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THE EFFECTS OF DIET AND CARCINOGEN ON THE NATURAL KILLER CELL ACTIVITY OF SPRAGUE DAWLEY RATS

The University of Oklahoma PH.D. 1981

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE EFFECTS OF DIET AND CARCINOGEN ON THE NATURAL KILLER CELL ACTIVITY OF SPRAGUE DAWLEY RATS

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THE EFFECTS OF DIET AND CARCINOGEN ON THE NATURAL KILLER CELL ACTIVITY OF SPRAGUE DAWLEY RATS

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DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Early Studies Which Led to the Discovery of Natural Killer Cells

One of the primary defenses of the host against tumor is thought to be the selective killing of tumor cells by tumor cell antigen-sensitized lymphocytes. This defense mechanism, known as cell-mediated immunity, was illucidated, in part, by the early studies of Mitchison and Dube (70,71), Southam and Dizon (109), Rosanau and Morton (95), Deckers et al. Wespic et al. (125), and Bard et al. (1), in which tumor-specific immunity could be conferred to a normal animal by transfer of lymphoid cells, and not sera, from tumor immune animals. Tumor-specific immunity to a wide variety of chemically and virally-induced cancers could also be induced by immunization with tumor cells. Tumor-specific immunity to a methylcholanthrene- induced tumor was first demonstrated by Foley (26), in which immunity was conferred by transplantation of growing tumor to C3H-He mice, and ligating the tumor two months later. Ligated tumors eventually disappeared, and the mice were immune to further tumor challenge by fresh tumor transplants. Sjogren et al. (107) reported that infection of mice with polyoma virus rendered the mice resistant to challenge with isogeneic, polyoma virus-induced tumors. In

a later study (106), Sjogren reported that the resistance to polyoma virus-induced tumors could be accomplished by immunizing mice with allogeneic polyoma virus-induced tumor cells. These mice, which naturally rejected the foreign tumor grafts, also developed resistance against syngeneic polyoma virus-induced tumor transplants. Resistance of these immunized mice from tumor cell challenge was an *in vivo* measurement of tumor-specific transplantation immunity. This immunity was thought to be associated with lymphoid cell reactions directed against tumor-specific transplantation antigens. An *in vitro* method of measuring this type of immunity was developed by Hellstrom (34). This technique, known as the colony inhibition assay, involved plating tumor cells into Petri dishes, and measuring the tumor cell colony formation, when cultured in the presence or absence of lymphoid cells. The general finding in human and animal studies (3,12,14,45,81,82,108) was that lymphocytes from tumor-bearing hosts reacted much stronger against their specific tumor (more inhibition of colony formation) as compared to the reaction of lymphoid cells taken from normal, non-tumor bearing hosts. In a later study, the Hellstroms *et al.* (35) reported that the lymphocytes from neuroblastoma patients reacted just as strongly against cultured neuroblastoma tumor cells as did the patient's own lymphocytes. They suggested, however, that some pre-sensitization might have taken place *in utero*. In a further study involving measurement of lymphocyte-mediated inhibition of tumor cell growth, one of the non-tumor bearing patient's lymphocytes demonstrated a strong inhibition of growth of a tumor cell culture (36). This natural cytotoxicity of lymphocytes from control animals was also observed with non-immune mouse spleen cells

cultured with Moloney sarcoma cells (50). Takasugi et al. (113) also demonstrated the growth inhibition activity of lymphocytes from normal and cancer patients. In a later study (114), lymphocytes from patients with various cancers were tested for cytotoxicity activity against cultured tumor cells. Takasugi found that the specificity of cell-mediated cytotoxicity was not associated with any histological type of cancer. More importantly, lymphocytes from some of the control patients also reacted against tumor cell targets. Heppner et al. (37) confirmed the inhibitory effects of lymphocytes from control patients on established cell lines, but not on short term, primary tumor cell cultures. Sendo et al. (101) also reported the natural occurrence of murine lymphocytes which were cytotoxic to BALB/c radiation-induced leukemia cells (RL01).

Svedmar and Hodes (111) reported that lymphoid cells from mice that were sensitized in vitro, were not only cytotoxic for fibroblasts of the same histocompatability, but were also cytotoxic for fibroblasts with different histocompatability antigens. They could not attribute this nonspecific "killer activity" as being a general toxic effect of sensitized lymphocyte products, since cell-free supernatants did not demonstrate the toxic effect directed against these different histocompatable type fibroblasts.

Keissling et al. (52) adopted the term "natural killer cell" (NK cell) for those naturally occurring lymphoid cells which had the capability of lysing tumor cell targets in vitro. The target cell used in their assays was a Moloney virus-induced leukemia cell line (the murine cell-line, YAC-1) which had been adapted to suspension culture (11). The assay involved labelling YAC-1 cells with ^{51}Cr (7) and mixing these

tumor cells with various numbers of effector cells (NK cells). Release of ^{51}Cr commenced in minutes, and the test was usually complete within 4 hours at 37°C. The measure of killing was the amount of ^{51}Cr released from the YAC-1, NK cell mixture, compared to the amount of ^{51}Cr released from the YAC-1 tumor cells cultured alone. This ^{51}Cr release assay is the standard by which many investigators measure NK cell activity. Other investigators, however, have used a post-labelling assay (21,72) in which NK cells and tumor target cells are allowed to react before a radioactive label is added. This type of assay measures the amount of radioactive label incorporated by tumor cells which have been exposed to NK cells, and compares this amount with the uptake of tumor cells cultured alone. This measurement is calculated using the following formula: Percent of Cytotoxicity =

$$1 \text{ minus } \frac{(\text{cpm of NK cells + tumor cells}) \text{ minus } (\text{cpm of NK cells alone})}{(\text{cpm of Tumor cells cultured alone})}$$

$$\times 100$$

Characterization of the NK Cell

Attempts to characterize NK cells as to cell lineage has been a difficult problem. Early studies by Takasugi et al. (112) and Kiuchi and Takasugi (54) postulated a non-T, non-B cell lineage for NK cells in humans. Granulocytes were shown to cause detachment of adherent tumor target cells and were, at first, thought to be the effector cell, however, nonspecific cytotoxicity remained in the granulocyte free cell syspensions. Fractionation of effector cell suspensions revealed that the "N" cell was neither a mature T-cell nor a mature B-cell, based on failure to form rosettes with sheep red blood cells and the inability

to form complement receptor rosettes. They were, however, retained on IgG coated columns, indicating the presence of an Fc receptor.

Most investigators ruled out the B-cell as being associated with NK cell activity by testing fractions of NK cells for the presence of complement receptors (54, 73, 126), membrane bound immunoglobulins (54, 102), and HLAB antigen in humans (51). Recent work by Koo and Hatzfield (58), demonstrated that mice failed to exhibit NK cell activity when treated with anti-LyB-2 antiserum, a specific marker for murine B-cells. The evidence for a non-B lineage, however, was challenged by the work of Eremin (12) in which he described human NK cells as either being an immature precursor of the B-cell lineage, or alternatively, a more mature B-lymphocyte. The study was based on a sensitive, direct anti-globulin rosetting reaction, which detected small amounts of surface immunoglobulin. However, this assay might have been detecting small amounts of cytophyllic antibody, rather than inherent membrane immunoglobulins. With the exception of the above data, most evidence is negative in regards to a B-cell lineage for NK cells (51,54,73,75, 110, 125).

Recent work by Timonen et al. (115) in the human and Reynolds et al. (89) in the rat, has indicated that the morphology of Giemsa stained blood and/or spleen cell smears might be useful in the identification of natural killer cells. Timonen et al. (115) demonstrated that Percoll density gradient centrifugation could be used to separate a population of peripheral blood lymphocytes which were highly enriched in cells that exhibited high cytoplasmic to nuclear ratios, reniform nuclei, and azurophillic granules in their cytoplasms. They described

these cells as large granular lymphocytes (LGL), and showed that they possessed NK cell activity. In addition, the cells were non-adherent, had Fc receptors, and could bind effectively to target cells. Reynolds et al. (89) similarly isolated and identified large granular lymphocytes from the peripheral blood and spleens of rats. These cells appeared to be similar in morphology to the large granular lymphocytes of humans. However, not all cells with the LGL morphology could bind effectively to target cells. This led to the conclusion that there might exist subpopulations of LGL's with varying abilities to bind and eventually kill certain tumor cell targets. However, the association of enhanced NK cell activity with cell populations enriched for cells of the LGL morphology was documented in both studies. Whether or not the LGL "marker" is characteristic for all natural killer cells remains to be determined.

In most of the characterization studies, the NK cell in the human, mouse, and rat appeared to be non-phagocytic, ruling out a macrophage lineage. Recent work however, by Lohmann-Matthes and Domzig (61) has demonstrated a promonocyte with natural killer cell type activity directed against the YAC-1 cell line. These promonocytes lacked adherence properties, but further differentiated into mature macrophages upon additional incubation at 37°C for 24 to 48 hours. They also possessed Fc receptors. Morphologically, they appeared similar to the large granular lymphocyte.

Recent work by Ojo et al. (79) however, has demonstrated that treatment of NK cell syspensions with complement and antiserum against Mph-1, an antigen known to constitute a marker for macrophages and

monocytes, resulted in a reduction in NK cell activity. There is not enough experimental evidence to rule out the promonocyte as a possible early precursor of NK cells. Furthermore, cell surface marker studies and studies of the function of promonocytes will help to determine if the morphological similarities exist between promonocytes and NK cells.

Early studies by West et al. (126) demonstrated the presence of low affinity receptors for sheep red blood cells, a human T-cell marker, in cultures which possessed NK cell activity. Further confirmation of the T-cell nature came from the work of Kaplan and Callewaert (51), in which they demonstrated that human NK cell activity could be abrogated by incubation of NK cell suspension with antiserum directed against human T-lymphocyte antigen (anti-HTLA), but not when incubated with antiserum against the human B-lymphocyte antigen (anti-HLAB). In mice, cells with NK cell activity were found to have low density of Thy 1 antigen, and treatment with anti-Thy 1 antiserum plus complement resulted in a decrease in cytotoxicity (41). In a recent study by Durdick et al. (19), it was demonstrated that antiserum against specific glycoproteins found on 30% of peripheral blood lymphocytes (non-T, non-B) could be used to abolish NK cell activity in the mouse. It was postulated that this glycoprotein (asialo-GM1) might be present as a unique marker of NK cells in the mouse. Studies by West et al. (126,127), Peter et al. (85), Pape et al. (82), and Pross and Jondal (86) have readily characterized the human NK cell as also having receptors for the Fc portion of IgG. Studies in rats (73,75,102) and mice (2,43,79) however, have indicated the presence of only a low affinity Fc receptor. The exact characterization of the NK cell as to its particular cell lineage is still

under investigation, however, recent studies (38) have indicated that NK cell most likely belongs to the T-cell lineage.

Augmentation of NK Cell Activity

There are a number of conditions and treatments that have been shown to augment NK cell activity. Bacterial adjuvants, such as Bacillus Calmette Guerin (BCG) and Corynebacterium parvum (C. parvum) have been demonstrated to augment NK cell activity. Wolfe et al. (128) was first to demonstrate that injections of viable BCG organisms into C57BL/6 mice caused an increase in the ability of peritoneal exudate cells to kill syngeneic EL-4 lymphoma cells and allogeneic P-815 mastocytoma target cells. He characterized the effector cells as having the following properties: (1) lack of adherent and phagocytic properties of macrophages, (2) no effect on lytic activity when treated with anti-θ antiserum, (3) no effect on lytic activity when treated with anti-mouse Fab antiserum and complement, and (4) ability to kill syngeneic and allogeneic target cells. From these characteristics, he concluded that the effector cells were natural killer cells. A similar augmentation of NK cell activity has been reported by Tracey (116) in which intraperitoneal injection of viable BGC organisms gave rise to an increase in the peritoneal exudate natural killer cell activity. This increase was shown to be mediated by macrophages and/or macrophage products, since in vivo treatment of mice with silica, which inactivates macrophages, markedly reduced the BCG-induced augmentation. C. parvum was shown by Oehler et al. (76) to augment levels of NK cell activity in strains of inbred rats with high or low levels of natural reactivity. Intraperitoneal (I.P.) injections of 7.0 mg per rat of C. parvum was

sufficient to boost spleenic NK cell activity within 24 hours. Reynolds and Herberman (88) demonstrated an *in vitro* augmentation of rat NK cell activity in macrophage-depleted spleen cell cultures. Normally low levels of NK cell activity were boosted when cultures were incubated with *C. parvum*. Gallagher *et al.* (17), reported that infant mice, which had normally low levels of NK cell activity, aged mice, which had similarly low levels of NK cell activity, and AKR mice, which had naturally low levels of NK cell activity throughout life, were stimulated to even higher levels of NK cell activity compared to normal responding control mice, when given I.P. injections of *C. parvum*. Peter *et al.* (84) have recently reported that NK cell activity of human NK cells could be augmented with *C. parvum*. This augmentation was confined, however, to purified T-cell populations.

Tumor cells, themselves, also have been shown to augment NK cell activity. Dawkins and Shellam (15) reported that injections of a syngeneic gross virus-induced lymphoma into W/Fu rats increased the levels of NK cell activity in the spleen. These lymphoma cells (W/FuG-1) which were naturally susceptible to lysis by NK cells, boosted the NK cell activity three fold. In another study, Dawkins and Shellam (16) reported similar augmentations of NK cell activity with injections of another tumor cell line, the murine RBL-5. However, rats injected with the murine P-815 cell line demonstrated augmented natural killer cell activity to the rat W/FuG-1 line only. Bloom *et al.* (4) studied the effects of injection of BHK-21 or BHK-VSV tumor cells into BALB/c mice on the natural killer cell activity. Their findings revealed that mice injected with BHK-21 cells did not show any significant spontaneous NK

cell activity compared to control mice. However, if these BHK cells were persistantly infected with a virus such as the measles, mumps, or vesicular stomatitis (VSV) virus, the infected cells induced an increased reactivity of NK cells directed towards not only the cell originally injected, but also to an antigenically, unrelated, persistantly infected cell line such as HeLa cells infected with measles or mumps virus.

Viruses also have been shown to stimulate NK cell activity. Oehler et al. (76) demonstrated that I.P. injections of lymphocytic choriomeningitis virus (LCMV) and the Kilham rat virus (KRV) into W/Fu rats, augmented the natural killer cell activity of peritoneal exudate cells, 72 hours following virus injection. This confirmed, for the rat, the earlier work of Herberman et al. (42) in mice, in which they demonstrated augmentation of spleen NK cell activity by injections of a wide variety of viruses including Sendai, Polyoma, mouse adenovirus, LCMV, xenotropic type C virus, and mouse hepatitis virus. Recently, Welsh and Kiessling (124) reported the effects of LCMV infection of mice, and the possible role of the NK cell in pathogenesis of the virus. Although they found that injection of LCMV virus augmented NK cell activity in the spleen against a number of target cells, as previously reported (42, 124), there was no apparent specificity towards LCMV virus infected cells. Their conclusion was that the experimental evidence failed to show any possible role for NK cells in the pathogenesis of the LCMV virus.

All of the above studies used agents which were inducers of interferons, and which have been postulated to be regulators of NK cell

activity. Trinchieri et al. (117,118) were one of the first groups of investigators to establish that interferon, induced by either tumor cells or viruses, was able to enhance the NK cell activity of human lymphocytes. Similar results were reported by Gidlund et al. (30), Djeu et al. (18), and Welsh (122) in mice, utilizing a number of non-viral agents that have been shown to increase levels of interferon such as C. parvum, pyran copolymer, BCG, tilorene, and statolon. Shellam et al. (103) reported that the in vitro cytotoxicity of rat NK cells was augmented by the incubation of normal spleen cells with a soluble factor derived from a 24 hour culture of spleen cells with tumor cells. This factor possessed anti-viral activity, and was postulated to be a type 1 interferon. Oehler and Herberman (74) reported that injections of polyinosinic-polycytidilic acid (poly IC), an interferon inducer, increased the NK cell activity of normal rats, and rats whose NK cell activity was depressed by x-irradiation or glucocorticoids. Enhancement of human NK cell activity in vivo by interferon has been recently reported by Einhorn (22) in which osteosarcoma patients, who had been treated with one injection of three million units of interferon, demonstrated an increase in NK cell activity, peaking at 24 hours. The level of NK cell activity dropped to near normal levels by 48 hours. In an extended study, forty patients with a variety of cancers were tested before, and after treatment with interferon. With few exceptions, most patients exhibited an increase in NK cell activity, 24 hours following interferon treatment. In a similar study by Reithmuller et al. (91), patients with chronic aggressive hepatitis had increased NK cell activity following daily treatment with 6 to 10 million units of interferon.

However, when the dosage was reduced to 2 million units per day, the NK cell activity decreased. This decrease was again noticeable when the dosage was changed to 4 million units, twice a week, which resulted in a decrease in NK cell activity to pre-treatment level. Pre-incubation of human lymphocytes *in vitro* with interferon inducers also could enhance NK cell activity. Trinchieri and Santoli (118) pretreated human lymphocytes with 10 units of interferon and reported a two-fold increase in NK cell activity. However, if target cells were treated *first* with interferon, they became resistant to lysis by NK cells (117,118).

A recent study by Welsh *et al.* (123) also reported the protection of normal and mouse tumor cells with fibroblast type 1 interferon from lysis by endogenous or virus activated mouse NK cells. Those cells that were not protected, were those that lacked mouse interferon receptors, namely F-9 teratoma cells, xenogenic Vero cells, and an interferon resistant L1210 line (123). This paradox might be explained by the fact that normal cells might have been protected from NK cell attack by low levels of interferon, while tumor cells or virus infected cells might not have had the ability to be protected by interferon, and thus be specifically lysed by interferon "activated" NK cells. On the other hand, Peter *et al.* (85) have reported that treatment of a human myelocytic leukemia cell line (K562) or an *in vitro* derived melanoma cell line (IGR3) with interferon, resulted in an increase of susceptibility to lysis by NK cells. This difference might have been due to the specific variant of K562 used. The dual role of interferon as an antagonist and as a protagonist, in relation to NK cell activity, is not understood. It is interesting to note that, as yet, no evidence for the protective

effects of interferon on autochthonous tumors has been reported.

Suppression of NK Cell Activity

There are a number of agents that may suppress NK cell activity either directly, or indirectly by an accessory cell mechanism. The addition of some types of prostaglandins, especially the E series, to NK effector cell mixtures, has been demonstrated by Brunda *et al.* (5,6) to have a direct suppressive effect on NK cell activity. Maximum suppression of NK cell activity in normal and euthymic mice occurred when prostaglandin was present throughout the entire assay period. Prostaglandin had no suppressive effect if the spleen cells were incubated overnight or 24 hours at 37°C. This suggested that prostaglandin receptors on spleen cells were lost during the incubation period. An *in vivo* correlate to prostaglandin-mediated suppression also was reported. Inhibitors of prostaglandin synthesis, indomethacin or aspirin, were injected into animals whose NK cell activity had been suppressed due to induction of tumors by the Moloney murine sarcoma virus. The NK cell activity of these treated animals was restored to near normal levels by either aspirin or indomethacin. The results of these studies suggested that prostaglandins might mediate NK cell activity *in vivo* and *in vitro*.

The effects of another agent, B-estradiol, on NK cell activity was recently reported by Seaman *et al.* (99,100). They demonstrated that the natural killer cell activity in spleens of B-estradiol treated mice was reduced from 45% to 10%, 6 weeks following estradiol treatment. Interestingly, the response of spleen cells to lipopolysaccharide (LPS), a B-cell mitogen, was slightly enhanced, while the response to T-cell mitogens such as phytohemagglutanin (PHA) and concanavalin A (con A)

was unaltered. Estradiol did not affect natural killer activity in vitro, and was not due to a soluble factor or as a cellular suppressor. It was postulated that the effects of estradiol on natural killing might be due to depletion of bone marrow precursors of NK cells due to osteosclerosis, brought on by estradiol treatment. Further evidence for bone marrow destruction-induced suppression of NK cell activity was recently reported by Parkinson et al. (83). Using leukemogenic split-dose irradiation which reduced prothymocytes in marrow, they demonstrated suppressed NK cell activity in radiation treated C57BL/6 mice. This suppression was not reversed by treatment with interferon, indicating permanent damage to precursor NK cells.

Levels of NK cell activity have been described to be influenced by circadian rhythms (24), age (80), and starvation (98). A recent report by Zeigler et al. (129) has described a deficiency in natural killer cell activity in patients with chronic lymphocytic leukemia (CLL). They reported that 9 out of 10 patients with advanced disease had no detectable NK cell activity. Two patients, with early diagnosed disease, had measurable, but low NK cell activity. In vitro treatment of lymphocytes from these two patients, with human fibroblast interferon or interferon inducer, poly I:C. resulted in enhancement of their NK cell activity. In vitro treatment of lymphocytes from later stage patients with interferon resulted in no enhancement of their NK cell activity. The percentage of cells in CLL patients that resembled NK cells based on the presence of receptors for SRBC's and the Fc portion of IgG, was 4 times higher in CLL patients compared to control patients. Although the number of NK cells of CLL patients that could bind to target cells was

equal to that of control patients, lysis of target cells by the CLL patients' NK cells did not occur.

Ojo et al. (78) reported that C. parvum could not only augment NK cell activity in peritoneal exudate cells of mice, but could suppress NK cell activity in the spleen. This dual role depended on the route of injection. Intraveneous (I.V.) injection of C. parvum resulted in a significant decrease of natural cytotoxicity of spleen cells toward tumor cell targets, but no effect on the natural cytotoxicity of peritoneal exudate cells. Intraperitoneal injection of of C. parvum, however, resulted in an increase of the natural cytotoxicity of spleen cells. A similar result was recently reported by Lotsova (63) in which she described suppression of NK cell activity by I.V. injection of C. parvum into B6DF mice. Cell mixing experiments, however, confirmed the presence of a non-adherent suppressor cell that was activated or induced by C. parvum to suppress NK cell activity of the spleen. The suppressor cell was resistant to various agents such as cortisone, silica, carrageenan, radiation, and cytoxan. Furthermore, the suppressor cell effect seemed to be dependent on mature T-cell function, since congenitally athymic mice failed to demonstrate suppressed NK cell activity, following C. parvum injections. In addition, removal of T-cells by Sephadex G-10 or Ficoll-Hypague centrifugation resulted in release of the C. parvum induced suppression. However, only a partial decrease in NK cell activity occurred when spleen cells were treated with anti- Thy 1.2. This result lead to the conclusion that even though a mature T-cell population was strongly indicated as the suppressor cell, other cell populations could not be entirely eliminated.

Macrophages have been shown to play a role in maintaining levels of NK cell activity in mice and rats. Oehler and Herberman (74) demonstrated that silica, which paralyzes macrophages, resulted in the suppression of NK cell activity of rat splenocytes. A suppressor cell of NK cell activity has been recently reported by Cudkowicz and Hochman (13). This cell, however, was activated by carrageenan, an anti-macrophage agent. Spleen cells of B6C3F mice were treated with 100g/ml of carrageenan for 5 days, then tested for NK cell activity against YAC-1 tumor cell targets. Carrageenan-treated spleen cells had no detectable NK cell activity. The NK cell activity was restored to normal levels when the carrageenan-treated spleen cells were filtered through Sephadex G-10. However, filtrations through glass wool or exposure to carbonyl iron plus a magnetic field, failed to restore NK cell activity. The specific adherence to Sephadex G-10 and non-adherence to glass wool or carbonyl iron plus a magnetic field was reconciled by the fact that adherence properties might be separated from phagocytic properties, according to the experimental conditions used. Furthermore, the insensitivity of the suppressor cell activity to radiation strongly suggested that the suppressor cells belonged to the macrophage-monocyte cell lineage. In addition, carrageenan was also shown to activate suppressor cells in splenocytes of athymic mice, indicating that the cells could not belong to the post-thymic pool of splenic T-lymphocytes. A final interesting result of this study was that supernatants from the carrageenan-treated splenocytes were able to suppress NK cell activity, again suggesting a macrophage origin for these suppressor cells. Santoni et al. (97) have presented further evidence of

the macrophage as being a regulator cell for the inhibition, as well as augmentation of NK cell activity. They reported that both agents, pyran copolymer, and adriamycin were able to either augment or suppress NK cell activity, depending on the dose or the route of injection. In pyran suppressed mice, the suppressor cell could be removed by carbonyl iron plus a magnetic field, was not inactivated by 2000 R of radiation, nor did it possess phygocytic properties. Similar results were obtained from the adriamcin-treated mice. Their conclusion was that the macrophage was the regulator of NK cell activity. Even though there is substantial evidence for the presence of an activated suppressor cell in treated mice, there is no evidence that naturally low responding strains of mice are deficient in NK cell activity, due the presence of naturally occurring suppressor cells (27).

Role of NK Cells In Vivo

There have been a number of studies which have indicated that NK cells might be important as anti-tumor effector cells. Herberman and Holden (40) and Keissling et al. (52) have observed that tumors that were sensitive to NK cell activity in vitro grew poorly in nude mice. Furthermore, tumor cells were more difficult to transplant into 5 to 10 week-old mice, the age that correlates with peak NK cell activity, compared to mice whose activity had declined with age (39). In addition, Riccardi et al. (90) reported that lethally irradiated mice could reject normal or drug-treated lymphoma cells. Haller et al. (33) demonstrated that one could transfer bone marrow precursor cells from high or low responding NK strains to lethally irradiated NK strains and produce mice whose NK cell activity and resistance to tumor was that of the

donor. An interesting note in these studies was that the ultimate test for in vivo NK cell activity was measured by resistance to a small innocula of tumor cells. This suggested that NK cells might play a role in the early stages of tumor cell growth (40). There are no present studies which have defined an in vivo role of NK cell activity in the development of spontaneous or chemically-induced tumors, or in combination with promoters. However, some evidence as to the effects of carcinogens or promoters on NK cell activity has recently been reported (20,31).

Erlich et al. (20) have recently obtained data on the effects of two chemical carcinogens, 7,12-dimethlbenz(α)anthracene (DMBA) and urethane, on the NK cell activity in mice. They found that the NK cell activity was suppressed for up to 6 weeks following DMBA administration. In addition, the number of cells per spleen was also decreased. In contrast, however, urethane treatment caused an increase in the NK cell activity of these mice. These apparent discrepancies, however, emphasized that carcinogens could produce early changes in NK cell activity, which might be important in early tumor development. The tumor promoter, phorbol-12myristate-13-acetate (PMA) has been recently shown by Goldfarb and Herberman (31) to inhibit mouse and human NK cell activity. They postulated that the ability of the PMA to inhibit NK cell activity might be related to its tumor promoting ability. The area of carcinogenesis and tumor promoting effects on NK cell activity, however, is not well known.

DMBA has been shown to be an effective inducer of mammary gland adenocarcinomas in the rat, especially by combining carcinogen treatment

with a diet of high, polyunsaturated fats (8,9,10,53). Since fats and fatty acids have been shown to be suppressive to the immune response (65,68), the combination of DMBA and polyunsaturated fat effects on immune function were recently examined by Kollmorgen et al. (57). They reported that polyunsaturated fats and DMBA were suppressive to the immune system, as measured by lectin-induced blastogenesis. Further, tumor incidence was highest in rats on polyunsaturated fat diets whose lymphocyte responses were low compared to the lower tumor incidence of rats on low fat diets, and whose lymphocytes responses were high. In another study, Kollmorgan et al. (55) reported that the methanol extraction residue of Bacillus-Calmette-Guerin (MER) protected rats against DMBA-induced mammary carcinomas when given before carcinogen administration. They postulated that the macrophage might play a pivotal role in directing lymphoid cell responses against tumor cells, and that this direction might be influenced by diet-related inhibitory factors via serum constituents. Other investigators have reported the effects of dietary polyunsaturated fats on immune functions and tumor promotion. Hillyard and Abraham (44) reported that dietary polyunsaturated fat promoted the growth of six transplantable rat and mouse mammary adenocarcinomas. They also reported that treatment of mice with inhibitors of prostaglandin synthetase prevented the growth-promoting effect of polyunsaturated fats. Their conclusion was that the growth enhancing effect of polyunsaturated fats might be mediated through the cellular immune system, with the involvement of prostaglandins in the regulatory process. Studies by Carroll and Khor (8) first reported that rats changed from a low fat diet to a high fat diet at the time of exposure

to DMBA, had the same tumor incidence compared to rats fed a high fat diet both before, and after exposure to DMBA. Conversely, tumor incidence in rats changed to the low fat diet at the time of carcinogen administration was not different compared to rats maintained on the low fat diet continuously. Ip (47) reported that feeding of a 20% polyunsaturated fat diet to older rats (150 days) which were more resistant to DMBA tumorigenesis than younger rats (50 days old), resulted in an increased incidence of tumors in the resistant rats. He also reported that rats which were maintained on high fat diets continuously, or were changed from a low fat diet to a high fat diet after carcinogen administration, had the highest incidence of tumors compared to rats fed low fat diets continuously, or changed from high fat to low fat diets, following carcinogen administration. He concluded that the diet fed after carcinogen might influence the promotional phase of carcinogenesis. In another study (48), Ip reported that transplantation of mammary gland explants, that had been exposed to DMBA *in vitro*, transplanted with a higher incidence into rats on high fat diets than on low fat diets.

Another dietary effect upon the induction of tumors was recently reported by Lombardi *et al.* (62) and Shinozuka *et al.* (104). They reported that rats fed choline deficient diets developed fewer tumors when exposed to 2-acetylaminofluorene or ethionine. Rats exposed to these two carcinogens and fed choline supplemented diets did not develop tumors. However, experiments were not continued long enough to determine if choline was acting in a protective manner, or if it was only delaying tumor formation.

One of the interrelationships between dietary fat, carcinogen, and choline definiency is that all of these factors have been shown to play an important role in the etiology of neoplastic disease. Although some of these factors have been demonstrated to cause suppression of various *in vitro* and *in vivo* immune responses, the relationship between immune dysfunction and the development of neoplasia influenced by these factors is not well known. If natural killer cells are important in the host's immune surveillence system, then it might be predicted that immune dysfunction caused by dietary and carcinogenic influences might be reflected in altered activity of natural killer cells.

Our previous studies have shown that rats maintained on high fat diets (20% corn oil) and given DMBA, have a greater incidence of mammary tumors, and a concurrent suppressed lymphocyte response to mitogens, compared to rats on low fat diets (2% linoleic acid) and similarly given DMBA. The present study was designed to develop techniques for the isolation and partial characterization of rat natural killer cells, and to study the relationship between the high fat dietary state, and suppressed levels of natural killer cell activity directed towards a relatively sensitive tumor target cell (YAC-1), and a target cell that was originally derived from rats fed high fat diets, and given DMBA. Furthermore, our assay for natural killer cell activity was designed to use low effector to target cell ratios, 20 hour incubation periods, and measurement of inhibition of incorporation of ^{3}H-thymidine by target cells cultured with effector (NK) cell populations. This assay was used, rather than the standard 4 hour, ^{51}Cr release assay, using low effector to target cell ratios. Our assay was designed to study *in*

vitro conditions of natural killer cell activity that might reflect conditions found *in vivo*, and therefore, possibly correlate levels of natural killer cell activity with dietary states which have been demonstrated to influence the development of DMBA-induced tumors.

CHAPTER II

MATERIALS AND METHODS

Animals

Female, albino, outbred, Sprague-Dawley rats were used for these studies. Weanlings (21 days old) were obtained from Charles Rivers Breeders (Portage, Mich.) which had been certified clean of SDA, Sendai, and Kilham rat viruses. Rats were then placed and maintained on one of four diets (ICN Pharmaceutical, Inc., Life Sciences Group, Cleveland, Ohio) as outlined in Table 1. Diets were fed ad libitum and were not isocaloric. However, based on caloric density and the amount of diet consumed per day, the daily caloric intake for all groups was similar. Previous studies (53) indicated that quantitative differences in dietary fat did not influence growth rate.

Exposure to Carcinogen

One-half of the rats in each dietary group was given carcinogen, while the other half of the rats served as control. Rats were given 10 mg of 7,12-dimethylbenz(α) anthracene (DMBA) (Sigma Chemical Co., St. Louis, Mo.) in 1.0 ml of stripped corn oil, with a stomach tube at 50 days of age.

Spleen Cell Preparation

Two rats from each dietary group, with and without DMBA, were killed with nitrogen on a weekly basis. Spleens were excised, weighed,

and transferred to a tissue homogenizer (Kontes Glass Co., Vineland, N.J.). Fifteen ml of RPMI 1640 media, supplemented with 5 ml of Penicillin (5,000 units/ml) and Streptomycin (5,000 mcg/ml) (Grand Island Biological, Grand Island, N.Y.) was added, and the spleen gently homogenized to obtain a single cell suspension. This spleen cell suspension was centrifuged for 10 minutes at 550 g. The supernatant was decanted, and the resulting cell pellet resuspended in 11 ml of RPMI 1640 with antibiotics. These spleen cell suspensions were placed on ice for 10 minutes to allow settling of cellular debris and connective tissue. The supernatant containing the spleen cells was removed to another tube, and the cells counted with a Coulter Counter (Model ZBI, Coulter Electronics, Hileah, Fla.). The cell concentration was adjusted to 1.0×10^7 cells/ml by dilution with RPMI 1640 media plus antibiotics.

Removal of Adherent Cells and Macrophages

Partial purification of lymphocyte suspensions was carried out according to modified techniques of Garvin (29) and Rabinowitz (87). Ten ml plastic pipettes (Falcon Products, Cockeyville, Md.) were filled with an 8 ml volume of plastic beads (Separ-Aid, J.T. Baker, Bethleham, Penn.). The columns were washed with 15 ml of RPMI 1640 with antibiotics and supplemented with 10% horse serum (Colorado Serum Co., Denver, Co.) at 37°C. The columns were loaded with 3 ml of spleen cells at a concentration of 2.0×10^7 cells/ml and stoppered immediately with plastic caps. The columns were incubated for 30 minutes at 37°C in a 10% CO_2 incubator, to allow adherent cells (macrophages) to attach. The stoppers were removed, and the resulting enriched lymphocyte suspension was collected in conical centrifuge tubes (Corning, Corning, N.Y.). Any

remaining non-adherent cells were collected by washing the columns with 5 ml of RPMI 1640 media with 10% horse serum. The resulting cell suspension was washed twice with RPMI 1640 without serum, and resuspended in 1.0 ml of RPMI 1640 media with antibiotics and 10% fetal calf serum (K.C. Biologicals, Lenexa, Ks.). The cell suspension was counted with a Coulter Counter, and the cell concentration adjusted to 3.0×10^7 cells/ml by diluting with RPMI 1640 with antibiotics and 10% fetal calf serum. Three columns were routinely used for each spleen, and 50% to 70% of the loaded cells were recovered. Non-adherent and whole spleen cell populations were stained for nonspecific esterase according to the method of Koski (57), (See Appendix, Table 1).

Cell Separation by Density

Cell suspensions were separated using Percoll discontinuous gradient centrifugation (Pharmacia, Pitscataway, N.Y.). Percoll was made isoosmotic by diluting 9 parts of Percoll with 1 part of 10X phosphate buffered saline. This solution was referred to as stock Percoll. Different percentages of Percoll solutions were made by diluting stock Percoll with RPMI 1640 with antibiotics and 10% fetal calf serum. The resulting percentage Percoll solutions were used to construct discontinuous gradients by carefully layering 2 ml of each percent Percoll solution, starting with the highest percentage at the bottom of the tube and continuing with each successive lower percentage Percoll solution. The constructed gradients consisted of Percoll percentage solutions of 26% or 40% at the top, followed by 45%, 50%, 55%, 60% and 65%. The density range of the gradient was determined to be from 1.048 g/ml to

1.086, as determined by density marker beads (Pharmacia, Pitscataway, N.J.), (See Appendix, Figure 1). One ml of the lymphocyte suspension at a concentration of 3.0×10^7 cells/ml was carefully layered onto the top layer of each gradient. The gradients were placed in a refrigerated centrifuge (Damon/IEC, Needham Hts., Mass.) and spun at 550 g at 15°C for 30 minutes. Bands of cells appeared at each Percoll density interface, and were collected by aspirating each Percoll layer, starting at the top of the gradient. Cells that passed through the gradient and formed a pellet at the bottom of the tube also were collected. In preliminary studies, lymphocytes from each interface, with the exception of the pelleted cells, were tested for their ability to inhibit ^{3}H-thymdine incorporation into YAC-1 and R_2T_2 tumor cells, at various effector to target cell ratios, and at different incubation times (See Appendix, Figures 2, 3, and 4). For the main study, gradients were constructed with 4 ml of the 26% Percoll solution at the top of the tube to serve as a cell cushion, followed by 2 ml of the 50%, 55%, 60%, and 65% Percoll solutions. Each band or combination of bands was collected by aspirating each Percoll layer, starting at the top of the gradient. The cell suspensions were counted, and the total recovery and percentage of each cell band contributing to the total number of cells recovered from the gradients was calculated. Cells from the 26% and 50% interfaces were combined and tested for NK cell activity. Cells from the 55% and 60% interfaces were tested separately, and as a combination. Cells from the 65% interface were also tested for NK cell activity (See Appendix, Figures 2, 3, and 4).

Analysis of Cell Morphology

Spleen cell populations from each density fraction of the Percoll gradients were adjusted to 1 x 10^6 cells/ml with RPMI 1640 media with antibiotics and 10% fetal calf serum. One ml of each cell suspension was centrifuged onto glass slides for 10 minutes at 900 rpm, using a Cytospin centrifuge (Shandon Southern Instrument Inc., Sewickey, Penn.). The preparations were air dried and then fixed for 10 minutes in cold absolute methanol. The slides were stained for 20 minutes with Giemsa stain (Harleco, Gibbstown, N.J.). Slides were examined by oil immersion microscopy, and photographed using an Olympus Vanox microscope with an Olympus PM-10A photographic system (Olympus, Inc., Tokyo, Japan).

Tumor Cell Targets

Tumor cells used as targets for the natural killer cell assay were the YAC-1, (a Moloney virus-induced murine lymphoma) and the R_2T_2 (originally obtained from a rat mammary adenocarcinoma, induced by DMBA while the rat was maintained on a high fat diet). The YAC-1 lymphoma (11) was kindly supplied by Dr. Ronald B. Herberman, NIH, Bethesda, Md., through Dr. Juneann Murphy, Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma. The R_2T_2 cell line was isolated and provided by Dr. Pal-Min Tseng Loh, University of Iowa, Iowa City, Iowa. The YAC-1 cell line was maintained as a suspension culture in RPMI 1640, supplemented with 10% fetal calf serum, penicillin, and streptomycin. Growth studies indicated that the YAC-1 cells had a doubling time of approximately 24 hours, between cell concentrations of 5 x 10^5 to 2 x 10^6 cells per ml. Cell densities of 3 x 10^6 cells/ml or higher resulted in a

slower growth rates, and were not used at this concentration as targets in the natural killer cell assays. Viability of log phase cells as measured by Trypan blue exclusion was consistantly greater than 97%. An initial cell density of 5×10^5 cells/ml was selected as the concentration to be used for the target cells to ensure use of tumor cell targets that were in logarithmic growth in day to day assays. Also, this number of tumor cells could be accurately counted with the Coulter Counter.

The R_2T_2 tumor cell line was maintained as a monolayer culture in T-75 tissue culture flasks (Falcon Products, Cockeysville, Md.) in Medium 199, supplemented with 10% fetal calf serum, penicillin, and streptomycin. These tumor cells had a doubling time of approximately 24 hours. Cell densities of 3×10^5 cells/ml represented a cell monolayer that was approximately 30% confluent in tissue culture flasks and microtiter test plate wells (Falcon Products, Cockeysville, Md.). Cell densities of 1×10^6 cells/ml or greater represented 100% confluency. To ensure that the R_2T_2 tumor cells were provided with optimal growth conditions for a 24 hour incubation period, stock cultures were initiated with 5×10^4 cells/ml, and were used 24 hours later in the natural killer cell assay. These cells had a consistant viability of between 96% and 100%, as determined by Trypan blue exclusion.

Determination of Killing Ability of NK Cells Based on Cell Viability

Spleen cells obtained from the natural killer cell enriched fraction of the Percoll gradients were cultured with YAC-1 tumor cells as outlined in the assay for natural killer cell activity. At the end of the incubation period, the cultures containing the NK cells and YAC-1

tumor cells were collected and stained with a 0.5% solution of Trypan blue. Wet preparations were examined under medium power (450x) and photographed using an Olympus Vanox microscope with a Nomarski differential interference contrast attachment and an Olympus PM-10A photographic system (Olympus, Inc., Tokyo, Japan). Killing ability of the NK cells against the R_2T_2 tumor cell line was not measured, since differentiation between R_2T_2 tumor cells and rat NK cells by their relative size was not possible.

Natural Killer Cell Assay Based on ^{3}H-thymidine Incorporation

In the natural killer cell assay, YAC-1 target cells at a concentration of 5×10^5 cells/ml were cultured alone, or in combination with various concentrations of Percoll separated or unseparated spleen cells. A volume of 0.1 ml of YAC-1 tumor cells (1×10^6 cells/ml) was added to triplicate wells of a microtiter test plate. To these wells was added a 0.1 ml volume of spleen cells obtained from the different density fractions of the Percoll gradients or from the unfractionated spleen cell populations. The numbers of spleen cells added to tumor cells were 0.1 ml volumes of cells at a concentration of 16×10^6 cells/ml. 8×10^6 cells/ml, 4×10^6 cells/ml, and 2×10^6 cells/ml. The numbers of spleen cells cultured with tumor cells were expressed as the ratio of effector (spleen) cells to target (tumor) cells. Effector cells and target cells cultured alone served as controls. Cells were cultured for time periods of 3, 5, or 16 hours, followed by the addition of .025 ml of (6-^{3}H)-thymidine (1.0μCi/well, spec, activ.>15 Ci/mmol, New England Nuclear, Boston, Mass.). Following an additional 3.5 hour

incubation, cells were harvested onto glass fiber filter strips (Whatman, Clifton, N.J.) using a Multiple Automated Sample Harvester (MASH II) (M.A. Bioproducts, Walkersville, Md.). The filter strips were dried, and the cells transferred to scintillation vials to which 10.0 ml of scintillation fluid was added (Fisher Scientific, Pittsburg, Penn.). The amount of ^{3}H-thymidine incorporated into the cells was determined by liquid scintillation counting using a Beckman LS-3155T liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). Natural killer cell activity was expressed as the inhibitory activity of spleen cells cultured with YAC-1 tumor cells as compared to the ^{3}H-thymidine incorporation of the YAC-1 tumor cells cultured along (control). This was calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen Cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{\text{cpm of YAC-1 cells cultured alone}} \times 100$$

For the natural killer cell assay using the YAC-1 tumor cells as targets, the following optimal conditions were used as the standard for the day to day assays. Spleen cell populations used for the Percoll density gradient centrifugation were obtained from plastic beaded column separated cells. Effector to target cell ratios were 4:1. Incubation time was 16 hours, followed by an additional 3.5 hour incubation with ^{3}H-thymidine (see Appendix, Figures 2 and 3).

The natural killer cell assay using the R_2T_2 cell line was set up in a similar manner, with the exception of using an effector to target cell ratio of 13:1. In addition, effector cells were removed before the ^{3}H-thymidine label was added. After the 3.5 hour incubation, the

R_2T_2 target cells were removed by a 10 minute incubation with trypsin, and EDTA (K.C. Biologicals, Lenexa, Ks.). The cells were harvested, and the radioactive label incorporation determined (see Appendix, Figure 4). Natural killer cell activity was expressed as inhibitory activity of spleen cells cultured with R_2T_2 tumor cells as compared to the ^{3}H-thymidine incorporation of the R_2T_2 tumor cells cultured alone (control). This was calculated using the following formula:

$$\% \text{ of Control } {}^3\text{H-thymidine Incorporation} = \frac{(\text{cpm of } R_2T_2 \text{ tumor cells cultured with spleen cells})}{(\text{cpm of } R_2T_2 \text{ tumor cells cultured alone})} \times 100$$

CHAPTER III

RESULTS

Morphology of Percoll Separated Spleen Cells

Plate 1a shows the spleen cell preparation before separation by Percoll density gradients. A lymphocyte having a large cytoplasmic to nuclear ratio, a reniform nucleus, and azurophillic granules in the cytoplasm can be seen in the middle of the field. Various other cell types such as polymorphonuclear leukocytes, macrophages, and small lymphocytes can also be seen.

Plate 1b shows the spleen cell preparation collected from the 55% and 60% fractions of the Percoll gradients. Numerous lymphocytes with large cytoplasmic to nuclear ratios, reniform nuclei, and azurophillic granules in their cytoplasms can be seen. Other cells of the same size, but lacking granules, can also be seen.

Plate 1c shows the spleen cell preparation collected from the 65% fractions of the Percoll gradients. These cells exhibit the morphology of medium to small lymphocytes, with no indication of granules in their cytoplasms, reniform nuclei, or large cytoplasmic to nuclear ratios.

Plate 1d shows a preparation of lymphocytes collected from the 55% and 60% fractions of the Percoll gradients which have been cultured with YAC-1 tumor cells. One YAC-1 tumor cell is shown surrounded by three natural killer cells. Trypan blue staining revealed that the YAC-1 tumor cell was non-viable, while the natural killer cells were viable.

Also in the field is shown another YAC-1 tumor cell that has no natural killer cells associated with it. Trypan blue staining revealed it to be viable. These results were from only one experiment, and therefore the numbers of NK cells that bound and killed YAC-1 tumor cells were not quantitated.

Inhibition of Incorporation of ^{3}H-thymidine into YAC-1 Tumor Cells Cultured with Spleen Cells from Rats Fed Low Fat or High Fat Diets

The ability of spleen cells to inhibit ^{3}H-thymidine incorporation into YAC-1 tumor cells was expressed as a percentage of the ^{3}H-thymidine incorporation of the YAC-1 tumor cells in the presence of spleen cells, compared to the ^{3}H-thymidine incorporation of YAC-1 tumor cells cultured alone (control).

Figure 1 compares the inhibitory activity of Percoll separated and unseparated spleen cells, as a function of age, in rats maintained on a low fat diet. Inhibition of ^{3}H-thymidine incorporation was high on day 48 (10% incorporation) when tumor cells were cultured with the NK fraction. A decrease in inhibitory activity was observed on day 55. Thereafter, the inhibitory activity increased, and, from day 69 to day 90, the ^{3}H-thymidine incorporation did not exceed 9% of control values. The lesser activity of the 65% fraction and the unseparated spleen cell populations followed the same general pattern, with maximum inhibition on days 60 through 76. However, this inhibitory activity decreased again on day 83. This "cyclic" inhibitory activity was observed in the 65% fraction and unseparated spleen cell populations, but not in the NK cell fraction.

Figure 2 compares the inhibitory activity of the three spleen cell populations from rats maintained on a high fat diet. Inhibition of ^{3}H-thymidine incorporation into YAC-1 tumor cells by the NK cell fraction was low on days 50 and 57 (48% and 50% incorporation, respectively). A rapid increase in inhibitory activity was observed on day 64 (11% incorporation). Following this, there was a gradual decrease in inhibitory activity through day 78 (45% incorporation). Thereafter, inhibitory activity increased to a maximum value of 9% incorporation by day 92. The lesser inhibitory activity of the 65% fraction and unseparated spleen cell populations followed the same general pattern as the NK cell fraction, with maximum activity on day 64 (15% and 44% incorporation, respectively). However, the maximum decrease in inhibitory activity of the 65% fraction and unseparated spleen cell populations occurred earlier (day 71) compared to the decrease shown by the NK cell fraction. Nonetheless, "cyclic" inhibitory activity was observed in all three spleen cell populations.

Figure 3 compares the inhibitory activity of only the NK cell fraction from rats fed a low fat diet, to rats fed a high fat diet. Inhibitory activity of the NK cell fraction from rats on a low fat diet was significantly higher compared to the inhibitory activity of the NK cell fraction from rats fed a high fat diet, on days 48-50, $p = .0001$; days 69-71, $p = .0018$, days 76-78, $p = .0001$, and days 83-85, $p. = .0260$. The results from Figures 1, 2, and 3 indicated that; (a) rats fed low fat diets generally had higher levels of inhibitory activity associated with their NK cell fraction compared to lower levels and "cyclic" inhibitory activity of their 65% and unseparated spleen cell populations.

(b) rats fed high fat diets also had high levels of inhibitory activity associated with their NK cell fraction compared to their 65% and unseparated spleen cell populations, however, all three spleen cell populations demonstrated "cyclic" levels of inhibitory activity and (c) the inhibitory activity of the NK cell fraction from rats on the low fat diet was generally higher compared with the inhibitory activity of the NK cell fraction from rats on a high fat diet. This difference in inhibitory activity was due to the "cyclic" behavior of the NK cell fraction of rats on the high fat diet.

Inhibition of Incorporation of ^{3}H-thymidine into YAC-1 Tumor Cells Cultured with Spleen Cells from Rats Fed Low or High Fat Diets and Given DMBA on Day 50

Figure 4 compares the inhibitory activity of the three spleen cell populations from rats fed a low fat diet and given DMBA on day 50. Inhibition of ^{3}H-thymidine incorporation into YAC-1 tumor cells by the NK cell fraction was relatively low on day 55 (35% incorporation) and gradually increased thereafter, reaching a maximum between days 69 to 76, as measured by only 8% incorporation. This inhibitory activity gradually decreased to its initial level by day 92. The lesser inhibitory activity of the 65% fraction and unseparated spleen cells demonstrated the same increasing trend towards maximum inhibitory activity on day 69 (25% and 30% incorporation, respectively). However, on days 76 and 83, inhibitory activity of the 65% and unseparated spleen cells returned to their initial levels. Inhibitory activity of both populations increased on day 92. This "cyclic" inhibitory activity of the 65% fraction and the unseparated spleen cell populations was not observed for the NK cell fraction.

Figure 5 compares the inhibitory activity of the three spleen cell populations from rats fed a high fat diet, and given DMBA on day 50. Inhibitory activity of the NK cell fraction was low on day 57, as measured by 63% incorporation by YAC-1 targets. The inhibitory activity increased by day 64 (15% incorporation. By day 17, however, the inhibitory activity had decreased slightly to 20% incorporation. On day 78, the inhibitory activity increased to a maximum value of 4% incorporation. On day 85, there was another decrease in inhibitory activity (52% incorporation), followed by another increase on day 92 (11% incorporation). The 65% fraction and the unseparated spleen cell populations followed the same general "cyclic" inhibitory pattern, with maximum values of inhibitory activity on day 64 (11% and 22% incorporation, respectively). The inhibitory activity of both spleen cell populations gradually decreased over the next three weeks over a range or 40% to 70% incorporation. A slight increase in inhibitory activity in both fractions was observed on day 92.

Figure 6 compares the inhibitory activity of only the NK cell fractions of rats fed a low fat diet and given DMBA, to rats fed a high fat diet and given DMBA. Inhibitory activity of the NK cell fraction from rats fed a low fat diet and given DMBA was significantly higher compared to the inhibitory activity of the NK cell fraction from rats fed a high fat diet and given DMBA, on days 55-57, $p = .0474$ and on days 83-83, $p = .0004$. The results from Figures 4, 5, and 6 indicated that; (a) rats fed low fat diets and given DMBA had generally higher levels of inhibitory activity associated with their NK cell fraction compared to the "cyclic" behavior of their 65% fraction and unseparated spleen cell

populations, (b) rats fed high fat diets and given DMBA had higher levels of inhibitory activity associated with their NK cell fraction compared to their 65% fraction and unseparated spleen cell populations, however, all three spleen cell populations demonstrated "cyclic" levels of inhibitory activity and, (c) the inhibitory activity of the NK cell fractions from rats fed a low fat diet, and given DMBA was greater, at only two time points, compared to the inhibitory activity of the NK cell fraction from rats fed a high fat diet, and given DMBA. This difference was due to the "cyclic" behavior of the NK cell fraction from rats fed a high fat diet, and given DMBA.

Inhibition of Incorporation of ^{3}H-thymidine by YAC-1 Tumor Cells Cultured with Spleen Cells from Rats Fed Low Fat or High Fat Diets Deficient in Choline

Figure 7 compares the inhibitory activity of the three spleen cell populations from rats fed a low fat, choline deficient diet. Inhibition of ^{3}H-thymidine incorporation into YAC-1 tumor cells by the NK cell fraction was low on day 49 (75% incorporation). This inhibitory activity, however, increased to a high level (5% incorporation) by day 70, and maintained a level that never exceeded 8% incorporation through day 84. On day 19, however, inhibitory activity decreased to 45% incorporation. The inhibitory activity of the 65% fraction and the unseparated spleen cell populations followed the same general initial pattern, with maximum inhibitory activity demonstrated on day 70. However, both of these spleen cell populations showed minimal activity, thereafter.

Figure 8 compares the inhibitory activity of the three spleen cell populations from animals fed a high fat, choline deficient diet. The

inhibitory activity of the NK cell fraction was high on day 51 (11% incorporation), and decreased to 29% incorporation by day 86. The lesser inhibitory activity of the 65% fractions followed the same type of "cyclic" pattern, while the unseparated spleen cell populations, after showing a slight decrease, maintained a relatively constant level of inhibitory activity, ranging from 55% to 65% incorporation.

Figure 9 compares the inhibitory activity of only the NK cell fractions from rats fed a low fat, choline deficient diet to rats fed a high fat, choline deficient diet. Significant differences were evident at only the very early (days 40-51, $p = .0001$) or late (days 84-84, $p = .0391$ and days 91-93, $p = .0001$) time points. The results from Figures 7, 8, and 9 indicated that; (a) rats fed low fat, choline deficient diets had generally higher levels of inhibitory activity associated with their NK cell fraction compared to the "cyclic" inhibitory activity of their 65% fraction and unseparated spleen cell populations, (b) rats fed high fat, choline deficient diets generally had higher levels of inhibitory activity associated with their NK cell fraction compared to their 65% fraction and unseparated spleen cell populations, however, this "cyclic" inhibitory activity was present only in the NK cell fraction and the 65% fraction, and (c) there was little difference in the inhibitory activity of the NK cell fractions from rats low fat, choline deficient diets to rats fed high fat, choline deficient diets. This was due to the fact that the NK cell fraction from rats fed high fat, choline deficient diets, did not demonstrate substantial losses in "cyclic inhibitory activity, over a majority of the time points tested, compared to the inhibitory activity of the NK cell fraction from rats

fed low fat, choline deficient diets.

Inhibition of Incorporation of ^{3}H-thymidine into YAC-1 Tumor Cells Cultured with Spleen Cells from Rats Fed Low Fat or High Fat Diets, Deficient in Choline, and Given DMBA on Day 50

Figure 10 compares the inhibitory activity of the three spleen cell populations from rats maintained on a low fat, choline deficient diet, and given DMBA on day 50. Inhibition of ^{3}H-thymidine incorporation into YAC-1 tumor cells by the NK cell fraction increased from 22% incorporation on day 56, to less than 1% incorporation by day 70. This level was maintained at a value of no greater than 20% incorporation through day 84. On day 19, however, the inhibitory activity decreased to 58% incorporation. Both the 65% fraction and the unseparated cell populations followed the same general increasing trend, with maximum inhibitory activity on day 70 (6% and 25% incorporation, respectively). This inhibitory activity, however, decreased on day 77, increased on day 84, and decreased on day 91. This "cyclic" inhibitory activity of the 65% and unseparated spleen cell populations was not evident in the NK cell fraction.

Figure 11 compares the inhibitory activity of the three spleen cell populations from rats fed a high fat, choline deficient diet, and given DMBA on day 50. On day 58, the NK cell fraction demonstrated little inhibitory activity (95% incorporation). This was followed by a slow increase in inhibitory activity over a three week period, reaching a peak of inhibitory activity of 20% incorporation by day 79. This was followed by a decrease to 41% incorporation on day 86, and an increase to a maximum inhibitory value of 5% incorporation by day 93. The lesser

inhibitory activity of the 65% fraction and the unseparated spleen cell populations followed the same general type of slow increase, reaching a peak of inhibitory activity of 20% incorporation by day 79. This was followed by a decrease to 41% incorporation on day 86, and an increase to a maximum inhibitory activity value of 5% incorporation by day 93. The lesser inhibitory activity of the 65% fraction and the unseparated spleen cell populations followed the same type of slow increase to maximum inhibitory activity between days 72 and 79, and a slight decrease between days 79 and 86, followed by a slight increase in inhibitory activity by day 93.

Figure 12 compares the inhibitory activity of only the NK cell fractions from rats fed a low fat, choline deficient diet, and given DMBA on day 50, to the inhibitory activity from rats fed a high fat, choline deficient diet, and given DMBA on day 50. The inhibitory activity of the NK cell fraction from rats fed the low fat, choline deficient diet and given DMBA was significantly higher, compared to the inhibitory activity from rats fed the high fat, choline deficient diet, and given DMBA on day 50, on days 56-58, $p = .0001$; days 63-65, $p = .0001$; days 70-72, $p = .0001$; and days 84-86, $p = .0003$. The results from Figures 10, 11, and 12 indicated that; (a) rats fed low fat, choline deficient diets and given DMBA on day 50, had higher levels of inhibitory activity associated with their NK cell fraction, compared to the "cyclic" activity of their 65% and unseparated spleen cell populations, (b) rats fed high fat, choline deficient diets, and given DMBA on day 50, had levels of inhibitory activity associated with their NK cell fractions that slowly increased over a period of three weeks, compared to the low levels

of inhibitory activity associated with their 65% and unseparated spleen cell populations, and (c) the inhibitory activity of the NK cell fraction from rats on the low fat, choline deficient diet, and given DMBA on day 50, was much greater, early on, (days 56 through 70) compared to the inhibitory activity of the NK cell fraction from rats on the high fat, choline deficient diet, and given DMBA on day 50. These differences were due to the slow development of inhibitory activity of the NK cell fraction from the rats on the high fat, choline deficient diet, and given DMBA on day 50.

Ranking of Inhibitory Activity of NK Cells According to Diet

To further clarify the role of diet and carcinogen on inhibitory activity of NK cells, data from each dietary group were analyzed, and the mean inhibitory activity expressed as the percent of control (YAC-1 cell proliferation, alone) was calculated. The data were ranked from the highest to lowest value, along with each corresponding dietary group. Those groups whose inhibitory activity was not significantly different from other groups were designated with the same group letter. Those groups whose inhibitory activity was significantly different were designated by a different letter. Testing was performed using the Duncan's Multiple Range Test, with an alpha value of 0.05. The data in Table 2 revealed that on the average, NK cells from rats fed low fat diets, had the greatest ability to inhibit YAC-1 tumor cell proliferation, while NK cells from rats fed high fat, choline deficient diets, and given DMBA on day 50, had the least ability to inhibit YAC-1 tumor cell proliferation. When the NK cell inhibitory activity of rats from these two dietary groups was compared as a function of age (Figure 13), the

data revaled that differences were evident at all time points tested, (days 55-57, p = .0158; days 69-71, p = .0001; days 76-78, p = .0158; days 83-85, p = .0001; and days 90-92, p = .2700). In addition, the data in Table 2 also indicated that, on the average, rats fed low fat diets had NK cells whose inhibitory activity was greater, compared to rats fed high fat diets. DMBA caused little additional suppression of NK cell activity of rats on either diet. These averaged results indicated that the mean values of NK cell activity of rats on the different diets could be used to rank dietary states which were correlated with different levels of NK cell activity, and that these mean values reflected the differences in NK cell activity when the data was previously compared with respect to age of the animal.

The Role of Low or High Fat Diets on NK Cell Number and Function

To determine the role that diet played in inhibitory activity of NK cells, inhibitory activity of the NK cell fraction of all low fat diet groups, regardless of treatment, was plotted as seen in Figure 14. The resulting graph illustrated that most low fat dietary groups exhibited a high level of inhibitory activity by their NK cell fractions from day 55 to day 84. The contrasting data (Figure 15) indicated that the inhibitory activity of rats fed the high fat diets appeared to be "cyclic" in nature, as previously noted, and, in addition, three out of four high fat dietary groups appeared to be synchronous in their "cyclic" responses. The data so far presented have indicated that high fat diets alone, and in combination with other factors such as DMBA and choline deficiency, resulted in animals whose inhibitory activity was "cyclic" and, on the average, lower than that of animals fed only low fat diets.

To further analyze the differences between the inhibitory activity of rats fed low or high fat diets, other criteria of the spleen cell populations used in these studies were examined for each combined low fat or high fat dietary group. As seen in Table 3, rats fed high fat diets had significantly greater numbers of spleen cells ($p = .0144$) and fewer percentages of NK cells (Fraction 2) compared to their low fat diet counterparts ($p = .0177$). In addition, rats fed high fat diets had more cells in the pellet (Fraction 4) compared to rats fed low fat diets ($p = .0116$). The smaller percentage of NK cells in spleens of rats fed high fat diets, however, was compensated by a larger total spleen cell number, and thus, when the total number of NK cells was calculated for each dietary group, the results, expressed on a per spleen basis, were equal. Thus, NK cell number could not account for the differences in inhibitory activity as seen in rats fed low fat diets, compared to rats fed high fat diets.

To further investigate the differences in inhibitory activity between rats fed low fat and high fat diets, a comparison of NK cell proliferation was made. In the natural killer cell assay, NK cells were cultured alone, without YAC-1 targets, to assay for any spontaneous proliferative response of the NK cells themselves. The data from all low fat dietary groups were combined, as well as all data from the high fat dietary groups. For each dietary group, the mean proliferative response of the NK cells cultured alone, for each animal, was ranked from the lowest to highest value, and the values divided into quartiles. The resulting data were analyzed for any correlations (see Table 4). Results revealed that an inverse correlation existed between the NK cell

proliferative activity, and the ability of NK cells to inhibit YAC-1 tumor cell proliferation. If proliferative activity of the NK cell fraction was low, the ability to inhibit YAC-1 proliferation was high. As the proliferative activity of the NK cell fraction increased, its corresponding ability to inhibit YAC-1 proliferation decreased. This inverse correlation was true for both dietary groups. In addition, some animals on the high fat diets had NK cells whose mean proliferative activity was twice that of low fat animals, and whose ability to inhibit YAC-1 proliferation was decreased approximately two-fold. These results indicated that some of the animals fed high fat diets had lower levels of inhibitory activity, which might be due to a higher proliferative activity of their NK cells, compared to animals fed the low fat diet.

Inhibition of Incorporation of ^{3}H-thymidine by YAC-1 Tumor Cells Cultured With Spleen Cells from Rats Fed Low Fat or High Fat Diets

To verify the effects of low fat or high fat diet on the ability of spleen cells to inhibit the ^{3}H-thymidine incorporation into YAC-1 targets, a second experiment was designed, using rats placed on either a low fat or high fat diet, and assaying for inhibitory activity, starting one week earlier (day 41) than the previous experiment. Data from the previous experiment were combined with the present experiment when the assay times fell on the same days.

Figure 16 compares the inhibitory activity of only the NK cell fractions from rats on a low fat diet, compared to rats fed a high fat diet. Rats fed a low fat diet had high levels of inhibitory activity early on, through day 48, never exceeding 10% incorporation. Starting on day 50, however, inhibitory activity decreased to a minimum value of

55% incorporation by day 57. The inhibitory activity then increased, beginning on day 62 (40% incorporation), and continued on days 69, 76, and 83 (20%, 5%, and 1% incorporation, respectively). The inhibitory activity of the NK cell fraction from high fat diet fed rats ranged from 2% to 35% incorporation, between days 41 and 50. On day 55, however, no inhibitory activity could be demonstrated. This lack of inhibitory activity in high fat diet fed rats was significantly lower than the inhibitory activity expressed by the low fat diet fed rats at the same time point ($p = .0500$). The inhibitory activity increased on days 57, 62, and 64, as shown by 58%, 50%, and 10% incorporation, respectively. This activity, however, decreased once again to 42% and 45% on days 76 and 78. This low level of inhibitory activity of rats fed high fat diets was significantly lower, compared to the inhibitory activity of rats fed low fat diets as measured on day 76 ($p = .0541$). On day 85, the inhibitory activity of the NK cell fraction from rats fed high fat diets increased to 21% incorporation. The results from Figure 16 substantiated the previous data indicating that; (1) rats fed low fat diets had NK cells whose inhibitory was, on the average, higher than the inhibitory activity of the NK cells from rats on high fat diets, (b) this inhibitory activity of NK cells from rats on low fat diets was high, early on, and never exceeded 55% incorporation, and (c) rats fed high fat diets had NK cells whose inhibitory activity appeared to be "cyclic" in nature, compared to the non-cyclic nature of NK cells from rats fed low fat diets.

Inhibition of Incorporation of ^{3}H-thymidine by R_2T_2 Tumor Cells Cultured with Spleen Cells from Rats Fed Low Fat or High Fat Diets

To determine the effects of diet on the inhibitory activity of rat spleen cells against a different target dell, rats were placed on either

low fat or high fat diets, and inhibitory activity of the three spleen cell populations was measured, using the rat mammary adenocarcinoma cell line, R_2T_2, as the source of target cells.

The data from Figure 17 demonstrated that although the NK cell fraction exhibited the highest level of inhibitory activity at some time points (days 41 and 62), there were no significant differences in the inhibitory activity of the three spleen cell populations at the majority of the time points tested.

Figure 18 compares the inhibitory activity of the three spleen cell populations from rats fed high fat diet. The NK cell fraction was somewhat better able to inhibit the ^{3}H-thymidine incorporation into R_2T_2 tumor cells, compared to the 65% fraction or the unseparated spleen cell populations. The highest level of inhibitory activity of the NK cell fraction occurred on day 57 (50% incorporation). Thereafter, this inhibitory activity gradually decreased to 100% incorporation by day 76. The inhibitory activity of the 65% and the unseparated spleen cell populations was, on the average, lower, compared to the inhibitory activity of the NK cell fraction. A similar decrease in inhibitory activity was also observed for both spleen cell populations on day 76.

Figure 19 compares the inhibitory activity of only the NK cell fractions from rats on low fat diets and rats on high fat diets. There were essentially no significant differences between the inhibitory activity of NK cell fractions from the low fat or high fat diet fed rats. The results from Figures 17, 18, and 19 indicated that diet did not influence the inhibitory activity of any of the spleen cell populations tested, when using the R_2T_2 tumor cell line as a source of target cells.

Resistance of R_2T_2 Tumor Cells to the Inhibitory Activity of Spleen Cell Fractions

To determine if R_2T_2 tumor cells were resistant to the inhibitory activity of spleen cell fractions, the R_2T_2 tumor cells were subjected to either one or two, 16 hour incubation periods with the 65% or NK fractions of spleen cells. Spleen cells were then removed, and the tumor cells allowed to continue growing for a total of 90 hours.

Figure 20 indicated that a 16 hour incubation of R_2T_2 tumor cells with the 65% or NK fraction of spleen cells, plus a 24 hour incubation period without spleen cells, reduced the number of viable R_2T_2 tumor cells from 3×10^4 to 2×10^4 for the 65% fraction, and from 3×10^4 to 1×10^4 for the NK cell fraction, by 40 hours. Further incubation without spleen cells resulted in a renewed growth rate for all R_2T_2 cells, regardless of exposure history. However, if R_2T_2 tumor cells were incubated with the 65% or NK cell fractions of spleen cells for an additional 16 hours, the resulting growth of the R_2T_2 tumor cells was inhibited. The Figure demonstrated that R_2T_2 tumor cells, with a 65% or NK cell fraction history, were further inhibited by the additional 16 hour incubation with either the NK or the 65% fractions of spleen cells. Furthermore, removal of spleen cell fractions after 40 hours of growth, resulted in a decline in subsequent growth of the tumor cells over an additional 48 hour incubation period. These data indicated that; (a) NK cell-mediated inhibition of growth of R_2T_2 tumor cells could be partially lifted if NK cells were removed after 16 hours, and tumor cells re-incubated and (b) NK cell-mediated inhibition of R_2T_2 tumor cell growth did not result in the emergence of resistant R_2T_2 tumor cells, since

re-incubation with additional NK cells and their subsequent removal, resulted in the establishment of R_2T_2 tumor cell cultures which were unable to continue their growth.

Table 1. Composition of diet for rats fed either low or high fat diets, with or without choline.

Table 1.

DIET COMPOSITION

	20% POLYUNSATURATED FAT DIET	2% LOW FAT DIET	2% LOW OR 20% POLYUNSATURATED FAT DIET CHOLINE DEFICIENT
	GM	GM	GM
CASEIN	23	23	23
FAT	20[1]	2[2]	20 OR 2
SUCROSE	46	64	46 OR 64
ALPHACEL (NON NUTRIENT BULK)	6	6	6
SALT MIXTURE[3]	4	4	4
VITAMIN MIXTURE[4]	1	1	[5]1

[1]CORN OIL; [2]LINOLEIC ACID; [3]HUBBELL SALT MIXTURE; [4]ICN VITAMIN MIXTURE (below) (below)

SALT MIXTURE	Percent
Calcium Carbonate	54.300
Magnesium Carbonate	2.500
Magnesium Sulfate	1.600
Sodium Chloride	6.900
Potassium Phosphate (monobasic)	21.200
Ferric Phosphate	2.050
Potassium Iodide	0.008
Manganese Sulfate (H_2O)	0.035
Sodium Fluoride	0.010
Aluminum Potassium Phosphate	0.017
Copper Sulfate	0.090
Zinc Chloride ($5H_2O$)	0.030

VITAMIN MIXTURE	grams per 100 lbs diet
Vitamin A Concentrate (200,000 units/gm)	4.5
Vitamin D Concentrate (400,000 units/gm)	0.25
Ascorbic Acid	45.0
Inositol	5.0
Choline Chloride[5]	75.0
Menadione	2.25
p-Aminobenzoic Acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine Hydrochloride	1.0
Thiamine Hydrochloride	1.0
Calcium Pantothenate	3.0
	mgms./100 lbs diet
Biotin	20.00
Folic Acid	90.00
Vitamin B-12	1.35

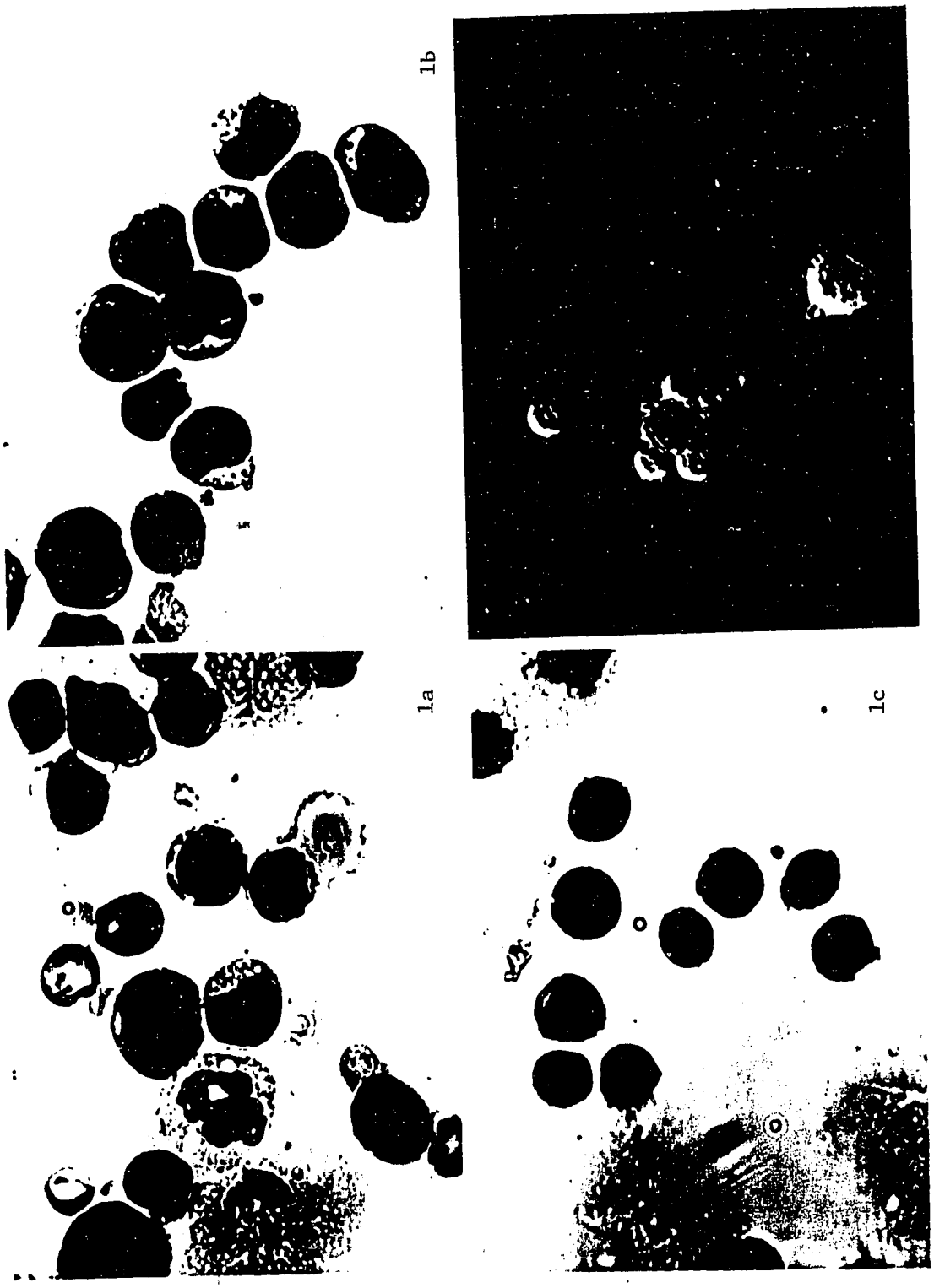
[5](Choline Chloride is ommitted from the vitamin mixture in diets that are deficient in choline).

Plate 1a. A representative field of unseparated spleen cells showing a large, granular lymphocyte, polymorphonuclear leukocyte, macrophages, and small to medium sized lymphocytes. Giemsa stain, 1000X.

Plate 1b. Appearance of cells obtained from the 55% and 60% fractions of Percoll density gradients. Numerous lymphocytes bearing a kidney-shaped nucleus, and azurophillic granules in the cytoplasm can be seen (large granular lymphocytes). Giemsa stain, 1000X.

Plate 1c. Appearance of cells obtained from the 65% fraction of Percoll density gradients. Morphology is that of medium sized lymphocytes. Giemsa stain, 1000X.

Plate 1d. YAC-1 tumor cells surrounded by three natural killer cells. YAC-1 tumor cell appears to be non-viable due to uptake of Trypan blue. YAC-1 tumor cell at lower right, not associated with natural killer cells, appears to be viable.

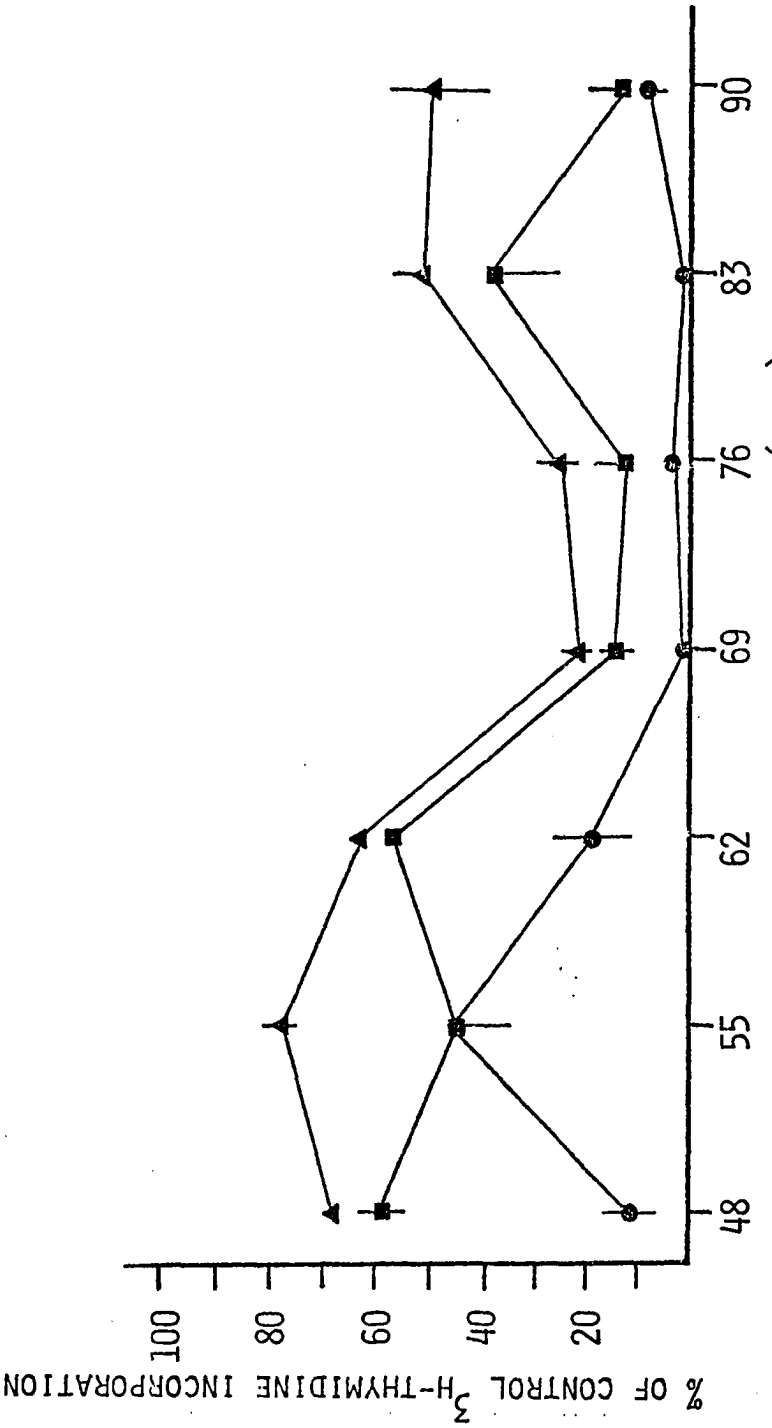


1a 1b 1c

Figure 1. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a low fat diet, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●——● designates spleen cells obtained from the NK fraction of Percoll from rats on low fat diets. ■——■ designates spleen cells obtained from the 65% fraction of Percoll from rats on low fat diets. ▲——▲ designates spleen cells not separated by Percoll obtained from rats on low fat diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.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AGE OF RATS (DAYS)

Figure 1.

Figure 2. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a high fat diet, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

O———O designates spleen cells obtained from the NK fraction of Percoll from rats on high fat diets. □———□ designates spleen cells obtained from the 65% fraction of Percoll from rats on high fat diets. Δ———Δ designates spleen cells not separated by Percoll obtained from rats on high fat diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

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Figure 2.

Figure 3. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK cell populations obtained from rats fed a low fat diet compared to rats fed a high fat diet, as a function of age. Tumor cells, spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●———● designates NK spleen cells obtained from rats on low fat diets. O———O designates NK spleen cells obtained from rats on high fat diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

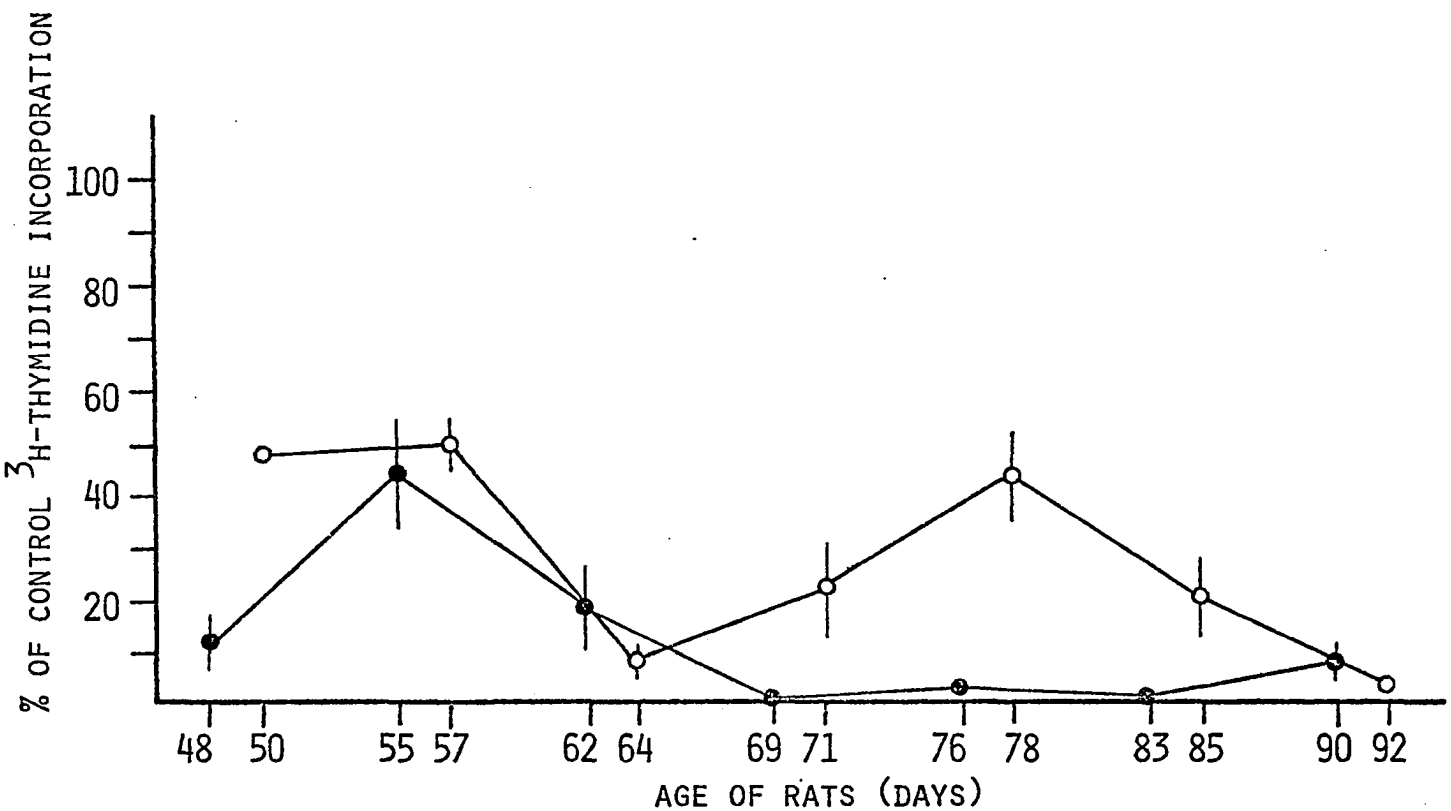
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Figure 3.

Figure 4. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a low fat diet and given DMBA at 50 days of age, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

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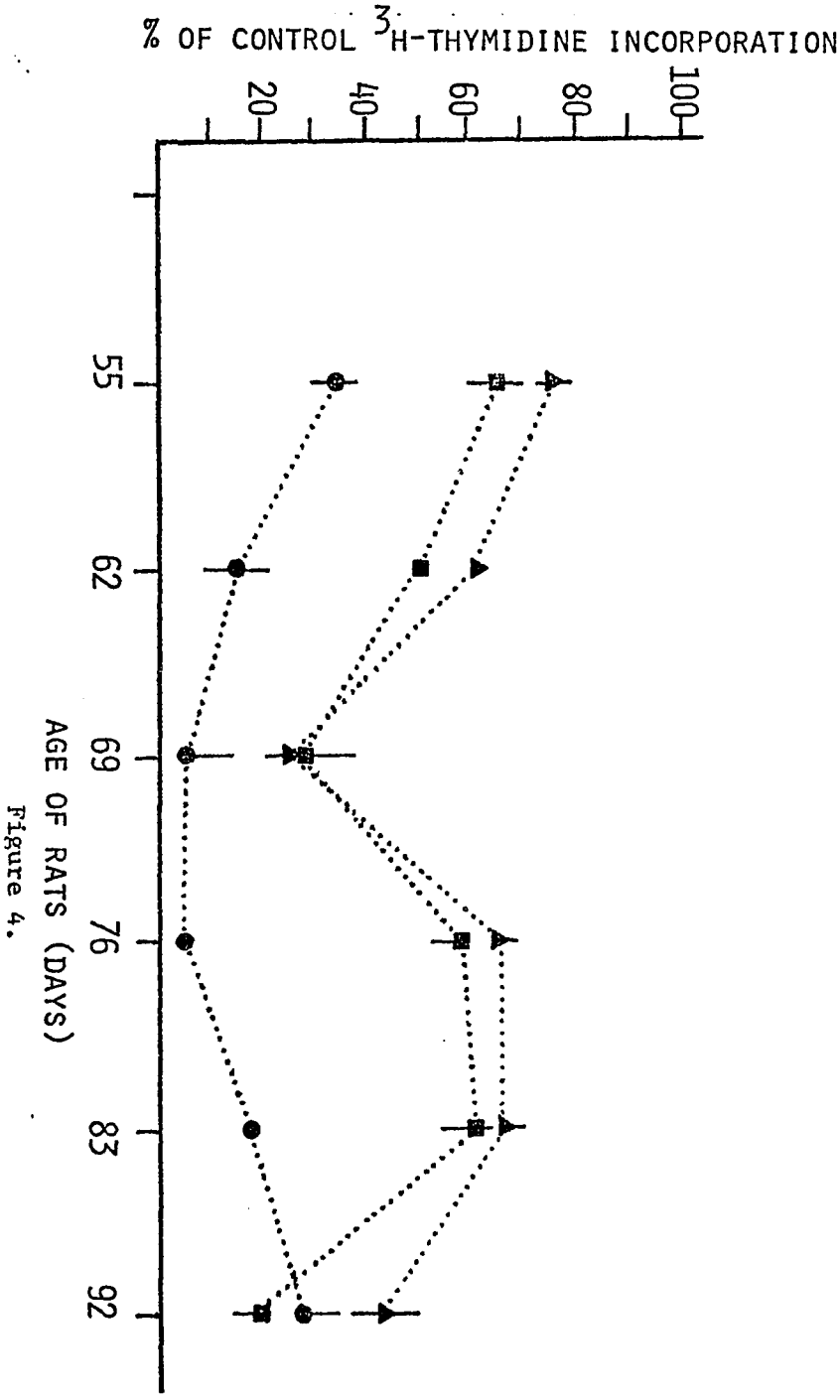
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Figure 4.

Figure 5. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a high fat diet and given DMBA at 50 days of age, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\%\ \text{of Control}\ ^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) - (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

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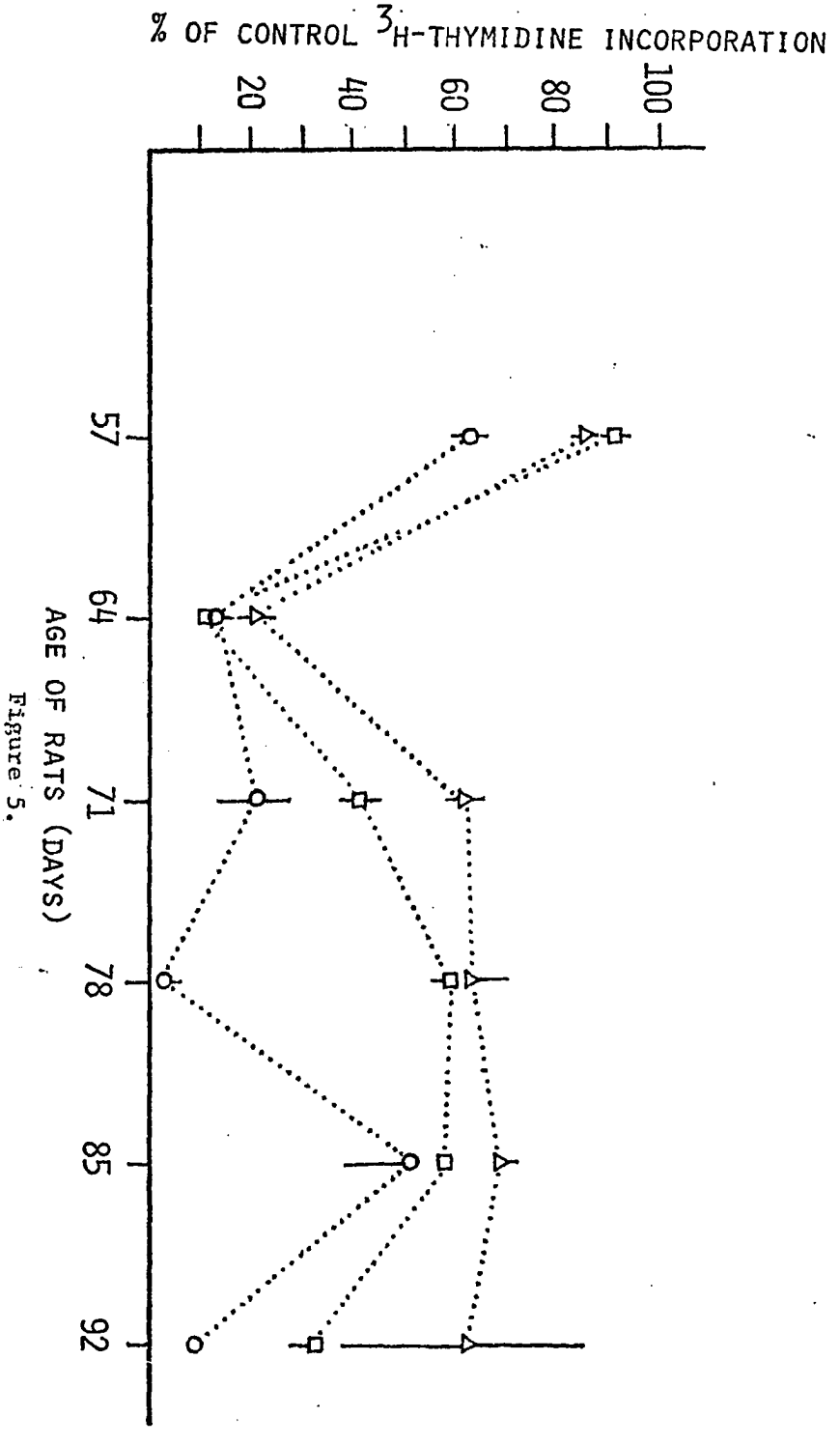
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Figure 5.

Figure 6. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK cell populations obtained from rats fed a low fat diet and given DMBA at 50 days of age compared to rats fed a high fat diet and given DMBA, as a function of age. Tumor cells, spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●.....● designates NK spleen cells obtained from rats on low fat diets and given DMBA. O.....O designates NK spleen cells obtained from rats on high fat diets and given DMBA. Each point represents the mean of six determination from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

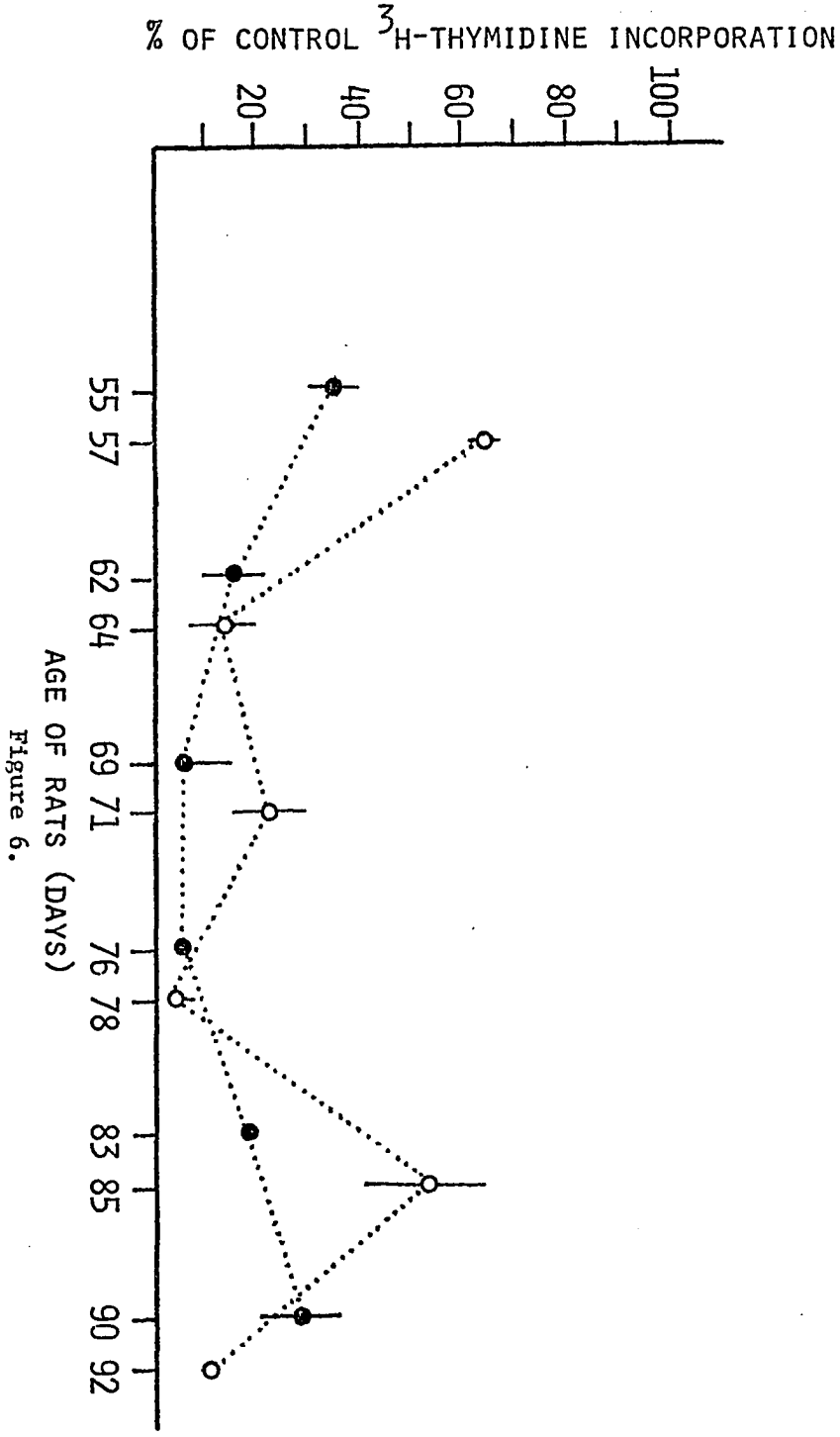
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Figure 6.

Figure 7. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a low fat, choline deficient diet, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

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●— — —● designates spleen cells obtained from the NK fraction of Percoll from rats on low fat, choline deficient diets. ■— — —■ designates spleen cells obtained from the 65% fraction of Percoll from rats on low fat, choline deficient diets. ▲— — —▲ designates spleen cells not separated by Percoll from rats on low fat, choline deficient diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown if when it exceeds the symbol's dimensions.

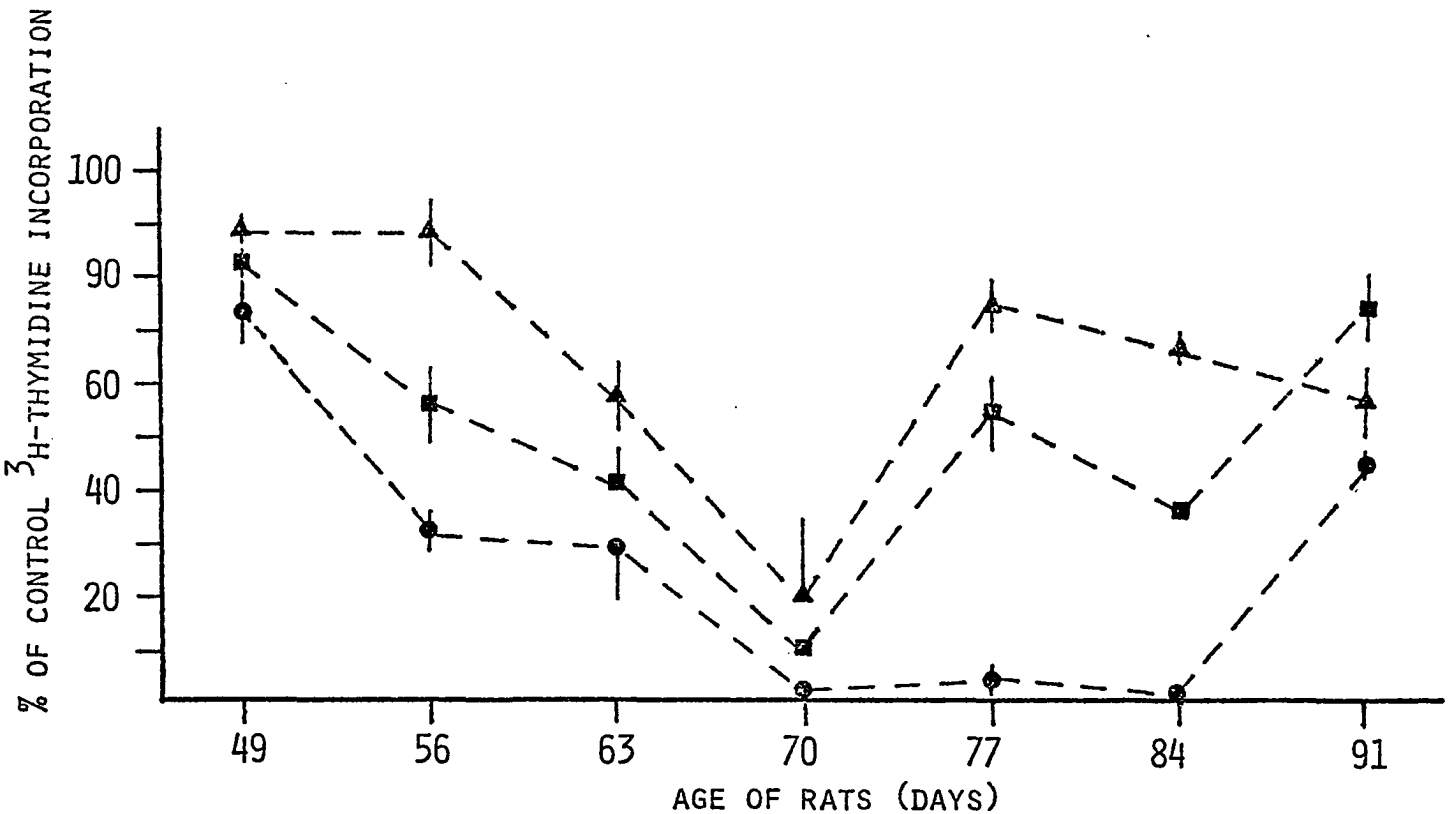
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Figure 7.

Figure 8. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a high fat, choline deficient diet, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

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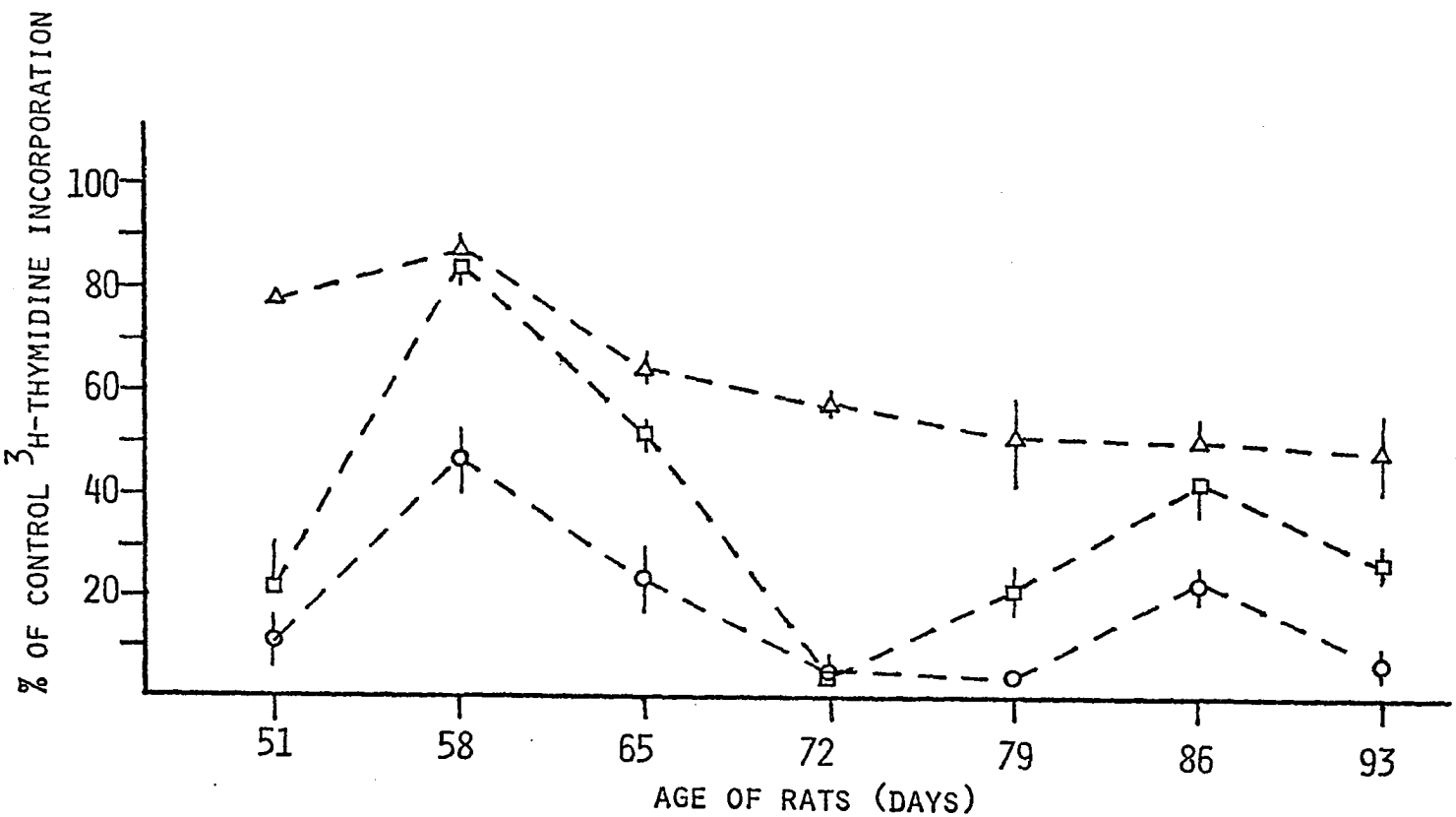
O— — —O designates spleen cells obtained from the NK fraction of Percoll from rats on high fat, choline deficient diets. ⊡— — —⊡ designates spleen cells obtained from the 65% fraction of Percoll from rats on high fat, choline deficient diets. Δ— — —Δ designates spleen cells not separated by Percoll from rats on high fat, choline deficient diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.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AGE OF RATS (DAYS)

Figure 8.

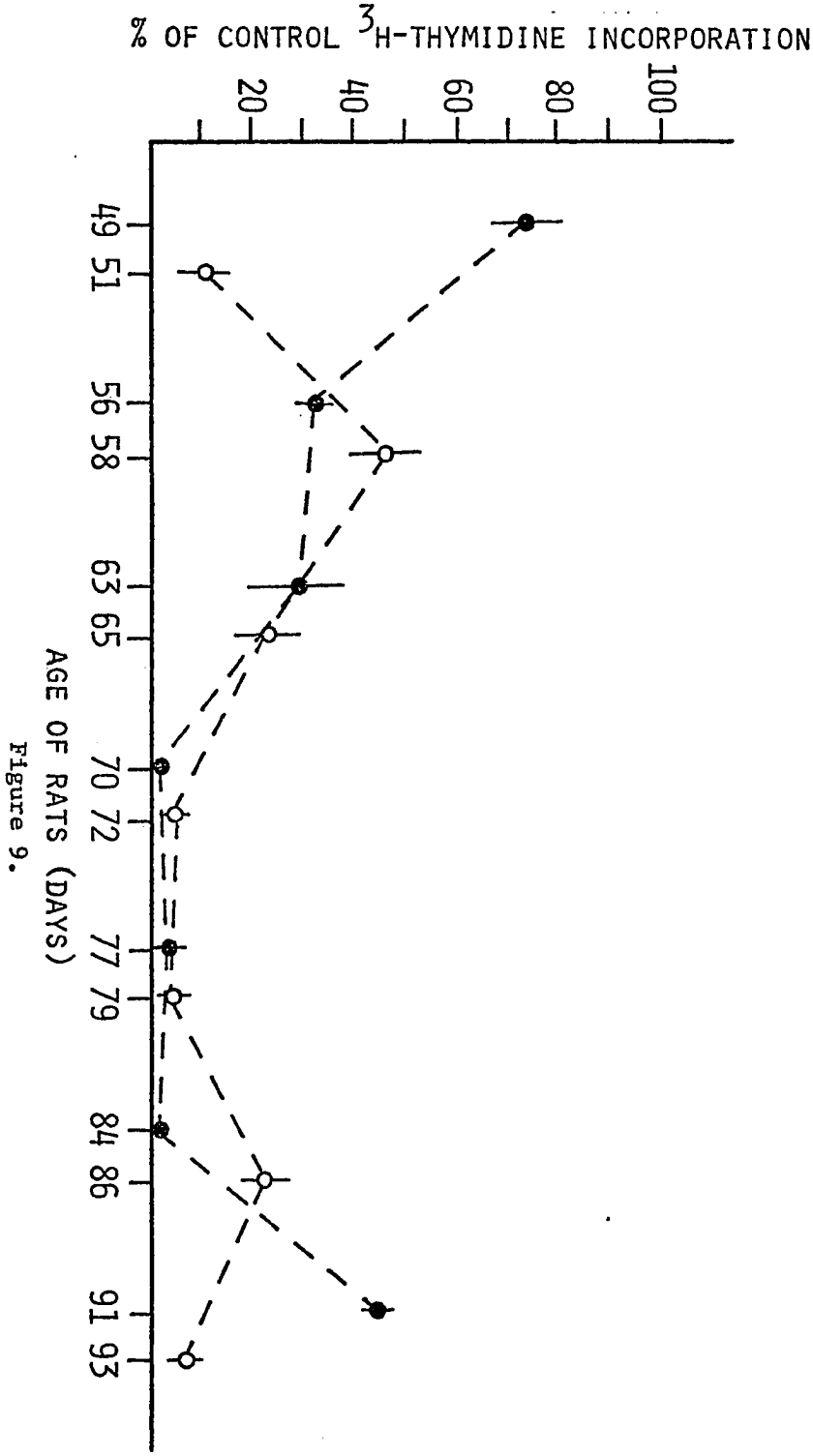
Figure 9. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK cell populations obtained from rats fed a low fat, choline deficient diet compared to rats fed a high fat, choline deficient diet, as a function of age. Tumor cells, spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control).. The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●— — —● designates NK spleen cells obtained from rats on low fat, choline deficient diets.

○— — —○ designates NK spleen cells obtained from rats on high fat, choline deficient diets.

Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.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AGE OF RATS (DAYS)

Figure 9.

Figure 10. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a low fat, choline deficient diet, and given DMBA on day 50, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●—·—·—● designates spleen cells obtained from the NK fraction of Percoll from rats on low fat, choline deficient diets and given DMBA. ■—·—·—■ designates spleen cells obtained from the 65% fraction of Percoll from rats on low fat, choline deficient diets and given DMBA. ▲—·—·—▲ designates spleen cells not separated by Percoll from rats on low fat, choline deficient diets and given DMBA. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

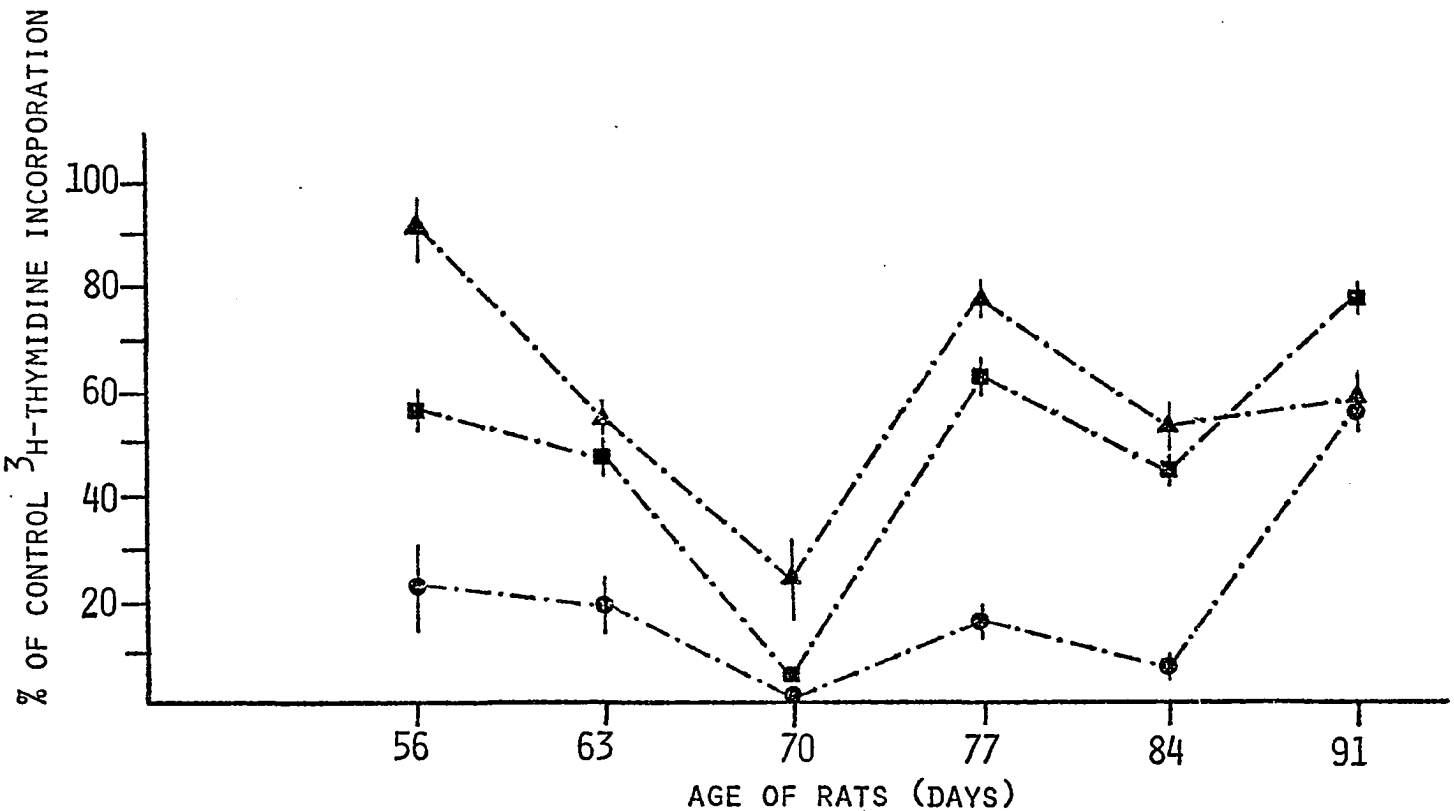
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Figure 10.

Figure 11. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with the three spleen cell populations obtained from rats fed a high fat, choline deficient diet, and given DMBA on day 50, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\%\ \text{of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

O—·—·—O designates spleen cells obtained from the NK fraction of Percoll from rats on high fat, choline deficient diets, and given DMBA. □—·—·—□ designates spleen cells obtained from the 65% fraction of Percoll from rats on high fat, choline deficient diets, and given DMBA. Δ—·—·—Δ designates spleen cells not separated by Percoll from rats on high fat, choline deficient diets, and given DMBA. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

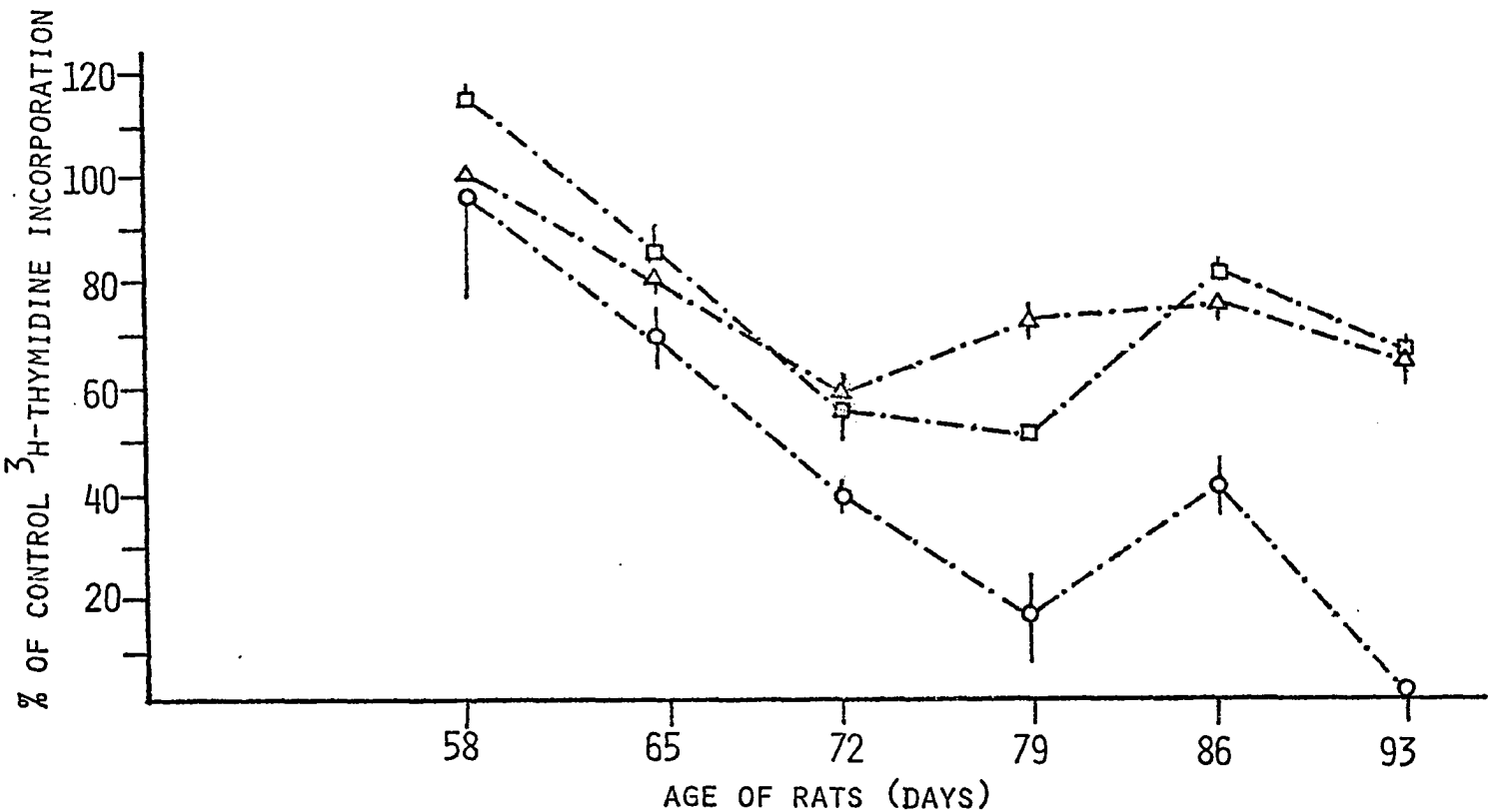
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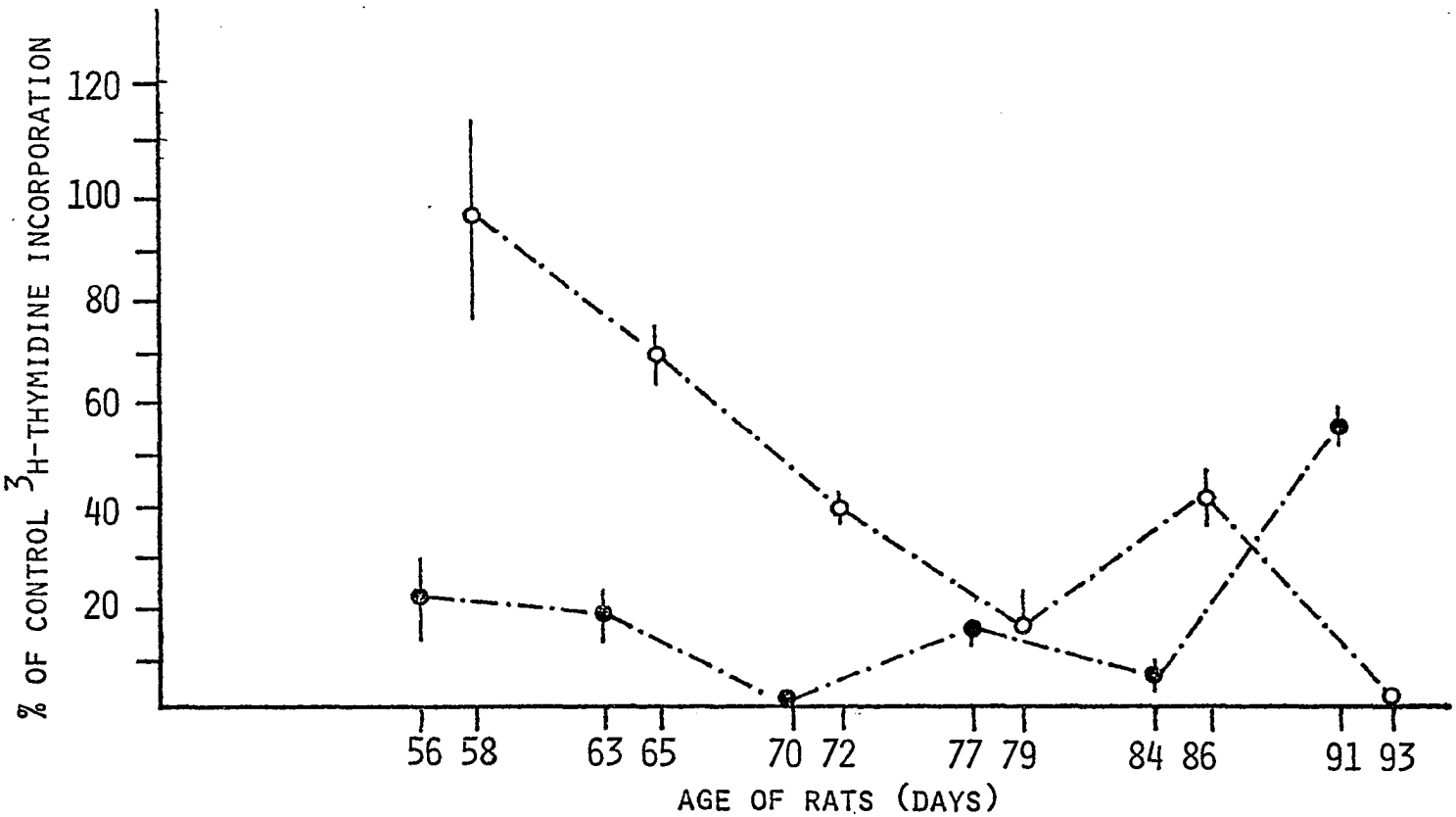
Figure 11.

Figure 12. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK cell populations obtained from rats fed a low fat, choline deficient diet, and given DMBA on day 50, compared to rats fed a high fat, choline deficient diet, and given DMBA on day 50, as a function of age. Tumor cells, spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\%\ \text{of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●—·—·—● designates NK spleen cells obtained from rats on low fat, choline deficient diets, and given DMBA. ○—·—·—○ designates NK spleen cells obtained from rats on high fat, choline deficient diets, and given DMBA. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown whan it exceeds the symbol's dimensions.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AGE OF RATS (DAYS)

Figure 12.

Table 2. Ranking of NK cell activity using Duncan's Multiple Range Test. Tumor cells, NK spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and were cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture and expressing it as a percentage of the incorporation of the YAC-1 tumor cells cultured alone. The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

The mean of the percent of control incorporation (proliferation) for all rats assayed in each dietary group was calculated and listed in increasing order along with its corresponding dietary group designation. The Duncan's Multiple Range Test was used to determine differences between the means of each group. Groups with the same letter were not significantly different at an alpha value of 0.05. Groups with the same letter were significantly different at an alpha value of 0.05.

Table 2.

RANKING OF NK CELL ACTIVITY USING
DUNCAN'S MULTIPLE RANGE TEST

RANK	DIET	PERCENT OF CONTROL PROLIFERATION	N	GROUP
1	LOW FAT	11.7	42	A
2	HIGH FAT MINUS CHOLINE	17.2	42	B
3	LOW FAT PLUS DMBA	17.9	36	B
4	LOW FAT MINUS CHOLINE PLUS DMBA	20.1	36	B
5	HIGH FAT	26.8	39	C
6	LOW FAT MINUS CHOLINE	28.9	39	C
7	HIGH FAT PLUS DMBA	29.1	33	C
8	HIGH FAT MINUS CHOLINE PLUS DMBA	44.2	36	D

(GROUPS WITH THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT,
GROUPS WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT,
$\alpha = 0.05$)

Figure 13. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK cell populations obtained from rats fed a low fat diet compared to rats fed a high fat, choline deficient diet, and given DMBA on day 50, as a function of age. Tumor cells, NK spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK-spleen culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●———● designates NK spleen cells obtained from rats on low fat diets. O—·—·—O designates NK spleen cells obtained from rats on high fat, choline deficient diets, and given DMBA. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

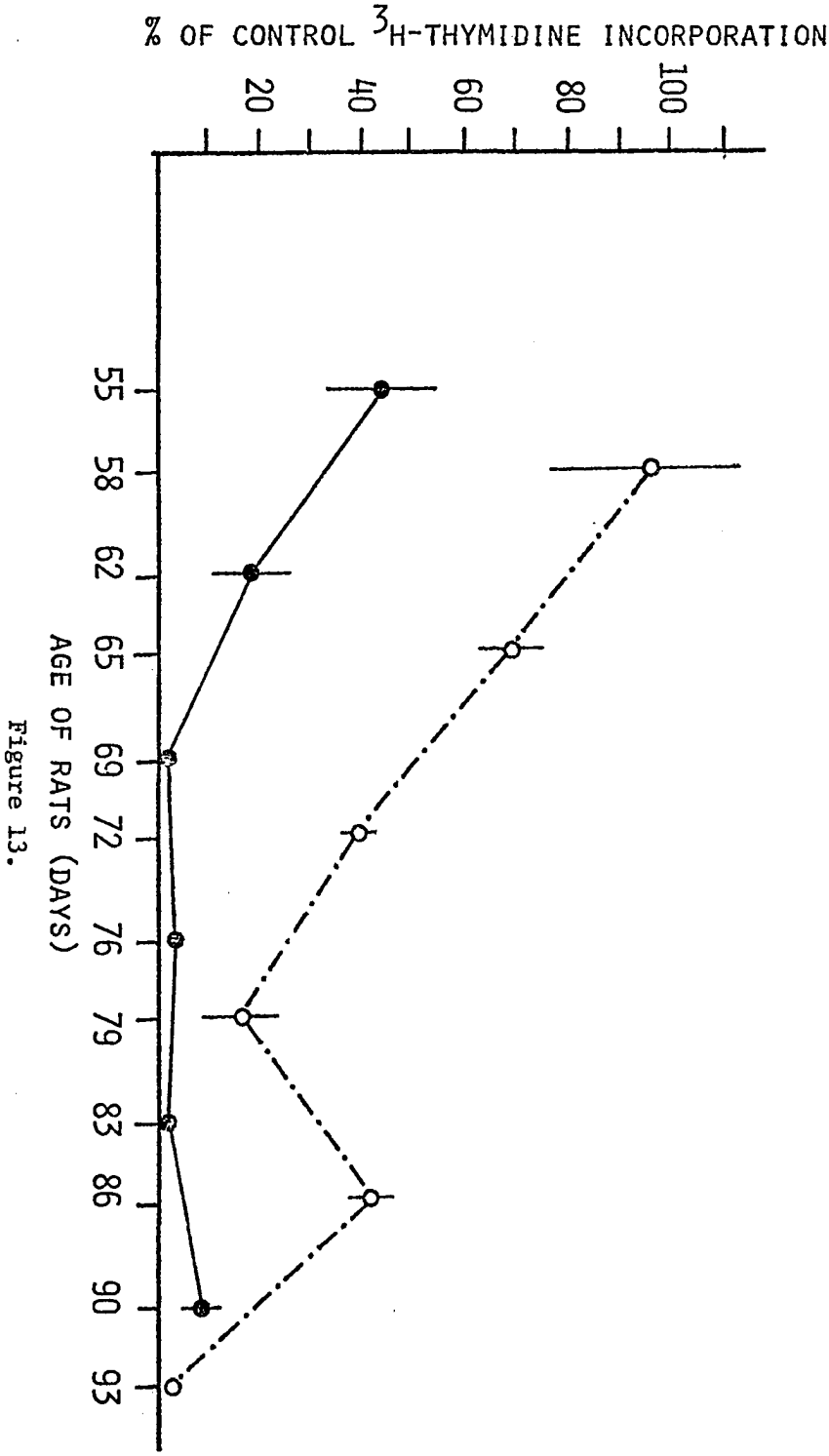
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Figure 13.

Figure 14. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK spleen cells obtained from rats on low fat diets, with or without choline, and with or without being given DMBA on day 50. Tumor cells, NK spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●————● designates NK spleen cells obtained from rats on low fat diets. ●— — —● designates NK spleen cells obtained from rats on low fat, choline deficient diets. ●·····● designates NK spleen cells obtained from rats on low fat diets and given DMBA. ●—·—·—● designates NK spleen cells obtained from rats on low fat, choline deficient diets, and given DMBA. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

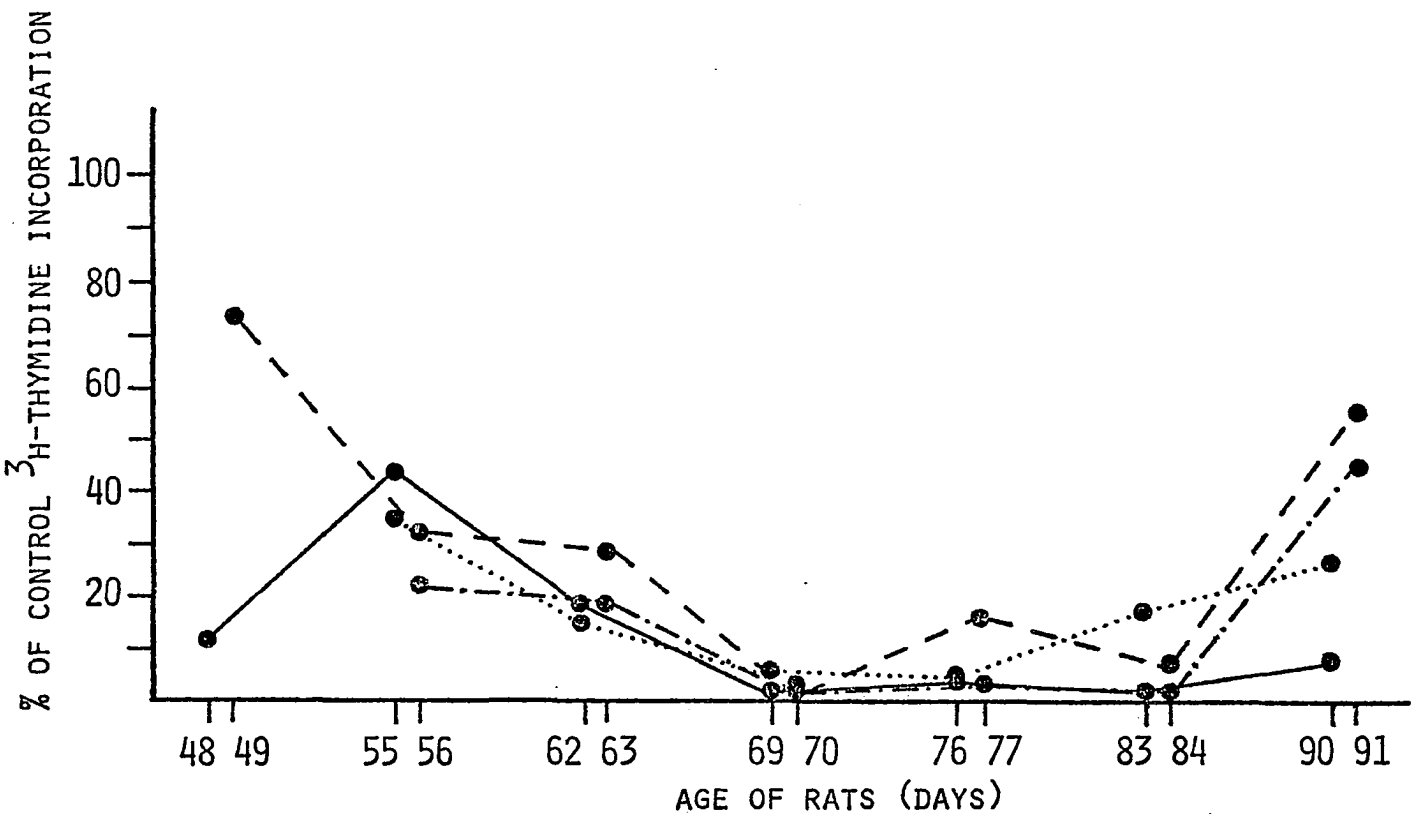
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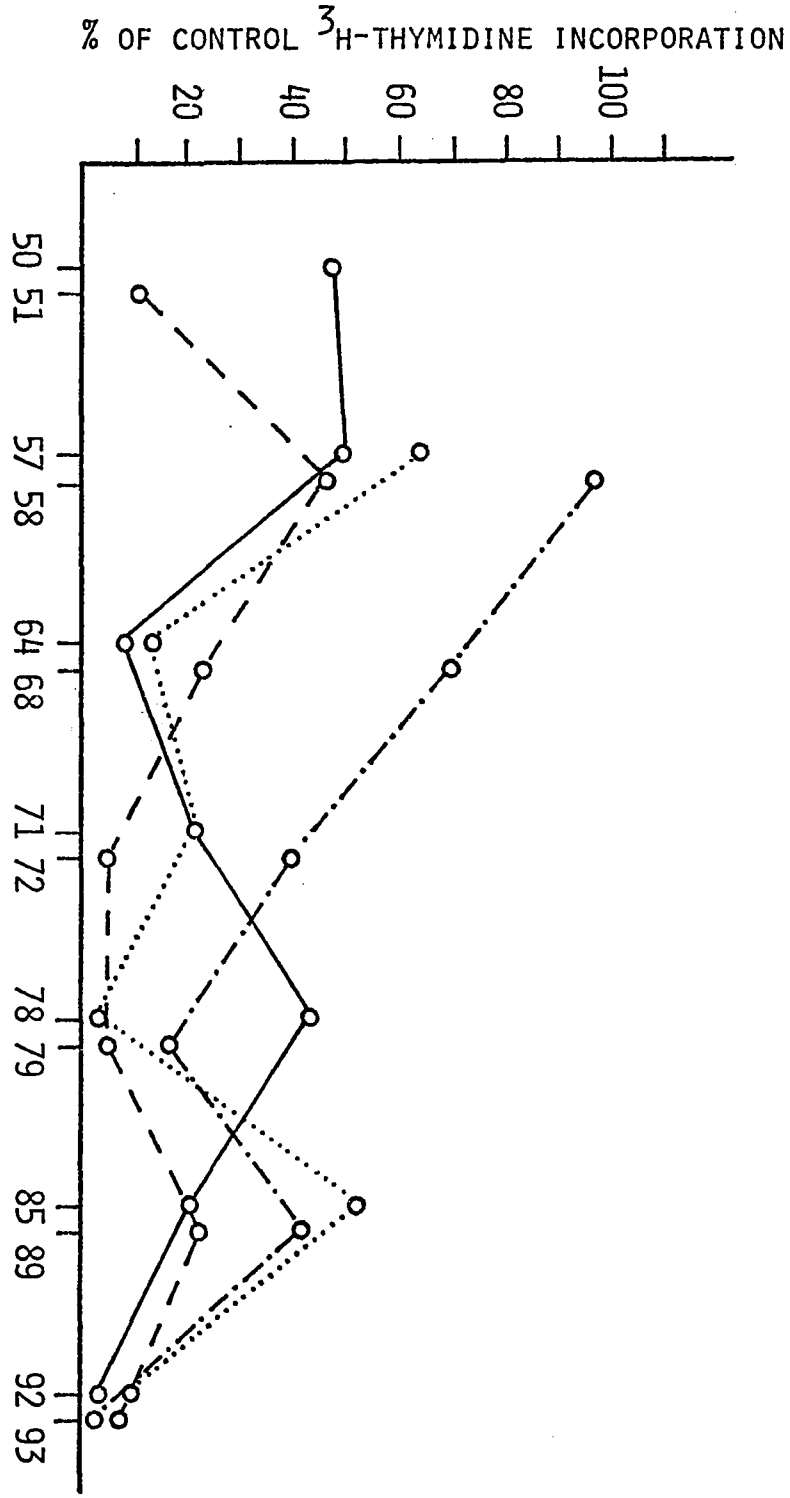
Figure 14.

Figure 15. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK spleen cells obtained from rats on a high fat diet, with or without choline, and with or without being given DMBA on day 50, as a function of age. Tumor cells, NK spleen cells, and tumor-NK-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK-spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

O———O designates NK spleen cells obtained from rats on high fat diets. O— — —O designates NK spleen cells obtained from rats on high fat, choline deficient diets. O·····O designates NK spleen cells obtained from rats on high fat diets and given DMBA. O—·—·—O designates NK spleen cells obtained from rats on high fat, choline deficient diets, and given DMBA. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.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AGE OF RATS (DAYS)

Figure 15.

Table 3. Characteristics of spleen cells taken from rats maintained on low fat or high fat diets. Diet groups were designated as either low fat or high fat, regardless of carcinogen treatment or choline deficiency. Mean values for each of the spleen cell characteristics were determined for each diet group. The Student's t-test was used to determine differences between the means.

Table 3.

CHARACTERISTICS OF SPLEEN CELLS TAKEN FROM RATS MAINTAINED ON LOW FAT OR HIGH FAT DIETS 48 - 93 DAYS OLD

MEAN VALUES	LOW FAT	HIGH FAT
SPLEEN WEIGHTS (GRAMS)	479.95 ± 11.85	475.56 ± 13.51
SPLEEN CELL NUMBER X 10^7	15.06 ± 0.67	17.69 ± 0.82*
PERCENT NON-ADHERENT TO PLASTIC BEADS	57.13 ± 1.49	57.43 ± 1.84
PERCENT RECOVERED FROM PERCOLL GRADIENT	70.13 ± 1.66	76.16 ± 1.91
DISTRIBUTION OF CELLS ON PERCOLL GRADIENT (PERCENT)		
FRACTION 1 (26 - 50)	7.74 ± 0.52	6.55 ± 0.41
FRACTION 2 (55 - 60)	23.11 ± 1.01	19.49 ± 1.11*
FRACTION 3 (65)	27.83 ± 1.19	23.31 ± 1.21
FRACTION 4 (PELLET)	38.73 ± 2.13	46.29 ± 1.98*
NUMBER OF CELLS PER SPLEEN OF FRACTION 2 ($\times 10^7$)	1.98	1.98

$P \leq 0.018$

Table 4. Correlation between NK cell proliferation and ability to inhibit YAC-1 proliferation. Tumor cells, NK spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and were cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture and expressing this as a percentage of the incorporation of the YAC-1 cells cultured alone (control). The results were calculated using the following formula:

$$\%\ \text{of Control}\ ^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells} + \text{YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

The cpm of NK cell proliferation was listed from the smallest to the largest value along with each corresponding NK + YAC-1 cpm value and percent of YAC-1 proliferation. All data was analyzed from all dietary groups, an was divided into either low fat or high fat diet categories, regardless of carcinogen treatment or choline deficiency. Each of the groups was further divided into four subgroups and the means calculated for each subgroup.

Table 4.

CORRELATION BETWEEN NK CELL PROLIFERATION AND ABILITY TO INHIBIT YAC-1 PROLIFERATION

LOW FAT

NK CELLS ALONE (CPM)	NK + YAC-1 (CPM)	PERCENT OF YAC-1 PROLIFERATION	N
1699 ± 230	44042 ± 17699	12.6 ± 5.4	12
2825 ± 142	66222 ± 25885	16.9 ± 6.3	14
3831 ± 137	84867 ± 36842	23.8 ± 8.2	14
6044 ± 582	85145 ± 37233	23.0 ± 11.0	12

HIGH FAT

846 ± 149	18196 ± 2665	5.2 ± 0.9	12
2457 ± 171	100403 ± 25515	22.8 ± 5.6	14
5415 ± 533	126211 ± 23381	33.6 ± 5.8	14
13167 ± 2481	189821 ± 44613	52.7 ± 12.5	12

Figure 16. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with NK cell populations obtained from rats on low fat or high fat diets, as a function of age. Tumor cells, NK spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. Inhibitory activity of each tumor-NK spleen cell culture was determined by measuring the amount of ^{3}H-thymidine incorporation of the YAC-1-NK spleen cell culture, and expressing this as a percentage of the incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of NK spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

●——● designates NK spleen cells obtained from rats on low fat diets. O——O designates NK spleen cells obtained from rats on high fat diets. Each point designated by an asterisk represents the mean of twelve determinations from four rats. All others represent the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

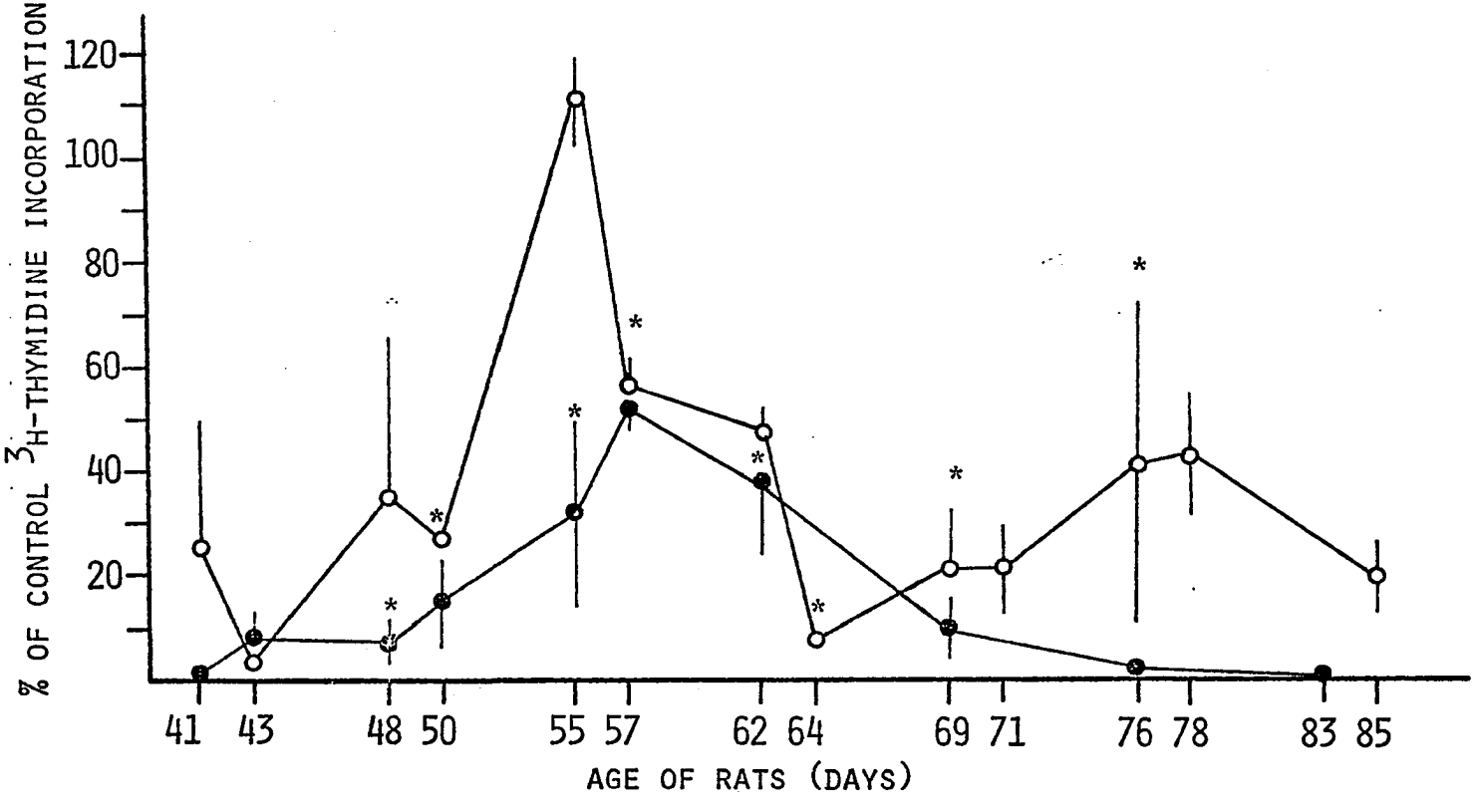
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Figure 16.

Figure 17. Inhibition of incorporation of ^{3}H-thymidine into R_2T_2 tumor cells cultured with the three spleen cell populations obtained from rats fed a low fat diet, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by removal of the spleen cells, and the addition of 1.0 uCi of ^{3}H-thymidine to the tumor cell monolayers. These cells were incubated for an additional 3.5 hours, removed with trypsin:EDTA, and harvested. Inhibitory activity of each tumor-spleen cell culture was measured by determining the amount of ^{3}H-thymidine incorporation of the R_2T_2 tumor cell-spleen cell culture and expressing this as a percentage of the incorporation of the R_2T_2 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of spleen cells} + R_2T_2 \text{ tumor cells})}{(\text{cpm of } R_2T_2 \text{ cells cultured alone})} \times 100$$

●——● designates spleen cells obtained from the NK fraction of Percoll from rats on low fat diets. ■——■ designates spleen cells obtained from the 65% fraction of Percoll from rats on low fat diets. ▲——▲ designates spleen cells not separated by Percoll obtained from rats on low fat diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

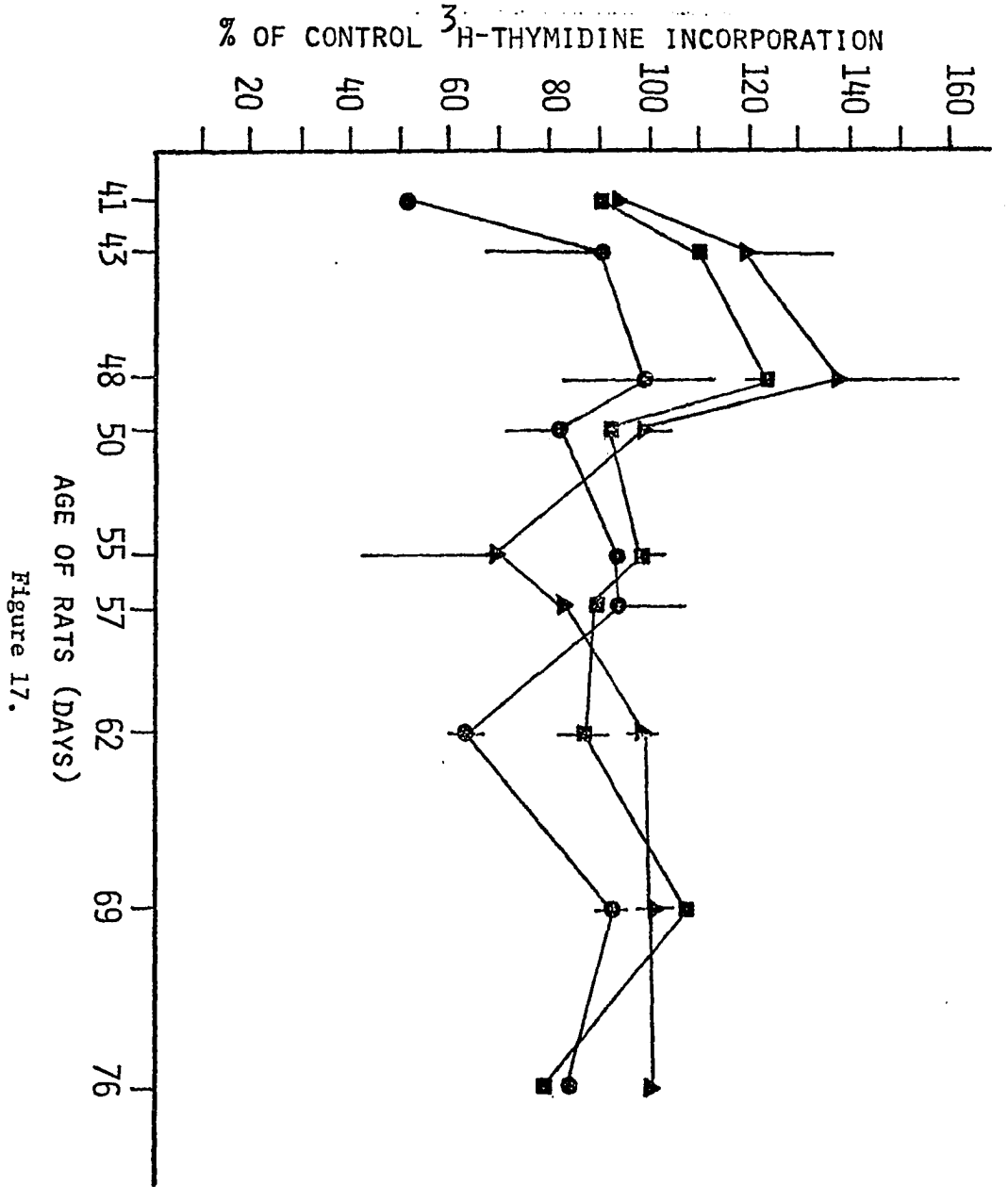
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Figure 17.

Figure 18. Inhibition of incorporation of ^{3}H-thymidine into R_2T_2 tumor cells cultured with the three spleen cell populations obtained from rats fed a high fat diet, as a function of age. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the removal of the spleen cells and the addition of 1.0 uCi of ^{3}H-thymidine to the tumor cell monolayers. These cells were incubated for an additional 3.5 hours, removed with trypsin:EDTA, and harvested. Inhibitory activity of each tumor-spleen cell culture was measured by determining the amount of ^{3}H-thymidine incorporation of the R_2T_2 tumor-spleen cell culture and expressing this as a percentage of the incorporation of the R_2T_2 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of spleen cells} + R_2T_2 \text{ tumor cells})}{(\text{cpm of } R_2T_2 \text{ cells cultured alone})} \times 100$$

O——O designates spleen cells obtained from the NK fraction of Percoll from rats on high fat diets. □——□ designates spleen cells obtained from the 65% fraction of Percoll from rats on high fat diets. △——△ designates spleen cells not separated by Percoll obtained from rats on high fat diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

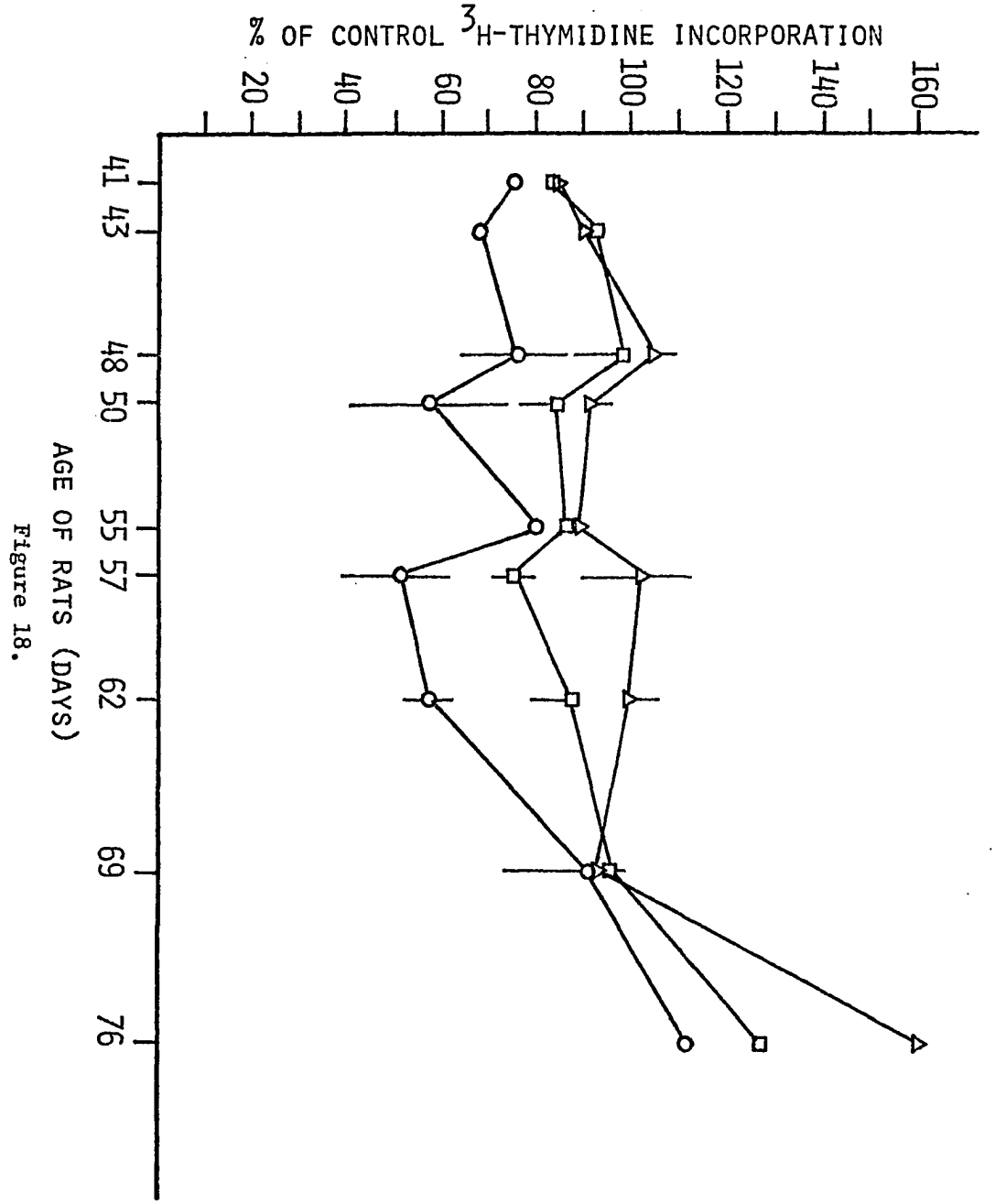
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Figure 18.

Figure 19. Inhibition of incorporation of ^{3}H-thymidine into R_2T_2 tumor cells cultured with NK spleen cell populations obtained from rats fed a low fat diet compared to rats fed a high fat diet, as a function of age. Tumor cells, NK spleen cells, and tumor-NK spleen cell cultures were grown for 16 hours, followed by the removal of the NK spleen cells and the addition of 1.0 uCi of ^{3}H-thymidine to the tumor cell monolayers. These cells were incubated for an additional 3.5 hours, removed with trypsin:EDTA, and harvested. Inhibitory activity of each tumor-NK spleen cell culture was measured by determining the amount of ^{3}H-thymidine incorporation of the R_2T_2 tumor-NK spleen cell culture and expressing this as a percentage of the incorporation of the R_2T_2 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of NK-spleen cells} + R_2T_2 \text{ cells})}{(\text{cpm of } R_2T_2 \text{ cells cultured alone})} \times 100$$

●——● designates NK spleen cells obtained from rats on low fat diets. ⊙——⊙ designates NK spleen cells obtained from rats on high fat diets. Each point represents the mean of six determinations from two rats. Standard error of the mean is shown when it exceeds the symbol's dimensions.

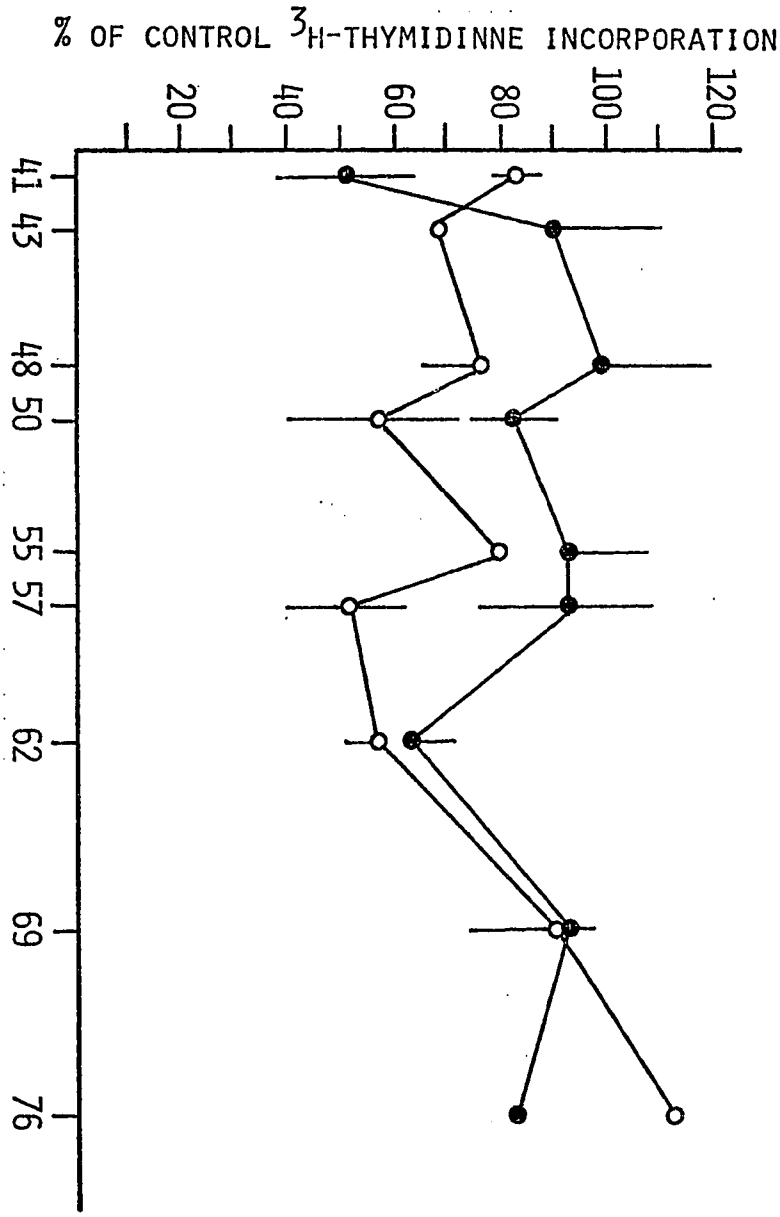
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Figure 19.

Figure 20. Number of viable R_2T_2 tumor cells after exposure to spleen cell populations as a function of time. R_2T_2 tumor cells were cultured with various spleen cell populations for 16 hours. Spleen cells were removed, and the cultures reincubated for 8 hours. NK cells were then added to some cultures, while media was added to others, and the cultures incubated for an additional 24 hours. NK cells were removed and replaced with fresh media. Numbers of viable R_2T_2 tumor cells were determined at subsequent time points.

●——● indicate R_2T_2 tumor cells cultured with media only.

■——■ indicate R_2T_2 tumor cells cultured for 16 hours with spleen cells obtained from the 65% fraction of Percoll from animals on low fat diets.

▲——▲ indicate R_2T_2 tumor cells cultured for 16 hours with spleen cells obtained from the NK fraction of Percoll from animals on low fat diets.

O— — —O indicate R_2T_2 tumor cells cultured with media for 16 hours plus NK cells for an additional 16 hours.

□— — —□ indicate R_2T_2 tumor cells cultured with the 65% fraction of spleen cells for 16 hours plus NK cells for an additional 16 hours.

Δ— — —Δ Indicate R_2T_2 tumor cells cultured with NK cells for 16 hours plus an additional 16 hour incubation with NK cells.

Viability was determined by Trypan blue.

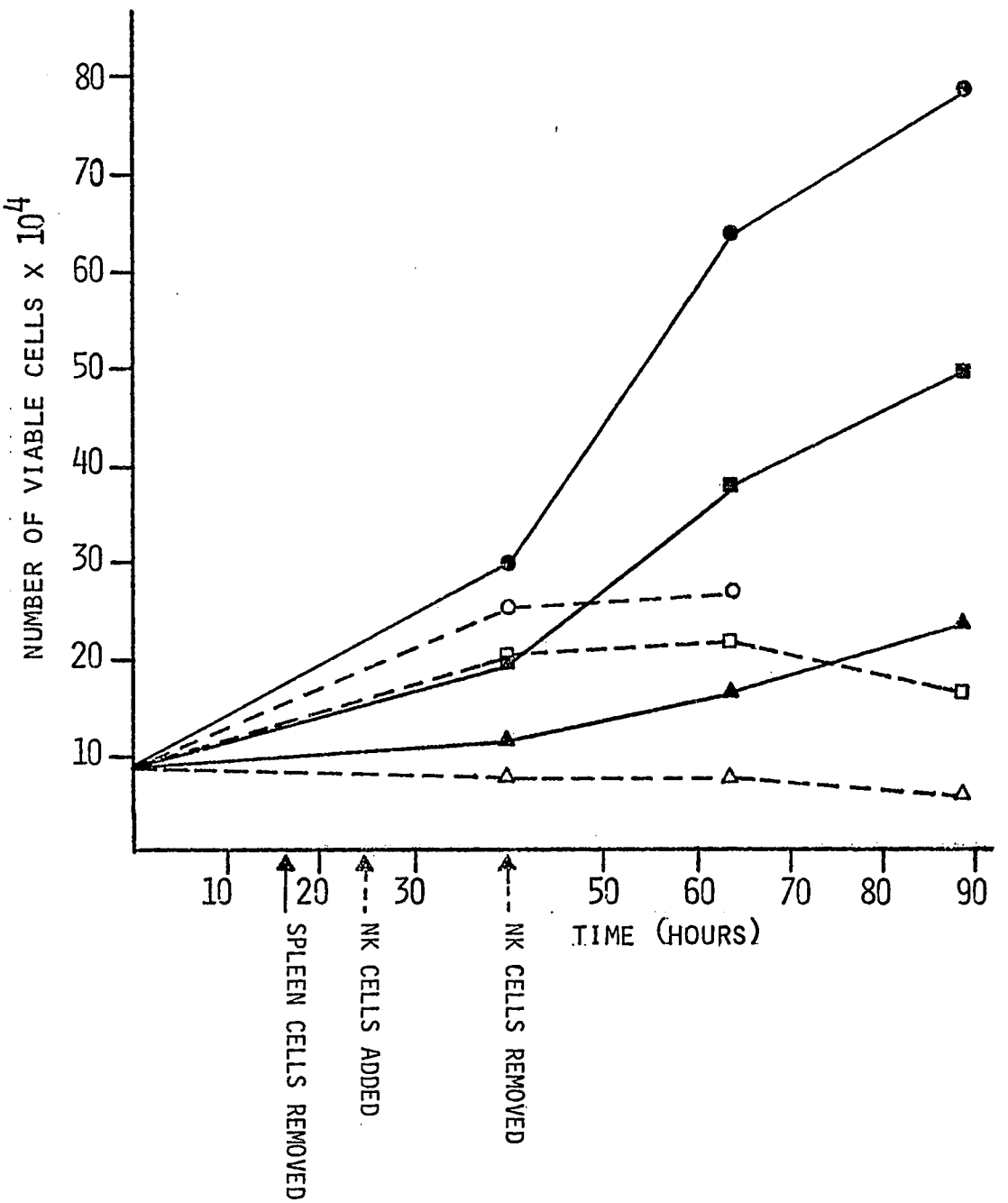
NUMBER OF VIABLE CELLS X 10^4
80
70
60
50
40
30
20
10
10
20
30
50
60
70
80
90
TIME (HOURS)
SPLEEN CELLS REMOVED
NK CELLS ADDED
NK CELLS REMOVED

Figure 20.

CHAPTER IV

DISCUSSION

The incidence of DMBA-induced mammary adenocarcinomas in female, Sprague-Dawley rats was dependent on the quantity and type of dietary fats (8,9,10,28). Rats fed low fat diets and given 10 mg of DMBA on day 50, began developing tumors 8 weeks following exposure to the carcinogen, reaching a maximum tumor incidence of 40% by 34 weeks following carcinogen. In addition, rats fed high fat diets had more tumors per tumor-bearing rat, compared to rats fed low fat diets.

The mechanism(s) by which high fat diets promote tumor growth are not known, however, results from some studies have suggested that high fat diets suppressed various in vivo aspects of the immune response. Feeding or injecting linoleic acid prolonged the skin allograft survival in rodents (46,67,92,120). More specifically, Hillyard and Abraham (44) reported that as little as 0.1% of cis-cis-linoleic acid added to a fat free diet, enhanced the transplantation of two mammary tumor cell lines into BALB/c mice. Other studies have indicated that polyunsaturated fats might be beneficial as an adjunct to immuno-suppressive theory, following renal transplantation (60,64,66,69.

The influence of fats on the immune response as measured by in vitro studies have indicated that the proliferation of lymphocytes by mitogens or allogeneic cells could be inhibited by both saturated fats polyunsaturated fatty acids (65,68,77). Recent studies in our laboratory have demonstrated that the concanavalin A-induced blastogenesis of spleen

lymphocytes was significantly inhibited when lymphocytes from rats on a high, polyunsaturated fat diet (20% corn oil) were compared to lymphocytes from rats on a low fat diet. This responsiveness was partially dependent on the source of serum used in the culture, since lymphocytes from rats fed a low fat diet were suppressed in serum from rats fed a high fat diet. Conversely, lymphocytes from rats fed a high fat diet responded better in serum obtained from rats fed a low fat diet compared to their autologous serum response. Although the response of high fat lymphocytes was improved when cultured in low fat serum, compared to autologous serum, the response did not equal the response of low fat lymphocytes cultured in their autologous serum (57). This suggested that lymphocytes from high fat diet fed rats were probably inherently defective. In another study (56), the combined effects of carcinogen (DMBA) and high fat diet were examined for their ability to inhibit lectin-induced blastogenesis. The results indicated that DMBA further suppressed lymphocyte responsiveness, regardless of type of diet fed.

There are several lines of evidence that suggest that natural killer cells might play a role in host defense against tumors. The premise of the immune surveillance theory is that tumor development should be associated with depressed immunity. Studies by Roder (94) et al. reported that patients with Chediak-Higashi syndrome had a marked deficit of NK cell activity and, in addition, had a high incidence of lymphomas. Roder and Duwe (93) also reported that a colony of beige mice, which had a genetically linked suppressed NK cell activity, also had a high incidence of lymphomas. Studies by Gorelick and Herberman (32) showed that the carcinogen, urethane, caused lung tumors in susceptible strains of

of mice which also had suppressed NK cell activity, while mice which were resistant to tumors caused by urethane, had normal levels of NK cell activity.

The effects of high fat diets on natural killer cell activity have not previously been reported. If high fat diets were shown to be suppressive to one type of in vitro immune response, the effects of high fat diet on natural killer cell function might be predicted.

Our studies indicated that rats fed high fat diets, generally had lower levels of inhibitory activity associated with their NK cell fractions, directed against YAC-1 tumor cell targets, compared to that of rats fed low fat diets. In addition, these lower levels appeared to be "cyclic" in nature, with a periodicity of approximately 3 weeks. The presence of "cyclic" immune phenomona has been reported by other investigators. An early study by Barrett and Hansen (2), reported that different groups of BALB/c mice which were challenged at various time points with allogeneic tumor cells, demonstrated varying degrees of tumor immunity, measured by tumor incidence. When the data was analyzed with respect to time, they found that these fluctuations in immunity appeared to be cyclic in nature, with a periodicity of about 2 weeks. Simpson and Beverley (105) reported a similar observation of "cyclic" responses of lymphocyte cytotoxicity toward a tumor xenograft in CBA mice. They reported that spleen lymphocytes obtained from CBA mice which had been injected with hamster tumor cells (Tr), demonstrated cyclic cytotoxicity towards these tumor cells. Levels of cytotoxicity peaked on days 10, 18, and 40. The response of lymphocytes from lymph nodes, however, showed only one peak response on day 10, followed by a gradual decline in

cytotoxicity through day 40. This differential, organ specific, activity of lymphocyte responsiveness in relation to its "cyclic" response pattern was postulated to be related to the formation of antigen-antibody complexes (105,121). The only other report of "cyclic" responses of natural killer cell activity, other than the present study, stem from work by Fernandes and Good (24), in which they describe a type of cycle associated with natural killer cell activity in male inbred, Fischer rats. This cycle was described as being circadian in nature, with maximum and minimal NK cell activities correlated with similar cyclic responses of rectal temperatures.

In the present study, the inhibitory activity of NK cells from rats on high fat diets appeared to cycle twice, while the inhibitory activity of NK cells from rats on a low fat diet cycled only once. These same types of cyclic responses were also observed in rats fed either low fat of high fat diets, and given 10 mg of DMBA at 50 days of age. Our studies also indicated that DMBA administration had no suppressive effect on the "cyclic" inhibitory activity of NK cells obtained from these rats compared to non-DMBA treated rats. In contrast, studies reported by Erlich et al. (20) indicated that 6 weekly intragastric feedings of 1 mg of DMBA to BALB/c mice resulted in suppression of their NK cell activity, measured against the YAC-1 lymphoma cell line. This suppression started 2 weeks after the last DMBA feeding, and continued for 6 weeks. There was also a corresponding loss in spleen cell number from 40% to 80% of the total number of spleen cells found in untreated animals of that age. By 7 weeks following the carcinogen, both the NK cell activity and the number of spleen cells had started to increase, with a return of normal

levels of NK cell activity and spleen cell numbers by 8 weeks of age. The lack of suppression of NK cell activity in our system might be due to the fact that a single exposure to carcinogen might not be as suppressive to NK cell activity compared to multiple exposures to carcinogen over an extended period of time. The interesting observation was that the major differences in inhibitory activity of high fat diet fed animals compared to low fat diet fed animals regardless of carcinogen, lay in the suppressed inhibitory activity of the high fat diet fed animals, following day 50. The interval between days 30 and 65 has been shown to be the critical time interval for carcinogen to be administered to Sprague-Dawley female rats, in order for maximum tumor induction to occur (49). Some investigataors have suggested that this time interval correlates with the rapid proliferation of cells in the rat mammary gland (96), and thus maximizes effects of DNA damage, brought about by an increase in DMBA binding affinity to DNA (49,96). The time interval of 64-76 days was the period in which our rats on high fat diets experienced a second cyclic decrease in inhibitory activity associated with their spleen cell fractions, compared to rats on a low fat diet, whose inhibitory activity was at a maximum. If NK cell activity *in vitro* correlates with an *in vivo* immune surveillance mechanism (40), the "cyclic" variations in inhibitory activity of NK cell fractions from high fat diet fed animals, might reflect defective NK cell function, during a critical time period in which tumor foci might be developing. The lack of functional NK cells during this time period might eventually result in the successful establishment and continued growth of these tumor foci. This defective NK cell function might also be related to the proliferative

capability of the NK cell itself. The data from table 4 demonstrates an inverse correlation between the amount of proliferation of NK cells cultured alone, with their corresponding ability to inhibit YAC-1 tumor cell proliferation. This data clearly demonstrated that when NK cells had low levels of proliferation, their corresponding inhibitory activity towards YAC-1 targets was high, while NK cells, whose proliferation was high, had low levels of inhibitory activity towards YAC-1 targets. NK cells obtained from animals on high fat diets which had highest levels of proliferation had correspondingly lowest levels of inhibitory activity towards YAC-1 targets. This suggested that NK cells which were in the proliferative pool, could not participate in NK cell-mediated immune reactions. Since animals on high fat diets had NK cells whose proliferative capacity was high, the consequence of being fed a high fat diet might be the production of more total NK cells to compensate for the fewer active NK cells. When the data on percentages of NK cells recovered from either low fat diet fed, or high fat diet fed animals was analyzed, however, there were significantly more cells in the NK cell fraction of rats on the low fat diet, compared to rats on the high fat diet. However, rats on the high fat diet also had a larger spleen cell number compared to the rats fed the low fat diet. When the number of NK cells was expressed on a per spleen basis, however, the number of NK cells in the low fat or high fat diet fed groups was identical.

There were no apparent differences in the inhibitory activity of NK cells from rats fed only low fat choline deficient diets, compared to rats fed only high fat choline deficient diets. However the combination of high fat diet, choline deficiency, and DMBA resulted in rats whose

inhibitory activity was severely suppressed and slow to develop, compared to rats fed low fat, choline deficient diets and given DMBA. This was interesting due to the fact that previous studies by Lombardi et al. and Shinozuka et al. (62, 104) have demonstrated that rats fed corn oil diets, deficient in choline, had a greater incidence of hepatomas induced by ethionine or 2-acetylaminofluorine compared to rats fed choline supplemented diets. In addition, a parallel study in this laboratory, measuring the tumor incidence of animals placed on the various test diets, demonstrated that those animals which were fed high fat, choline deficient diets, and given DMBA on day 50, developed palpable mammary tumors, two weeks earlier than those animals on the high fat diet and similarly given DMBA on day 50, although the final tumor incidence, number of tumors per tumor bearing rat, and the tumor mass were identical in rats fed high fat or high fat, choline deficient diets at 20 weeks of age.

The combined conditions of high fat diet, choline deficiency and DMBA further suggested a synergistic relationship of suppressive effects on NK cell activity, since rats fed this diet regimen were the lowest in their ability to inhibit YAC-1 tumor cell proliferation compared to any other dietary condition tested.

The early appearance of tumors in the parallel study, and the suppressed NK cell activity of rats fed high fat, choline deficient diets and given DMBA reported here, again suggested a possible relationship between early suppression of NK cell activity and the ability of tumor foci to develop. However, a direct cause and effect relationship between suppressed NK cell activity and development of tumors was not demonstrated by this data.

One of the reasons for using the YAC-1 murine lymphoma for rat NK cell assays was that the YAC-1 cell line is the standard tumor cell target used to assay for NK cell activity in rats and mice (5,6,13,18,24, 30) due to its relative sensitivity to NK cell lysis (38,39,40,41,42,43, 52,63,78,79,81). The series of experiments using the R_2T_2 rat mammary adenocarcinoma cell line was an attempt to demonstrate a similar suppressive fat effect, using a target cell that was more closely related to the actual tumor that these animals developed when exposed to the carcinogen. Data was collected and analyzed for inhibitory activities of spleen cell fractions against the R_2T_2 tumor cell line, from rats fed either a low fat diet or a high fat diet. Data from DMBA and/or choline deficient diet fed rats was not included because early tumor foci that might be developing in DMBA treated animals might sensitize lymphocytes in the spleen. If this were the case, the assays would be measuring not only NK cell activity against the R_2T_2 cell line, but also specific cytotoxicity of sensitized lymphocytes towards the R_2T_2 tumor cells. The data revealed that although there were differential inhibitory effects of the three spleen cell populations on the incorporation of ^{3}H-thymidine into the R_2T_2 tumor cell targets, there was no significant difference in the ability of NK cells from rats on a low fat diet, to inhibit the incorporation of ^{3}H-thymidine by R_2T_2 targets compared to the inhibitory activity of NK cells from rats fed high fat diets. In addition, the inhibitory activity of NK cells towards R_2T_2 targets was never lower than 55% of control ^{3}H-thymidine incorporation compared to the greater inhibition of proliferation of the more sensitive YAC-1 target. There are several reasons that could explain low levels of inhibitory activity

of NK cells against R_2T_2 tumor targets. First, the R_2T_2 cell line is a non-lymphoid cell line. Several investigators (43,52,101) have pointed out that lymphoid cell lines are more sensitive to NK cell activity than non-lymphoid cell lines. Secondly, the R_2T_2 cell line grows as an adherent monolayer. Stutman et al. (110) have recently reported that YAC-1 cells grown in artificial monolayers were resistant to natural killer cell attack, but were lysed when grown as suspension cultures. The adherence of R_2T_2 cells to plastic substrate might at least, in part, protect some parts of each cell from NK cell binding and subsequent inhibitory activity. The third reason for observing low levels of inhibitory activity against R_2T_2 tumor cells may be inadequate incubation periods with NK cells. Studies using R_2T_2 tumor targets used incubation times which were sufficient to inhibit the growth of the sensitive YAC-1 tumor cell. A longer incubation time might be required to demonstrate higher levels of inhibitory activity of NK cells against R_2T_2 tumor cells. Finally, the fourth reason for low NK cell activity against R_2T_2 tumor cells might be in the nature of the tumor cell line itself. The in vitro culturing of the original cell line may have selected for certain populations of tumor cells that no longer reflect the characteristics of in vivo tumor cells, and thus are insensitive to NK cell attack. Our studies however, did indicate that if R_2T_2 tumor cells were exposed to two 16 hr periods of culture with NK cells, their subsequent growth, after removal of NK cells, was inhibited for an additional 50 hours. However, a growth curve with zero slope could be interpreted in two ways; (1) NK cells could be cytostatic against R_2T_2 tumor cells or (2) NK cells could be cytolytic with cell division cell and death of R_2T_2 tumor cells being equal.

The correlation between inhibition of ^{3}H-thymidine uptake and actual killing of tumor cells by NK cells is a more difficult question to answer. The standard assay used for demonstrating NK cell activity utilizes a 4 hour, ^{51}Cr release assay to detect lysis of ^{51}Cr labelled tumor cell targets by NK cell populations. In addition, high effector to target cell ratios are used (100:1) to ensure detection of target cell killing as measured by release of ^{51}Cr into the media by lysed target cells. This method however, is limited due to the fact that some tumor cell lines release ^{51}Cr spontaneously into the media if longer incubation times (8 to 12 hours) are employed (7). The inhibition assay employed in the present study may indeed reflect a combination of cytostasis and cytolysis in as far as the YAC-1 cell line is concerned. This is based on the observations that some YAC-1 tumor cells bound to NK cells fail to exclude trypan blue, indicating that cytolysis has taken place. However, some NK cells also were observed to be bound to YAC-1 cells which appeared to be viable. These results however were based on a limited amount of data obtained from YAC-1 NK cell cultures.

The separation of spleen cell populations using Percoll density gradient centrifugation appeared to be a convenient method to enrich spleen cell populations for natural killer cell activity. This method was very similar to the method recently described by Reynolds et al. (89). One main difference between the present study and their work is the natural killer cell activity in nylon wool passed rat spleen cells was enriched in the 44.2% and 47.5% fraction of Percoll, compared to the enrichment in inhibitory activity of our NK cells in the 55% and 60% Percoll fractions.

The interesting observation in studies was that the 55% and 60% fractions of Percoll separated cells were enriched also with large granular lymphocytes (LGL), described by Reynolds et al. (89), as being present in their 44.2% and 47.5% Percoll fractions. Although LGL morphology has yet to be designated as a unique marker for NK cells (89,115), the appearance of these cells in our 55% and 60% Percoll fractions indicated that the inhibitory activity of these types of NK cells reported in this study, probably are the same cells which have been described as "killers" in ^{51}Cr release assays and further lend support to the idea that natural killer cell activity can be related to inhibition of ^{3}H-thymidine uptake by tumor cell targets.

The present study indicated a relationship of diet to inhibitory activity of natural killer cells. The correlation of low inhibitory activity of NK cells with rats fed high fat, choline deficiency diets, and given DMBA could be further extended to animals fed high fat diets, regardless of carcinogen or choline deficiency. The presence of proliferating NK cells in NK cell fractions of rats fed high fat diets could also be correlated with low inhibitory activity of these NK cell populations. These correlations in the decreased ability of NK cells from rats fed high fat diets, to inhibit ^{3}H-thymidine incorporation into tumor cell targets, were only valid for reactions involving the YAC-1 tumor cell line. The inability to show diet related differences in NK cell inhibitory activity against the R_2T_2 tumor cell line probably was related to a number of factors, including the nature of the target cell itself. The association of LGL morphology to NK cell fractions confirmed the earlier work of Reynolds et al. (89) in the rat, and Timonen et al. (115)

in the human. The significance of NK cells in protection of animals from primary tumors, induced by carcinogenic agents is not known. However, recent work by Flannery et al. (25) has shown suggestive evidence that NK cells may play an important in vivo role in host response to chemically-induced, solid tumors. He reported that NK cells were present as infiltrates into two 3-methylcholanthrene induced sarcomas which had been transplanted into syngeneic Wistar rats. These NK cells had cytolytic reactivity towards a number of in vitro derived cell lines tested. There was no evidence of specific cytotoxicity against the tumor of origin.

Whether or not NK cells play an in vivo role in the host response to DMBA-induced rat mammary tumors is currently under investigations. Our present studies have attempted to correlate some of the factors, such as high fat diet, choline deficiency, and DMBA, which have been demonstrated to be important in the establishment of rat mammary tumors, with suppressed in vitro NK cell activity. Further studies are necessary to determine whether or not specific augmentation of suppressed NK cell activity in rats on high fat, choline deficient diets and given DMBA, would result in protection of these animals from subsequent tumor growth, and thus define an in vivo role for natural killer cells in host defense against DMBA-induced mammary tumors.

CHAPTER V

SUMMARY

The incorporation of ^{3}H-thymidine into YAC-1 lymphoma cells was inhibited when Percoll separated, rat spleen cell fractions and unseparated spleen cells were cultured with YAC-1 tumor cells. The greatest inhibition occurred with spleen cells obtained from the 55% and 60% Percoll fractions. These cells were characterized as being large granular lymphocytes which were non-adherent to plastic, and hence, were designated as natural killer (NK) cells.

The inhibitory activity of NK cells was dependent on the quantity of fat in the diet of the rats from which these cells were obtained. Rats which were fed a 20% corn oil diet (high fat) generally had lower levels of inhibitory activity against YAC-1 tumor cells compared to rats fed a diet containing 2% linoleic acid (low fat). While choline deficiency or treatment with DMBA did not alter NK cell activity in either dietary group, the combination of both conditions caused a significant reduction in NK cell activity when rats were maintained on a high fat diet.

The proliferative activity of NK cells was generally higher in rats on the high fat diet compared to rats on the low fat diet. NK-induced inhibition of YAC-1 cell proliferation correlated inversely with the proliferative activity of the NK cells. Some of the rats fed high fat diets had NK cells whose proliferative activity was approximately

twice that of rats fed low fat diets, and whose corresponding inhibitory activity against YAC-1 tumor cells was one-half that of rats on low fat diets.

NK cell activity was not constant during the period of study, but tended to display cyclic responses, particularly in rats on the high fat diet.

There was no difference in the level of inhibitory activity of NK cells from rats on a low fat or a high fat diet, when the R_2T_2 tumor cell line was used as the target cell. The amount of inhibitory activity of the NK cells from rats on both diets was relatively low, compared to the response against YAC-1 tumor cells, but was not apparently due to the presence of NK cell-resistant tumor cells. The data suggested that the response against R_2T_2 tumor cells was cytostatic, but not necessarily cytolytic.

While the ability of NK cells to inhibit proliferation of YAC-1 tumor cells *in vitro* was diet dependent, the inability to show diet dependency on the ability of NK cells to inhibit the proliferation of the R_2T_2 tumor cells *in vitro* did not necessarily rule out an *in vivo* role for NK cells in the resistance of rats to DMBA-induced tumors.

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APPENDIX

YAC-1 Tumor Cells

The YAC-1 tumor cell line was used as one of the targets to assay for natural killer cell activity. Their original characteristics, and culture conditions were previously described in Materials and Methods. Studies indicated that cell cultures initiated with 5×10^5 cells/ml of viable, YAC-1 tumor cells, grew logarithmically for 48 hours, with an approximate doubling time of 24 hours. After 48 hours, the growth rate slowed, and no further increase in cell number was observed by 72 hours. Viability of cells in logarithmic growth was consistently between 97% and 100%, as measured by Trypan blue exclusion. To ensure that YAC-1 cell targets maintained logarithmic growth, stock cultures were prepared each day, using an initial concentration of 5×10^5 cells/ml. These stock cultures were used 24 hours later at a concentration of 1×10^6 cells/ml in the natural killer cell assays. In addition, 5×10^5 cells/ml of YAC-1 tumor cells typically incorporated from 300,000 cpm to 350,000 cpm after being cultured for 16 hours with an additional 3.5 hour pulse of ^{3}H-thymidine. This measurement served as an additional method to monitor whether or not the YAC-1 cells were in logarithmic growth on a daily basis.

R_2T_2 Tumor Cells

The R_2T_2 tumor cell line was used as another source of target cell to assay for natural killer cell activity. Their origin, characteristics,

and culture conditions were previously described in Materials and Methods. Studies indicated that 3 x 10^5 cells/ml of R_2T_2 tumor cells represented a cell monolayer that was approximately 30% confluent in both microtiter test plate wells, and in T-75 tissue culture flasks. Doubling time of these cells was approximately 24 hours. Cell concentrations of 1 x 10^6 cells/ml represented 100% confluent monolayers. Viability was consistantly between 96% and 100%, as measured by Trypan blue exclusion. To ensure that R_2T_2 tumor cells were provided with proper growth conditions for a 24 hour culture period, stock cultures were initiated with 3 x 10^5 cells/ml, and were used 24 hours later to assay for natural killer cell activity. In addition, 3 x 10^5 cells/ml of R_2T_2 tumor cells cultured for 16 hours, followed by an additional 3.5 hour incubation with ^3H-thymidine, typically incorporated between 60,000 and 70,000 cpm. This method served as an additional way to determine whether or not the R_2T_2 cells were in logarithmic growth on a daily basis.

Nonspecific Esterase Staining

To determine the relative percentage of macrophages in unseparated and plastic beaded column separated spleen cell populations, spleen cell suspensions from rats on low fat or high fat diets, before and after column separation, were stained for nonspecific esterase, according to the method of Koski (59). Briefly, spleen cell suspensions were applied to slides that had been previously coated with fetal calf serum. The slides were then air dried and fixed in cold formaldehyde-acetone fixative for 30 seconds. The slides were then incubated for 45 minutes at 37°C in a solution of hexazotized pararosanaline and α-naphthyl butyrate.

The slides were then washed with distilled water and counterstained with 0.6% methyl green. Slides were examined under oil immersion. Cells containing nonspecific esterase were distinguished by the presence of multiple, intensely red stained granules in their cytoplasm, compared to the uniform green staining of nonspecific esterase negative cells. Approximately 200 cells were counted per preparation. Five animals from each diet were used for the evaluation. The results in Table 1 indicated that separation of spleen cells by plastic beaded columns reduced the number of esterase positive cells from 21.1% to 1.2% in animals on low fat diets, and from 22.3% to .9% in animals on high fat diets. There were no significant differences in the percentages of esterase positive cells removed by the plastic beaded columns between animals on the low fat or high fat diet.

Separation of Cells by Percoll Density Gradients

Discontinuous Percoll density gradients were prepared in accordance with procedures previously described in Materials and Methods. Figure 1 illustrates the composition of a typical gradient. Gradients were prepared by pipetting 2 ml of each percentage Percoll solutions into 15 ml conical centrifuge tubes, starting with the highest percentage Percoll solution, followed by successive lower percentages. Discrete interfaces were formed between each percentage Percott solution. Spleen cells (3×10^7) were carefully layered onto the top of each gradient, and the gradients centrifuged at 450 g for 30 minutes. The result was the formation of distinct cell bands at each Percoll percentage interface. Cells were collected from each interface by aspiration with a pipette and were assayed for natural killer cell activity. The use of density

marker beads in a corresponding control tube established the density range of the gradient as being from 1.048 gm/ml to 1.087 gm/ml.

Optimization of Natural Killer Cell Assay (YAC-1)

Figure 2 illustrates an experiment in which various Percoll separated fractions of spleen cells were assayed for their ability to inhibit the ^{3}H-thymidine incorporation of YAC-1 tumor cells. Whole spleen cell populations and non-adherent cell populations (obtained by plastic beaded column separation, see Materials and Methods) were used in this study. Both spleen cell populations were separated by Percoll density gradient centrifugation as described above. A volume of 0.1 ml of spleen cells obtained from the different interface fractions of the Percoll gradients was added to triplicate wells of microtiter test plates. In addition, spleen cell populations not separated by Percoll were also added to triplicate wells. The number of spleen cells added to tumor cells was either 2×10^{5} or 1×10^{5}, yielding an effector (spleen cell) to target (tumor cell) ratio of 2:1 and 1:1, respectively. Spleen cells or tumor cells were also cultured alone. Spleen cells, tumor cells, and spleen-tumor cell cultures at E:T ratios or 2:1 and 1:1 were incubated at 37°C for 16 hours in 10% CO_2, followed by the addition of 1 μCi/well of (^{3}H-6)-thymidine, and cultured for an additional 3.5 hours. The cell cultures were harvested, and the amount of ^{3}H-thymidine incorporation determined (see Materials and Methods). The amount of ^{3}H-thymidine incorporation of the YAC-1 tumor cell-spleen cell cultures was expressed as a percentage of the ^{3}H-thymidine incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

Figure 2 illustrates that the ^{3}H-thymidine incorporation by YAC-1 calls was more inhibited by effector to target cell ratios of 2:1 compared to ratios of 1:1. Non-adherent cells, either separated by Percoll, or used as unseparated (U) cells were more effective in inhibiting YAC-1 ^{3}H-thymidine incorporation than were whole spleen cell populations subjected to the same separation procedures. All spleen cell fractions, however, inhibited the ^{3}H-thymidine incorporation of YAC-1 cells to some degree. The 55% and 60% Percoll fractions of spleen cells inhibited the ^{3}H-thymidine incorporation of YAC-1 cells to the greatest degree. In all subsequent experiments, these two fractions were combined and designated as the NK cell fraction. Since non-adherent cells, separated by Percoll gradients, were more effective in inhibiting the ^{3}H-thymidine incorporation compared to whole spleen cell populations separated by the same techniques, only non-adherent cells were used in all subsequent assays.

Figure 3 represents an experiment in which the 65%, NK, and (U) spleen cell populations were used to further optimize the natural killer cell assay, based on incubation time and E:T ratios. The E:T ratios of 16:1, 8:1, 4:1 and 2:1 were obtained by culturing 16 x 10^{5}, 8 x 10^{5}, 4 x 10^{5}, and 2 x 10^{5} spleen cells with 1 x 10^{5} YAC-1 tumor cells in the wells of microtiter test plates. Spleen cells and tumor cells were also cultured alone. The incubation times were 3 hours, 6 hours, and 16 hours, with each incubation being followed by the addition of 1.0 μCi of (^{3}H-b)-

thymidine, and an additional 3.5 hour incubation. Cells were harvested and the ^{3}H-thymidine incorporation determined (see Materials and Methods). Figure 3 demonstrates that maximum inhibition of ^{3}H-thymidine incorporation of YAC-1 tumor cells occurred when NK cells were incubated with target cells for 16 hours at an E:T ratio of 4:1 or greater. At E:T ratios of 8:1 and 16:1, the 65% and unseparated spleen cells also showed significant inhibition at each of the three time points. Based on these results, NK cell activity against YAC-1 tumor cells was measured in all subsequent experiments, using an incubation time of 16 hours, with an E:T: ratio of 4:1.

Optimization of Natural Killer Cell Assay (R_2T_2)

Figure 4 demonstrates an experiment designed to determine the optimal E:T ratio when assaying for NK cell activity of the three spleen cell populations described above, against the R_2T_2 cell line. The E:T ratios of 13:1, 8:1, 4:1, and 2:1 were obtained by culturing 4 x 10^5, 2 x 10^5, 1.3 x 10^5 and 6.5 x 10^4 spleen cells with 3 x 10^4 R_2T_2 tumor cells in triplicate wells of microtiter test plates. Spleen cells and tumor cells were also cultured alone. The standard 16 hour incubation time was used so that results could be compared to previous experiments. After the initial incubation period, spleen cells and media were removed from the spleen cell-R_2T_2 cultures, leaving the adherent R_2T_2 tumor cells in the wells. All cultures were refed with media, and 1.0 μCi of (^{3}H-6)-thymidine was added to each well. Cells were harvested, the R_2T_2 tumor cells being removed by a 10 minute incubation with 0.25% trypsin containing 0.02% EDTA, and the amount of ^{3}H-thymidine incorporation determined

(see Materials and Methods). The amount of ^{3}H-thymidine incorporation of the R_2T_2 tumor-spleen cell cultures was expressed as a percentage of the ^{3}H-thymidine incorporation of the R_2T_2 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of spleen cells} + R_2T_2 \text{ tumor cells})}{(\text{cpm of } R_2T_2 \text{ cells cultured alone})} \times 100$$

The results indicated that maximum inhibition of ^{3}H-thymidine incorporation occurred when NK cells were incubated with R_2T_2 tumor cells at an E:T ratio of 13:1. The 65% and unseparated spleen cells also showed significant inhibition at this ratio. Based on these results, NK cell activity against R_2T_2 tumor cells was measured in all subsequent experiments using an incubation time of 16 hours, and an E:T ratio of 13:1.

Table 1. Percent of nonspecific esterase positive spleen cells before and after plastic beaded column separation, from rats on low fat or high fat diets. Mean of five rats per diet group.

Table 1.

EFFECTS OF COLUMN SEPARATION ON THE PERCENT OF ESTERASE POSITIVE SPLEEN CELLS FROM RATS ON LOW OR HIGH FAT DIETS

	BEFORE COLUMN		AFTER COLUMN	
	DIET		DIET	
	LOW	HIGH	LOW	HIGH
PERCENT OF CELLS STAINED POSITIVE FOR NON-SPECIFIC ESTERASE	21.1 ±2.0	22.3 ±4.0	1.2 ±.7	0.9 ±.3

Figure 1. Discontinuous Percoll density gradient prepared by by pipetting different percentages of iso-osmotic Percoll solutions into a conical centrifuge tube. Spleen cells (3×10^7) were added to the top of the 40% Percoll layer. The gradient was centrifuged at 450 G for 30 minutes. Spleen cells formed distinct bands at each Percoll density interface. Cells were collected by aspiration from each density band. Density of the gradient ranged from 1.048 gm.ml to 1.087 gm.ml as determined by density marker beads.

Figure 1.

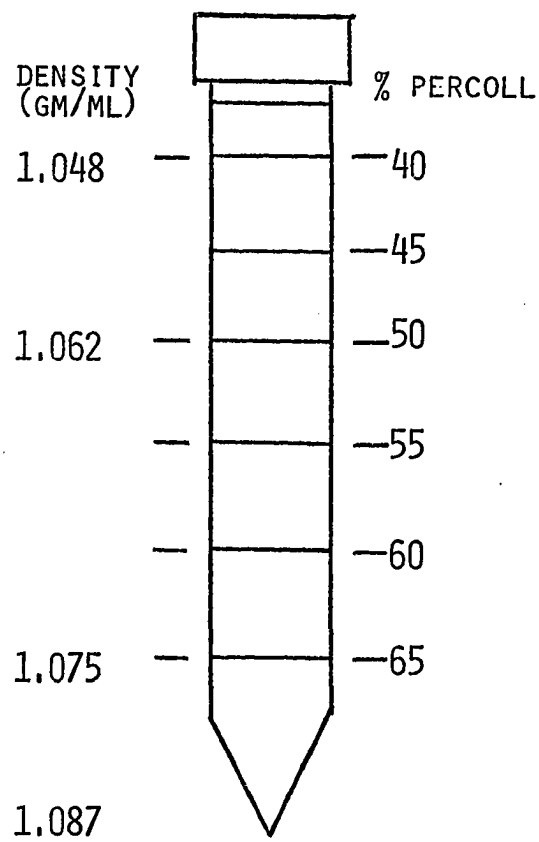

Figure 2. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with various Percoll separated and unseparated spleen cells at different effector to target cell ratios. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine and cultured for an additional 3.5 hours. Cells were harvested and the amount of ^{3}H-thymidine incorporation determined. The ^{3}H-thymidine incorporation of the YAC-1 tumor cells cultured with the various spleen cell populations was expressed as a percentage of the ^{3}H-thymidine incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) \text{ minus } (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

■———■ ▲———▲ designates whole spleen cells used for Percoll separation.

□— — —□ △— — —△ designates non-adherent cells used for Percoll separation.

U = spleen cells not separated by Percoll.

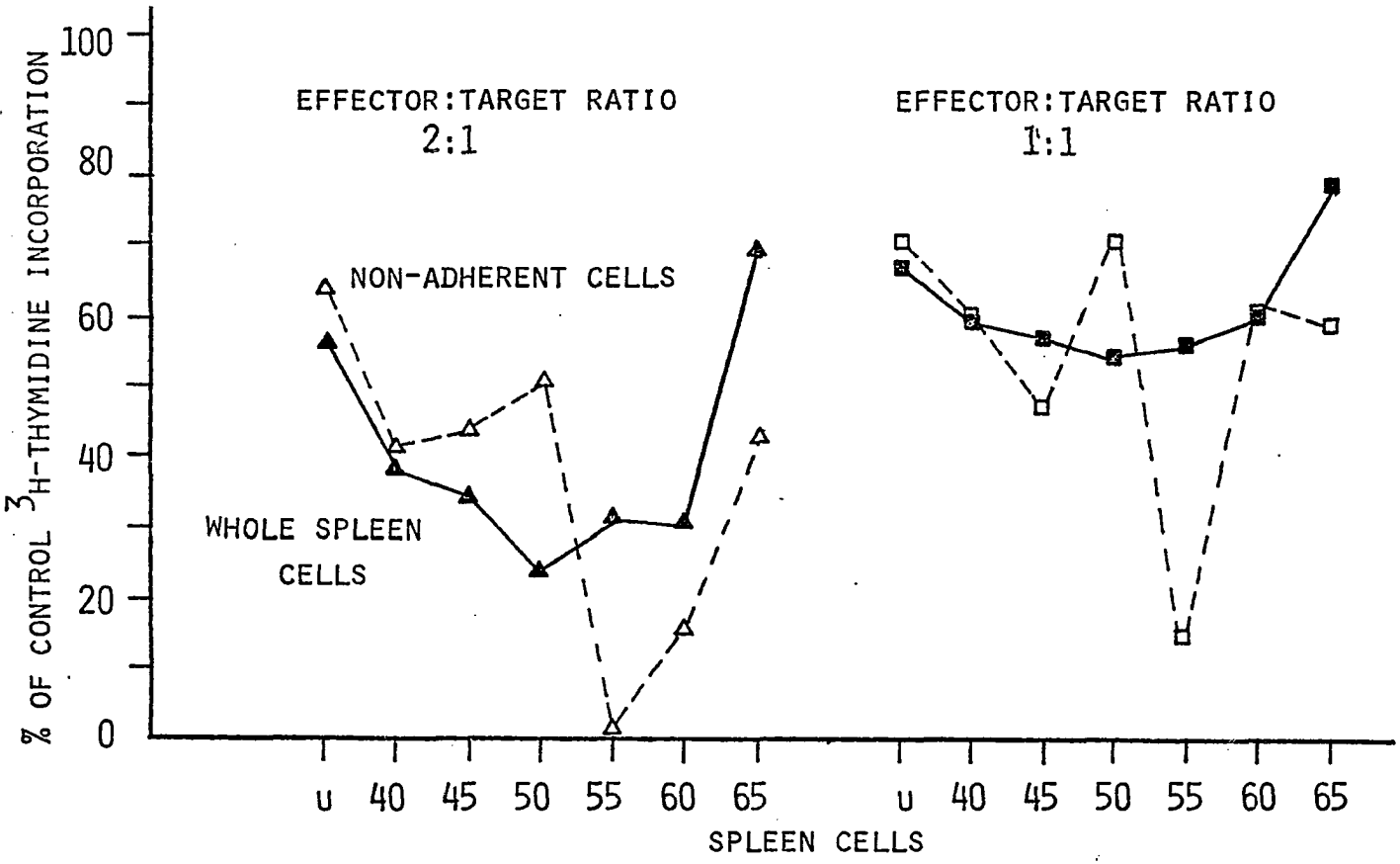
% OF CONTROL ^{3}H-THYMIDINE INCORPORATION
100
80
60
40
20
0
EFFECTOR:TARGET RATIO
2:1
EFFECTOR:TARGET RATIO
1:1
NON-ADHERENT CELLS
WHOLE SPLEEN CELLS
u 40 45 50 55 60 65
u 40 45 50 55 60 65
SPLEEN CELLS

Figure 2.

Figure 3. Inhibition of incorporation of ^{3}H-thymidine into YAC-1 tumor cells cultured with various Percoll separated and unseparated spleen cells for different periods of time, and different effector to target cell ratios. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 3, 5, or 16 hours, followed by the addition of 1.0 uCi of ^{3}H-thymidine, and cultured for an additional 3.5 hours. Cells were harvested, and the amount of ^{3}H-thymidine incorporation determined. The ^{3}H-thymidine incorporation of the YAC-1 tumor cells cultured with various fractions of spleen cells was expressed as a percentage of the ^{3}H-thymidine incorporation of the YAC-1 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\%\ \text{of Control}\ {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of Spleen cells + YAC-1 cells}) - (\text{cpm of Spleen cells cultured alone})}{(\text{cpm of YAC-1 cells cultured alone})} \times 100$$

■———■ designates 3 hours of growth. ●—·—·—● designates 5 hours of growth. ▲— — —▲ designates 16 hours of growth. U = spleen cells not separated by Percoll. NK = spleen cells obtained from the 55% and 60% fractions of Percoll. 65 = spleen cells obtained from the 65% fraction of Percoll.

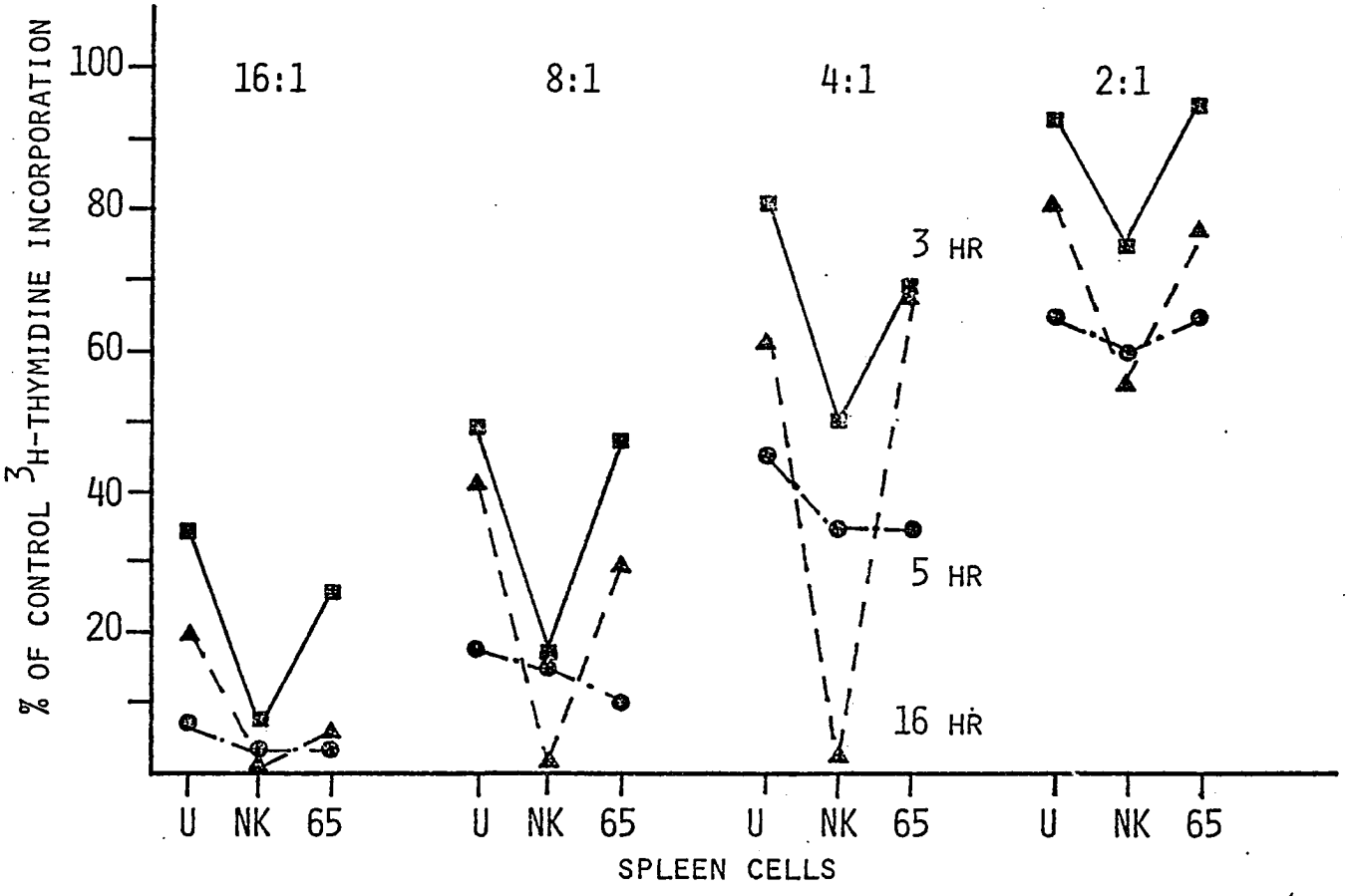
% OF CONTROL ^{3}H-THYMIDINE INCORPORATION
100
80
60
40
20
16:1
8:1
4:1
2:1
3 HR
5 HR
16 HR
U NK 65
U NK 65
U NK 65
U NK 65
SPLEEN CELLS

Figure 3.

Figure 4. Inhibition of incorporation of ^{3}H-thymidine into R_2T_2 tumor cells cultured with various Percoll separated and unseparated spleen cells at different effector to target cell ratios. Tumor cells, spleen cells, and tumor-spleen cell cultures were grown for 16 hours, followed by removal of the spleen cells, and the addition of 1.0 uCi of ^{3}H-thymidine to the tumor cell monolayers. These cells were incubated for an additional 3.5 hours, removed with trypsin:EDTA, and harvested. Inhibitory activity of each tumor-spleen cell culture was measured by determining the amount of ^{3}H-thymidine incorporation of the R_2T_2 tumor-spleen cell culture and expressing this as a percentage of the incorporation of the R_2T_2 tumor cells cultured alone (control). The results were calculated using the following formula:

$$\% \text{ of Control } {}^{3}\text{H-thymidine Incorporation} = \frac{(\text{cpm of spleen cells} + R_2T_2 \text{ tumor cells})}{(\text{cpm of } R_2T_2 \text{ cells cultured alone})} \times 100$$

U = spleen cells not separated by Percoll. NK = spleen cells obtained from the 55% and 60% fractions of Percoll. 65 = spleen cells obtained from the 65% fraction of Percoll.

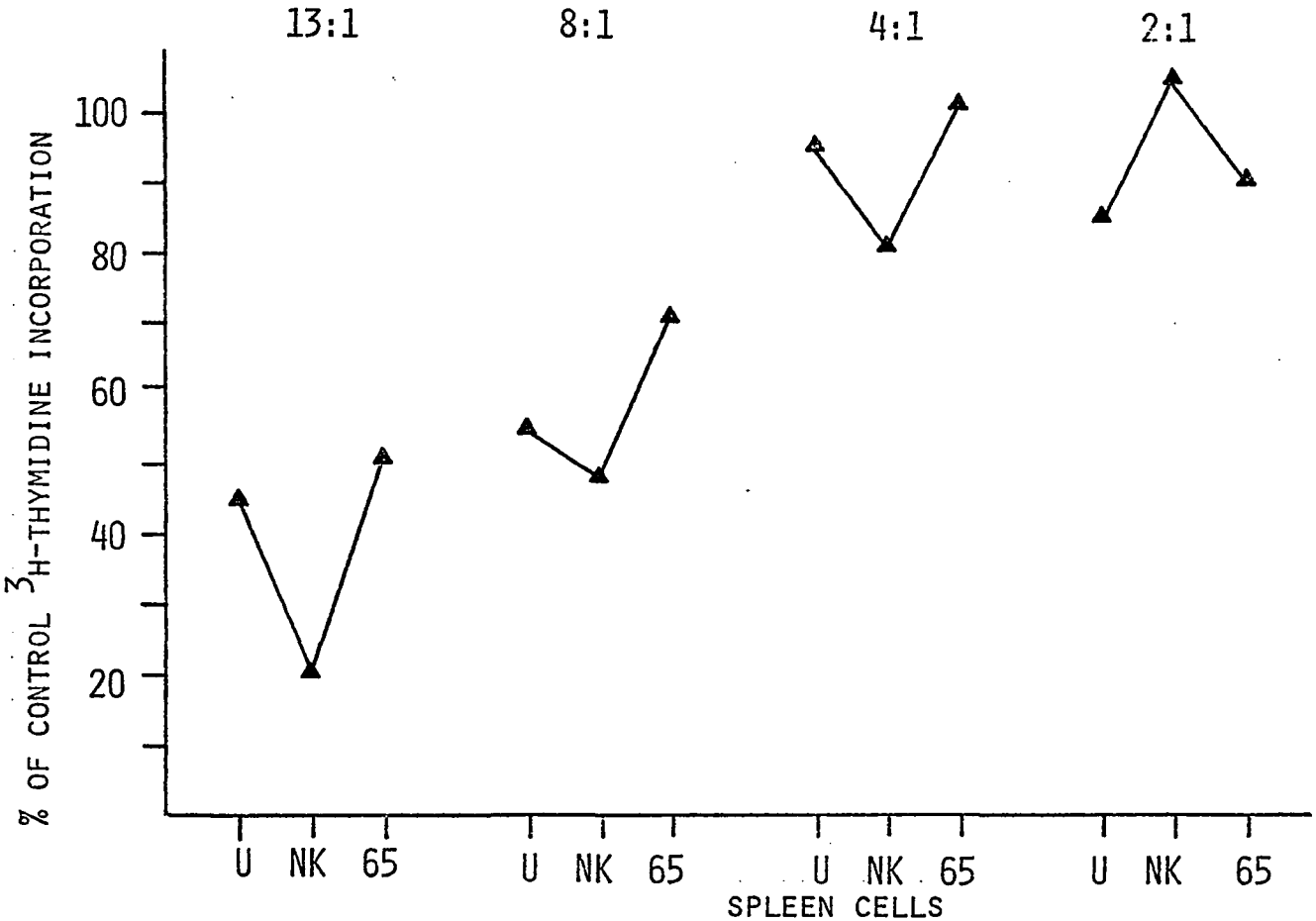
13:1
8:1
4:1
2:1
100
80
60
40
20
% OF CONTROL ^{3}H-THYMIDINE INCORPORATION
U NK 65
U NK 65
U NK 65
U NK 65
SPLEEN CELLS

Figure 4.