured by radioimmunoassay as previously described (Weinberg and Emerman, 1989). Briefly, 30 µl of plasma was extracted in 270 µl absolute ethanol and total corticosterone was then measured by standard RIA. Antiserum and tracer were obtained from Radioassay Systems Laboratories, Inc. (Carson, C.A.). Samples were counted in Formula 989 (New England Nuclear) .

In Vitro Hormone Responsiveness of Tumor Cells. For these studies, mice from GI (largest tumors, n=6) and IG (smallest tumors, n=10) conditions were terminated at 17 d post injection, when the fastest growing tumors had reached a mass of approximately 3 g. Tumors were removed and dissociated to single cell suspensions as described in General Methods. Cells from 5 mice (IG) or 3 mice (GI) were pooled and resuspended at a concentration of 1.6×10^5 cells/ml in DMEM and either 2% dextran-coated charcoal-treated fetal bovine serum (DCC FBS; Grand Island Biology Co, Burlington, Ontario) alone or 2% DCC FBS plus either 10^{-7} M DHT (Sigma) or 10^{-6} M HC (Sigma). The hormone concentrations chosen were those shown previously to provide maximal stimulation of cell growth using tumors from mice housed under standard control conditions (Jiang *et al.*, submitted). Cells were plated in 96 well plates, 100 ml/well, 12 wells/condition, and incubated for 5 d at 37°C, 95% CO_2, with complete media changes on d 2 and 4. On d 5, numbers of viable cells were determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) colorimetric assay (Carmichael *et al.*, 1987, Mosmann, 1983).

Statistical Analyses. Statistical analyses were performed using appropriate analyses of variance (ANOVA) for the factors of Group, Tumor and Day where appropriate. Significant main effects and interactions were further analyzed by Tukey post-hoc tests ($p<0.05$).

RESULTS

Plasma Levels of Testosterone. The ANOVAs revealed significant main effects of Day ($F(2,186) = 9.189$, $P<0.001$) and Group ($F(3,186) = 4.499$, $P<0.005$) as well as a Group x Day interaction ($F(6,186) = 3.102$, $P<0.01$) (Figure . 24). Post hoc analysis of the Group x Day interaction revealed that, at 1 d post tumor cell- or vehicle-injection and group formation, GG and II animals had significantly greater basal testosterone levels than IG animals ($p<0.05$). Mice in the GI group also had marginally elevated plasma testosterone levels compared with mice in the IG group ($p<0.10$). At 3 d and 7 d, plasma testosterone levels in GI animals were significantly elevated over levels of animals in all other groups ($p<0.05$). The presence of a tumor did not significantly affect plasma testosterone levels, ($F(1,186) = 1.169$, $P=0.281$).

Plasma Levels of Corticosterone. The ANOVAs revealed significant main effects of Day ($F(2,182) = 6.101$, $P<0.005$) and Group ($F(3,182) = 44.252$, $P<0.001$). Post hoc comparisons of group effects revealed that IG animals had significantly elevated basal levels of plasma corticosterone compared with animals in all other groups at all time points measured, $p<0.05$ (Figure . 25). Overall, plasma corticosterone levels showed significantly greater elevations at 1 d post injection than at 1 wk post injection ($p<0.05$). As with testosterone, plasma corticosterone levels were not affected by the presence of a tumor ($F(1,182) = 0.051$, $P=0.822$).

Effect of Social Status (in Group Housed Mice) on Plasma Hormone Levels. Anovas revealed that, in mice of the GG group there was no effect of dominance on plasma hormone

Figure 24. *Plasma testosterone levels.* Mice from the 4 experimental housing groups (as described in figure 4) were injected with tumor cells (2×10^6) or vehicle and terminated on d 1, 3 or 7 post injection. To insure that hormone levels measured were basal values, mice were rapidly decapitated immediately following disturbance of their cage. Points represent mean ± SEM. At 1 d, mice in the II and GG conditions had significantly increased basal testosterone levels compared with IG mice ($p<0.05$). On d 3 and 7, GI mice had significantly greater basal levels than all other mice ($p<0.05$).

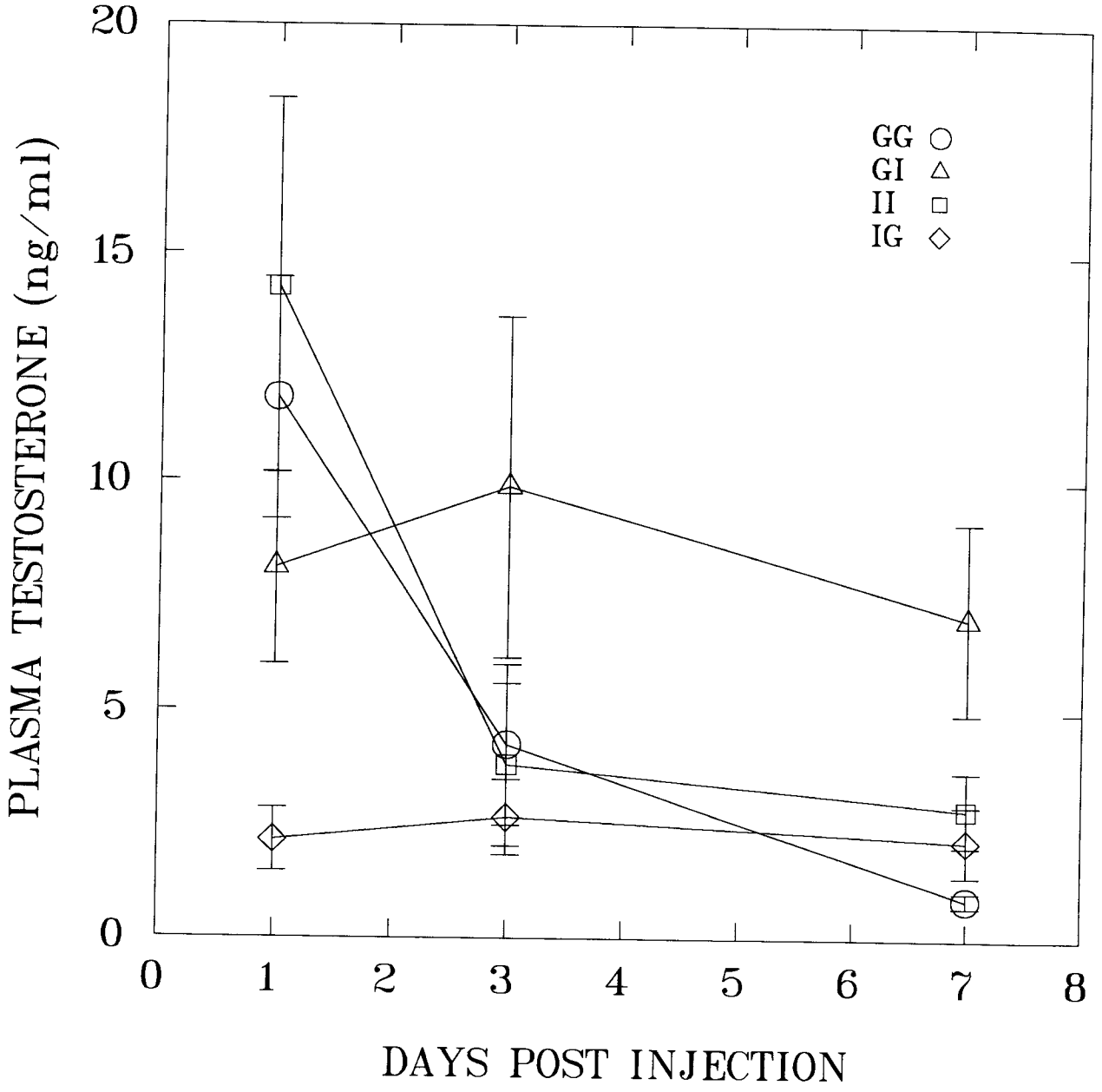

20
15
10
5
0
PLASMA TESTOSTERONE (ng/ml)
GG
GI
II
IG
0
1
2
3
4
5
6
7
8
DAYS POST INJECTION

Figure 25. *Plasma corticosterone levels.* Mice from the 4 experimental housing groups (as described in figure 4) were injected with SC115 tumor cells (2×10^6) or vehicle and terminated on d 1, 3 or 7 post injection (as described in figure 24). Points represent mean ± SEM. IG mice had significantly greater basal corticosterone levels than mice in all other conditions at all time points measured ($p<0.05$).

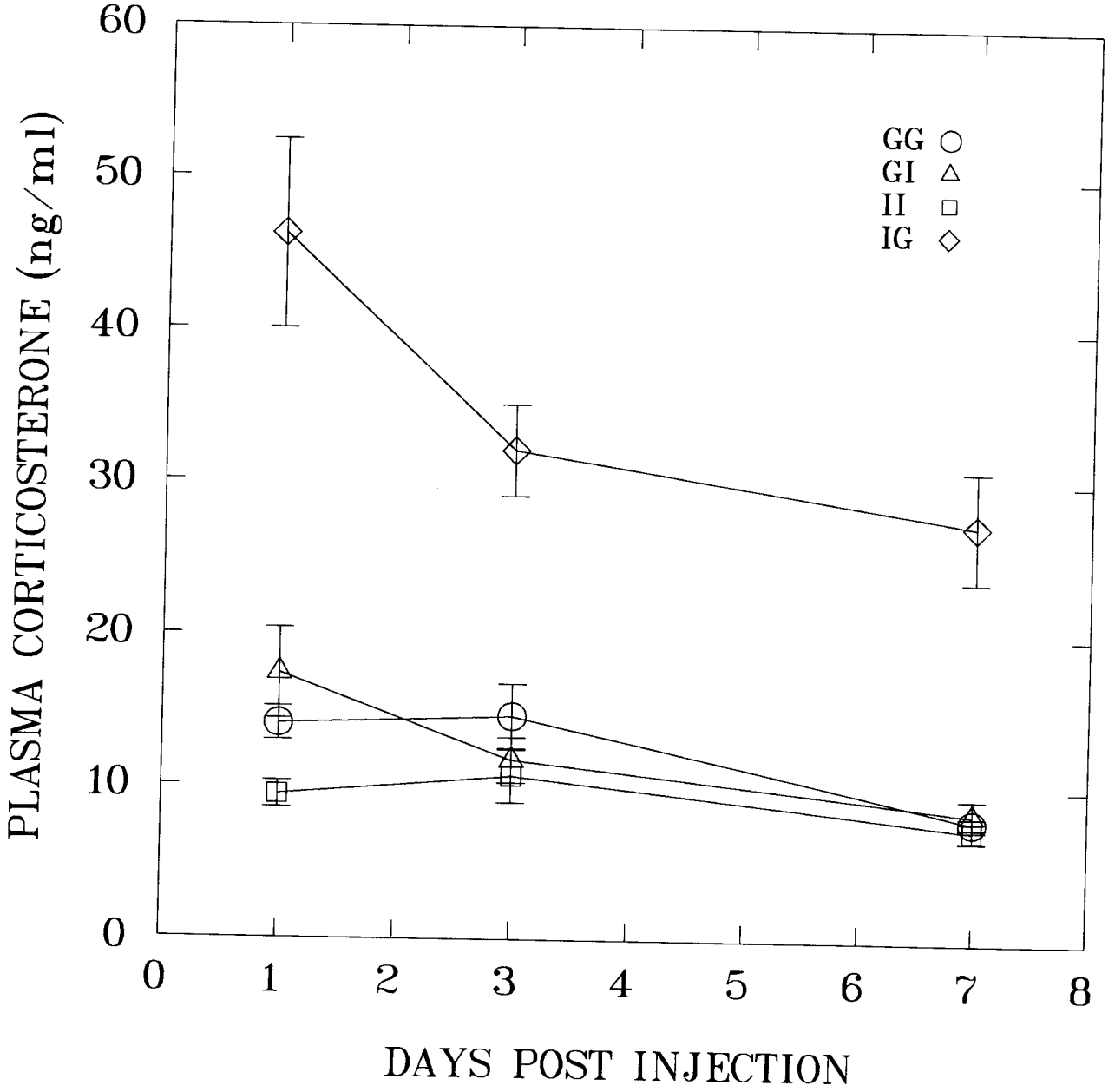

PLASMA CORTICOSTERONE (ng/ml)
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
DAYS POST INJECTION
GG
GI
II
IG

Table 4. *Effect of Social Status (in Group Housed Mice) on Plasma Hormone Levels.* Mice of the GG and GI groups (as described in figure 4) were injected with SC115 tumor cells (2×10^6) or vehicle and terminated on d 1, 3 or 7 post injection (as described in figure 24). Dominance status was assessed by examining the relative frequency of tail wounds. The mouse with the fewest or no wounds, in each group, was assessed as dominant and all other mice in the group were classified as subordinant. In mice of the GG group there was no effect of dominance on plasma hormone levels. In mice of the IG group, subordinate mice had significantly lower levels of plasma testosterone and higher levles of plasma corticosterone than did the dominant mice ($p<0.05$).

Table 4

Plasma Testosterone (ng/ml)

	GG		IG	
	Dominant	**Subordinate**	**Dominant**	**Subordinate**
Day				
1	7.56 [1] ± 6.09	12.67 ± 3.00	5.08 ± 2.35	1.19 ± 0.32
3	6.22 ± 3.76	3.49 ± 2.02	4.03 ± 1.82	2.09 ± 0.89
7	0.67 ± 0.13	0.97 ± 0.24	3.77 ± 3.15	1.69 ± 0.46

Plasma Corticosterone (ng/ml)

	GG		IG	
	Dominant	**Subordinate**	**Dominant**	**Subordinate**
Day				
1	13.86 ± 5.82	14.12 ± 1.12	30.84 ± 7.66	50.54 ± 7.44
3	15.97 ± 2.53	14.20 ± 2.76	26.54 ± 6.27	33.81 ± 3.37
7	8.61 ± 0.32	7.68 ± 0.48	17.84 ± 3.90	30.03 ± 4.31

[1] Mean ± sem.

levels. In contrast, in mice of the IG group, there was a significant effect of dominance status on plasma levels of both corticosterone ($F(1,72)=5.002$, $P<0.03$) and testosterone($F(1,70)=6.299$, $P<0.02$). Post hoc analysis revealed that, in mice of the IG group, subordinate mice had significantly lower levels of plasma testosterone and higher levels of plasma corticosterone than did the dominant mice ($p<0.05$, Table 4).

In Vivo Growth of Tumor for the In Vitro Hormone Responsiveness Study. At 17 d post injection, tumors of mice in the GI group were significantly larger than tumors of mice in the IG group (Figure . 26). These data are consistent with our previous findings using this model (Weinberg and Emerman, 1989).

In Vitro Hormone Responsiveness. *In vitro* proliferation of tumor cells from GI and IG animals in response to DHT and HC was examined. As shown in Figure 27, cells from both GI and IG mice were significantly stimulated by both DHT ($F(1,68) = 386.630$, $P<0.001$) and HC ($F(1,68) = 673.335$, $P<0.001$). Interestingly, a significant main effect of group for both DHT ($F(1,68) = 52.598$, $P<0.001$) and HC ($F(1,68) =30.130$, $P<0.001$) indicated that tumor cells from IG mice had a significantly greater response to DHT and HC stimulation than did tumor cells from GI mice.

Discussion

This study demonstrates that alterations in plasma levels of testosterone and corticosterone in the first several days post tumor cell- /vehicle-injection and group formation may play a role in mediating the differential tumor growth rates observed in mice housed under the different conditions of our model. As well, the study indicates that the slow growth rate of

Figure 26. *Tumor Growth in Mice of The GI and IG Groups.* Mice of the GI (n=12) and IG (n=20) groups were injected with SC115 tumor cells (2×10^6). Points represent mean ± SEM. Tumors were significantly larger in GI mice than in IG mice on day 17 ($p<0.05$).

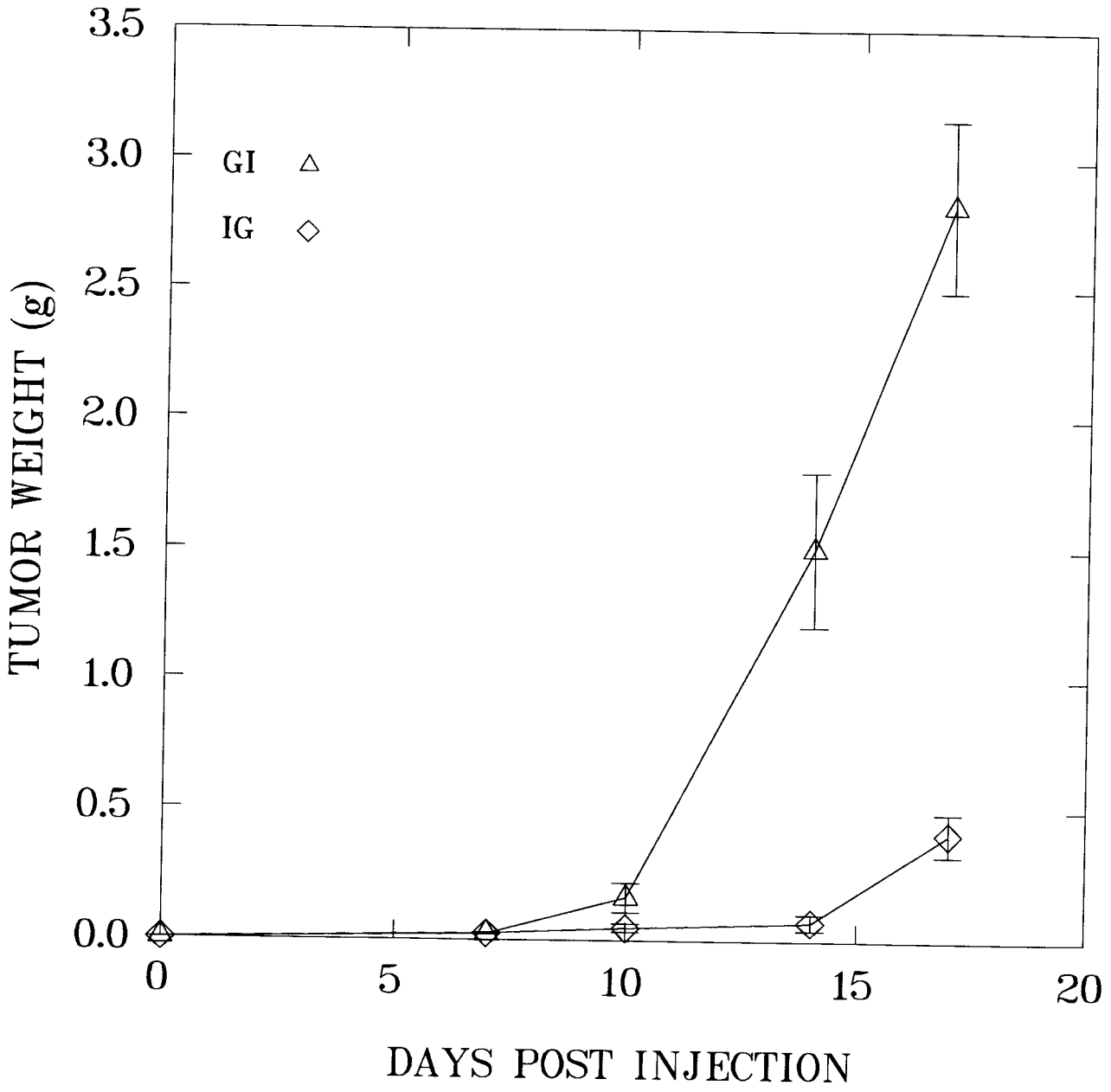
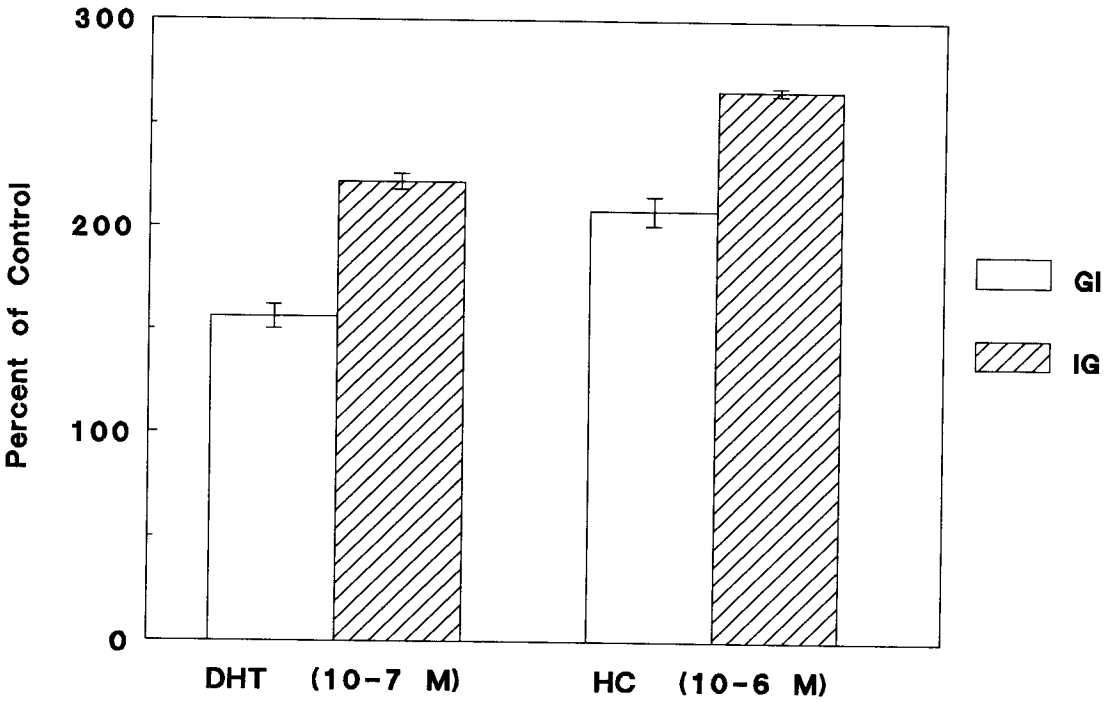

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
TUMOR WEIGHT (g)
GI
IG
0
5
10
15
20
DAYS POST INJECTION

Figure 27. *In vitro hormone response of tumor cells from IG mice and GI mice*. Mice from the GI and IG housing conditions were injected with SC115 tumor cells (2×10^6) and terminated on d 18. Tumors were removed, dissociated to a single cell suspension and cultured on collagen-coated 96-well tissue culture plates in medium containing 2 % dextran charcoal-treated fetal bovine serum alone or in combination with either 10^{-8} M dihydrotestosterone or 10^{-7} M hydrocortisone (doses previously shown to provide optimal stimulation of SC115 cells in our hands (Jiang *et al.*, submitted)). After 5 d, cultures were terminated and cell growth determined by the MTT assay. Bars represent mean ± SEM. Cell growth was expressed as percent of cells in control cultures. Tumor cells from IG mice were significantly more responsive to both DHT and HC than cells from GI mice ($p<0.05$).


300
200
100
0
Percent of Control
DHT (10-7 M)
HC (10-6 M)
GI
IG

tumors in mice of the IG group was not due to the selection for a hormone-independent subpopulation of tumor cells.

Plasma testosterone levels were elevated in mice of the GG, GI and II groups, but not in mice of the IG group, at 1 d post injection and group formation. At 3 d and 7 d, testosterone levels of mice in the GG and II condition had declined, whereas the levels of mice in the GI condition remained elevated. The elevation of plasma testosterone levels 1 d post injection and group formation may be associated with an increase in level of arousal induced by this procedure. Frankel and Ryan (1981) have shown that, in rats, anesthetization causes a rise in plasma testosterone levels which is followed by a suppression 4 to 8 h later. In our model, the stimulation of testosterone secretion was of a much longer duration, and the suppressive effect was not apparent. However, our procedure involved transporting mice to an adjacent laboratory, a brief anesthetization, injection of tumor cells/vehicle, placing animals in a clean cage and, in some cases, a change of housing condition (GI and IG). While any of these factors alone would probably be a mild stressor for the animal, their combination could result in a longer lasting effect.

Although mice in both the GI and II conditions were housed individually following tumor cell- /vehicle-injection, they did not demonstrate similar patterns of testosterone secretion. Testosterone levels remained elevated for 7 d in mice of the GI condition, whereas they declined by 3 d in mice of the II condition. It has been shown that isolated mice are hyper-responsive to external stressors (Bronson, 1967). It is possible that mice which were moved from group to individual housing were more sensitive to external stressors than were mice raised as individuals. Thus, mice in the GI condition may adapt more slowly to the effects of the acute daily novelty stress and continue to display acute elevations of testosterone in response to the stress. To our knowledge, differences in the endocrine responses to stress of mice moved from group to individual housing compared to those of mice that remain individually housed from weaning have not been explored previously. Interestingly, mice of the GI and II groups also differed in their

levels of splenic NK cell activity at 3 d post tumor cell-injection. Thus, it is likely that the prior housing condition significantly affects mice housed individually for the experiment.

Animals in the IG condition, which have the slowest tumor growth rates, did not show an increase in plasma testosterone levels 24 h post injection and group formation. It is likely that, for mice of the DD/S strain, a density of 5 mice per cage constitutes crowding. In rodents, crowding causes a suppression of testosterone secretion (Koike and Noumura, 1989) and decreases the influence of stressors on plasma testosterone levels (Armario and Lopez-Calderon, 1986). Although 5 mice per cage is a lower density than is traditionally used for crowding studies (Ortiz *et al.*, 1985), the DD/S strain of mice appears to be more aggressive than other strains. Males of other strains are routinely housed in groups of 5-7 per cage (Brain and Nowell, 1970); however we are unable to house DD/S males in groups of 5 for periods longer than 6-8 wk since wounding and occasionally even death will result. Observation of male DD/S mice housed in groups of 5 revealed that, in a 45 min period after lights out, there may be as many as 6-10 fights per cage (Sault *et al.*, in preparation). Furthermore, a period of isolation prior to crowding is known to increase the aggression of male mice (Valzelli, 1973) and crowding effects have been reported with mice housed 4 per cage when the mice were isolated for several wk prior to group housing (Bronson, 1973). Thus, it is possible that mice of the IG condition did not exhibit increased testosterone levels at 1 d post group formation because the suppressive effects of crowding compete with the stimulatory effects of anesthetization and/or tumor cell- /vehicle-injection.

During the first wk post tumor cell- /vehicle-injection and group formation, basal levels of plasma corticosterone were significantly elevated in IG mice compared with mice in all other groups. It has been reported that crowding elevates plasma glucocorticoid levels in male mice (Bronson, 1973, Peng *et al.*, 1989). This effect has been attributed to the fighting involved in the establishment of dominance hierarchies (Brain, 1975, Peng *et al.*, 1989). Since mice of the IG condition fight significantly more than mice of the GG condition (Sault *et al.*, in preparation) this

could explain their increased plasma corticosterone levels. Interestingly, further analysis of our data indicated that for mice of the IG condition, subordinate mice had significantly suppressed basal testosterone and increased basal corticosterone levels compared to dominant mice. Such differences were not observed in mice of the GG condition. The importance of dominance in modulating tumor growth rate is currently being investigated.

Importantly, glucocorticoid levels have been shown to exert a regulatory influence on testosterone levels (Sapolsky, 1986) . Chronically elevated glucocorticoid levels are thought to depress plasma testosterone levels by inhibiting the actions of LH on Leydig cells (Sapolsky, 1986). Thus, the pituitary-adrenal axis may be indirectly involved in the regulation of tumor growth rates by exerting a modulatory action on plasma testosterone secretion. In our model, basal corticosteroid levels were elevated in IG mice and testosterone levels were low. Conversely, basal corticosterone levels were low in all other groups and testosterone levels were high at 1 d post injection. Consistent with our results, Hiraoka *et al* (1987) demonstrated *in vivo* that doses of corticosterone comparable to those seen in IG mice (10^{-7}M) inhibited the growth-stimulating effect of physiological doses of androgen.

Finally, our data indicate that the differential tumor growth rates observed in our model are not due to altered hormone sensitivity of the tumor cells. *In vivo*, tumors in IG mice had a slower growth rate than tumors in GI mice, whereas *in vitro*, cells from tumors of IG mice consistently grew faster and were actually more responsive to DHT and HC than cells from tumors of GI mice. These data suggest that the slower growth rates of tumors in mice of the IG group result from alterations in the internal environment of the mice rather than from a decrease in the tumor cell's ability to respond to hormones. These data are consistent with our previous findings on tumor morphology (study 4A. of this chapter). The previous study indicated that tumors grown in male mice of the 4 housing groups were similar to each other in morphological characteristics and were also similar in morphology to SC115 tumors grown in male mice raised

under the standard laboratory conditions. In contrast, tumors grown in female mice, a condition known to select for androgen-independent subpopulations of tumor cells, demonstrated a morphology which differed considerably from that observed in the male mice. Thus, these data suggest that selection for an androgen-independent phenotype did not occur in mice of the IG group. Further, we have demonstrated that tumors cells grown in mice from the 4 housing conditions did not differ in terms of their of androgen or glucocorticoid receptor levels (Rowse *et al.*, 1992). Together, these data provide strong evidence that the differential tumor growth rates observed in this model are not due to the selection of subpopulations of SC115 cells with different hormone responsiveness but rather are due to alterations in the environment of the cells (i.e. altered plasma hormone levels and altered NK cell activity).

In summary, we have demonstrated that alterations in basal levels of plasma testosterone and corticosterone occur in the first wk following tumor cell/vehicle injection and group formation. These alterations likely represent an important factor modulating the differential tumor growth rates observed in our model. Future studies will examine this issue using males from the 4 housing conditions that have been castrated and/or adrenalectomized and implanted with osmotic pumps to maintain chronically high or chronically low basal hormone levels.

CHAPTER 5 DISCUSSION

This thesis examined several potential physiological mediators of psychosocial stressor effects on the growth of the SC115 tumor. In our animal tumor model, the growth of the androgen-responsive SC115 tumor is modulated by an animal's social housing condition. Mice which are raised in groups from weaning age to adulthood and are subsequently housed as individuals for the course of the experiment (GI) have significantly increased tumor growth rates compared with those in mice who remain group housed for the experiment (GG). In contrast, mice who are raised as individuals and are subsequently rehoused in groups for the experiment (IG) have significantly decreased tumor growth rates compared with mice of the GG group. Previous evidence suggested that the growth of the SC115 tumor may be influenced by both immune (Watanabe et al., 1982, Nohno et al., 1986) and endocrine (Emerman and Worth, 1985, Omukai et al., 1987) variables. Thus, this dissertation examined the role of specific immune and endocrine variables in modulating the differential tumor growth rates observed in this model. The data demonstrate that both immune and endocrine variables are likely to play an important role in modulating the growth of the SC115 tumor.

Our studies of the immune system demonstrated that the presence of the SC115 tumor stimulated NK cell activity both in the spleen (Rowse et al., 1990) and at the tumor site. Interestingly, at both sites, NK cell activity was found to be greater in mice of the GI group, those mice who develop the largest tumors, than in mice of the IG group, those mice who develop the smallest tumors. One interpretation of these data is that NK cells may actually stimulate rather than inhibit the growth rate of the SC115 tumor. This hypothesis was investigated by modulating (increasing or decreasing) *in vivo* NK cell activity in mice of the GI and IG groups and monitoring tumor growth rate. Modulation of *in vivo* NK cell activity did not affect the growth rate of tumors in mice of the IG condition. Importantly, however, it was discovered that in mice of the

GI group, *in vivo* modulation of NK cell activity was accompanied by a corresponding change in tumor growth rate. That is, protocols which increased NK cell activity also stimulated the growth rate of the SC115 tumor relative to that observed in mice with suppressed NK cell activity. Therefore, the data on NK cell activity in mice from the different housing groups support the hypothesis that NK cells play a role in stimulating growth of the SC115 tumor in mice of the GI group. This finding is consistent with a study by Wei and Heppner (1987) which demonstrated that NK cells increased the malignant transformation of preneoplasmic mouse mammary epithelial cells. The mechanism by which NK cells may stimulate the transformation or growth of malignant mammary epithelial cells in not known. However, it is possible that cytokines or peptide hormones secreted by activated NK cells may stimulate the growth of the mammary tumor cells.

The endocrine system also appears to play a role in mediating the effects of psychosocial stressors on the differential tumor growth rates observed in our model. The data indicate that, in the first 7 d post group formation, there is a strong correlation between plasma levels of testosterone and tumor growth rate. Mice of the GI group, who develop the largest tumors, have elevated basal levels of plasma testosterone throughout the first 7 d post group formation. In contrast, mice of the IG group, who develop the smallest tumors, maintain low basal levels of plasma testosterone through out the first 7 d of the experiment. Mice of the GG and II groups, who develop tumors of intermediate size, have elevated basal levels of plasma testosterone at 1 d post group formation, but, by 3 d, plasma testosterone levels decline to the low basal levels of mice of the IG group. Altered plasma levels of testosterone are likely to effect the growth rate of the SC115 tumor as these cells possess functional androgen receptors and are stimulated to proliferate in the presence of physiological levels of testosterone *in vitro* (Bruchovsky and Rennie, 1978, Emerman and Worth, 1985, Hiraoka et al., 1987). Interestingly, basal plasma levels of corticosterone, although elevated in all groups at 1 d compared with 3 and 7 d post group formation, were significantly elevated in mice of the IG group compared with all other groups during the first 7 d of the experiment. The elevated plasma corticosterone levels could affect

tumor growth in several ways. These include the possibility that elevated levels of corticosterone could interfere with the ability of testosterone to stimulate the growth of the SC115 tumor (Hiraoka et al., 1987). These conditions occur in mice of the IG group who have low basal levels of plasma testosterone and high basal plasma levels of corticosterone. In addition, elevated plasma corticosterone levels have been shown to decrease the secretion of testosterone by a direct effect on the Leydig cells of the testis (Sapolsky, 1986). Thus, it appears very likely that alterations in the secretion of testosterone and corticosterone play an important role in mediating the effects of psychosocial stressors on the differential tumor growth rates observed in this model.

Interestingly, all available data suggest that selection for a slow-growing hormone-independent SC115 cell subpopulation, similar to that observed in female DD/S mice, does not occur in mice of the IG group. This suggestion is supported by 3 independent lines of evidence. First, data from this dissertation demonstrate that, *in vitro*, SC115 cells from tumors grown in mice of the IG group exhibited greater rates of proliferation in response to DHT or HC than did cells from tumors grown in mice of the GI group (Rowse et al., 1992). Second, it was previously demonstrated by our laboratory that SC115 cells from mice of the 4 experimental housing groups did not differ in their maximum binding capacity or binding affinity for both the androgen and the glucocorticoid receptor (Rowse et al, 1992). Finally, as demonstrated by the data in this thesis, tumors grown in mice from the 4 housing groups exhibited morphological characteristic similar to each other and to tumors from mice reared in standard housing conditions and different from the morphology of androgen-independent tumors grown in female mice (Rowse *et al.*, 1990). Thus, we conclude that the differential tumor growth rates observed in this model probably do not arise as a result of the selection for a subpopulation of SC115 cells with altered hormone sensitivity. Rather, *in vivo* alterations in the environment of the cells, such as plasma hormone levels and NK cell activity, may be involved in modulating tumor growth rate. However, heterogeneity for other characteristics than hormone responsiveness may also be involved in mediating the differential tumor growth rates observed in this model.

The data presented in this thesis suggest that several physiological mechanisms may interact to produce the differential tumor growth rates observed in this model. It is possible that, in mice of the GI group, the increased rate of tumor growth results from the combined stimulatory actions of elevated plasma levels of testosterone and of activated NK cells at the tumor site. In contrast, the decreased tumor growth rate of mice in the IG group may result from the low plasma testosterone levels and an inhibitory action of the elevated plasma levels of corticosterone. Although these studies have revealed several physiological mechanisms by which psychosocial stressors may affect the growth rate of the SC115 tumor, many questions still remain.

1.) Further investigation is required to confirm the role of altered plasma levels of testosterone and corticosterone in mediating the differential tumor growth rates. As mentioned previously, although the alterations in plasma levels of testosterone are strongly correlated with the changes in tumor growth rate, it is not clear if the magnitude of these changes is sufficient to cause the differential tumor growth rates. This question could be investigated by monitoring tumor growth in adrenalectomized and castrated male mice implanted with osmotic pumps containing low or high doses testosterone and/or corticosterone. This would demonstrate the effect of maintaining chronically high, chronically low or no plasma testosterone, both alone and with either chronically high or chronically low basal corticosterone levels. Such an experiment would reveal if the alterations in plasma hormone levels observed in the current study are of a sufficient magnitude to induce the differential tumor growth rates observed with this model. As well, this experiment would allow the assessment of the relative contributions of plasma corticosterone and testosterone to the differential tumor growth of the model.

2.) It is possible that other hormones may also be involved in mediating the differential tumor growth rates observed in this model. One interesting candidate is opioid peptides. Studies in our laboratory have indicated that opioid peptides cause a suppression of DHT-, HC-, and

bFGF- stimulated SC115 tumor cell growth *in vitro* (Jiang *et al.*, submitted). Further, it is known that various stressors induce the secretion of the opioid beta-endorphin from the pituitary and enkephalins from the adrenal gland (Rossier et al., 1977, Viveros et al., 1979). Thus, it is possible that endogenous opioids may mediate the suppression of SC115 tumor growth rate observed in mice of the IG condition. Another category of hormones which could potentially play a role in modulating the differential tumor growth rate observed in our model are the lactogenic hormones. As mentioned in the introduction, lactogenic hormones such as prolactin and growth hormone are known to affect the growth and differentiation of normal mammary epithelial cells (Imagawa *et al.*, 1990). As the SC115 tumor originated from the malignant transformation of breast epithelium of a female mouse and it may retain responsiveness to the growth promoting effects of lactogenic hormones. This possibility is supported by the finding that the androgen-responsive SC115 tumor subline used in these studies possesses functional estrogen receptors and respond to estrogen (Nohno et al., 1982). The effects of lactogenic hormones on the growth of the SC115 tumor should be assessed both *in vivo* and *in vitro*.

3.) It is possible that, in addition to NK cells, other immune effector cell populations play a role in mediating the effects of pyschosocial stressors on the differential tumor growth rates observed in our model. Data from several experiments in our laboratory suggest that CTL may be stimulated by the presence of the SC115 tumor. First, as reported in this thesis, there is a marked increase in the relative percentage of lymphocytes and monocytes in white blood cells infiltrating tumor cell-injected sponges 7 d post injection compared with that in vehicle-injected sponges. This finding suggests that a specific cell-mediated immune response could be occurring in these animals. This possibility is supported by the findings of Hoffman (1988), who demonstrated that there was a significant increase in the percentage of lymphocytes infiltrating polyurethane sponges during an active immune response against allogeneic peritoneal cells. Furthermore, our laboratory has demonstrated that antitumor immune-activity against [^{51}Cr]-labeled SC115 cells may be generated by culturing splenic lymphocytes with mitomycin C inactivated SC115 cells for 6 d *in*

vitro (G. Rowse, unpublished observations). Importantly, this antitumor immune-activity was only observed when spleens of mice previously injected with SC115 cells 9 or 14 d prior to harvesting of the splenocytes were used as the effector cells in the 6 d *in vitro* culture. Such activity was not generated in 6 d cultures of splenocytes from vehicle-injected mice or from mice injected with SC115 cells 21 d prior to the harvesting of the splenocytes (G. Rowse, unpublished observations). These findings strongly suggest that CTL may play a role in modulating the growth of the SC115 tumor. Future studies could examine the role of CTL in mediating the effects of psychosocial stressors on tumor growth rate in our model by measuring the ability of tumor-infiltrating lymphocytes to lyse [^{51}Cr]-labelled SC115 cells and by directly inhibiting *in vivo* CTL activity (using Lyt 2 antibody).

In addition to these future lines of research, it is of interest to examine the implications of the current studies for human breast cancer. Of course, caution must be exercised when extrapolating data from animal models to the human condition. Biological differences may exist between humans and rodents in the regulation of physiological mechanisms. Further, it is difficult to compare directly the psychological experiences of experimental animals with the psychological experiences of humans. Nevertheless, with these cautions in mind, certain correlations between the current studies and findings in human studies are evident. Many studies in humans have suggested the existence of a link between stressors and the altered growth rate of cancer. The most compelling of these studies are the works of Grossarth-Maticek (1989) and Spiegel (1989), who independently demonstrated that psychological counselling could significantly extend the life expectancy of women with advanced breast cancer. Several large prospective studies provide support for these findings, indicating that decreased levels of meaningful social contact are associated with increased susceptibility to cancer and poorer prognosis (Kaplan and Reynolds, 1990, Ell, 1992, Waxler-Morrison, 1991). Our animal model also demonstrates that the level and type of social contacts can significantly influence the growth rate of tumors in mice. The physiological mechanisms by which psychological counseling affects the survival of women with

advanced breast cancer are not known. Our model indicates that, in mice, NK cell activity and altered plasma hormone levels are involved in mediating the effects of psychosocial stressors on breast tumor growth rate. It is possible that similar physiological processes mediate the effects of psychological councelling in women with breast cancer as well.

We observed that stimulation of NK cell activity is accompanied by increased growth of the SC115 tumor. Interestingly, it has been shown that NK cells stimulate the malignant transformation of hyperplastic alveolar nodules in the mammary glands of mice to a neoplastic state (Wei and Heppner, 1989). These studies suggest that NK cells may stimulate the growth of tumor cells in the early stages of breast tumor development. However, many studies, in both humans and animals, suggest that NK cells are important in the destruction of tumor cells and especially in the control of blood-borne metastases (Aslakson et al., 1991, Johnson et al., 1990, Greenberg et al., 1987). Prospective studies in humans demonstrate that greater levels of NK cell activity in peripheral blood lymphocytes are a positive prognostic indicator for increased survival of women with breast cancer (Levy et al., 1985). Further, studies in humans demonstrate that suppression of immune reactivity of lymphocytes in tumor-draining lymph nodes occurs as tumors grow in size (Reiss et al., 1983) and studies of lymphocytes infiltrating human breast cancers indicate that there is a suppression of NK cell activity in this cell population compared with that of the peripheral blood (Bonilla et al.,1988). Thus, studies in humans indicate that NK cells may be involved in suppressing tumor growth and metastases, i.e. the development of the tumor may be associated with suppression of NK cell activity. These data are consistent with our findings in the SC115 model, however, as we have demonstrated that, in addition to a positive correlation between NK cell activity and tumor growth rate in early tumor development, NK cell activity declines as the tumor grows in size.

Currently it is not known if our observation that NK cells stimulate the growth of a transplantable murine breast tumor early in the development of the tumor is also applicable to

other tumors in rodents and or human tumors. It is possible that NK cells may play different roles in modulating the growth of breast tumors depending on the site of action considered. In the primary tumor, NK cells could stimulate the early development of the tumor. This effect could result if the tumor cells were relatively resistant to lysis by the NK cells but were stimulated to proliferate by a cytokine or hormone-like peptide produced by the activated NK cells. Thus, for the tumor, the proliferation of the tumor cells in response to cytokines or hormones released by NK cells may outweigh the lysis of tumor cells by NK cells. In contrast, in the blood, the concentration of tumor cells may be much lower allowing more NK cells to bind to these tumor cells, thus increasing the ability of the NK cells to lyse the tumor. Furthermore, the concentration of cytokines and NK-derived hormones in the blood would be diluted and may be insufficient to stimulate the growth of the tumor cells.

Studies examining the effects of *in vivo* NK cell activity on the early stages of tumor development in humans have not been performed. However, the effect of NK cells on the growth of early tumors in humans could be assessed by growing normal human breast epithelial cells in collagen gels to produce epithelial organoids (Imagawa 1990, Lawler *et al.*, 1983) and then transforming the cells by *in vitro* treatment with carcinogens (Ethier, 1987, Ganguly *et al.*, 1982). These transformed breast epithelial organoids could then be cultured with NK cells isolated from the donor's peripheral blood lymphocytes.

Similarly, it is also likely that alterations in plasma hormone levels play a significant role in mediating the stressor-induced alteration in tumor incidence and growth rate observed in human studies. We have demonstrated that, in our murine model, the alteration of plasma testosterone and corticosterone levels are likely to play an important role in mediating the effects of psychosocial stressors on tumor growth rate. As discussed previously, stressors are known to alter the secretion of many hormones in humans. Further, most human breast cancers are thought to develop initially as hormone-responsive tumors (Dickson *et al.*, 1992) and the alteration of

plasma hormone levels is thought to play an important role in the induction of breast cancer in humans (Dickson *et al.*, 1992, Secreto *et al.*, 1991). Thus, stressor-induced alterations in plasma hormone levels may be universally important in mediating the effect of stressors on tumor growth.

In summary, these experiments have demonstrated that stressors may alter the growth rate of tumors in animals. Further, in our model it was shown that alterations in NK cell activity and plasma hormone levels may play an important part in mediating the effects of psychosocial stressors on the growth of the SC115 tumor. Studies in humans suggest that similar mechanisms may be operating in the stressor-induced alteration in tumor incidence and progression.

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