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The University of North Carolina at Greensboro PH.D. 1984

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IN VIVO AND IN VITRO EFFECTS OF RETINOIDS ON THE HISTOLOGICAL CHANGES IN COLORECTAL TISSUE

by

Patricia Bowling Brevard

A Dissertation Submitted to
the Faculty of the Graduate School at
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Doctor of Philosophy

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1984

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Directed by Dr. Laura Anderton and Dr. Aden Magee. Pp. 123

The purpose of this study was to determine the histological changes induced by high physiological levels of dietary retinoids on rat colorectal tissue in vivo and on human colorectal adenomas and adenocarcinomas in vitro. For the in vivo study, young male rats were fed stock diets supplemented with 6, 30, or 60 Retinol Equivalents (RE) of retinol (Retinol A, B, and C, respectively) or β-carotene (β-carotene A, B, and C, respectively) for two weeks. The animals were sacrificed and a segment of colorectal tissue was fixed at the end of the experiment. The High Iron Diamine-Alcian Blue and Periodic Acid Thionin/Potassium Hydroxide/Periodic Acid Schiff stains were used to determine the numbers of goblet cells and mast cells and amount, type, and location of mucus produced by goblet cells and connective tissue.

The in vitro study was performed on human tumors exposed to retinol (75 or 250 µg/100 ml medium, Retinol 1 and 2, respectively) and β-carotene (250 or 500 µg/100 ml medium, β-carotene 1 and 2, respectively), for six days. Histological stains and dependent variables were the same as the in vivo study. Number of plasma cells and lymphocytes were also determined.

There were no significant differences between the effects of β-carotene and retinol, but differences could be attributed to the level of either form of the vitamin. Increasing dietary retinol or β-carotene from level A to B resulted in a significant increase in number of goblet cells in rat tissue and, although not significant, the same trend was seen in vitro. In tissue culture, the mucus produced by the tissue was significantly more sulfated in the Retinol 1 group and both β-carotene

groups compared to the control group. Although not significant, the same trend was seen *in vivo*. There was a significant change in the chemistry of sialomucins produced in goblet cells and connective tissue in human tissue and in connective tissue in rat colorectal segments. Sialic acids with OH groups substituted at C_8 increased in all groups receiving vitamin A over control *in vitro* and from level A to level C *in vivo*.

The number of mast cells significantly increased when the level of vitamin A was increased from A to C *in vivo* and in both β-carotene groups compared to control *in vitro*. There was an increase in number of plasma cells and lymphocytes in the β-carotene 1 group compared to the control group *in vitro* which approached statistical significance.

Although mucin histochemistry of cancerous tissue has previously been reported, the sialomucin and sulfated mucin content of colorectal tumors has not until now been determined after exposure to retinoids in tissue culture.

Results of this study indicate that vitamin A induces changes in the type of mucus produced and stimulates the immune response of colorectal tissue. It appears that vitamin A influences the genomic regulatory mechanisms in sensitive cells and causes genes controlling production of immunocompetent cells, goblet cells, and sulfated mucins to be turned on.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Colorectal cancer is one of the three leading cancers in the United States in annual new cases. It is estimated that 126,000 new cases will be detected this year and 58,000 deaths will be attributed to colorectal cancer. It is second only to lung cancer in rate of occurrence (Cancer Facts and Figures, 1983). Americans begin to be at significant risk for colorectal cancer between the ages of 40 and 50, and there is a doubling of age-specific incidence rates each decade after age 50. Males and females have the same risk for colon cancer, but males have a much higher risk for rectal cancer. Most colorectal cancers arise from adenomas or polyps. There is a greater risk of cancer with increase in size or number of these tumors. Once a colonic adenoma or colorectal cancer occurs, the mucosa transforms into a premalignant mucosa with altered cell kinetics (Winawer, 1983). Although many forms of cancer have actually declined in frequency of occurrence in the last few years, the frequency of cancer of the colon has remained constant throughout the 1900's (Newell & Ellison, 1981).

The cause of colorectal cancer is not known, but epidemiological studies suggest that several environmental factors, including diet, can accentuate or decrease the development of colon cancers. Vitamin A is one of the nutrients which is currently being studied as a factor in the prevention of certain types of cancer. Several studies have suggested a relationship between dietary intakes of vitamin A active compounds and

the risk of cancer development (Kummet & Meyskens, 1983). The exact mechanisms whereby vitamin A prevents the development of cancer, however, are unknown.

Vitamin A plays an important role in the maintenance of epithelial tissue and apparently has control over the cell's differentiation and proliferation and influences genetic expression in some cells. In a vitamin A deficiency state, the epithelial tissues lose the ability to secrete mucus and the ability to produce goblet cells. Lacking a protective layer of mucus, such tissues would be vulnerable to infections and disease causing agents, including carcinogens.

There are many questions that arise in regard to vitamin A intake and colorectal cancer incidence; thus it seems important that further studies be conducted to investigate the possibilities. The present study was designed to answer questions that are currently being asked about vitamin A and cancer. There are several unique aspects of this study that have not previously been investigated. Although many forms of vitamin A have been used in past studies, the use of β-carotene in tissue culture and in rat diets in vivo has not been attempted, and β-carotene has not been used in investigations which determined histological changes. High physiological levels of retinol and β-carotene have not been used in the past in organ culture with colorectal adenomas and adenocarcinomas.

It is hypothesized that high physiological levels of dietary vitamin A such as retinol and β-carotene can induce histological changes in colorectal tissues both in vivo in rats and in vitro in human adenomas and adenocarcinomas. A second hypothesis is that since vitamin A has been shown to affect mucus secretion and also the immune system, the effects will

be measurable in number of goblet cells, mast cells, plasma cells, and lymphocytes as well as the type of mucus present and location of mucus. In order to investigate these hypotheses, the following objectives were established:

1. To compare the response of young rats fed different forms and levels of vitamin A with respect to the following variables: amount of weight gained, number of goblet cells and mast cells[1] in colorectal tissue, and amount, type and location of mucus secreted in colorectal tissue.
2. To determine whether retinol or β-carotene have an effect *in vitro* on the histological development of adenomas and adenocarcinomas of human colorectal tissue and, if so, to clarify their mechanisms of action at a cellular level by measuring the following variables: number of goblet cells, mast cells, plasma cells, and lymphocytes, and amount, type and location of mucus secreted in colorectal tissue.
3. To determine adequate levels of vitamin A required to stimulate formation of a normal mucous covering for the inner lining of the human colon in organ culture.
4. To test the effects of vitamin A on the production of sulfated mucins (associated primarily with normal colon) and sialomucins (associated primarily with malignant tumors) in adenomas and adenocarcinomas of the colon, and in rat colorectal tissue.

[1]There is presently controversy about staining of mast cells, and those cells counted could also be macrophages. They are carefully defined in Chapter 3, and are referred to as mast cells throughout the text.

5. To determine effective levels of vitamin A necessary in organ culture of the human colon to inhibit growth or stimulate reversion from a malignant state to a benign state as determined by the HID-AB stain.

CHAPTER II

REVIEW OF LITERATURE

Since its inception in the early 1900's, the theory that vitamin A is related to cancer inhibition has been investigated with increasing interest. Although progress has been made in this area, the specific roles and/or mechanisms whereby vitamin A may inhibit cancer development in certain tissues remain an enigma.

Results of several epidemiological studies (Hirayama, 1979; Kark, Smith, Switzer, & Hames, 1981; Peto, Doll, Buckley, & Sporn, 1981) have suggested a definite relationship between dietary intake of vitamin A active compounds and cancer development. In general, the risk of developing several types of cancer appears to be lessened as the dietary intake of Vitamin A, primarily from vegetable sources, is increased. Smith and Jick (1978), however, reported that dietary supplements of vitamin A did not protect against the development of cancer in high-risk individuals. However, Kolonel, Nomura, Hinds, Hirohata, Hankin, and Lee (1983) reported a decreased risk of cancer development with increased usage of vitamin A supplements. Many studies have also suggested that individuals with low serum retinol levels show an increased tendency toward cancer development (Atukorala, Basu, Dickerson, Donaldson, & Sakula, 1979; Basu, Donaldson, Jenner, Williams, & Sakula, 1976; Bjelke, 1975; Kark et al, 1981; Wald, Idle, & Boreham, 1980).

A number of studies have been conducted to determine the effectiveness of different vitamin A active compounds in the prevention and/or

cure of cancer. Many of these studies, however, reveal varying degrees of effectiveness of natural forms of vitamin A in preventing cancer (Newell & Ellison, 1981). Toxic effects have been noted when high levels of vitamin A were used as pharmacologic treatment doses for malignancies ("Vitamin A and Cancer", 1980). Since vitamin A toxicity has been a problem in many cancer studies, investigations have been undertaken to determine the effectiveness of synthetic vitamin A analogs in cancer prevention (Clamon, 1980). Approximately 1500 different retinoids have been synthesized, and many have been tested to determine their effect on carcinogenesis (Peck, 1981). Both the amount and the form of vitamin A are important because either extremely high or low levels of vitamin A can have adverse effects. The form of vitamin A that is most effective has not been determined.

The mechanism whereby vitamin A prevents or reverses carcinogenesis are still largely unknown. Since vitamin A active compounds have control over cell differentiation and glycoprotein synthesis, there is the possibility that the anticarcinogenic effect of these compounds occurs at the cellular level (Marchok, Clark, & Klein-Szanto, 1981; Pawson, 1981; Sporn, 1977). In a vitamin A deficiency, there is a decrease in the number of goblet cells which ultimately results in an inability of mucus to be secreted in epithelial tissue and a decrease in number of these cells could lead to a decrease in mucus secreting ability of such tissue (DeLuca, Schumacher, & Wolf, 1970; Kleinman & Wolf, 1974). Rojanapo, Lamb, and Olson (1980) reported the rate of differentiation of sensitive goblet cells from oligo-mucus cells and other precursors appeared to be blocked in a vitamin A deficiency state. Since differentiation occurs after the cell undergoes division, interference in cell

differentiation could have a subsequent effect on mucus synthesis, goblet cell production, mucus secretion, migration, and exfoliation. However, high physiological levels of vitamin A that are possible to obtain by increasing dietary sources have not been used to determine effects on number of goblet cells.

When tumors were induced by a chemical carcinogen in laboratory animals, those animals fed high levels of vitamin A had fewer and later forming tumors, and in many cases the tumors which did develop underwent regression in the presence of vitamin A. A higher indicence of tumors have been reported in vitamin A deficient animals (Kalamegham & Krishnaswamy, 1980; McCormick, Burns, & Albert, 1981; Morré, Kloppel, Rosenthal, & Fink, 1980; Newberne & Suphakarn, 1977; Rogers, Herndon, & Newberne, 1973; Wattenberg, 1980). Mathews-Roth (1980) also reported a significant decrease in carcinogen induced skin tumors on mice treated with carotenoids. It has recently been reported that retinoids inhibit mammary gland carcinogenesis by modulating the mitogenic effects of peptide hormones such as prolactin and insulin, which may be a mechanism of action of retinoids in mammary tumors as well as other target cells (Welsch, DeHoog, Scieszka, & Aylsworth, 1984).

Some cancers are preceded by long periods of preneoplastic epithelial changes before the development of invasive malignancy, and several vitamin A active compounds have been shown to be effective in arresting and/or reversing such changes (Sporn, 1977). The zone surrounding the neoplasia undergoes many changes. The excessive regenerative growth noted by Usubachi, Kudo, and Sato (1981) may be to compensate for important functions of cells damaged by oncogenic viruses. Benign tumors have a small

amount of regeneration in transitional areas while malignant ones have a markedly excessive regeneration. Preneoplastic lesions are usually present as metaplasia or dysplasia for many years prior to the development of invasive cancer but some can regress (Clamon, 1980).

Clark & Marchok (1979) found that the ability of an explant in cell culture to secrete mucous glycoprotein into the surrounding medium and the production of this mucin fraction depended on the vitamin A status of the explant. Marchok et al. (1981) also reported that adenomas responded better to retinyl acetate stimulation than did squamous cell carcinomas. This response would result in increased mucin glycoproteins and a shift toward secretory epithelium. Many cell lines have been found to be sensitive to the inhibiting effects of various retinoids on carcinogen-induced neoplastic transformation. Several different retinoids have been used to reverse malignant tumors, inhibit or block proliferation of these cells, promote differentiation, suppress transformation, or extend the latency period between exposure to carcinogens and development of malignancy (Bertram, Mordan, Blair, & Hui, 1981; Lotan, Neumann, & Lotan, 1980; Meyskens & Fuller, 1980; Wilson & Dowdle, 1980).

Many cell lines are apparently sensitive to the inhibition by retinoids of carcinogen-induced neoplastic transformation. Reversability as well as inhibition has been shown in vitro. The action of cancer cells in vitro without vitamin A may give insight to the mechanisms of action of vitamin A. Cancer may cause changes in mucus production because colonic tissue both remote from and adjacent to tumors have been shown to contain abnormally high levels of sialomucins and low levels of sulfated mucins (Dawson & Felipe, 1976). Normal colon tissue contains

predominately sulfated mucins and only small amounts of sialomucins. The amount of sialic acid may be directly associated with the stage of tumor development since there is an increase in sialomucins as tumors become malignant. Kalus (1972) found that in cell culture, adenomatous colorectal polyps often become carcinoma *in situ*, with invasive features similar to cultures of colonic adenocarcinoma. Thus, according to Kalus, polyps have a malignant potential of becoming histologic and biologic cancers and adenomatous polyps first become carcinoma *in situ* which then becomes invasive carcinoma. The effect of vitamin A on sialomucins and sulfated mucins in colonic tissue has not previously been studied.

Vitamin A could have an effect on the mucus produced by tissue. Clark and Marchok (1979) found that tracheal explants secreted mucous glycoproteins into the medium but the production of certain mucins depended on the vitamin A status of the explant. Retinol is a carrier of monosaccharide units in glycosyltransferase reactions during glycoprotein synthesis (Peck, 1981). *In vitro* studies have shown that vitamin A enhanced the number of cells engaged in deoxyribonucleic acid (DNA) synthesis, increased transcription of new messenger ribonucleic acid (mRNA) for induction and suppression, and enhanced cell proliferation in various cell lines (Nettesheim, 1980; Tchae, 1980). Vitamin A seems to block a step of tumor promotion (Sugimura, 1982). In addition to inhibiting tumor-promoting agents, vitamin A has been shown to significantly inhibit the proliferation of tumors and various tumor cell lines and to stimulate differentiation toward secretory epithelium. Squamous metaplasia which was induced by vitamin A deficiency with carcinogens was rapidly reversed (Lotan et al., 1980; Meyskens & Fuller, 1980; Peck,

1981; Sporn & Newton, 1979; Wilson & Dowdle, 1980). Vitamin A decreased the ability of human melanoma cells to form colonies (Meyskens & Salmon, 1979). Some cell lines (i.e., those of epithelial origin) accumulate in G-1 and do not enter the S phase of the cell cycle with retinoid treatment (Peck, 1981).

There is also the possibility that vitamin A has a beneficial effect on the immune system by stimulating both cellular mediated immunity and humoral responses (Beisel, 1982). A vitamin A deficiency is associated with decreased lysozyme synthesis which can reduce the resistance of the body to infection. Vitamin A stimulates labilization of lysosomal membranes which causes a release of lysosomal enzymes from cells, and thus contributes to the differentiated state in epithelial tissues. A depletion of lysosomes stimulated proliferation of lymphoid cells (Israel & Aguilera, 1980). There is a marked depletion of lymphoid tissue in chickens fed vitamin A deficient diets. Adding vitamin A back to the diet stimulated proliferation of lymphocytes and production of humoral antibodies. One hundred times more virus was recovered from throat swabs of vitamin A-deficient birds innoculated with virus than from normally fed infected birds (Abb, Abb, & Deinhardt, 1982; Abb & Deinhardt, 1980; Bang, Bang, & Foard, 1972, 1975). Host stem cells seem unable to regenerate normally which could ultimately lead to loss of lymphocytes and failure of stem cell reproduction from lack of vitamin A. Enlargement of the thymus and peripheral lymph nodes and curtailment of skin allografts have been noted with administration of vitamin A at high physiological and toxic levels, which suggests that retinoids may activate or induce natural killer cells. Vitamin A seems to interfere with the

metabolic transformation of oncogenic hydrocarbons or decrease the susceptability to tumors (Medawar, 1981; Rivlin, 1982). With vitamin A deficiency, there is a decreased capacity for splenic lymphocytes to transform when exposed to a T-cell mitogen (Newberne & Suphakarn, 1977). Cytotoxic effects of killer T cells and the tumoricidal effects of macrophages are stimulated by retinoids (Jurin & Tannock, 1972; Mohanram, Reddy, & Mishra, 1974; Peck, 1981; Trechsel, Dew, Murphy, & Reynolds, 1982). Although the number of lymphocytes, plasma cells, mast cells and other immunocompetent cells has been determined in several kinds of cancerous tissues, these cell numbers have not been determined in normal or cancerous colorectal tissue after exposure to retinoids in tissue culture.

Although there is evidence that vitamin A has beneficial effects in cancer development, the results are still inconclusive. Very few studies have been conducted using β-carotene, yet review articles and lay articles continually advocate an increased dietary intake of β-carotene from foods such as carrots (Graves, 1982; Peto et al., 1981; Ross, 1983). It has been speculated that β-carotene is the active form of vitamin A in cancer prevention and treatment ("Dietary Carotene and the Risk of Lung Cancer", 1982; Wolf, 1982). β-carotene acts as an antioxidant in the diet and could be important in protecting body fat and lipid membranes against oxidation. Antioxidants have inhibited tumorigenesis in certain tumors after exposure to carcinogens (King & McCay, 1983). Carotenoids are free-radical traps which are efficient at quenching singlet oxygen, a mutagen, and this may be one mechanism of action of β-carotene as an anticarcinogen in humans (Ames, 1983). A recent report by the National

Cancer Institute (Greenwald, 1981) stressed the fact that β-carotene is likely to be involved in cancer prevention and/or inhibition, and because of the scanty data using β-carotene, they recommended further expansion of basic research in this area.

The present study was designed to include several aspects of the research cited above, especially the effects of vitamin A on the immune system and the type of mucus produced with vitamin A stimulation. The present study is unique in that β-carotene is used in tissue culture medium for human tumors and concurrently in a parallel study in rat diets in vivo. There are no previously reported studies that have determined changes in type of mucus or numbers of immunocompetent cells following exposure to retinoids. These parameters, which are present in characteristic amounts in cancerous tissue were included in the present study. There are many indications that there is a correlation between retinoids and the inhibition of cancer. However, the precise timing has not been determined when retinoids are either the most or the least effective in the developmental stages of malignant transformation. In addition, the most effective type of retinoid for prevention of colon cancer remains to be determined. Information on how vitamin A influences epithelial changes in cancer is not known, and the mechanism of action of retinoids at the cellular level are still unknown. Thus, further research in this area would be a justifiable endeavor.

CHAPTER III

EXPERIMENTAL PROCEDURES

This investigation was divided into two phases. One phase was an *in vivo* study to determine the effects of retinol and β-carotene on the intestinal epithelium and submucosa of young rats. Criteria used for the evaluation included the number of goblet cells and mast cells and the amount, type, and location of mucus secreted. The second phase of the investigation was to determine whether retinol or β-carotene have *in vitro* effects on the histological development of human colon adenomas and adenocarcinomas and to identify the mechanism of action of each retinoid at the cellular level. Criteria used to evaluate this phase of the investigation were the numbers of goblet cells, mast cells, plasma cells, and lymphocytes, and type and location of mucus secreted.

In Vivo Phase

Forty-eight male weanling rats[1] were used in the *in vivo* experiment. The animals were assigned to stainless steel wire-bottom cages in a completely randomized design. The animals were maintained on the experimental diets for two weeks and had free access to food and water. Initial and final weights of each animal were taken. Food consumption records for each animal were also maintained.

Six experimental diets were used in the study. Three of the diets contained 6, 30, and 60 µg or retinol equivalents (RE) of retinol[2] per gram

[1]Sprague-Dawley rats averaging 57 grams in weight initially. Purchased from Holtzman Company, Madison, Wisconsin.

[2]Retinol, Sigma Chemical Company, St. Louis, Missouri.

of diet (Retinol A, B, & C, respectively). The other three diets contained 36 µg (6 RE), 180 µg (30 RE), and 360 µg (60 RE) of β-carotene[3] per gram of diet (β-carotene A, B & C, respectively). Retinol and β-carotene were combined for analysis as three levels (See Table 1). These levels are approximately twice, five times, and ten times the equivalent of the RDA for vitamin A for humans. High physiological levels were desirable to determine the histological changes that occur when dietary vitamin A is manipulated.

Table 1

Levels of Vitamin A Resulting When Retinol and β-carotene are Combined

Level of Retinoid	Retinol Equivalents (RE)	Amount of Retinoid (µg)	Amount of β-carotene (µg)
A	6	6	36
B	30	30	180
C	60	60	360

The other ingredients of each diet included 67 percent cornstarch[4], 15 percent protein (casein[5]), 10 percent fat[6], 4 percent mineral mix[7], 2 percent vitamin mix[8], and 2 percent cellulose[9]. Composition of the diets, mineral mix and vitamin mix are given in Appendix A.

[3]β-carotene, Type I, Sigma Chemical Company.

[4]Cornstarch, Teklad Test Diets, Madison, Wisconsin.

[5]Vitamin test casein, ICN Pharmaceuticals, Inc., Cleveland, Ohio.

[6]Hydrogenated vegetable oil, Teklad Diets, Madison, Wisconsin.

[7]Wesson Modified Osborne-Mendel Mineral mix, Teklad Test Diets, Madison, Wisconsin.

[8]Ingredients purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio.

[9]Alphacel, ICN Pharmaceuticals, Inc., Cleveland, Ohio.

At the end of two weeks, each animal was placed under ether until dead. A 5 cm segment of the colon, measured from the anus, was excised, and the fecal pellets present were removed. The segment was split longitudinally and placed in a 10 percent Earles' Balanced Salt[10] solution at 4°C for approximately one minute. Each colon segment was then placed in a 10 percent Phosphate Buffered Formalin solution[11] and kept in this solution until histological techniques could be performed on the segments.

Each colorectal segment was cut in half longitudinally and routinely embedded in paraffin (Appendix Table B-1). Five micron thick sections were cut from each paraffin embedded segment and floated onto grease-free slides which had been cleaned with Sparkleen[12] and absolute ethanol. Gelatin[13] (1 tablespoon/2 liters water) was used in the water bath to float sections onto slides to prevent loss of sections due to the corrosive nature of some of the chemicals used in the staining process.

Two slides were made from each block of rat tissue. One slide was stained using a modification of the High Iron Diamine-Alcian Blue (HID-AB) method (Dawson & Filipe, 1976; Dawson, Patel, & Filipe, 1978; Filipe, 1969; Filipe & Branfoot, 1974; Jones & Reid, 1973; Lev, 1965; Spicer, 1965). The second slide was stained with Periodic Acid-Thionin/Potassium Hydroxide/Periodic Acid Schiff (PAT/KOH/PAS) stain (Culling, Reid, Worth, & Dunn, 1977; Filipe & Fenger, 1979). See Appendix Tables B-2, B-3, and B-4 for procedures and solutions used for these stains.

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[10]Grand Island Biological Company (GIBCO), Grand Island, New York.
[11]Fisher Scientific Company, Raleigh, North Carolina
[12]Fisher Scientific Company, Raleigh, North Carolina.
[13]Knox Gelatin, Inc., Johnstown, New York.

After slides were stained, five areas of each slide were chosen for close investigation. One section from each corner of the slide and one from the middle, or an area close to these, was chosen wherever possible. An ocular micrometer with a square was used to count the number of cells within a given area. For all rat tissue, 0.7 cm^2 was used. A single investigator counted all cells in order to decrease variability and criteria were set up for each type counted to insure consistency in counting cells. Cells were counted within the area of the square, excluding any cells that were on the lines. To check accuracy, two additional investigators counted identical sections. Cell counts from all investigators were the same.

Goblet cells were counted in each of the five areas and reported as number of goblet cells per 0.7 cm^2. The HID-AB stain was used to determine the amount of sulfated mucins and sialomucins in the tissue. The sulfated mucins stained black, while the sialomucins stained blue. Each of the five areas on the slide was evaluated for uptake of the stains. The sulfated mucins which take up the diamine stain are black or purple-gray, and the sialomucins, which have affinity for the alcian blue at a pH of 2.5, are bright blue. Each of the five selected areas was evaluated for color and coded as shown in Table 2 for both goblet cells and connective tissue surrounding the crypts.

Location of the uptake of the black and blue stains was also recorded, and coded as shown in Table 3. Some of the code categories were combined for discussion of the data (see Chapter IV).

Table 2

Color Coding for HID-AB Stain

Code	Color	Type of Mucus
1	Predominantly black, dark black	Mostly sulfated mucins
2	75% black, 25% blue, lighter black or purple-gray	Mostly sulfated, some sialomucins
3	50% black, 50% blue	Mixed sulfated mucins and sialomucins
4	75% blue, 25% black, lighter blue	Mostly sialomucins, some sulfated mucins
5	Predominantly blue, dark blue	Mostly sialomucins

Table 3

Location Codes for HID-AB Stain

Code	Location
1	Connective tissue, goblet cells
2	Mucus only
3	Goblet cells
4	Connective tissue, columnar epithelium, goblet cells
5	Mucus, connective tissue
6	None
7	Columnar epithelium
8	Mucus, columnar epithelium
9	Columnar epithelium, goblet cells, mucus
10	Connective tissue
11	Connective tissue, columnar epithelium, mucus
12	Mucus, goblet cells
13	Mucus, goblet cells, connective tissue
14	All cells and tissue, generalized staining in all areas of the section

Criteria that were used for counting goblet cells were as follows:

1) large cells found in crypts of Lieberkühn

2) shaped like a goblet, bulged at lumenal surface, narrower at area adjacent to basement membrane

3) often contain droplets of mucus

4) stain either black, gray, or blue, depending on type of mucus produced

5) size varies with amount of mucus, but normally somewhat wider than columnar epithelial cells.

The PAT/KOH/PAS stain was used to determine the amount of sialic acid present in mucus and the point of acylation of the sialic acid. Red staining identifies sialic acids with hydroxyl groups substituted at the C_8 position while blue staining indicates either unsubstituted sialic acids or sialic acids substituted at the C_7 or C_9 positions. Purple staining indicates a mixture. The onset of malignancy in colorectal cancers in humans has been shown to be accompanied by an increase in blue staining, or a change in the chemistry of the mucus when tissue was fixed after removal from the body without maintaining tissue in culture (Culling et al., 1977). Normal colon is predominantly red, with very little blue or purple. There is an increase in sialomucins and a decrease or absence of sulfated mucins with malignancy. Each of five areas on every slide was evaluated to determine the color of goblet cells and also of the connective tissue surrounding the crypts. The color codes for the PAT/KOH/PAS staining pattern are given in Table 4.

Cells that were thought to be mast cells were also counted in each of five areas. According to Spicer (1965), mast cells take up the black stain and are easily detected in the connective tissue. On this basis, those cells that stained black are called mast cells here. Uptake of

Table 4

Color Coding for PAT/KOH/PAS Stain

Code	Color	Type of Sialomucin
1	Blue	OH substituted at C_7, C_9, or unsubstituted (found with cancer)
2	Purple	Mixture (very little found in normal colorectal tissue
3	Red	OH substituted at C_8 (predominant in normal colorectal tissue)

this stain maybe due to the sulfur content of heparin found in mast cells. It is possible that a change in number of mast cells which stained with these dyes is an indication of the effect of vitamin A on immune responses. At the present time, there is controversy as to whether macrophages could be mistaken for mast cells. They have similar characteristics and, depending on whether they take up the diamine stain, it is possible that some of these mast-like cells could be macrophages. However, for the present study they will be referred to as mast cells. Criteria for counting mast cells were as follows:

1) granular cytoplasm
2) large, darkly-stained cells that take up black stain
3) found in the connective tissue
4) about the size of goblet cells, larger than most other cells in the area
5) often found in groups clumped together
6) often found around blood vessels
7) irregular shapes, sometimes amoeboid, some are elongated.

In order to verify that these were mast cells, the toluidine blue stain was used and gave a positive test (Lamb & Lumsden, 1982). See Appendix Tables B-4 and B-5 for solutions and techniques used for this stain.

In Vitro Phase

In order to investigate the effects of vitamin A on colorectal cancer, tumors of the colon and rectum were examined after exposure to vitamin A in tissue culture to determine the histological changes that occurred. Tissue was received from the Pathology Departments of Wesley Long Community Hospital and The Moses H. Cone Memorial Hospital in Greensboro. Since bacterial contamination is a problem in tissue culture and interferes with the experiment if present, every effort was made to prevent such contamination from occurring. The tissue was cooled immediately after removal from the body to lower tissue metabolism until the tissue could be placed in a 37°C incubator with medium containing antibiotics.

After being excised from the body, a tissue sample was placed in physiological saline or culture medium at 4°C at the hospital until a member of the research team could pick it up. Upon returning to the tissue culture laboratory, the tissue was placed in the refrigerator until medium could be mixed. The tissue was first placed in a solution with a high level of antibiotics for thirty minutes to one hour (see Table 5). The amount of antibiotic was five times the amount in regular medium. The tissue was dissected in this solution to eliminate bacterial contamination by instruments. Sterile iridectomy scissors and a sterile scalpel were used to cut the tissue into small pieces. A piece of the tissue was fixed immediately in a 10% Calcium-formol solution (see

Table 5

Contents of Medium Used in Tissue Culture

	Amount and Type of Medium Used		
	Regular Medium	Medium With 2X Antibiotics	Medium With 5X Antibiotics
Eagle's MEM without L-Glutamine[14]	92 ml	83 ml	15 ml
Fetal Bovine Serum[14]	5 ml	5 ml	-
L-Glutamine[14]	1 ml	1 ml	-
Non-essential Amino Acids[14]	1 ml	1 ml	-
Fungizone[14]	1 ml	2 ml	5 ml
Sodium Bicarbonate[15]	50 mg	60 mg	-
Gentamycin Sulfate[16]	10 mg	15.2 mg	38 mg

Appendix Table B-6) and six other pieces of tissue, each approximately .5 cm^2 were placed on gelfoam sponges[17] in Corning 25 cm^2 flasks[18] with screw caps in an incubator[19] at 37°C. The first medium used was one containing two times the normal amount of antibiotics (see Table 5). This period of acclimation lasted 24 hours. The medium was then changed and medium with varying levels of retinoids was added to flasks. Eagle's Minimum Essential Medium was used as the base medium, and other substances added are listed in Table 5. The culture medium was not mixed until needed because antibiotics tend to break down after approximately

[14]GIBCO
[15]Mallinckrodt Chemical Works, St. Louis, Missouri
[16]Sigma Chemical Company, St. Louis, Missouri
[17]The Upjohn Company, Kalamazoo, Michigan
[18]Fisher Scientific Company
[19]National Appliance Company, Portland, Oregon, Model 3241

three days. This medium was changed on the third day, and tissue samples were fixed on the sixth day. Total exposure time to retinoids ranged from 94 to 140 hours.

The types and amounts of retinoids added to the basic culture medium are shown in Table 6. The lower levels were chosen because they represent high physiological levels found in the serum of normal humans, and are possible to obtain with diet. The high levels of retinol and β-carotene represent serum levels that are only possible with pharmacologic doses of vitamin A.

Table 6

Type and Amount of Retinoids Added to Culture Medium

Experimental Group	Contents of Medium
Control	Regular basic medium
Control with diluent	Medium with Propylene Glycol
Retinol 1	Medium with 75 µg retinol/100 ml medium
Retinol 2	Medium with 250 µg retinol/100 ml medium
β-carotene 1	Medium with 250 µg β-carotene/100 ml medium
β-carotene 2	Medium with 500 µg β-carotene/100 ml medium

Ten mg powdered retinol were dissolved in 2 ml absolute alcohol and the solution was stirred with a glass rod for about 30 minutes until the retinol had dissolved. A stock solution of retinol was made by adding 38 ml of sterile distilled water to the retinol-alcohol mixture. Absolute alcohol has been used successfully as a vehicle for dissolving

retinol (Kelly, 1975). Ten mg of β-carotene was mixed with 2 ml of propylene glycol and was then mixed with 38 ml sterile distilled water for the stock solution. A control was mixed with 2 ml of propylene glycol added to 38 ml sterile distilled water to make the stock solution for the control with diluent. Propylene glycol is an inert substance and has been used in tissue culture as a vehicle in past studies, but has not been used with β-carotene (Wangh, Osborne, Hentschel, & Tilley, 1979). All stock solutions were stored at 4°C, away from light until needed.

In order to insure sterility and to prevent contamination by bacteria, mycoplasma, fungi, viruses or other vertebrate cell contaminants via the glassware used in mixing medium, all glassware and containers used in mixing medium were rinsed five times immediately after use and stored filled with tap water until washed. The glassware was boiled in a 1% solution of Liquinox[20] for one hour, and glassware was scrubbed with brushes used only for tissue culture, and rinsed twenty times with tap-water and three times with running distilled water to remove any traces of Liquinox. The glassware was dried in a Despatch oven[21] for two hours at 170°C. The oven was turned off after two hours and containers were allowed to cool to room temperature. Containers were autoclaved 20 minutes at 121°C and 15 p.s.i., dried in the Despatch oven for two hours, and then cooled to room temperature. Bottle caps were then tightened and taped with masking tape, and bottles were stored in closed cabinets in the tissue culture room until needed.

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[20]Fisher Scientific Company, Raleigh, North Carolina
[21]Despatch Oven Company, Minneapolis, Minnesota

Used pipets were immediately placed in a 1% Liquinox solution in a plastic pipet holder, washed in warm tap water containing an Alcotab[22] for one hour, and rinsed in cold tap water for two hours in a Nalgene[23] pipet washer. The washed pipets were dried at 170°C for two hours and cooled to room temperature in a Despatch drying oven. The cooled pipets were plugged with sterile cotton, placed in glass pipet canisters with air vent caps[24], autoclaved for twenty minutes at 121°C and 15 p.s.i., and dried at 170°C for two hours. The pipets were allowed to cool to room temperature in the drying oven.

All metal instruments were wiped clean after use, placed in autoclave bags, and autoclaved at 121°C and 15 p.s.i. for twenty minutes. All used medium was decontaminated in the autoclave oven and then discarded.

A sterile hood[25] was used for the mixing of medium and all other solutions used in tissue culture. An ultraviolet light in the hood was used to kill bacteria for 20 minutes before use. All utensils and pipets were flamed both before and after placing in any solution used in tissue culture. The hood was wiped with 70% ethanol after use. Falcon flasks (Corning) were used with screw caps which were tightened in an attempt to allow carbon dioxide to build up and maintain pH at 7.2, which is normal for colorectal tissue. Actual pH fluctuated during some experiments for a limited period of time and ranged from 7.0 to 7.6, but most flasks remained between 7.2 and 7.4. Thirteen tumors were collected and cultured. After six days' exposure to retinoids, tissue samples were placed

[22]Alconox Inc., New York
[23]Nalge Sybron Corporation, Rochester, New York
[24]Bellco Glass Inc., Vineland, New Jersey
[25]Germfree Laboratories, Miami, Florida

in Phosphate Buffered Saline[26] for 15 minutes, and then placed in 10% Calcium-Formol until histology could be done.

Human tissue samples were treated the same as rat tissue for embedding and staining. In addition to cells counted and colors coded with the two stains used in rat tissue, plasma cells and lymphocytes were found to stain well with the HID-AB stain. These were also counted. The area used on the ocular micrometer for human tissue was 0.3 cm^2. Criteria for counting plasma cells were as follows:

1) oval-shaped cell with clear or blue-staining cytoplasm, often has a negative Golgi apparatus

2) nucleus is at one end and stains darkly, often resembling an ice cream cone

3) chromatin material is clumped within nucleus and resembles a cartwheel shape

4) often found in groups but more frequently as a single cell.

Criteria for counting lymphocytes were as follows:

1) darkly-staining with black stain

2) halo seen surrounding cell is a narrow rim of cytoplasm which is best seen by focusing up and down

3) round or bean-shaped nucleus, varying in size from small to large

4) often found in lymph nodules and near crypts.

Analysis of Data

The VAX computer system was used for the analysis of the data. Statistical analyses included multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA). Tukey's or Duncan's Multiple Range tests (Keppel, 1982) were used to determine differences between individual

[26]GIBCO

means when the statistical analyses revealed significant differences in treatments. Statements of significance were based on the .05 level of probability. The overall experiment-wise α for both the *in vivo* and *in vitro* studies was .05.

In Vivo Study

The SPSS-X computer package was used for the analysis of the data from this phase of the investigation. Dependent variables entered into the MANOVA included weight gain, number of goblet cells, number of mast cells, type of mucus with HID-AB stain, type of mucus in goblet cells with PAT/KOH/PAS stain and type of mucus in connective tissue with PAT/KOH/PAS stain. The dietary effects of retinol or β-carotene on the above-mentioned variables were determined by ANOVA. Tukey's test was used to determine differences between individual means.

In Vitro Study

MANOVA and ANOVA by means of the SAS computer package, were used for the statistical analyses of the *in vitro* data. Tests of significance between individual means were determined with Duncan's Multiple Range test.

Literature Search

In addition to a careful perusal of current relevant journals, articles in *Current Contents* from 1981 through 1983 were identified and studied. A Medline search was also utilized to find information on plasma cells and mast cells and their role in cancer. Medline indexes articles from over 3,000 international journals.

CHAPTER IV

RESULTS AND DISCUSSION

The present study was conducted to determine the histological changes induced by vitamin A both *in vivo* and *in vitro*.

Results of In Vivo Study

Description of Sample and Analysis

Multivariate Analysis of Variance

Data were analyzed by groups to see if responses of animals receiving varying levels of retinol were significantly different from groups receiving equivalent amounts of β-carotene. Data from retinol and β-carotene were combined over the three levels (i.e., retinol A and β-carotene A were grouped together to form one level, etc.) to determine the effects of level or amount of vitamin A and also to determine whether vitamin by level had an interaction effect. When retinol and β-carotene were combined, three levels of vitamin A resulted (see Table 1, page 14). Levels in this discussion, therefore, refer to 6 RE, 30 RE, or 60 RE.

Multivariate analysis of variance (MANOVA) analyzes the effects of all dependent variables simultaneously on each group. Dependent measures were weight gain, number of goblet cells, number of mast cells, type of mucus with HID-AB stain, and type of mucus with PAT/KOH/PAS stain in goblet cells and connective tissue. When MANOVA (Appendix Table D-1) was performed on the data, there was a highly significant overall effect of level of vitamin A when all dependent variables were considered simultaneously ($F[12,74]=2.81$, $p=.003$) whereas MANOVA effects were not

significant for vitamin by level ($F[12,74]=.76$, $p=.692$), nor when β-carotene was compared to retinol ($F[6,37]=.92$, $p=.495$). These findings indicate that the three levels of vitamin A fed to rats do have an effect and produce differences among groups, but there is no difference in effects of retinol compared to β-carotene.

Analysis of Variance

ANOVAs were run to determine whether vitamin source and/or vitamin level had a significant effect upon weight gain, number of goblet cells, number of mast cells, type of mucus with HIB-AB, type of mucus with PAT/KOH/PAS in goblet cells and connective tissue. ANOVA for level of vitamin A showed significant differences for three dependent measures: number of goblet cells, number of mast cells, and uptake of PAT/KOH/PAS stain by connective tissue ($F[2,45]=3.84$, $p=.029$; $F[2,45]=7.15$, $p=.002$, and $F[2,45]=3.47$, $p=.040$, respectively). ANOVAs for weight gain and type of mucus with the HID-AB stain approached significance ($F[2,45]=2.33$, $p=.109$; $F[2,45]=2.93$, $p=.064$, respectively) at the .05 level of significance (see Appendix Table D-2). Tukey's post hoc analyses at .05 level of significance, were run on all ANOVAs that were significant and also on those that approached significance.

None of the ANOVAs for retinol by β-carotene, nor for vitamin by level were significant at the .05 level of significance (see Appendix Tables D-3 and D-4).

Retinoid Consumption and Weight Gain in Rats

Retinoid consumption and weight gains of animals fed varying levels of retinol or β-carotene are presented in Table 7 and Appendix Tables C-1 and C-2. Increasing the dietary level of either retinoid resulted

Table 7

Total Retinoid Consumption and Weight Gains of Young Rats[1] Fed Varying Levels of Retinol and β-carotene

Dietary Retinoid	Amount Consumed[2] (μg)	Weight Gain[2] (g)
Retinol A	872 ± 34	66 ± 3[3a]
Retinol B	4429 ± 129	71 ± 2[a]
Retinol C	9060 ± 318	72 ± 3[a]
β-carotene A	5420 ± 205	64 ± 5[a]
β-carotene B	27315 ± 1240	69 ± 3[a]
β-carotene C	58050 ± 2072	72 ± 3[a]

[1]Weanling rats averaging 57 grams in weight initially
[2]Each value is the mean of eight animals ± SEM
[3]Means not sharing common superscript letters are significantly different ($p \leq .05$).

in marked increases in total retinoid consumption which was expected. The difference in weight gain associated with higher intakes of either retinoid approached significance at the .05 level of probability, and based on this observation a power function was calculated (see Appendix Tables D-2 and D-5). The power of the test was .20 with 8 animals and increased to .91 with 15 animals. Thus, it is possible that significant differences between weight gains of animals fed higher levels of retinoids and those fed the low levels of these compounds could have been detected if the number of animals used had been increased.

Number of Goblet Cells and Mast Cells

The number of goblet cells and mast cells observed in the tissues removed from rats fed either retinol or β-carotene are shown in Table 8. Because ANOVA was significant for number of goblet cells, post hoc tests were conducted and revealed that there was a significant difference between levels A and B or between the 6 and 30 RE levels of vitamin A ($F[2,45]=3.84$, $p=.0289$) (see Appendix Tables D-2 and D-5). Increasing the level of retinol or β-carotene from A to B resulted in increased numbers of goblet cells (see Figures 1 and 2).

Table 8

Number of Goblet Cells and Mast Cells Found in Colorectal Tissue of Rats Fed Varying Levels of Retinol and β-carotene

Dietary Retinoid	Number of Cells per 0.7 cm^2 Tissue Goblet Cells[1]	Mast Cells[1,2]
Retinol A	64 ± 5[3a]	8 ± 0.6[a]
Retinol B	79 ± 7[b]	9 ± 0.7[ab]
Retinol C	75 ± 7[ab]	12 ± 0.9[b]
β-carotene A	69 ± 4[a]	9 ± 0.9[a]
β-carotene B	87 ± 9[b]	11 ± 1.0[ab]
β-carotene C	80 ± 6[ab]	12 ± 0.7[b]

[1]Each value is the mean number of cells for eight animals ± SEM
[2]For definition of mast cells, see page 20
[3]Means not sharing common superscript letters are significantly different ($p \leq .05$).

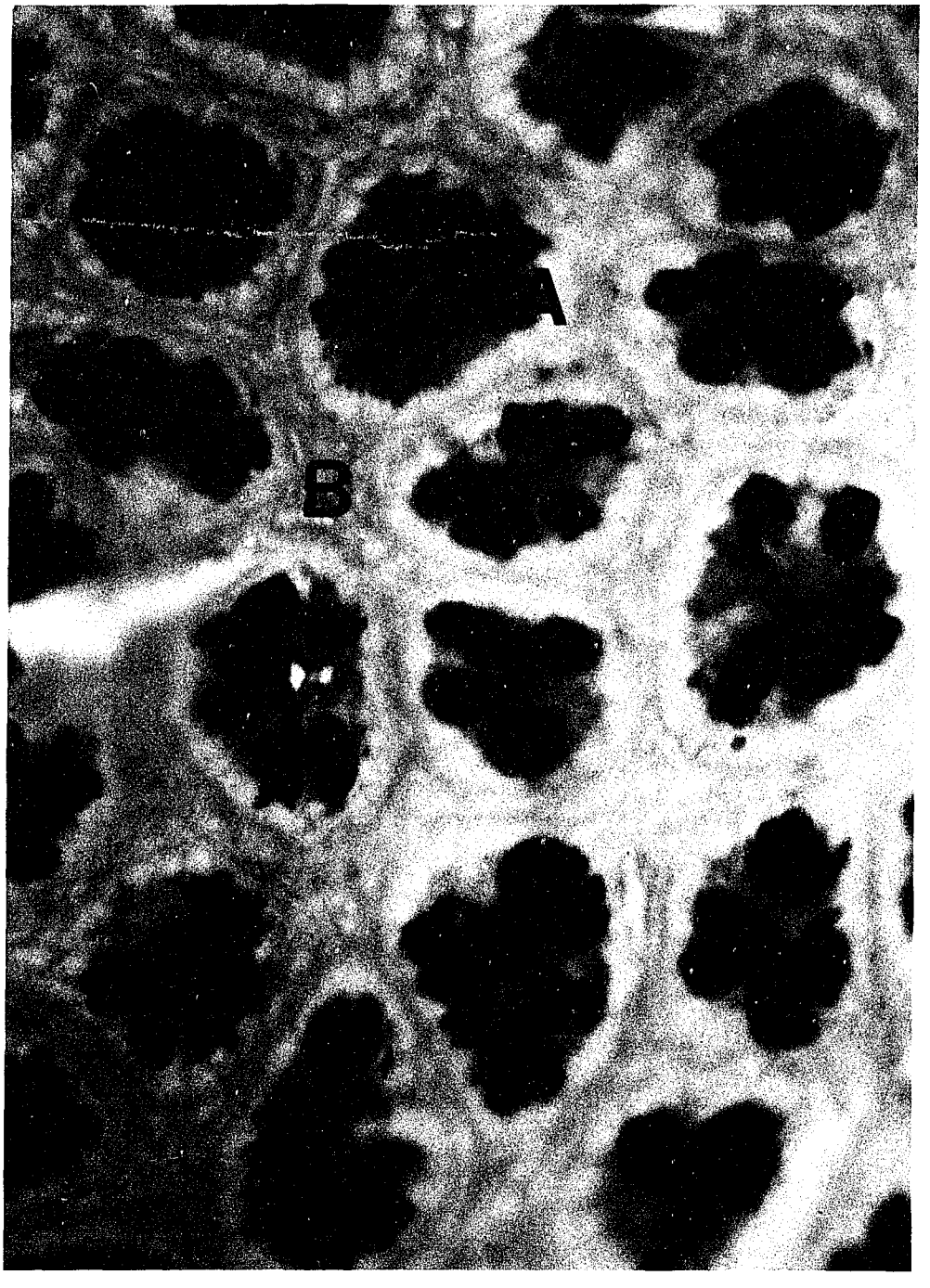

Figure 1. Color Photograph of Goblet Cells and Staining Patterns With The PAT/KOH/PAS Stain in Rat Tissue With Low Levels of Vitamin A. Goblet Cells (A) stained darker purple and connective tissue (B) had more bluish-purple, indicating more OH groups that were mixed or substituted at C_7, C_9, or unsubstituted. Number of goblet cells increased with increase in amount of Vitamin A. 320X microscopic magnification.

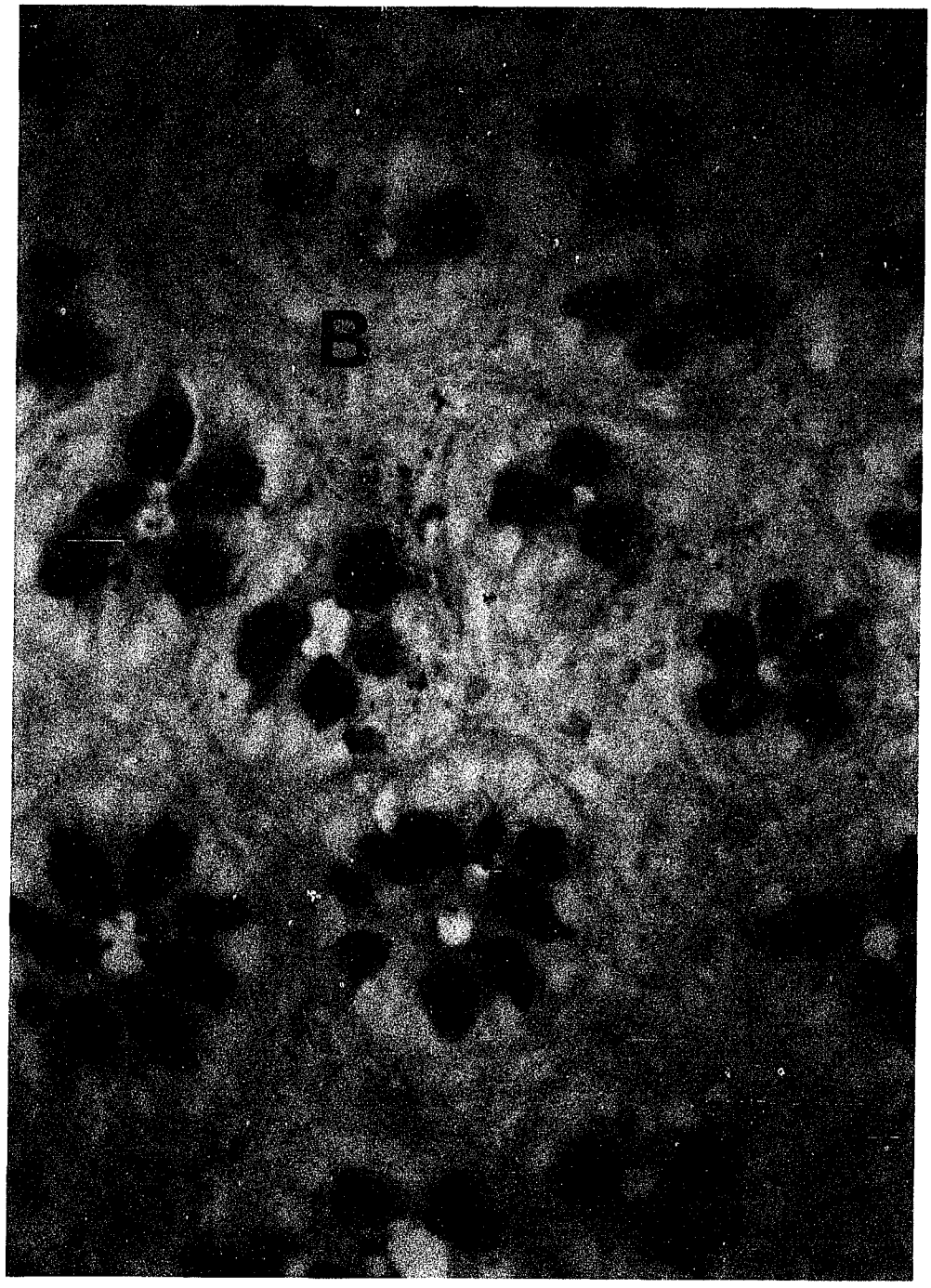

Figure 2. Color Photograph of Goblet Cells and Staining Patterns With the PAT/KOH/PAS Stain in Rat Tissue With High Levels of Vitamin A. Goblet Cells (A) stained red with high levels of vitamin A, indicating that sialomucins have more OH groups substituted at C_8 or mixed. Connective tissue (B) is more red-purple, again indicating mixed sialomucins. 320X microscopic magnification.

Number of mast cells was significantly different between levels A and C or between the 6 and 60 RE levels of vitamin A, and the number of mast cells increased with an increase in vitamin A ($F[2,45]=7.148$, $p=.002$) (see Figure 3). Although not significant, between levels A and B and B and C, there was a trend toward increasing numbers of mast cells with each increase in level of dietary vitamin A (see Table 8 and Appendix Tables D-2 and D-5). The number of mast cells increased with each increase in retinol or β-carotene. The number of mast cells in animals in the level C retinol group were essentially the same as the number found in animals in the level C β-carotene group.

As levels of dietary retinoids increased, the numbers of goblet and mast cells per 0.7 cm^2 tissue tended to increase (see Table 9). In groups receiving the higher levels of β-carotene and retinol, there were greater percentages of goblet and mast cells, a trend which is shown in Table 9.

Type of Mucus Found in Tissue

Because treatment differences in the type of mucus with the HID-AB stain approached significance ($F[2,45]=2.93$, $p=.0635$), a post hoc test was done and a power function was calculated. The post hoc test revealed that the differences were between levels A and B. Mucus became more sulfated with an increase in amount of vitamin A (see Table 10 and Appendix Tables D-2 and D-5) (see Figure 3). There was, however, a decrease in sulfated mucins and a subsequent increase in sialomucins when retinol was increased from level B to level C, although sulfated mucins continued to predominate in tissues of rats in the level C β-carotene group.

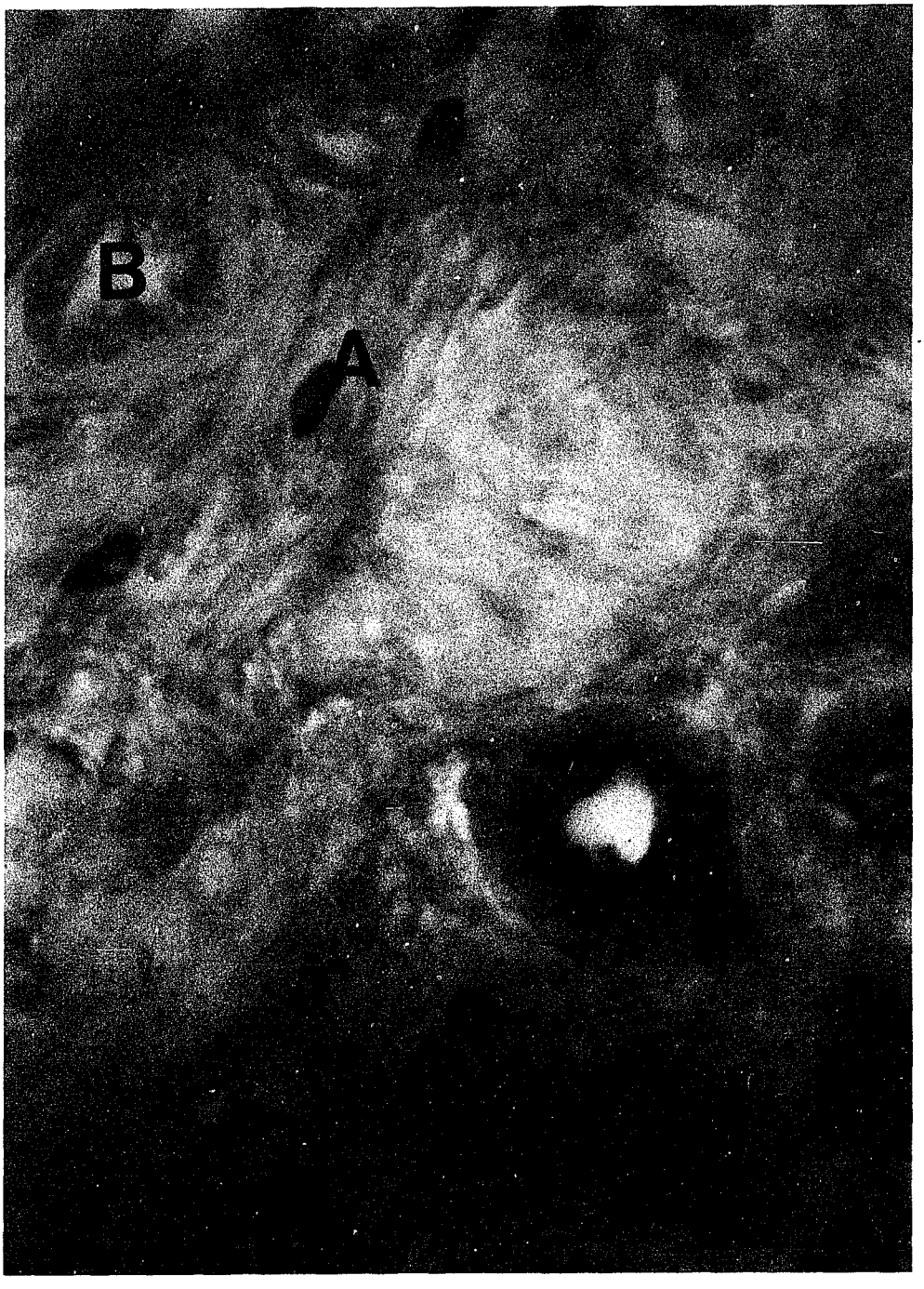

Figure 3. Color Photograph of Mast Cells, Sulfated Mucins and Sialomucins With the HID-AB Stain in Rat Tissue With High Levels of Vitamin A. Mast cells (A) increased in number with an increase in amount of vitamin A. Mast cells are often found around blood vessels (B). Sulfated mucins (C), which stain black, increase with an increase in vitamin A and sialomucins (D), which stain blue, decrease with vitamin A treatment. 320X microscopic magnification.

Table 9

Numbers of Goblet Cells and Mast Cells by Group

Retinoid	Retinol						β-carotene					
Dietary Level	A		B		C		A		B		C	
Number of Cells per 0.7 cm^2	f[1]	%[2]	f	%	f	%	f	%	f	%	f	%
	Number of Goblet Cells											
50-69	6	75	4	50	4	50	5	62.5	2	25	3	37.5
70-89	2	25	2	25	3	37.5	3	37.5	4	50	2	25
90-109	0	0	2	25	0	0	0	0	1	12.5	3	37.5
110-129	0	0	0	0	1	12.5	0	0	0	0	0	0
130-143	0	0	0	0	0	0	0	0	1	12.5	0	0
	Number of Mast Cells[3]											
5-7.9	6	62.5	3	37.5	0	0	3	37.5	2	25	0	0
8-10.9	3	37.5	4	50	3	37.5	1	12.5	2	25	3	37.5
11-13.9	0	0	1	12.5	4	50	4	50	3	37.5	4	50
14-15.9	0	0	0	0	1	12.5	0	0	1	12.5	1	12.5

[1]frequency
[2]percentage
[3]For definition of mast cells, see page 20

Table 10

Color Score[1] for Rat Colorectal Tissue Sialomucins and Sulfated Mucins as Shown by the HID-AB Stain

Group	Group Mean[2]	Distribution of Individual Means[3]					
		1.0-1.9		2.0-2.9		3.0-3.9	
		f	%	f	%	f	%
Retinol A	1.9 ± 0.3[4a]	5	62.5	1	12.5	2	25
Retinol B	1.3 ± 0.1[a]	8	100	0	0	0	0
Retinol C	1.8 ± 0.3[a]	5	62.5	2	25	1	12.5
β-carotene A	1.8 ± 0.3[a]	4	50	4	50	0	0
β-carotene B	1.3 ± 0.2[a]	7	87.5	1	12.5	0	0
β-carotene C	1.3 ± 0.2[a]	6	75	2	25	0	0

[1]See Table 2, page 17 for color scores
[2]Average of mean color score for eight rats ± SEM
[3]Distribution of mean color scores from five areas of each slide evaluated for individual animals within each group of eight
[4]Values with the same superscripts are not significantly different ($p \leq .05$).

A power function was calculated for the type of mucus with HID-AB stain because this variable approached significance. Power for the number of subjects used in the present study (eight) was .55. When the number of subjects was increased to 15, power increased to .97. Increasing the number of subjects would result in increased power which could have been sufficient to obtain significant results for this variable.

Composition of Sialomucins in Goblet Cells and Connective Tissue

The PAT/KOH/PAS stain gave additional information about sialomucins. With this stain sialic acids with OH groups substituted at C_8 were red, which is normal for colonic tissue, and those with mixed sialic acids were purple while those substituted at C_7 or C_9, or those unsubstituted were blue. Goblet cells and connective tissue were each observed for staining patterns.

There was no significant difference in uptake of the PAT/KOH/PAS stain by goblet cells (see Appendix Tables D-2, D-3, and D-4). With β-carotene uptake of this stain shifted from predominantly red ($\underline{M}$=2.8) with level A to more purple staining ($\underline{M}$=2.6) with the level B and back to predominantly red with level C ($\underline{M}$=3.0) (see Table 11). This indicates that there is a change from sialomucins substituted at C_8, to mixed sialomucins and then back to sialomucins substituted at C_8, with each increase in vitamin A (see Figure 2). With retinol, sialomucins had more OH groups substituted at C_8 when retinol was increased from level A to level B and remained the same for level C. The uptake of the PAT/KOH/PAS stain in connective tissue was significantly different between levels B and C. Sialic acids had more OH groups substituted at C_8 with an increase in vitamin A ($\underline{F}[2,45]=3.47$, $\underline{p}=.0395$) (see Table 11 and Appendix Tables D-2 and D-5) (see Figure 1).

Table 11

Color Score[1] for Rat Colorectal Tissue Sialomucins as Shown by the PAT/KOH/PAS Stain

Group	Group Mean[2]	Distribution of Individual Means[3] 2.0-2.9 f	%	3.0-4.0 f	%
		Goblet Cells			
Retinol A	2.6 ± 0.18[4a]	6	75	2	25
Retinol B	2.9 ± 0.17[a]	3	37.5	5	62.5
Retinol C	2.9 ± 0.20[a]	4	50	4	50
β-carotene A	2.8 ± 0.16[a]	3	37.5	5	62.5
β-carotene B	2.6 ± 0.15[a]	7	87.5	1	12.5
β-carotene C	3.0 ± 0.18[a]	4	50	4	50
		Connective Tissue			
		1.0-1.9		2.0-3.0	
Retinol A	1.5 ± 0.11[ab]	6	75	2	25
Retinol B	1.1 ± 0.05[b]	8	100	0	0
Retinol C	1.5 ± 0.14[a]	7	87.5	1	12.5
β-carotene A	1.5 ± 0.21[ab]	6	75	2	25
β-carotene B	1.3 ± 0.17[b]	7	87.5	1	12.5
β-carotene C	1.7 ± 0.22[a]	6	75	2	25

[1]See Table 4, page 20 for color scores
[2]Group mean is average for eight rats ± SEM
[3]Distribution of mean color scores for individual animals within each group of eight
[4]Values with the same superscripts are not significantly different ($p \leq .05$). Retinol and β-carotene were combined to form three levels for analysis.

The uptake of this stain was similar for both retinol and β-carotene and shifted from more purple staining, implying mixed sialomucins (M=1.5) with level A, toward more blue uptake of stain with level B (M=1.1 and 1.3, respectively). This implies sialomucins in these groups were substituted at C_7, C_9, or unsubstituted. The trend was a shift back toward purple staining with level C of retinol and β-carotene (M=1.5 and 1.7, respectively), indicating mixed sialomucins.

Location of Mucins in Tissue

The location of the sialomucins and sulfated mucins was also recorded using the HID-AB stain. Location of black and blue staining for each group was noted. There were five places on each slide, from each rat, which were observed for the location of mucins. Therefore, there was a total of 40 observations for each group of 8 rats. These will be referred to as number of observations from each group. Some sections of tissue had no uptake of stain, and this was referred to as "none" in location codes. Likewise, some sections of tissue had an uptake of one of the stains in all parts of the tissue. This was referred to as "all" in location codes. These observations were for descriptive purposes and were not analyzed statistically.

Sulfated mucins were concentrated in the goblet cells, while the intra-lumenal mucus, connective tissue, and columnar epithelial cells contained predominantly sialomucins for animals in all groups. Increasing the level of dietary retinoid appeared to have little effect on the location of sulfated mucins. Animals receiving level B of β-carotene, however, appeared to have more sulfated mucins in intra-lumenal mucus than did animals in levels A or C of β-carotene groups (see Table 12).

Table 12

Area on Slide Where Sialomucins and Sulfated Mucins in Tissue Took up HID-AB[1] Stain

	Number of Observations[2] of Sulfated Mucins		Number of Observations of Sialomucins	
Group	Location	Frequency	Location	Frequency
Retinol A	Goblet cells and C.T.[3]	39	Goblet cells	8
	Mucus, Goblet cells, C.T.	1	Mucus only	17
	None	0	C.T. only	2
			Mucus, C.T., C.E.[4]	9
			None	4
Retinol B	Goblet cells and C.T.	40	Goblet cells	0
	Mucus, Goblet cells, C.T.	0	Mucus only	24
	None		C.T. only	0
			Mucus, C.T., C.E.	9
			None	7
Retinol C	Goblet cells and C.T.	40	Goblet cells	0
	Mucus, Goblet cells, C.T.	0	Mucus only	15
	None	0	C.T. only	0
			Mucus, C.T., C.E.	24
			None	1

Table 12 (continued)

Group	Number of Observations[2] of Sulfated Mucins		Number of Observations of Sialomucins	
	Location	Frequency	Location	Frequency
β-carotene A	Goblet cells and C.T.	40	Goblet cells	0
	Mucus, Goblet Cells, C.T.	0	Mucus only	25
	None	0	C.T. only	0
			Mucus, C.T., C.E.	15
			None	0
β-carotene B	Goblet cells and C.T.	30	Goblet cells	0
	Mucus, Goblet cells, C.T.	10	Mucus only	23
	None	0	C.T. only	0
			Mucus, C.T., C.E.	11
			None	6
β-carotene C	Goblet cells and C.T.	40	Goblet cells	0
	Mucus, Goblet cells, C.T.	0	Mucus only	22
	None	0	C.T. only	0
			Mucus, C.T., C.E.	7
			None	11

[1]See location codes, Table 3, page 18
[2]There were a total of five observations from each slide, or 40 observations per group
[3]C.T. = Connective tissue
[4]C.E. = Columnar epithelial cells

With the exception of retinol A, all groups showed an absence of sialomucins located in goblet cells and connective tissue. With retinol, there was an increase in sialomucins in mucus from levels A to B and then a decrease with level C. However, with β-carotene, the number of observations where sialomucins were located in mucus remained relatively constant over all levels with only a slight decrease. There was an increase in sections having no sialomucins with an increase from level A to level B of retinol and then a decrease with level C. As level of β-carotene increased there was an increase in numbers of observations having no sialomucins. With retinol, there was an increase in sialomucins located in mucus, connective tissue, and columnar epithelium, or a generalized observation of blue staining indicating sialomucins located in all tissues which remained constant from level A to level B of retinol but increased with level C. There was a gradual decrease in sialomucins with each increase in β-carotene.

As seen in Table 12, there is, in general, a decrease in sialomucins as retinoids are increased which indicates that vitamin A stimulates a decrease in production of sialomucins. This supports the data in Table 10 which indicates that there is an increase in sulfated mucins with an increase in β-carotene, and with an increase in retinol from level A to level B.

Results of In Vitro Study

Description of Sample

Data by Group

Each tumor that was received was divided into seven pieces. Six pieces received one of the treatments and the seventh piece was fixed without treatment and used for diagnostic and comparative purposes. The control with diluent and the control with regular medium were compared to determine if there were differences that could be attributed to the diluent. Table 13 shows a summary of the cell numbers and type of mucus for the three control groups. These figures are very similar for the two controls grown in tissue culture, and are higher for the control that was fixed without treatment. This could be due to the fact that many types of cells may have been killed in the excision process. In addition, it is generally accepted that some blood cells and connective tissue cells die after a few days in culture. The controls that were treated in vitro were very similar for all variables observed, therefore, in the analyses only the control with regular medium was used. Two groups were treated with retinol at 75 and 250 µg/100 ml medium (Retinol 1 and Retinol 2, respectively) and two groups received 250 and 500 µg β-carotene/100 ml medium (β-carotene 1 and β-carotene 2, respectively).

Analysis of In Vitro Study

Multivariate Analysis

This study was a repeated measures design because each tumor was divided and each of the treatments was repeated for all tumors. The MANOVA procedure was run on all data to determine if there was an overall effect of the following factors: ID, group, level, vitamin, and level by

Table 13

Average Numbers of Goblet Cells, Mast Cells, Plasma Cells, and Lymphocytes and Type of Mucus With HID-AB and PAT/KOH/PAS Stains in the Three Control Groups

Group	Goblet Cells[1]	Mast Cells[1,2]	Plasma Cells[1]	Lymphocytes[1]	HID-AB[3] Stain	PAT/KOH/PAS[4] (Goblet Cells)	PAT/KOH/PAS[4] (Connective Tissue)
Control Fixed Without Treatment	7.4 ± 2.3	4.3 ± 0.3	4.0 ± 0.4	20.2 ± 3.3	2.88 ± 0.43	2.15 ± 0.12	1.47 ± 0.08
Control With Regular Medium	3.1 ± 1.7	3.5 ± 0.1	2.7 ± 0.3	12.3 ± 1.2	3.78 ± 0.26	1.53 ± 0.09	1.15 ± 0.06
Control With Diluent	2.5 ± 1.8	3.6 ± 0.3	2.7 ± 0.4	13.9 ± 1.9	3.68 ± 0.31	1.55 ± 0.17	1.28 ± 0.11

[1]Average number of cells for the group ± SEM. Mean is calculated from five observations per slide, or 60 per group.
[2]For definition of mast cells, see page 20.
[3]Average of color codes indicating type of mucus from five observations per slide, or 60 per group. See color codes for HID-AB stain, Table 2, page 17.
[4]Average of color codes indicating type of mucus from five observations per slide, or 60 per group. See color codes for PAT/KOH/PAS stain, Table 4, page 20.

vitamin. Dependent variables were as follows: number of goblet cells, mast cells, plasma cells, and lymphocytes, type of mucus with HID-AB stain, uptake of PAT/KOH/PAS stain by goblet cells and connective tissue. The .025 level of significance was selected because the data were subjected to two sets of tests. The overall experiment-wise α was .05. The multivariate effects for ID and group were significant ($F[99,265]=3.64$, $p=.0001$ and $F[36,136]=2.05$, $p=.0017$, respectively, see Appendix Table D-6). Thus, when all dependent measures were considered simultaneously, each one has effects of group and ID independently of the others. Multivariate effects for level, vitamin, and level by vitamin were not significant, meaning the dependent measures were highly correlated, causing difficulty in interpreting these results separately.

Analysis of Variance

The analysis of variance procedure was used to determine differences in each of the dependent variables tested. Number of goblet cells, mast cells, plasma cells, and lymphocytes, and type of mucus with HID-AB stain and with PAT/KOH/PAS stain were each tested (see Appendix Tables D-7 and D-8).

Number of Goblet Cells, Mast Cells, Plasma Cells, and Lymphocytes

Table 14 shows the effects of retinoid compared to controls on the number of goblet cells, mast cells, plasma cells, and lymphocytes of human tumor tissue used in this study. Number of goblet cells increased from Retinol 1 to Retinol 2, but decreased from β-carotene 1 to β-carotene 2. Both levels of β-carotene are higher than retinol groups, and all vitamin A groups were higher than the control, but these differences were not significant. The number of mast cells was significant for group

Table 14

Mean Cell Numbers[1] in Groups Receiving Vitamin A Compared to Control

Group	Goblet Cells	Mast Cells[2]	Plasma Cells	Lymphocytes
Control	3.1 ± 1.7[3a]	3.5 ± 0.1[3a]	2.7 ± 0.3[3a]	12.3 ± 1.2[3a]
Retinol 1	4.1 ± 2.2[a]	4.9 ± 0.4[a]	3.1 ± 0.3[a]	18.4 ± 3.0[a]
Retinol 2	4.6 ± 2.3[a]	4.6 ± 0.3[a]	3.2 ± 0.4[a]	18.8 ± 3.0[a]
β-carotene 1	5.1 ± 2.3[a]	5.5 ± 0.5[a]	3.8 ± 0.4[a]	22.2 ± 4.0[a]
β-carotene 2	4.7 ± 2.1[a]	5.7 ± 0.2[a]	3.6 ± 0.5[a]	18.4 ± 4.0[a]

[1]Each value is the mean of 12 samples ± SEM.
[2]For definition of mast cells, see page 20.
[3]Figures not sharing common superscript letters are significantly different ($p \leq .025$).

($F[4,44]=6.03$, $p=.0006$). Duncan's multiple range test at the .01 level of significance revealed that the differences were between the control group and β-carotene 1 group and also between the control group and the β-carotene 2 group, which suggests an effect of β-carotene (see Figure 4). The number of mast cells decreased from Rentinol 1 to Retinol 2 and increased from β-carotene 1 to β-carotene 2 (see Figure 5). Again, both levels are higher for the β-carotene group. This could indicate that any further increase beyond a certain optimal level of β-carotene has no advantage in increasing cell numbers.

Figure 4

Duncan's Multiple Range Test for Differences in Number of Mast Cells[1]

Group	Control	Retinol 1	Retinol 2	β-carotene 1	β-carotene 2
Control		-	-	*	*
Retinol 1			-	-	-
Retinol 2				-	-
β-carotene 1					-
β-carotene 2					

Note: - indicates not significant, * indicates significance, $p \leq .01$
[1]For definition of mast cells, see page 20.

Number of plasma cells was higher in β-carotene groups than retinol groups, but all are higher than control. Number of lymphocytes was highest in β-carotene 1 group, but again all groups were higher than the control group. Number of plasma cells and lymphocytes approached

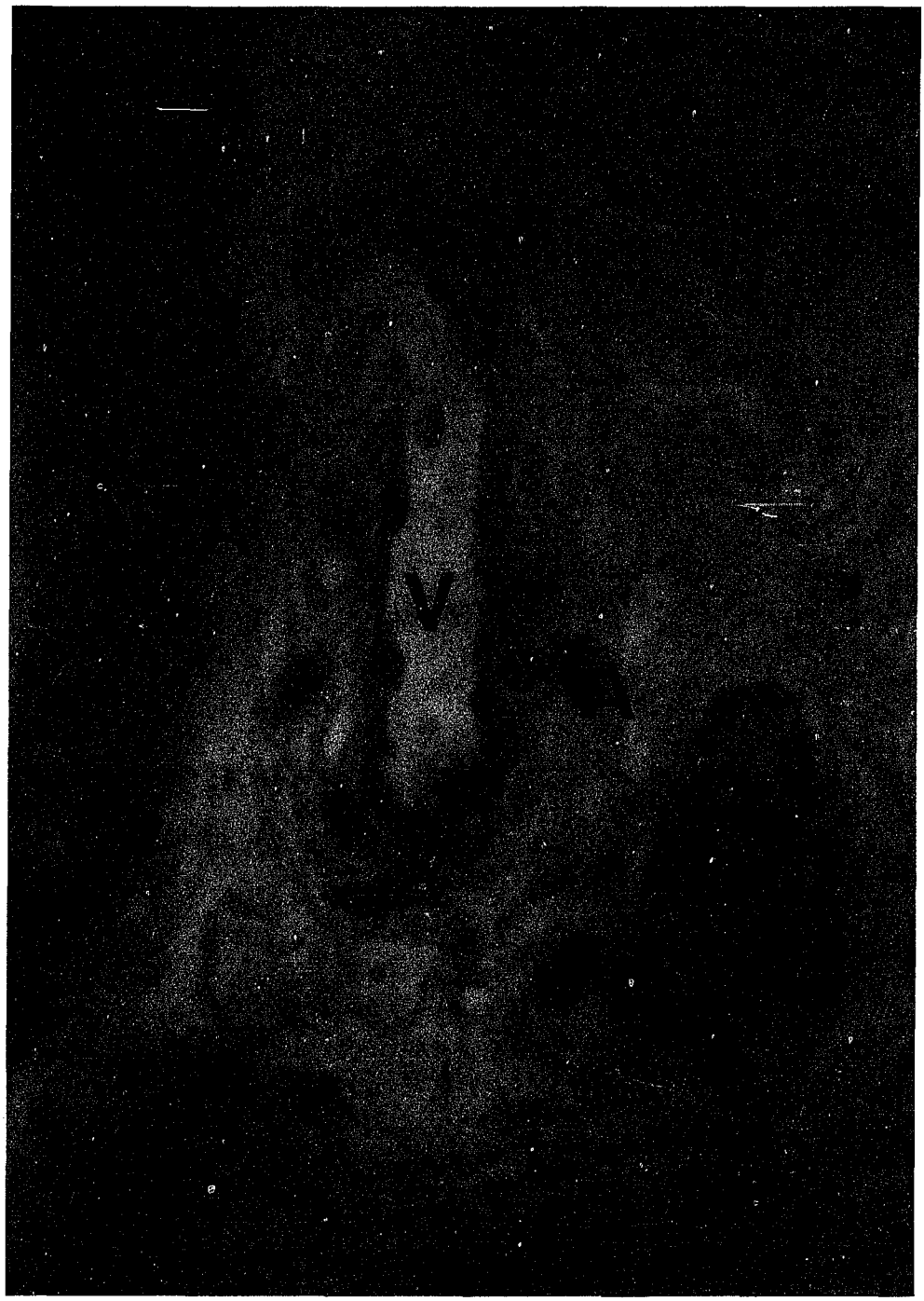

Figure 5. Color Photograph of Mast Cells, Sulfated Mucins, and Sialomucins With the HID-AB Stain in Human Tissue. With an increase in Vitamin A, number of mast cells (A) increases, sulfated mucins (B), which stain black, increase and sialomucins (C), which stain blue, decrease. Mast cells are often found surrounding blood vessels (V). 320X microscopic magnification.

significance for group ($F[4,44]=2.63$, $p=.0472$ and $F[4,44]=2.47$, $p=.0586$, respectively). At the .01 level of significance, Duncan's multiple range test revealed that plasma cell and lymphocyte averages were different between the β-carotene 1 group and control, again indicating an effect of β-carotene on the host's immune response. When a power function was performed on the data, it was found that with the number of subjects used for analysis (12), power was .62 for plasma cells and .75 for lymphocytes at the .01 level of significance, and by increasing the sample size to 15, power was raised to .92 for plasma cells and .90 for lymphocytes. Thus, it is possible that, considering the p values and power, with a larger sample size these variables could have been significant.

Type of Mucus Found in Tissue

The type of mucus stained with the HID-AB stain was highly significant for both ID and group (see Appendix Tables D-7 and D-8). Duncan's multiple range test showed that the differences were between the control group and the Retinol 2 group, the control group and the β-carotene 1 group and between the control group and the β-carotene 2 group (see Figure 6). The type of mucus as determined by the HID-AB stain becomes slightly more sulfated with an increase in retinol but remains the same with the β-carotene groups. Both groups receiving β-carotene are more sulfated than the groups receiving retinol (see Table 15). The control group had more sialomucins whereas the groups containing β-carotene and retinol had more sulfated mucins (see Figure 5).

The type of mucus with the PAT/KOH/PAS stain was significant for both group and ID for uptake of stain by goblet cells and connective tissue ($F[4,44]=4.24$, $p=.0054$; $F[11,44]=4.29$, $p=.0002$; $F[4,44]=3.81$,

Figure 6

Duncan's Multiple Range Test for Differences in Type of Mucus With HID-AB Stain

Group	Control	Retinol 1	Retinol 2	β-carotene 1	β-carotene 2
Control		-	*	*	*
Retinol 1			-	-	-
Retinol 2				-	-
β-carotene 1					-
β-carotene 2					

Note: - indicates not significant, * indicates significance, $p \leq .01$.

Table 15

Mean Color Scores Indicating Mucus Type in Human Colorectal Tumor Tissue

Group	Type Mucus With HID-AB[1,2]	Type Mucus With PAT/KOH/PAS (Goblet Cells)[2]	Type Mucus With PAT/KOH/PAS (Connective Tissue)[2]
Control	3.8 ± 0.26[3a4]	1.5 ± 0.09[a]	1.2 ± 0.06[a]
Retinol 1	3.3 ± 0.24[ac]	1.8 ± 0.11[ac]	1.5 ± 0.1[b]
Retinol 2	3.1 ± 0.17[bc]	2.0 ± 0.14[bc]	1.5 ± 0.11[b]
β-carotene 1	2.7 ± 0.15[bc]	2.0 ± 0.12[bc]	1.5 ± 0.12[b]
β-carotene 2	2.7 ± 0.18[bc]	2.0 ± 0.15[bc]	1.5 ± 0.12[b]

[1]Average of color codes indicating type of mucus from five observations per slide, or 60 per group. See color codes for HID-AB stain, Table 2, page 17.
[2]Type of mucus is the mean for each group ± SEM.
[3]Average of color codes indicating type of mucus from five observations per slide, or 60 per group. See color codes for PAT/KOH/PAS stain, Table 4, page 20.
[4]Values with the same superscript are not significantly different ($p \leq .025$).

p=.0096; F[11,44]=4.11, p=.0004, respectively). Duncan's multiple range test showed that for uptake of the PAT/KOH/PAS stain by goblet cells, the differences were between the control group and Retinol 2, control and β-carotene 1, and control and β-carotene 2 (see Figure 7). Mucus in goblet cells contained more mixed sialic acids with an increase in retinol but remained the same with β-carotene (see Figure 8).

Uptake of this stain by the connective tissue was significantly different between the control group and Retinol 1, control and Retinol 2,

Figure 7

Duncan's Multiple Range Test for Differences Between Groups in Uptake of PAT/KOH/PAS Stain by Goblet Cells

Group	Control	Retinol 1	Retinol 2	β-carotene 1	β-carotene 2
Control		-	*	*	*
Retinol 1			-	-	-
Retinol 2				-	-
β-carotene 1					-
β-carotene 2					

Note: - indicates not significant, * indicates significance, $p \leq .01$.

Figure 8. Color Photograph of Sialomucins Stained With the PAT/KOH/PAS Stain in Human Tissue. Tissue contained more sialomucins which stained purple-red with vitamin A treatment, indicating an increase in OH groups substituted at C_8 or mixed sialomucins. 320X microscopic magnification.

control and β-carotene 1, and control and β-carotene 2 (see Figure 9). Sialomucins in connective tissue of all vitamin A treated groups contained significantly fewer OH groups substituted at C_7, C_9, or unsubstituted than control. There is a shift in both goblet cells and connective tissue toward more mixed sialomucins with groups receiving vitamin A than controls, and away from the sialomucins substituted at C_7, C_9, or those unsubstituted.

Figure 9

Duncan's Multiple Range Test for Differences Between Groups in Uptake of PAT/KOH/PAS Stain by Connective Tissue

Group	Control	Retinol 1	Retinol 2	β-carotene 1	β-carotene 2
Control		*	*	*	*
Retinol 1			-	-	-
Retinol 2				-	-
β-carotene 1					-
β-carotene 2					

Note: - indicates not significant, * indicates significance, $p \leq .01$.

The type of mucus with HID-AB stain showed a significant vitamin effect (see Appendix Table D-8), indicating that there is a difference between retinol and β-carotene. The group averages show that the β-carotene groups are more heavily sulfated than the retinol groups (see Table 15). The number of plasma cells also showed a significant vitamin

effect. The β-carotene groups had a higher mean number of plasma cells than retinol groups (see Table 14).

In spite of the fact that these two parameters showed trends toward having a vitamin effect, because of the nonsignificant MANOVA, there could be an interaction in the data that is difficult to interpret. However, the group means do indicate that this significant vitamin effect is valid.

Location of Sialomucins and Sulfated Mucins

In order to determine the location where each type of mucus was found, five observations were made on each slide. With each observation, the location of sulfated mucins and the location of sialomucins were noted. All observations were compiled and will be discussed descriptively for each group. The total number of observations for each group are referred to as "number of observations". In some cases, the sections observed contained either sialomucins or sulfated mucins in all cells in that section. These cases will be referred to as observations of mucus located in "all" tissues or cells. In some cases there was no mucus of one of the types being observed. These observations are referred to as "none" for that location. Table 16 shows the number of observations from each group for sialomucins and sulfated mucins.

In both retinol groups, sulfated mucins were found predominantly in columnar epithelial cells. In β-carotene groups, goblet cells and connective tissue were the most frequent locations, whereas in the control group there were no sulfated mucins in most observations. This indicates an increase in total amount of sulfated mucins. When compared with the control group, all vitamin A groups had fewer sections

Table 16

Location of Mucins in Groups Receiving Vitamin A Compared to Control

Group	Number of Observations Sulfated Mucins		Number of Observations Sialomucins	
	Location	Frequency	Location	Frequency
Control	None	30	None	1
	All	0	All	45
	Goblet Cells, C.T.[1]	19	Goblet Cells, C.T.	5
	C.E.[2]	16	Mucus, C.T.	13
Retinol 1	None	21	None	0
	All	2	All	39
	Goblet Cells, C.T.	15	Goblet Cells, C.T.	1
	C.E.	27	Mucus, C.T.	25
Retinol 2	None	8	None	3
	All	5	All	44
	Goblet Cells, C.T.	23	Goblet Cells, C.T.	13
	C.E.	29	Mucus, C.T.	5
β-carotene 1	None	3	None	5
	All	17	All	32
	Goblet Cells, C.T.	23	Goblet Cells, C.T.	5
	C.E.	19	Mucus, C.T.	22
β-carotene 2	None	3	None	9
	All	10	All	29
	Goblet Cells, C.T.	25	Goblet Cells, C.T.	18
	C.E.	22	Mucus, C.T.	4

[1]C.T. = connective tissue
[2]C.E. = columnar epithelium

containing no sulfated mucins and more sections were all cells contained sulfated mucins. This supports the contention that vitamin A does cause an increase in amount of sulfated mucins in tissue (see Table 15). When β-carotene and retinol groups are compared, there are fewer sections in β-carotene groups where sulfated mucins are absent and more observations where sulfated mucins are located in all cells, which indicates that β-carotene further increases sulfated mucins.

In all groups, the primary location of sialomucins was in all cells. This could be due to the fact that this was abnormal tissue and total amount of sialomucins was increased over normal. However, the total number of observations where sialomucins were located in all tissues was greater for the control than the vitamin A groups. In β-carotene groups, there were fewer observations where sialomucins were found in all cells compared to retinol groups. In both retinol and β-carotene there was a slight increase in number of observations where there were no sialomucins present with an increase in vitamin A, which implies there was a decrease in sialomucins. This decrease is greater in β-carotene than retinol groups. There was a decrease in the number of sections where sialomucins were located in mucus and connective tissue in retinol 1 compared to retinol 2 and β-carotene 1 compared to β-carotene 2. There was an increase in observations where sialomucins were located in goblet cells and connective tissue in both β-carotene 1 and Retinol 1 when compared to β-carotene 2 and Retinol 2. At the same time there was a decrease in the number of observations of sialomucins in mucus and connective tissue with an increase in either β-carotene or retinol.

These findings support the data that show an increase in sulfated mucins and a decrease in sialomucins with vitamin A treatment either as β-carotene or retinol over controls and a further increase in sulfated mucins and a decrease in sialomucins with β-carotene compared to retinol (see Table 15 and Figure 6).

Data by Individual Tumor

All variables but number of mast cells had a significant effect for ID (see Appendix Tables D-7 and D-8). Because there was a wide variety in types of tumors and site of tumor before removal from the intestines, there are differences in response of each individual tumor to vitamin A. For this reason, each individual tumor will be considered in its response to vitamin A. Each tumor was given a number, which will be referred to in this section as ID or tumor number. Table 17 shows that, when averages of all variables for each tumor are obtained, there are differences. Some tumors vary more than others in cell numbers and type of mucus produced. For example, tumors number 9 and 10 have more goblet cells than the other tumors. Number of mast cells and plasma cells are similar for all tumors, but tumors 10, 11, and 12 have a greater number of lymphocytes than other tumors.

The type of mucus was more sulfated in tumor 9 than other tumors, but sialomucins that were present were primarily substituted at C_7 or C_9 or were unsubstituted. There is very little difference between tumors in the type of mucus as indicated with the PAT/KOH/PAS stain.

In order to determine if there are changes with vitamin A in each individual tumor, descriptive information was compiled by ID or by tumor in addition to compiling the data by group (see Table 18). In tumors 3 and 12 there were no goblet cells and in numbers 11 and 13 there were .2

Table 17

Description of Variables by ID

ID Number	Number of				Type of Mucus		
	Goblet Cells	Mast Cells[1]	Plasma Cells	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)
1	4.2[2]	4.3	3.4	12.0	2.8	2.0	1.7
2	5.3	4.9	2.1	11.1	2.9	1.7	1.2
3	2.2	5.1	2.8	16.7	3.7	2.3	1.4
4	4.2	5.1	2.7	15.4	3.4	2.1	1.5
5	0.6	3.8	3.0	14.2	3.4	2.0	1.2
6	5.1	5.9	3.8	16.5	3.0	1.7	1.3
7	2.4	4.7	5.1	14.9	3.2	2.1	1.5
8	4.4	4.6	2.8	16.4	2.9	1.6	1.2
9	9.9	5.7	3.0	11.0	1.8	1.2	1.0
10	22.9	4.1	3.4	36.4	2.1	2.0	1.6
11	0.1	4.7	3.4	26.5	3.8	1.9	1.5
12	0.0	4.4	4.7	27.8	3.3	2.0	1.8
13	0.2	3.4	4.0	19.7	3.6	2.2	1.7

[1]For definition of mast cells, see page 20

[2]Each value is the mean of five observations per tumor.

Table 18

Response of Individual Tumors to Vitamin A

Subject	Number of Cells[1]				Type of Mucus			Primary Location Black	Primary Location Blue
	Goblet	Mast[2]	Plasma	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)		
ID 1									
Control	0	3.4	3.6	7.8	3.8	1.6	1.2	CE	All
Retinol 1	19.6	5.4	2.4	10.8	2.0	2.0	1.8	Goblets,CT	Mucus,CT
Retinol 2	1.2	3.8	1.6	7.2	3.0	2.6	2.0	CE	All
β-carotene 1	0	4.6	4.6	15.6	2.6	2.0	1.8	CE	All
β-carotene 2	0	4.4	4.8	18.8	3.0	2.0	1.6	CE	All
ID 2									
Control	2.2	3.4	1.0	8.4	3.2	1.4	1.0	Goblets	CT
Retinol 1	0	6.8	2.2	9.0	3.6	2.2	1.6	None	All
Retinol 2	6.4	4.4	.4	11.4	3.0	1.8	1.2	Goblets,CT	Goblets,CT
β-carotene 1	5.6	2.6	2.2	8.8	3.0	1.8	1.2	Goblets,CT	Goblets, CT
β-carotene 2	9.0	6.4	2.4	8.0	2.8	1.2	1.0	All	All

Table 18 (continued)

Subject	Number of Cells[1]				Type of Mucus			Primary Location Black	Primary Location Blue
	Goblet	Mast[2]	Plasma	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)		
ID 3									
Control	0	3.4	2.0	18.2	4.0	1.8	1.6	CE	All
Retinol 1	0	4.0	2.6	19.6	3.4	1.6	1.0	CE	All
Retinol 2	0	4.4	4.4	19.4	3.4	2.4	1.2	CE,CT	All
β-carotene 1	0	7.8	2.2	16.2	3.4	2.6	1.6	All	All
β-carotene 2	0	6.0	1.8	9.8	3.0	2.6	1.6	All	All
ID 4									
Control	0	3.8	1.6	15.6	4.8	1.6	1.0	None	All
Retinol 1	.6	4.4	3.0	19.8	3.0	1.8	1.4	CE	CT
Retinol 2	20.2	7.4	3.0	9.6	4.0	2.4	1.2	All	All
β-carotene 1	0	5.4	2.4	10.8	3.0	2.2	2.0	CE	CT
β-carotene 2	0	4.4	2.0	9.2	3.6	2.2	1.8	CE	All
ID 5[3]									

Table 18 (continued)

Subject	Number of Cells[1]				Type of Mucus			Primary Location Black	Primary Location Blue
	Goblet	Mast[2]	Plasma	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)		
ID 6									
Control	0	3.8	2.0	6.4	4.0	1.4	1.2	CE	All
Retinol 1	0	5.6	4.0	7.2	3.8	1.6	1.4	CE	All
Retinol 2	0	5.4	5.0	32.2	3.8	2.0	1.4	CE	All
ß-carotene 1	4.4	10.0	3.6	24.0	3.2	1.4	1.0	CT,Goblets	All
β-carotene 2	5.2	5.2	2.2	13.2	2.0	1.0	1.0	All	None
ID 7									
Control	1.6	3.6	5.0	11.6	4.4	2.0	1.4	None	All
Retinol 1	0	5.2	4.0	21.0	3.4	2.2	1.8	CE	All
Retinol 2	0	4.2	4.8	18.4	3.2	2.4	2.0	CE	All
β-carotene 1	6.2	4.6	6.8	11.0	2.2	1.8	1.0	Goblets,CT	All
β-carotene 2	6.8	6.2	8.0	11.8	2.0	2.0	1.6	Goblets,CT	CT

Table 18 (continued)

Subject	Number of Cells[1]				Type of Mucus			Primary Location Black	Primary Location Blue
	Goblet	Mast[2]	Plasma	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)		
ID 8									
Control	7.2	3.6	2.8	10.0	2.6	2.0	1.0	All	All
Retinol 1	0	4.4	1.8	17.2	4.0	1.4	1.4	None	All
Retinol 2	0	5.0	3.2	14.4	3.2	1.2	1.0	CE	All
β-carotene 1	10.8	5.0	3.2	23.4	2.0	1.8	1.2	Goblets,CT	CT
β-carotene 2	0	5.4	3.0	24.8	2.8	1.6	1.0	CE	CT
ID 9									
Control	6.8	3.6	2.2	8.8	2.0	1.0	1.0	All	Mucus
Retinol 1	13.2	8.2	3.2	12.6	2.0	1.0	1.0	All	CT
Retinol 2	7.2	4.0	2.8	11.0	2.0	1.0	1.0	All	Mucus
β-carotene 1	11.0	7.8	2.0	10.4	2.0	1.0	1.0	All	CT
β-carotene 2	11.6	6.8	1.8	11.2	2.0	1.6	1.0	All	CT

Table 18 (continued)

Subject	Number of Cells[1]				Type of Mucus			Primary Location Black	Primary Location Blue
	Goblet	Mast[2]	Plasma	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)		
ID 10									
Control	19.4	3.0	3.6	17.4	3.0	1.0	1.0	All	Mucus
Retinol 1	19.8	3.6	3.6	30.2	2.6	1.2	1.0	Goblets,CT	Mucus
Retinol 2	24.2	4.0	2.6	51.2	2.0	2.0	1.8	Goblets,CT	None
β-carotene 1	28.6	4.8	3.6	43.0	2.0	2.4	2.0	Goblets,CT	CT
β-carotene 2	23.6	6.0	3.4	53.8	2.0	2.8	2.0	Goblets,CT	Mucus,CT
ID 11									
Control	0.2	4.0	2.0	16.8	4.8	1.4	1.0	CE	All
Retinol 1	0	3.8	2.8	10.2	4.6	2.0	1.8	None	All
Retinol 2	0	4.2	2.4	14.6	3.0	2.2	1.4	All	All
β-carotene 1	0	5.2	5.4	43.8	3.0	2.2	1.6	All	All
β-carotene 2	0	6.4	4.6	21.6	2.4	2.0	1.8	All	All

Table 18 (continued)

Subject	Number of Cells[1]				Type of Mucus			Primary Location Black	Primary Location Blue
	Goblet	Mast[2]	Plasma	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)		
ID 12									
Control	0	3.4	4.0	14.6	4.0	1.6	1.4	None	All
Retinol 1	0	4.8	5.0	38.6	2.6	2.0	2.0	CE	CT
Retinol 2	0	4.2	5.0	22.6	2.6	2.0	1.8	CE	CT
β-carotene 1	0	4.0	5.8	52.0	2.6	2.4	2.0	CE	CT
β-carotene 2	0	6.2	4.4	22.4	3.4	2.0	2.0	CE	All
ID 13									
Control	0.2	3.0	2.2	11.6	4.4	1.6	1.0	None	All
Retinol 1	0	2.8	3.8	37.0	3.6	2.2	2.0	None	All
Retinol 2	0	3.0	3.8	18.2	3.0	2.6	2.0	CE	All
β-carotene 1	0	4.2	4.8	16.4	3.4	2.2	1.8	CE	All
β-carotene 2	0	4.6	4.8	15.8	3.4	2.4	2.0	CE	All

[1]Each value is the mean of five observations from the tissue sample.
[2]For definition of mast cells, see page 20.
[3]Because of missing data, ID number 5 was deleted.

in control group but none in other groups. However, in numbers 1, 2, 4, 6, 7, 8, 9, and 10 there was some increase in number of goblet cells from control to one or more of the vitamin A groups. ID numbers 2, 4, and 10 had increased numbers of goblet cells with an increase in amount of retinol, and ID numbers 2, 6, 7, and 9 had increased numbers of goblet cells with an increase in amount of β-carotene. ID numbers 2, 6, 7, 8, 9, and 10 had slightly more goblet cells with β-carotene than with retinol. Number of plasma cells increased from control groups to groups containing vitamin A for all tumors. There was an increase in number of plasma cells from average to high levels of retinol in tumors 3, 6, 7, and 8 and an increase in number of plasma cells with increases from average to high levels of β-carotene in tumors 1, 2, and 7. However, there was a decrease in number of plasma cells from average to high levels of β-carotene in tumors 3, 4, 6, 8, 9, 10, 11, and 12.

Number of mast cells showed an increase in all tumors from control groups to one or more of the vitamin A groups. Number of mast cells increased with an increase in retinol in ID numbers 3, 4, 8, 10, 11, and 13, and with an increase in β-carotene in ID numbers 2, 7, 8, 10, 11, 12, and 13.

Number of lymphocytes increased from control to one or more groups receiving vitamin A in all tumors. There was an increase in number of lymphocytes with an increase in retinol in tumors 2, 6, 10, and 11, and a similar trend with β-carotene in tumors 1, 7, 8, 9, and 10. However, there were decreases in number of lymphocytes from average to high levels of retinol in tumors 1, 3, 4, 7, 8, 9, 12, and 13. The same trend was seen in β-carotene in tumors 2, 3, 4, 6, 11, 12, and 13. Again, this

could indicate that there is an optimal level of vitamin A beyond which further increases are of no additional benefit.

All tumors became more sulfated with one or more groups of vitamin A than control groups except number 9, which remained the same for all groups. Tumors that showed more sulfated mucins with an increase of either retinol or β-carotene from average to high levels were numbers 2, 3, 6, 7, 8, 10, 11, and 13.

Although there are differences in the actual values for variables, the trends seen in individual tumors appear to be similar to trends seen in each group. They all demonstrate beneficial effects of vitamin A over control. This indicates that vitamin A may be more effective at different stages of carcinogenesis, and with some types or sites of tumors. These stages and types of tumors where vitamin A has an optimal effect have not been determined.

Summary of In Vitro Data

When all data for groups receiving vitamin A are compared with the selected control group, there are differences in all variables. Table 19 gives a summary for all groups. There is an increase in number of goblet cells, mast cells, plasma cells, and lymphocytes in groups receiving vitamin A compared with the control group. The type of mucus is more sulfated in all vitamin A groups than in the control group, as seen with the HID-AB stain, and sialomucins contain more hydroxyl groups substituted at C_8 in vitamin A groups compared with the control, as seen with the PAT/KOH/PAS stain. There were also more observations with no sulfated mucins in control than in groups containing vitamin A, and fewer observations with all tissues containing sialomucins in groups

Table 19

Summary of Data From All Groups

Group	Average Number of				Type of Mucus		
	Goblet Cells	Mast Cells[1]	Plasma Cells	Lymphocytes	HID-AB	PAT/KOH/PAS (Goblets)	PAT/KOH/PAS (C.T.)
Control	3.1	3.5	2.7	12.3	3.8	1.5	1.2
Retinol 1	4.1	4.9	3.1	18.4	3.3	1.8	1.5
Retinol 2	4.6	4.6	3.2	18.8	3.1	2.0	1.5
β-carotene 1	5.1	5.5	3.8	22.2	2.7	2.0	1.5
β-carotene 2	2.7	5.7	3.6	18.4	2.7	2.0	1.5

Group	Number of Observations With			
	No Sulfated Mucins	All Sulfated Mucins	No Sialomucins	All Sialomucins
Control	30	0	1	45
Retinol 1	21	2	0	39
Retinol 2	8	5	3	44
β-carotene 1	3	17	5	32
β-carotene 2	3	10	9	29

[1]For definition of mast cells, see page 20.

receiving vitamin A than in the control group. This indicates that vitamin A induces an increase in sulfated mucins and a decrease in sialomucins. Vitamin A also increases number of immunocompetent cells in the area of the adenoma or adenocarcinoma.

Discussion of Results

Implications of This Study

A variety of retinoids have been found *in vivo*, *in vitro*, and in epidemiological studies to have anticancer activity, although the mechanisms of action are still not clear (Moon, McCormick, & Mehta, 1983). Previous investigators have speculated that vitamin A inhibits tumor promotion and also that it interferes with the initiation phase of carcinogenesis (Bertram et al., 1981). The present study addresses several factors which are unique. Although many forms of vitamin A have been used *in vivo* and *in vitro* in past studies, the addition of β-carotene to tissue culture medium and to rat diets has not been attempted, and the effect of β-carotene on histological changes has not been determined. The hypothesis that β-carotene has effects similar to retinol in its influence on cell numbers and mucin content is supported by data in the present study. These changes suggest some ideas for the mechanisms of action of β-carotene and other retinoids. The data also support the hypothesis that β-carotene may be effective as a preventive measure in cancer development.

The use of levels of retinoids that could be achieved with diets high in vitamin A-rich foods (i.e., without vitamin supplements) has not been attempted in previous research. Many studies have used vitamin A deficient diets and levels of vitamin A needed to support normal growth.

Studies have not been conducted to determine the histological changes that occur with levels of vitamin A that are possible by feeding naturally occurring forms of vitamin A (retinol and β-carotene). Some authors have hypothesized that natural retinoids are toxic in culture to many cell lines so use of synthetic forms has increased recently. High physiological levels of retinol and β-carotene have not been used in organ culture using colorectal adenomas nor have they been added to diets of rats to determine histological changes of colorectal tissue. The hypothesis of the present study that dietary vitamin A can induce histological changes in colorectal tissue is confirmed by the data in this study. Levels of retinoids used in this study were not found to be toxic.

In the present study retinol and β-carotene had the same effects on tissue in vivo but there was a trend toward more favorable effects with β-carotene in vitro. This could be due to the fact that abnormal tissue was used in vitro whereas normal tissue was used in vivo. This may also indicate that β-carotene has effects on cancer development that may not be seen with normal tissue. These differences might also be explained by the variation between rats and humans. The fact that there was no difference in retinol and β-carotene in vivo could support the results of epidemiological research. In the typical U.S. diet, 80% of the total vitamin A intake is from carotene sources rather than retinol (Kolonel et al., 1983). β-carotene could thus be given credit for causing low risk of cancer because of its predominance in the diet whereas retinol could cause these same beneficial effects if consumed in equal quantities in the diet. One hypothesis, that vitamin A induces histological changes both in normal and cancerous colorectal tissue, is supported by the changes noted in the present study.

The effects of vitamin A upon the histological development of colorectal tissue seem to be centered around two major areas: type of mucus produced by the tissue and effects on immune responses. These will be discussed separately.

Type of Mucus Produced

With the HID-AB stain, sialomucins and sulfated mucins can be seen simultaneously in the same section. The *in vivo* effect of increased vitamin A either as retinol or β-carotene is an increase in sulfated mucins, the mucus found predominantly in normal colorectal tissue. This same trend was seen *in vitro*. Both β-carotene groups and the group receiving the highest level of retinol were significantly more sulfated than the control group. This supports research reporting that vitamin A effects cancer prevention and/or cure. Vitamin A may be causing mucus to become more sulfated, the condition which exists in normal mucosa, which could influence cancer development. There is a shift in groups receiving vitamin A from a malignant condition to a benign condition as diagnosed by a change in the type of mucus produced by the tissue (Filipe & Branfoot, 1974). Vitamin A appears to regulate gene activation, changing the activity of genes coding for mucus production from sialomucins to sulfated mucins. Although mucin histochemistry of cancerous tissue has been reported, the sialomucin and sulfated mucin content of colorectal adenomas and adenocarcinomas has not been determined after exposure to retinoids in tissue culture. The coding for location of stain uptake by tissue was also unique to this study.

The increase in number of goblet cells seen with vitamin A stimulation in the present study has been demonstrated by others (DeLuca et al.,

1970; Kleinman & Wolf, 1974). However, the effects of β-carotene on number of goblet cells has not previously been shown. Other studies have used deficiency levels, but the effects of high levels of vitamin A in rat diets and human tissue in culture on number of goblet cells has not been previously determined. The hypothesis that number of goblet cells will increase with an increase in vitamin A from deficiency to normal levels *in vivo* has been supported by several studies. The hypothesis in the present study that the number of goblet cells will increase with high levels of vitamin A both *in vivo* and *in vitro* is supported by data in this investigation. The number of goblet cells was significantly higher in dietary level B than level A of both retinol and β-carotene in the *in vivo* study. Although not significant, *in vitro* there was an increase in number of goblet cells with each of the groups receiving vitamin A compared to the control group. Since goblet cells are the primary source of mucus production in the intestines, and goblet cells contain the majority of sulfated mucins produced (see Tables 12 and 16), an increase in number of goblet cells could lead to an increase in amount of mucus produced. This would be the means by which sulfated mucins increase. An increase in mucus concentration could increase the protection of the intestinal lining from invasive substances that may be present in the lumen. Thus, vitamin A has an effect not only on the type of mucus, but also on the amount of mucus produced by colonic tissue.

Carcinogenesis brings about changes in each tissue that it affects. In the colon, there is an increase in sialomucins produced by the tissue and a decrease in sulfated mucins. Sialomucins contain mono-, di-, and tri-O-acyl substituents. With cancer development there is a change in

the structure of sialic acids. The hydroxyl groups substituted on O-acylated sialic acids at C_8 change and become unsubstituted or substituted at C_7, or C_9. There is an overall decrease in amount of heavily substituted sialic acids that occurs with carcinogenesis. In the present study, vitamin A caused an increase in sialic acids with hydroxyl groups substituted at C_8, the type of sialic acids present in normal tissue. Vitamin A could provide or carry these hydroxyl groups in a manner similar to the way it carries monosaccharide units for glycoprotein synthesis (Peck, 1981).

The PAT/KOH/PAS stain gives information about the sialic acids in mucus. The in vivo study showed no significant differences in the uptake of this stain by goblet cells with increasing amounts of vitamin A. However, there was a trend toward more red staining, or sialic acids substituted at C_8, with the high levels of vitamin A. The in vitro study showed this same trend with significantly higher levels of sialic acids substituted at C_8 in both ß-carotene groups and the higher retinol group compared to the control group.

Evaluation of stains for mucin content in goblet cells and connective tissue as two separate areas has not previously been done. Uptake of the PAT/KOH/PAS stain by connective tissue showed the same trend in both studies. The in vivo study showed a significant increase in sialomucins substituted at C_8 or mixed sialomucins with an increase in vitamin A from level B to level C. The in vitro study revealed a significant increase in these same mucins from control to each of the four groups treated with vitamin A. Again, this indicates that vitamin A may have a role in the chemical changes of mucins, and the changes in chemistry

may be a shift toward mucus found in normal tissue. Vitamin A induces an increase in hydroxyl groups substituted at C_8 in sialic acids, a chemical change which could be very important in the role that mucus plays in the intestines. Thus, a direct effect of vitamin A on DNA is implied.

Frequently as colonic tissue undergoes precancerous changes, mucus secretion decreases. At times the layer of mucus becomes clumped and stringy and does not form a continuous sheath protecting epithelium as it does in normal tissue (Chantler, Elder, & Elstein, 1982). Results of the present study demonstrate that vitamin A induces a change in the type of mucus produced, causing an increase in heavily sulfated mucins. Vitamin A is a carrier for monosaccharide units in glycosyltransferase reactions during glycoprotein synthesis. After malignant transformation, the glycosylation of many glycoproteins is altered and is related to an increased sialylation of oligosaccharide moieties (Honey & Shows, 1983). The availability of glycosyltransferases during glycoprotein synthesis determines the pathway of mucin synthesis (Peck, 1981). Since some transferases compete with other enzymes for a single substrate, sialic acid transferase may prevent or inhibit the action of glycosyltransferases, and vice versa. Vitamin A apparently increases the number of oligosaccharide assembly units that can be used for the production of heavily sulfated mucins by the action of glycosyltransferases. It has recently been shown that the composition of the diet of rats influences glycosyltransferase activity (Biol et al., 1984).

Sialic acid, however, is a ligand for some viruses, and these viruses require the sialyl 2-3 α-galactose sequence as a host cell receptor determinant (Chantler et al., 1982). Sialomucins bind with the virus or

antigen and protect it from immunocompetent cells (Filipe & Branfoot, 1974). Protection of these antigens could result in an increase in substances available to induce carcinogenesis. There is an increase in sialomucins associated with cancer development. Vitamin A, in addition to stimulating increased glycosyltransferase activity, may also inhibit cancer development by inhibiting sialic acid production. If sulfated mucins are predominant and few sialic acids are present, the cancer cells can not be protected and, therefore, may not survive. Since the mucus coat serves as a protective barrier against bacteria, viruses, and foreign agents in normal intestinal epithelium, the presence of more sulfated mucins stimulated by vitamin A could provide for a more protective mucus coat and prevent it from clumping which can occur with an increase in sialomucins due to the chemical properties of sialic acid. Thus, carcinogens and other invasive substances could be prevented from coming in contact with the epithelium.

Results of the present study indicate that the effects of vitamin A on the number of goblet cells and amount and type of mucus are apparently mediated by the influence of vitamin A on gene regulation. The fact that vitamin A is involved in genetic regulation is supported by the fact that cellular retinol binding protein (CRBP) binds retinol to the nucleus (Newell & Ellison, 1981). CRBP interacts with the nucleus and delivers the retinol specifically into the nucleus (Arnott, Van Eys, & Wang, 1982). Recent work indicates that retinoids with a high affinity for the binding protein have higher biological activity which could be important in chemoprevention (Sani, Dawson, Hobbs, Chan, & Schiff, 1984).

Vitamin A seems to have effects on tissues similar to the steroid hormones. They influence changes by initiating DNA transcription which results in mRNA being exported. Ribosomal translation of the message leads to synthesis of unique proteins which mediate their action (Williams, 1981). The mechanism of action of vitamin A could be similar. Prutkin and Bogart (1970) found labeled vitamin A to be bound to the loose chromatin and localized in the nucleus of the keratinocyte in the keratoacanthoma, an epithelial tumor. By binding to the loose chromatin, the portion of the nucleus responsible for synthesis of RNA, vitamin A may act similarly to the steroid hormones and produce an alteration in the genomic regulatory machinery. Vitamin A could activate a region of the genome involved in transcription of a mRNA coded for translation of components of mucins (Audette & Page, 1983; Kummet & Meyskens, 1983; Prutkin & Bogart, 1970).

Retinoids have also been shown to affect cells which do not have binding proteins (Sporn & Roberts, 1983). In these cells, the action of retinoids is similar to the peptide hormones. The action of vitamin A in the nucleus produces an alteration in gene expression via protein kinase activity (Bollag & Matter, 1981), both cAMP-dependent and cAMP-independent. Retinoids increase cAMP-dependent protein kinase activity and also induce a calcium- and phospholipid-dependent, cAMP-independent protein kinase activity (Sporn & Roberts, 1983). Malignant transformation decreases the amount of cAMP in cells and hormone receptors are also thought to decrease following transformation. Since retinoids can function in cells with or without a binding protein and in the presence or absence of cAMP, they could continue to induce changes in malignantly

transformed cells even though cAMP is decreased (Honey & Shows, 1983). Retinoids also control the expression of many proteins that are either direct constituents of the cytoskeleton and extracellular matrix or participate in the formation of the cytoskeleton and matrix. This would influence differentiation by causing the cell to produce specific proteins that would be important in determining the cell's function.

Vitamin A induces cellular differentiation in vitro and in vivo (Clark & Marchok, 1979; Wilson & Dowdle, 1980). Retinoids have control over cellular proliferation and differentiation, which are processes that are closely related. Vitamin A, in many cell lines in vitro, causes neoplastic cells to stop proliferating and to differentiate terminally. This anti-proliferative effect of retinoids is important in carcinogenesis (Sporn & Roberts, 1983). This hypothesis is supported in the present study because stem cells are terminally differentiated as shown by the increase in number of goblet cells. The increase in number of goblet cells results in fewer cycling cells and it appears that some of the cycling cells have been shunted out of the cell cycle into a pathway of differentiation. Thus, retinoids have an anti-proliferative effect as well as an inducing effect on differentiation.

The influence of retinoids on genetic regulation is seen in the present study with the increase in number of goblet cells, the change from sialomucins to sulfated mucins with retinoid treatment, and the change in the chemistry of sialic acids. This supports previous work which demonstrated that vitamin A influences cellular differentiation, cellular proliferation, and mucin biosynthesis (Clark & Marchok, 1979; Sporn & Roberts, 1983).

Malignant Transformation in Cells

A recent hypothesis for the mechanism of carcinogenesis is that initial DNA damage occurs from one of many possible processes. Then there is a generation of genomic and phenotypic diversity engineered by transpositional elements that are activated by the damage to DNA. Following changes in cellular phenotypes, those capable of further growth favor tumor formation and progression (German, 1983). Tumors are the result of chromosomal and biochemical changes and reflect the altered expression of many genes. Often tumor cells express chromosomal changes, such as loss or gain of a whole chromosome, translocations, or deletions of parts of chromosomes (Honey & Shows, 1983). It is thought that these chromosomal rearrangements result in the oncogene of one chromosome attaching to the active site of another chromosome, which could cause oncogene activation (Gold, 1983). Another hypothesis is that alkylation of DNA at specific positions by carcinogenic agents produces compounds which may be related to carcinogenesis. These cause miscoding, which occurs at crucial parts of the DNA molecule and produces a carcinogenic mutation (Klopman & Ray, 1982). Vitamin A may play a protective role that prevents this from occurring.

Developmental defects, an increased mutation rate, and increased chromosome instability are all associated with neoplastic proliferation (German, 1983). Some of the oncogenes associated with transformation may be the same genes involved normally in control of proliferation and differentiation (German, 1983). Since retinoids inhibit tumor promotion and initiation (Bertram et al., 1981), they apparently prevent the damage to DNA from occurring or in their control over proliferation and

differentiation, prevent malignant transformation from occurring. Because vitamin A is known to initiate chemical changes within the cell, such as changing the position of OH groups in sialic acids, it is speculated that vitamin A may have a role in prevention of alkylation and mutations via chemical reactions that it induces.

Effects on the Immune Response

Vitamin A has been demonstrated to enhance both humoral and cellular immunity (Clamon, 1980). In the present study, the number of mast cells significantly increased with increases in amount of β-carotene *in vitro*, and also with increases in retinol and β-carotene in the *in vivo* study. Mast cells increase in number with the development of some cancers, but the present *in vitro* study showed an additional increase when vitamin A was added to culture medium for tumors compared with controls not receiving vitamin A. Mast cells produce histamine, bradykinin, and serotonin which are powerful vasodilators and also increase capillary permeability (Gadebusch, 1979). They increase the capillary pore size which allows leakage of fluid and plasma proteins into the tissues. Mast cells are involved in the inflammatory response. All of these conditions would delay the spread of harmful substances throughout the body. Mast cells also produce heparin, an anticoagulant that stimulates the removal of lipoproteins from the blood. A vitamin A-stimulated increase in number of mast cells could cause an increase in blood flow, which would increase the rate of delivery of blood and nutrients to tissues. The delivery of phagocytic cells may also be increased which would enhance the inflammatory response of tissues, a situation which would inhibit tumor growth. There is usually an increase in blood flow

to the tumor area and the cancerous cells rob the body of nutrients. Increased numbers of mast cells could, in these ways, strengthen host resistance to carcinogens.

Phagocytic cells generate H_2O_2 which reacts with adjacent mast cells and stimulates release of granules, which damages tumor cells, thus playing a role in the host defense against neoplasms (Henderson, Chi, Jong, & Klebanoff, 1981). One study reported that patients in the pre-tumor stage and cancer patients had a degradation of mast cells with a reduction and disappearance of mucopolysaccharide saturated mast cells (Kimura, 1979; Uspenskii & Grinevich, 1981), and a disappearance of mature granulated mast cells. Vitamin A may have a role in mast cell metabolism similar to that described for glycoprotein synthesis. Heparin is a conjugated polysaccharide, and 10% of the D-glucosamine residues are N-acetylated while 90% are N-sulfated (Thunberg, Hook, Lindahl, Abildgaard, & Langholm, 1980). The fact that sulfated mucins also contain a sulfur group could indicate a role of sulfur which vitamin A mediates. SH groups are the components that acts as regulatory agents in normal cell mitosis. For example, in developing sea urchin eggs with each cleavage sulfhydryl groups are formed, keeping track of the number of cell divisions and signaling when the cell should die (Dan & Ikeda, 1971). Regulatory genes in cancer cells are apparently lost, suggesting a possibility that the gene for producing sulfate groups or sulfhydryl bonds might be lost in some forms of cancer. The increased number of sulfate bonds could initiate more sulfated mucins in mucus and form a more continuous protective coat of mucus, which is not present in cancerous tissue.

Increases in number of plasma cells and lymphocytes with an increase in vitamin A in the *in vitro* study approached statistical significance, and are worthy of discussion, and possibly of further study. Plasma cells participate in humoral immunity while lymphocytes are known to be involved in cell-mediated immunity. They could have a role in the prevention and also in the cure of cancer by eliminating carcinogens and destroying cancerous cells. Plasma cells secrete antibodies which attack antigens produced by cancerous cells or tumor promoters. An increase in number of plasma cells could strengthen the body's response to antigens. Lymphocytes are phagocytic cells and an increase in number of lymphocytes strengthens the immune response to such substances as carcinogens, co-carcinogens, tumor promoters, and cancerous cells.

Although the number of mast cells, plasma cells, and lymphocytes have been determined in many kinds of cancerous tissue, these cell numbers have not been determined in adenomas and adenocarcinomas of colorectal tissue after exposure to retinoids in culture medium. The hypothesis that these cell numbers may change after exposure to vitamin A is supported by the data in the present study showing increases in numbers of these cells in vitamin A-treated groups compared with controls.

The exact mechanisms of action of retinol and β-carotene are still unclear but it is certain that these actions do bring about histological changes which appear to be beneficial to the host. However, the possibility remains that the cancer cells themselves might be directing and using the increase in mast cells, plasma cells, and lymphocytes to their own advantage in destroying the host.

Many substances which are carcinogenic to humans are detoxified by the immune system. An increase in the various cells of the immune system, such as the mast cells, plasma cells, and lymphocytes, could provide the body with a more efficient preventive mechanism against carcinogens. In some types of cancer, a decrease in number of plasma cells and mast cells has been reported with cancer development (Chomette, Auriol, & Tereau, 1979; Uspenskii, & Crinevich, 1980, 1981; Von Gumberg & Siefert, 1980), and a depression of the immune system was seen during metastasis (Palma, Mercure, & DiLorenzo, 1981). Several kinds of cancer, however, are associated with an increased infiltration of mast cells, plasma cells, lymphocytes, and immunoglobulins with malignancy or the premalignant condition (Fox, Bull, & Guz, 1981; Sienski, 1980; Svennevig, 1980; Svennevig, Lunde, & Holter, 1982). These cells seem to be selectively attracted to the tumor site (Svennevig et al., 1982). This infiltration of immunocompetent cells could result from the immune system's attempt to destroy the cancer, or be attributed to the cancer cells' attempt to survive.

In some tumors, there is a translocation which involves chromosomes number 14, 2, and/or 22. These contain genes for immunoglobulin light and heavy chains, and are often involved in tumorigenesis (Honey & Shows, 1983). This could explain the increase in immunoglobulin formation with some types of cancer (Sienski, 1980; Svennevig, 1980). Also, some chromosomes undergo genetic changes or mutations of the oncogenes that control lymphocyte growth and development which could also explain the increase in lymphocytes with cancer (Svennevig et al., 1982). An increase in number of lymphocytes could decrease the number of antigens from

microorganisms allowed to survive in a tissue. Several authors have found an increase in number of lymphocytes due to vitamin A stimulation (Abb & Deinhardt, 1980; Abb et al., 1982; Bang et al., 1972, 1975). Plasma cells produce antibodies that attack microorganisms that are antigens or carcinogens of a protein nature. This supports the fact that a local immune reaction seems to be initiated by the tumor and results in an increased number of plasma cells at the tumor site (Svennevig, 1980).

Although the human body has an immunological surveillance system that destroys many of the cancers that develop within the body, there is occasionally a failure of the immune response and tumor progression occurs. This may be due to a restricted access of immune lymphocytes to tumor cells because an intact capillary endothelium is usually interposed between the tumor cell and immune lymphocytes. Factors such as inflammation, antigen-antibody reactions, or tissue necrosis could enhance major breaches in capillary walls so that lymphocytes are exposed to tumor cells. When the lymphocyte to tumor cell ratio is low, tumor growth is stimulated but when the ratio is high, tumor growth is inhibited (Jeejeebhoy, 1975). Because mast cells induce capillary permeability, they help to stimulate this response by lymphocytes.

Vitamin A also increases the labilization of lysosomal membranes and the release of lysosomal enzymes which are toxic to antigens. Increased release of lysosomal enzymes would help destroy antigens and carcinogens. Sialidases are also released which can change the pattern of metastasis and immune recognition of tumor cells. Lysosomal activation also enhances macrophage activation and activation of killer T cells (Bollag & Matter, 1981), whereas depletion of lysosomes stimulates

proliferation of lymphoid cells. Thus, a vitamin A-stimulated activation of lysosomes would result in an enhanced activation of macrophages and killer T cells which are also effective in destroying carcinogens.

Vitamin A has effects on hematopoietic cells in the bone marrow and may have a role in their proliferation and differentiation as is seen in epithelial cells. Retinol deficiency or excess causes failure of mesenchymal cells to proliferate and differentiate to form the vascular system in one-day old chick embryos. A common stem cell type is a precursor to both blood cells and those that form the vascular system (Sporn & Roberts, 1983). Thus, retinoids play a role in the control of proliferation and differentiation of mesenchymal precursor cells, and because these cells are precursors of the cells in the immune system, immune responses are affected by vitamin A in this way. As vitamin A increases cellular proliferation of hematopoietic cells and induces differentiation of lymphocytes, it has an effect on the immune status of the host. The pluripotential hematopoietic stem cell generates lymphoid stem cells, which stimulate the pre-B stem cells. These become immature B cells, then mature B cells, and then activated B cells which become memory B cells and then plasma cells (Kuehl, 1982).

Retinoids stimulate production of humoral antibodies (Jurin & Tannock, 1972). This could be via an increase in number of plasma cells. Another possible mechanism is activation of the genes which would increase the number of antibodies produced by each cell. Also, an increase in number of lymphocytes could result in an increase in number of plasma cells because B lymphocytes are precursors of plasma cells. Therefore, it is likely that retinoids have effects on the immune system

that may involve gene activation which results in cellular differentiation and an increase in amount of antibody formed by each plasma cell.

Immunodeficiency is one of six features classically associated with neoplastic proliferation (German, 1983). Viral oncogenesis is more frequent and is greatly facilitated when immune responses are depressed and tumors often appear earlier and grow better when the immune response is depressed (Jeejeebhoy, 1975). Thus, a vitamin A-stimulated increase in number of immunocompetent cells could serve as protection against immunodeficiency and subsequently prevent the development of cancer that is associated with immunodeficiency.

The effects that vitamin A has on the immune system and mucus production could be interrelated, and could be due to chemical groups that vitamin A provides or transports within the body. The mechanisms of action of vitamin A are still not totally clear, but it is certain that vitamin A does bring about changes in the histological development of colorectal tissue. The levels where retinoids are most effective are yet to be determined, but it appears that amounts that could be provided with dietary alterations are sufficient to bring about many beneficial changes. It appears that supplementation beyond a certain point is of no further benefit to the tissue. Thus, with additional studies in this area of nutrition, it is possible that these amounts could be determined, and recommendations for dietary changes could be made.

CHAPTER V

SUMMARY AND CONCLUSIONS

Vitamin A had several definite effects on the histology of colorectal tissue both *in vivo* and *in vitro*. In both studies there was no significant differences in the effects of β-carotene compared to retinol, although *in vitro* there was a trend toward increased cell numbers and more sulfated mucus with β-carotene. However, significant differences were found in the amount or level of either form of vitamin A. The major significant findings related to the type of mucus produced and the effects on the cells involved in the immune system.

Mucus Production

There was a significant increase in the number of goblet cells with an increase from level A to level B of vitamin A *in vivo*. Goblet cell numbers then decreased from level B to level C. This same trend was seen *in vitro*, although not significant, and there was an increase in number of goblet cells in all groups receiving vitamin A compared to controls. Because goblet cells are the primary source of mucus secretion in the colon, this affects the amount and type of mucus produced.

In vitro there was a significant increase in sulfated mucins with Retinol 2 and both levels of β-carotene compared to controls. The same was seen *in vivo*, although not significant. There was an increase in sulfated mucins from level A to level B of either retinol or β-carotene. As the amount of sulfated mucins increased there was a decrease in the sialomucins found in tissues, but there was a significant change in the

chemistry of these sialomucins. The in vitro study showed a significant increase in the sialic acids in goblet cells with OH groups substituted at C_8 in Retinol 1 and both β-carotene groups compared to the control group, which contained more mixed sialomucins. Although not significant, the same trend was seen in vivo. The sialomucins in connective tissue contained significantly more OH groups substituted at C_8 in vivo and in vitro with the higher levels of vitamin A.

The increase in number of goblet cells, the subsequent increase in sulfated mucins, and the change in chemistry of sialomucins are changes toward production of a more protective, normal type of mucus. This indicates that vitamin A does affect mucus production. The shift toward more normal mucus production, which is mediated by changes in the genetic expression of vitamin A-responsive cells, is important in cancer development. Tissues change from a malignant to a benign condition with vitamin A treatment, as diagnosed by the type of mucus produced. This supports the hypothesis that vitamin A is important in the prevention, cure, inhibition and/or reversal of cancer.

Immune Response

The number of mast cells both in vivo and in vitro significantly increased with exposure to vitamin A. In vivo, there was a significant increase from level A to level B of either retinol or β-carotene, whereas in vitro, the increase in number of mast cells was found in both β-carotene groups compared with the control group. The increase in number of plasma cells and lymphocytes approached significance with the β-carotene 1 group over the control group in vitro. Although not significant, the trend in these experiments was that both retinol and β-carotene caused

an increase in numbers of plasma cells and lymphocytes over control groups. The increase in number of immunocompetent cells induced by vitamin A is important in the development of cancer. These cells are involved in the surveillance and destruction of foreign substances that are carcinogenic, and against cancer cells themselves. Thus, vitamin A, via a strengthened immune system, has a role in the control of cancer.

Suggestions for Further Study

As indicated in Chapter IV, because of the heterogeneous sample in the *in vitro* study, there might have been some other effects of vitamin A that were not found with this experimental design. It would be of interest to repeat this experiment and limit the tumors to a specific site and type to decrease variability. Also, in order to increase accuracy, it would be advantageous to use a densitometer for precision in classifying type of mucus found in tissues. It would also be advantageous to add a synthetic retinoid such as 13-cis-retinoic acid to see if there are differences between natural and synthetic retinoids. Because the *in vivo* phase of the present study indicated that there was no difference in retinol and β-carotene but the *in vitro* portion showed a trend toward beneficial effects with β-carotene, it would be advisable to do additional studies testing the two forms of vitamin A.

The number of goblet cells first increased and then decreased *in vivo* with each increase in vitamin A, and although with retinol *in vitro* there was an increase from low to high levels, a decrease was seen with β-carotene in the highest level. Use of a dose-response curve would be helpful in order to determine an optimal level of retinol and β-carotene both *in vivo* and *in vitro*.

It is important for future studies to continue experiments with β-carotene on the cells involved in the immune system and in the type of mucus produced. Other cells involved in the immune system such as neutrophils, macrophages, monocytes, and natural killer cells should be tested for increases with vitamin A. A more complete study of the mast cell populations which stain with the HID-AB and PAT/KOH/PAS stains is needed. A study designed to use the electron microscope would give additional information that could be helpful in determining effects of vitamin A. Also, a study of the role of these stained mast cells in cancer is needed. It would be advantageous to know if heparin or histamine take up the stains used, and to determine more completely the mechanisms of action of vitamin A on the metabolism of these mast cells.

Since high levels of vitamin A are indeed causing mucus to become more sulfated in addition to causing a change in the chemistry of sialomucins, this could have an effect on cancer. The total amount of sulfated mucins decrease and sialomucin content increases and contains more OH groups substituted at C_7 or C_9 or unsubstituted with cancer development.

Further work on the effect of vitamin A on the immune system and on mucus production is necessary before conclusive remarks can be made concerning vitamin A. However, at the point this dissertation is complete, it would not be harmful to encourage an increase in the dietary intake of β-carotene as a preventive measure, although this practice can not be advocated on a wide-scale basis without further studies. β-carotene is readily available in vegetables and has not been shown to have toxic medical effects. Since it does indeed strengthen the immune system, and cause mucus to become a more protective type, it could be beneficial to consider carefully the about of β-carotene recommended in the diet.

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Appendix A

Table A-1

Composition of Basal Diet Used for *in vivo* Phase of Study

Ingredient	gm/kg Diet	%
Starch	654	67
Protein	166	15
Vegetable oil	100	10
Mineral mix	40	4
Vitamin mix	20	2
Cellulose	20	2

Retinoid Added to Basal Diet	µg/g Diet	%
Retinol (level A)	6	-
Retinol (level B)	30	-
Retinol (Level C)	60	-
β-carotene (level A)	36	-
β-carotene (level B)	180	-
β-carotene (level C)	360	-

Table A-2

Composition of Vitamin Mixture[1]

Constituents	Amount per 100 gm Mix
	mg
Vitamin B_{12}	0.1
Biotin	1.0
Folic acid	5.0
Thiamin HCl	25.0
Pyridoxine HCl	25.0
Menadione (2-methyl-napthaquinone)	25.0
Riboflavin	50.0
Nicotinic acid	50.0
Ca pantothenate	150.0
p-Aminobenzoic acid	500.0
	gm
Inositol	5.0
Choline chloride	7.5
DL-methionine	30.0
Corn starch[2]	56.6

[1]All vitamins and methionine were purchased from ICN Nutritional Biochemicals, Cleveland, Ohio.

[2]Teklad Test Diets, Madison, Wisconsin.

Table A-3

Composition of Wesson Modification of Osborne-Mendel Mineral Mix[1]

Constituents	%
Calcium carbonate	21.000
Cupric sulfate (5 H_2O)	0.039
Ferric pyrophosphate	1.470
Manganeous sulfate (anhyd.)	0.020
Magnesium sulfate (anhyd.)	9.000
Aluminum potassium sulfate (12 H_2O)	0.009
Potassium chloride	12.000
Potassium dihydrogen phosphate	31.000
Potassium iodide	0.005
Sodium chloride	10.500
Sodium fluoride	0.057
Tricalcium phosphate	14.900

[1]Teklad Test Diets, Madison, Wisconsin

Appendix B

Table B-1

Technique for Embedding Tissue in Paraffin

Fixation

Store tissue in fixative until embedding process begins

Dehydration

Solution	Amount of Time
Water	1/2 hour
35% ethanol	1/2 hour
50% ethanol	1/2 hour
70% ethanol	1/2 hour (may be stored here)
95% ethanol	1/2 hour
Absolute ethanol	1/2 hour
Absolute ethanol	1/2 hour
Clearing	
Absolute ethanol:xylol (50:50)	1/2 hour
Xylol I	1/2 hour
Xylol II	1/2 hour
Xylol:paraffin (50:50)	1 hour
Embedding	
Paraffin I	1 hour
Paraffin II	1 hour

Remove tissue from embedding oven and place in water at 4°C until cooled and paraffin hardens. Immerse completely in water to loosen tissue blocks from dishes.

Table B-2

Histological Technique for High Iron Diamine-Alcian Blue Stain (HID-AB)

Fix in: 10% Neutral Formol-Calcium or 10% Formalin-2% Calcium Acetate or Carnoy's

Embed in paraffin, 5 μ sections

Staining Procedure:	Minutes
Xylol I	10
Xylol II	5
Xylol:Absolute ethanol	2
Absolute ethanol	2
95% ethanol	2
70% ethanol	2
50% ethanol	2
Water	2
1% Periodic Acid (H_5IO_6)	10
Tap water (change several times)	5
Diamine Solution	18 hours
Water	quick rinse
Alcian Blue (8GX)	30
Water	quick rinse
Dry with hot air	30 seconds
95% ethanol	quick rinse
Absolute ethanol	2
Absolute ethanol:Xylol	2
Xylol I	5
Xylol II	5
Mount in Permount	

Stains:
Brown-black: sulfated mucins
Alcianophilia: non-sulfated acid mucins or sialomucins

Normal colonic goblet cells secrete a mixture, but sulphomucins predominate in deep half of crypt and surface epithelium
Adjacent to carcinoma: replaced sulfated mucins by sialomucins. More sialomucins indicates increased invasiveness of cancer.

Table B-3

Histological Technique for Periodic Acid-Thionin Schiff/Potassium Hydroxide/Periodic Acid-Schiff Stain (PAT/KOH/PAS)

Fix in 10% Formol-Calcium

Embed in paraffin, 5 μ sections

Staining Procedure:	Minutes
Xylol I	10
Xylol II	5
Xylol:Absolute ethanol	2
Absolute ethanol	2
95% ethanol	2
70% ethanol	2
50% ethanol	2
Water	2
1% Periodic Acid (freshly prepared)	30
Tap water (change several times)	10
Thionin Schiff Reagent	30
Tap water (change several times)	10
70% ethanol	rinse
5% KOH in 70% ethanol	30
Tap water, rinse gently	rinse
1% Periodic Acid (freshly prepared)	10
Tap water (several gentle changes)	10
Standard Schiff Reagent	30
Tap water (several changes)	10
50% ethanol	2
70% ethanol	2
95% ethanol	quick rinse
Absolute ethanol	2
Absolute ethanol:Xylol	2
Xylol I	5
Xylol II	5
Mount in Permount	

Stains;

Blue - indicates presence of C_7, C_9 or unsubstituted O-acylated sialic acid (increases with adenocarcinoma, a good sign of malignancy)

Purple - villous lesions, mixed sialomucins

Red - normal colon, identifies sialic acids substituted at C_8

Table B-4

Solutions Used for HID-AB Stain

Periodic Acid:
1 g. periodic acid
100 ml. water

Alcian Blue
1 ml. glacial acetic acid
99 ml. distilled water
1 g. Alcian Blue 8GX

Ferric Chloride Solution:
mix 40 g. ferric chloride with 100 ml. water for 40% solution
mix 10 ml. 40% ferric chloride with 30 ml. water, this gives a 10% ferric chloride solution

Diamine Solution:
measure 120 mg. meta-diamine
20 mg. para-diamine
add at once to:
50 ml. water
pour into a coplin jar with:
1.4 ml. of 10% ferric chloride solution

Solutions Used for PAT/KOH/PAS Stain

Periodic Acid: (same as above)

Thionin Schiff Reagent:
dissolve 1 g. thionin in 100 ml. distilled water with heat, cool
add .75 ml. thionyl chloride, leave overnight
add 2 g. activated charcoal
shake and filter immediately, store in refrigerator 3 to 4 weeks

KOH Solution:
.5 g. KOH
100 ml. 70% ethanol

Solutions Used for Toluidine Blue Stain

Toluidine Blue Solution:
.5 g. toluidine blue
200 ml. water
1 ml. glacial acetic acid

Filter before use, keeps indefinitely

Table B-5

Histological Technique for Toluidine Blue Stain for Metachromasia

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Fix in Formalin, Embed in paraffin, 5 μ sections

Staining Procedure:	Minutes
Xylol I	10
Xylol II	5
Xylol:Absolute ethanol	2
Absolute ethanol	2
95% ethanol	2
70% ethanol	2
50% ethanol	2
Water	2
Toluidine blue	1-5
Water	rinse
70% ethanol	2
95% ethanol	quick rinse
Absolute ethanol	2
Absolute ethanol	2
Xylol:Absolute ethanol	2
Xylol I	5
Xylol II	5
Mount in permount	

Stains:

Orthochromatic color (nuclei, cytoplasm of some cells, Nissl substance of neurons) blue, mast cells stain blue

Metachromatic color - red.

Table B-6

Formula for 10% Calcium Formalin Fixative

Solution	Amount
40% Formalin	10 ml
Calcium chloride (anhydrous) 10% aqueous solution (10g/100ml water)	10 ml
Distilled water	80 ml

Appendix C

Table C-1

Weight Gains of Young Rats Fed Varying Levels of Retinol or β-Carotene

Animal Number	Retinoid Source and Dietary Level					
	Retinol			β-Carotene		
	A	B	C	A	B	C
	2 Weeks weight gain (gm)					
1	81	64	75	66	72	81
2	58	77	71	71	83	66
3	58	70	78	44	71	67
4	69	62	53	62	74	83
5	80	68	68	72	55	65
6	65	81	76	69	66	64
7	61	72	75	45	69	61
8	59	77	76	80	65	85

Table C-2

Retinoid Consumption of Young Rats Fed Varying Levels of Retinol or β-Carotene

Animal Number	Retinoid Source and Dietary Level					
	Retinol			β-Carotene		
	A	B	C	A	B	C
	Total Amount of Vitamin A Consumed (μg)					
1	852	4200	8400	5580	26460	63000
2	774	4500	9900	5760	32400	52200
3	768	3900	9000	4824	31500	57600
4	990	4140	7500	5520	29160	61200
5	930	4440	8640	5400	23580	48600
6	990	5100	10200	5580	23400	61200
7	762	4500	9840	4392	27720	54720
8	912	4650	9000	6300	24300	65880
	Average Amount of Vitamin A per Day (μg)					
1	61	300	600	399	3150	4500
2	55	321	707	411	2314	3729
3	55	279	643	345	2250	4114
4	71	296	536	373	2083	4371
5	66	317	617	386	1684	3471
6	71	364	729	399	1671	4371
7	54	321	703	314	1980	3909
8	65	332	643	450	1736	4706

Appendix D

Table D-1

MANOVAs for *in vivo* Study

Variable	Degrees of Freedom	F Value	p Value	Significance
Level	12,74	2.81	.0030	**
Vitamin	6,37	.92	.4950	NS
Level x Vitamin	12,74	.76	.6920	NS

Note: NS indicates not significant, * is significant ($p \leq .05$); ** is highly significant ($p \leq .01$).

Table D-2

ANOVA Tables for *in vivo* Study by Level

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Significance[1]
			Weight Gain			
Total	47	4118				
Between Groups	2	386	193	2.33	.1091	NS
Within Groups	45	3732	83			
			Number of Goblet Cells			
Total	47	16232				
Between Groups	2	2366	1183	3.84	.0289	*
Within Groups	45	13866	308			
			Number of Mast Cells[2]			
Total	47	340				
Between Groups	2	82	41	7.15	.0020	**
Within Groups	45	258	6			
			Type of Mucus With HIB-AB Stain			
Total	47	23				
Between Groups	2	3	1.30	2.93	.0635	NS
Within Groups	45	20	.45			
			Uptake of PAT/KOH/PAS Stain by Goblet Cells			
Total	47	10.59				
Between Groups	2	.32	.16	.64	.5300	NS
Within Groups	45	10.27	.24			
			Uptake of PAT/KOH/PAS Stain by Connective Tissue			
Total	47	10.2				
Between Groups	2	1.4	.67	3.47	.0395	*
Within Groups	45	8.8	.19			

[1]NS indicates not significant, * is significant ($p \leq .05$); ** is highly significant ($p \leq .01$).

[2]For definition of mast cells, see page 20.

Table D-3

AVOVA Tables for *in vivo* Study by Vitamin

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Significance[1]
		Weight Gain				
Total	43	3715.58				
Model	1	30.08	30.08	.34	.5610	NS
Error	42	3685.50	87.75			
		Number of Goblet Cells				
Total	43	13834.16				
Model	1	394.45	394.45	1.23	.2730	NS
Error	42	13439.71	319.99			
		Number of Mast Cells[2]				
Total	43	244.38				
Model	1	18.25	18.25	3.39	.0730	NS
Error	42	266.13	5.38			
		Type of Mucus With HID-AB Stain				
Total	43	19.66				
Model	1	.40	.40	.88	.3540	NS
Error	42	19.26	.46			
		Uptake of PAT/KOH/PAS Stain by Goblet Cells				
Total	43	10.28				
Model	1	.01	.01	.03	.8620	NS
Error	42	10.27	.24			
		Uptake of PAT/KOH/PAS Stain by Connective Tissue				
Total	43	8.66				
Model	1	.12	.12	.59	.4470	NS
Error	42	8.54	.20			

[1]NS indicates not significant.
[2]For definition of mast cells, see page 20.

Table D-4

ANOVA Tables for In Vivo Study for Vitamin by Level

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Significance[1]
		Weight Gain				
Total	44	3701.67				
Model	2	16.17	8.08	.09	.9120	NS
Error	42	3685.50	87.75			
		Number of Goblet Cells				
Total	44	13471.23				
Model	2	31.52	15.76	.05	.9520	NS
Error	42	13439.71	319.99			
		Number of Mast Cells[2]				
Total	44	239.83				
Model	2	13.70	6.85	1.27	.2910	NS
Error	42	226.13	5.38			
		Type of Mucus With HID-AB Stain				
Total	44	19.91				
Model	2	.65	.32	.71	.5000	NS
Error	42	19.26	.46			
		Uptake of PAT/KOH/PAS Stain by Goblet Cells				
Total	44	10.92				
Model	2	.65	.32	1.32	.2780	NS
Error	42	10.27	.24			
		Uptake of PAT/KOH/PAS Stain by Connective Tissue				
Total	44	8.64				
Model	2	.10	.05	.23	.7930	NS
Error	42	8.54	.20			

[1]NS indicates not significant.
[2]For definition of mast cells, see page 20.

Table D-5

Post Hoc Tests for Significance in Selected Variables in *In Vivo* Study

Variable	Group Mean	F Value	Degrees of Freedom	p Value	Significance[1]
Weight Gain		2.3	2,45	.1091	NS
Level A	65[a2]				
Level B	70[a]				
Level C	72[a]				
Number of Goblet Cells		3.8	2,45	.0289	*
Level A	67[a]				
Level B	83[b]				
Level C	78[ab]				
Number of Mast Cells[3]		7.1	2,45	.0020	**
Level A	8.6[a]				
Level B	9.7[ab]				
Level C	11.7[b]				
Type of Mucus with HID-AB Stain		2.9	2,45	.0635	NS
Level A	1.9[a]				
Level B	1.3[b]				
Level C	1.6[ab]				
PAT/KOH/PAS Stain in C.T.		3.5	2,45	.0395	*
Level A	1.5[ab]				
Level B	1.2[a]				
Level C	1.6[b]				

[1]* indicates significance ($p \leq .05$); ** indicates significance ($p \leq .01$); NS indicates no significant difference.
[2]Values with the same letter are not significantly different ($p \leq .05$).
[3]For definition of mast cells, see page 20.

Table D-6

MANOVAs for *in vitro* Study

Variable	Degrees of Freedom	F Value	p Value	Significance
ID	99	3.64	.0001	**
Error	265			
Group	36	2.05	.0017	**
Error	136			
Level	9	.40	.8761	NS
Error	3			
Vitamin	9	.71	.6976	NS
Error	3			
Level x Vitamin	9	.79	.6573	NS
Error	3			

Note: NS indicates not significant, ** indicates significance ($p \leq .01$).

Table D-7

ANOVAs for *in vitro* Study, ID by Group

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Significance[1]
		Number of Goblet Cells				
Total	59	3270.87		7.75	.0001	**
ID	11	2334.92	212.26	10.40	.0001	**
Group	4	38.22	9.55	.47	.7586	NS
Error	44	897.73	20.40			
		Number of Mast Cells[2]				
Total	59	127.29		2.86	.0034	**
ID	11	27.46	2.50	1.70	.1041	NS
Group	4	35.36	8.84	6.03	.0006	**
Error	44	64.47	1.47			
		Number of Plasma Cells				
Total	59	120.98		5.45	.0001	**
ID	11	68.56	6.23	6.48	.0001	**
Group	4	10.11	2.53	2.63	.0472	*
Error	44	42.31	.96			
		Number of Lymphocytes				
Total	59	7974.14		4.36	.0001	**
ID	11	4045.09	367.74	5.04	.0001	**
Group	4	719.94	179.99	2.47	.0586	NS
Error	44	3209.11	72.93			
		Type of Mucus With HID-AB Stain				
Total	59	36.82		5.63	.0001	**
ID	11	14.54	1.32	4.61	.0001	**
Group	4	9.67	2.42	8.44	.0001	**
Error	44	12.61	.29			
		Uptake of PAT/KOH/PAS Stain by Goblet Cells				
Total	59	13.37		4.28	.0001	**
ID	11	5.83	.53	4.29	.0002	**
Group	4	2.10	.53	4.24	.0054	**
Error	44	5.44	.12			

Table D-7 (continued)

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Significance
	Uptake of PAT/KOH/PAS Stain by Connective Tissue					
Total	59	8.89		4.03	.0002	**
ID	11	3.85	.35	4.11	.0004	**
Group	4	1.30	.33	3.81	.0096	**
Error	44	3.74	.09			

[1]NS indicates not significant, * is significant ($p \leq .05$), ** is highly significant ($p \leq .01$).
[2]For definition of mast cells, see page 20.

Table D-8

ANOVAs for *in vitro* Study ID by Level by Vitamin

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Significance[1]
Number of Goblet Cells						
Total	47	2876.80		3.36	.0179	*
ID	11	2044.40	185.55	8.53	.0007	**
Level (L)	1	.40	.40	0.02	.8942	NS
Vitamin (V)	1	2.25	2.25	0.10	.7538	NS
Level X Vitamin (L X V)	1	5.60	5.60	0.26	.6221	NS
ID X Level (I X L)	11	241.08	21.92	1.01	.4963	NS
ID X Vitamin (I X V)	11	343.35	31.21	1.43	.2806	NS
Error	11	239.72	21.79			
Number of Mast Cells[2]						
Total	47	100.78		0.87	.6427	NS
ID	11	30.97	2.82	1.19	.3915	NS
L	1	.19	.19	.08	.7840	NS
V	1	9.19	9.19	3.87	.0750	NS
L X V	1	1.02	1.02	.43	.5256	NS
I X L	11	17.96	16.33	.69	.7277	NS
I X V	11	15.32	1.39	.59	.8052	NS
Error	11	26.13	2.38			
Number of Plasma Cells						
Total	47	99.90		5.82	.0018	**
ID	11	59.39	5.40	11.91	.0001	**
L	1	.16	.16	.36	.5605	NS
V	1	3.20	3.20	7.07	.0223	*
L X V	1	.32	.33	.74	.4095	NS
I X L	11	3.80	.35	.76	.6705	NS
I X V	11	28.04	2.55	5.62	.0040	**
Error	11	4.99	.45			
Number of Lymphocytes						
Total	47	7216.47		3.10	.0243	*
ID	11	4546.95	413.36	7.03	.0015	**
L	1	70.08	70.08	1.19	.2983	NS
V	1	21.87	21.87	.37	.5544	NS
L X V	1	56.34	56.34	.96	.3488	NS
I X L	11	974.13	88.56	1.51	.2543	NS
I X V	11	900.14	81.83	1.39	.2966	NS
Error	11	646.99	58.82			

Table D-8 (Continued)

Source of Variation	Degrees of Freedom	SS	MS	F Value	p Value	Signif-icance
Type of Mucus With HID-AB Stain						
Total	47	20.64		3.45	.0162	*
ID	11	9.44	.86	5.62	.0040	**
L	1	.12	.12	.79	.3944	NS
V	1	2.08	2.08	13.64	.0035	**
L X V	1	.12	.12	.79	.3944	NS
I X L	11	3.16	.29	1.88	.1548	NS
I X V	11	4.04	.37	2.40	.0808	NS
Error	11	1.68	.15			
Uptake of PAT/KOH/PAS Stain by Goblet Cells						
Total	47	10.65		7.28	.0006	**
ID	11	6.14	.56	14.31	.0001	**
L	1	.19	.19	4.81	.0508	*
V	1	.04	.04	1.05	.3283	NS
L X V	1	.30	.30	7.71	.0180	*
I X L	11	1.06	.10	2.48	.0741	NS
I X V	11	2.49	.23	5.80	.0035	**
Error	11	.43	.04			
Uptake of PAT/KOH/PAS Stain by Connective Tissue						
Total	47	7.11		5.46	.0023	**
ID	11	4.49	.41	11.91	.0001	**
L	1	.00	.00	.00	1.000	NS
V	1	.00	.00	.10	.7609	NS
L X V	1	.00	.00	.10	.7609	NS
I X L	11	.58	.05	1.54	.2429	NS
I X V	11	1.66	.15	4.40	.0106	*
Error	11	.38	.03			

[1]NS indicates not significant, * is significant ($p \leq .05$), ** is highly significant ($p \leq .01$).

[2]For definition of mast cells, see page 20.