contains GTP than when it contains ATP. In a GTP reassembly buffer the dependence of equilibrium microtubule polymerization on pressure is similar to that in Fig. 3A, but for correspondingly lower temperatures. For example, the decreases in equilibrium absorbance induced by pressure at 21°C in 0.5 m*M* GTP buffer is nearly equivalent to the decrease at 29.5°C in 1.0 m*M* ATP buffer, and the decrease at 16°C in GTP buffer is nearly equivalent to the decrease at 21°C in ATP buffer.

It should also be noted that little high-molecular-weight protein copolymerizes with the microtubules prepared in the 1.0 m*M* ATP buffer (Fig. 1A). Copolymerizing, high-molecular-weight proteins account for 15 to 20 percent, by weight, of microtubules purified in 0.5 m*M* GTP buffer (*6, 7*). The significance of these copolymerizing proteins, however, is not yet clear.

As is shown above, pressure depolymerizes brain microtubules in vitro, and this process is reversible. The results are quantitatively consistent with those of experiments on the depolymerization of microtubules by various agents in vivo. The similar behavior of microtubules in response to pressure in vitro and in vivo further substantiates the conclusion (*11*) that pressure affects microtubule assembly in vivo directly through the large positive volume change associated with polymerization.

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9. Tubulin was purified from rabbit brain in a reassembly buffer composed of 0.1 *M* MES [2-(*N*-morpholino)ethane sulfonic acid; *p*H 6.4], 0.1 m*M* DDT (dithiolthreitol), 1 m*M* EGTA [ethylene-bis-(oxyethylenenitrilo)tetraacetate], 1 m*M* $MgCl_2$, and 1 m*M* ATP. Approximately 8 g of brain was minced and washed in buffer. Cold buffer (10 ml) was added, the brain was homogenized, and the mixture was centrifuged at 40,000*g* for 1 hour at 4°C. Glycerol was added to the supernatant to make a solution 25 percent glycerol by volume, and this solution was incubated at 37°C for ½ hour. After centrifugation at 100,000*g* for 1 hour at 30°C, the pellets were either used immediately or stored on ice overnight. Before an experiment a pellet was suspended in cold buffer and cleared by centrifugation at 100,000*g* for 1 hour at 4°C. Microtubules were assembled by incubating a solution of approximately 2.5 mg of protein per milliliter at 37°C for 15 minutes. The resulting change in absorbancy upon polymerization was about 0.2 at 403 nm. Although it is possible that small amounts of glycerol remain attached to the tubulin even when no glycerol is present in the experimental reassembly buffer, the consequent effects on the observed polymerization of microtubules are probably very small.
10. A temperature-controlled pressure spectrophotometer cell, similar to the microscope pressure chamber of Salmon and Ellis (*15*), was constructed with a 1-cm internal space between the optical glass windows. The temperature control and pressure generating equipment was the same as described in (*15*). The chamber was mounted in its temperature control stage on a Zeiss WL microscope equipped with two Leitz UM 20× objectives, one used as a condenser. The aperture diaphragms were adjusted to give a 2-mm diameter beam, significantly smaller than the 3-mm apertures of the chamber ports. A tungsten lamp (line voltage regulated) with 403-nm interference filter provided light. Voltage changes in the output of a silicon solar cell placed above the ocular were proportional to changes in light intensity and were read directly into a Beckman 10-inch (1 inch = 2.54 cm) Lin-Log potentiometric recorder.
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Inhibition of the Growth and Development of a Transplantable Murine Melanoma by Vitamin A

Abstract. *Vitamin A, given either orally or intraperitoneally, has a dramatic inhibitory effect on the growth and development of a highly immunogenic murine melanoma. This effect may be due to enhancement of antitumor immunity by vitamin A.*

Dresser (*1*) and others (*2*) have shown that vitamin A is capable of acting as an immune adjuvant. This and work with other immune stimulants suggests that vitamin A might also be active in inhibiting tumor growth. Vitamin A has been capable of decreasing tumor incidence following administration of certain viruses (*3*) and chemical carcinogens (*4*). To date, however, multiple attempts to show that vitamin A inhibits the growth of transplanted tumor cells has been unsuccessful. Specifically, Bollag (*5*) was unsuccessful in six transplantable tumor systems in preventing tumor growth by pretreatment with vitamin A in mice. Meltzer and Cohen (*6*) were able to show enhancement of bacillus Calmette-Guérin (BCG) effects against transplantable mouse tumors with vitamin A but found no effect of vitamin A alone on the growth and development of a transplanted methylcholanthrene-induced murine sarcoma. We now describe experiments demonstrating that vitamin A given either orally or intraperitoneally has a dramatic inhibitory effect on the growth and development of a highly immunogenic murine melanoma.

In all experiments S-91 (Cloudman) melanoma was used. This murine melanoma arose spontaneously in the tail of a DBA mouse and has subsequently been passed in BALB/c mice. This tumor grows progressively in untreated BALB/c mice. The tumor was maintained by serial transplantation with single cell suspension. In each experiment, a single cell suspension was prepared, the cells were counted, and 10^5 or 5×10^5 cells were injected between the scapulae in the subcutaneous tissue of each mouse. Tumors developed during the third to fifth week after inoculation, and grew progressively in size to cause death of the affected animals. No tumors developed during the subsequent observation period of 3 to 6 months.

A series of experiments were performed to determine the value of pretreatment with oral vitamin A. Ten- to 12-week-old male BALB/c mice were divided into two groups. The experimental group received vitamin A palmitate (Aquasol A, USV Pharmaceutical, Tuckahoe, N.Y.) in their drinking water (625 units per milliliter) with an average daily intake of 5 ml of water, or approximately 3100 units of vitamin A palmitate. The control group received ordinary drinking water, which was freely available. All animals in these experiments received NIH Open Formula rat and mouse ration, which contains 15 international units of vitamin A palmitate per gram of food. After 2 weeks of vitamin A palmitate or normal drinking water, each group was challenged with either 10^5 or 5×10^5 tumor cells. Mice were maintained

Table 1. Comparison of the inhibition of tumor development by varying doses of intraperitoneal vitamin A. The significance of the differences was measured by the chi-square test. For the saline (S) versus the A_2 group, $P < .01$. For the saline versus the A_3 group, $P < .005$.

Group	Dose of vitamin A (units/day × 5 days)	No. of mice with tumor/ No. of mice inoculated
S	0	20/25
A_1	2500	16/28
A_2	3500	9/22
A_3	5000	4/17

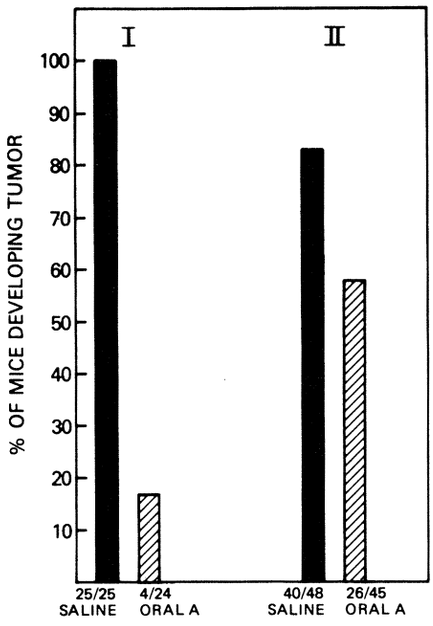

Fig. 1. Effect of oral vitamin A on the percent of challenged mice developing tumor. Experiment I: tumor challenge of 10^5 viable cells. Experiment II: tumor challenge of 5×10^5 viable cells.

on vitamin A drinking water until 2 weeks after the first tumors began to appear and were then given free access to normal water. Weekly measurements of tumor size were performed. Lesions over 3 mm in diameter always grew progressively. In experiment I, control mice challenged with 10^5 tumor cells developed tumor in 100 percent (25/25) of the mice challenged while the mice receiving oral vitamin A developed tumor in only 17 percent (4/24) of the challenged animals. This difference was significant at the .001 level (Fig. 1). When mice were challenged with 5×10^5 tumor cells in experiment II, 83 percent of the control animals (40/48) compared to 58 percent of the animals treated with vitamin A (26/45) developed tumor ($P < .01$) (Fig. 1).

In order to evaluate more accurately the exact dosage of vitamin A required to prevent the development of the transplanted melanoma, studies were carried out using vitamin A palmitate via the intraperitoneal route. In experiment III, test animals received 5000 units of vitamin A intraperitoneally per day for 5 days and were then challenged with tumor cells on the fifth day. Control animals received saline in a similar fashion. With 10^5 tumor cells, vitamin A significantly inhibited the development of tumors in the experimental group ($P < .001$.). Five percent of the animals treated with vitamin A (2/41), compared to 76 percent of the control animals (19/25), developed tumor (Fig. 2). When animals were challenged with 5×10^5 tumor cells, 26 percent of the mice treated with vitamin A (18/68), compared to 76 percent of the control mice (61/80), developed tumor ($P < .001$) (experiment IV, Fig. 2). The size of the tumors in the group treated with vitamin A significantly lagged behind the size of the tumors in the saline-treated group during the first 4 weeks of measurement (Fig. 3). A dose relationship between intraperitoneal vitamin A and tumor incidence was determined by pretreating groups of mice with either 0, 2500, 3500, or 5000 units of vitamin A intraperitoneally per day for 5 days and then challenging the mice with 5×10^5 tumor cells on the fifth day. A dose of 3500 or 5000 units per day for 5 days was required to significantly protect the mice from the development of the Cloudman melanoma (Table 1). A similar study administering 5000 units of vitamin A intraperitoneally per day for 1 to 4 days demonstrated that a total dose of more than 15,000 units of vitamin A was required to protect the mice ($P < .01$).

The exact mechanism by which vitamin A enhanced resistance to tumor in this system is not known. There is evidence in other systems, however, suggesting that vitamin A may work through an immune mechanism. Cohen and Cohen (*7*), Florsheim and Bollag (*8*), and Taub *et al.* (*9*) have all shown that vitamin A can enhance serum- or cell-mediated immunity in mice. Vitamin A has also been shown to enhance the antitumor effect of another immune adjuvant, BCG (*10*). Tannock *et al.* (*11*) have demonstrated that vitamin A can reduce the dose of radiation required to control a murine tumor, and that this effect is abrogated by immune suppression with total body irradiation.

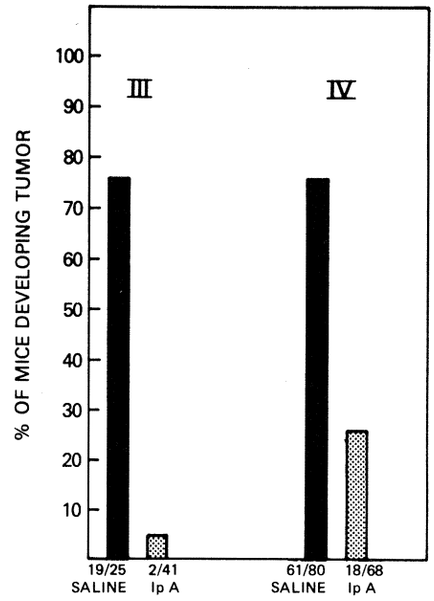

Fig. 2. Effect of intraperitoneal vitamin A (5000 units per day, times 5 days) on the percent of challenged mice developing tumor. Experiment III: tumor challenge of 10^5 viable cells. Experiment IV: tumor challenge of 5×10^5 viable cells.

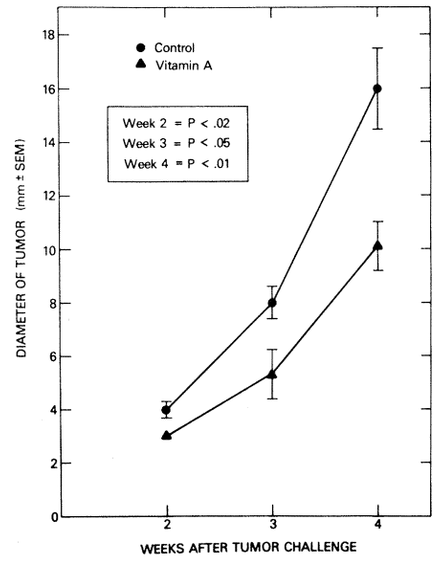

Fig. 3. Effect of intraperitoneal vitamin A (5000 units per day, times 5 days) on the size of developing tumors as measured by the greatest diameter in millimeters.

We have found in our system that rabbit antiserum to mouse lymphocytes (ALS), which itself enhances tumor growth, abrogates the protection afforded by vitamin A ($P < .005$), and that vitamin A can reverse the enhancement of tumor growth that follows ALS treatment. The effects of vitamin A and ALS appear to be antagonistic, suggesting that the antitumor effect of vitamin A in this system is at least partly due to enhancement of the antitumor immune response. Other known effects of vitamin A such as lysosomal labilization, suppression of neovascularity in skin, and changes in epithelial differentiation may, however, play a role in vitamin A's antitumor effect. In summary, these studies demonstrate for the first time that vitamin A can prevent growth and development of transplanted tumor cells and that this effect may be due to enhancement of antitumor immunity by vitamin A. The fact that we have not observed a similar effect of vitamin A in two methylcholanthrene-induced murine tumor systems suggests to us that the highly immunogenic nature of the Cloudman melanoma tumor may be necessary to demonstrate a vitamin A effect against transplanted tumor cells.

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12. We acknowledge valuable conversations with Dr. Benjamin E. Cohen.

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DNA Hybridization and Evolutionary Relationships in Three Osmunda Species

Abstract. *Molecular hybridization techniques have been used to estimate the degree of DNA base sequence homology between some members of the fern genus* Osmunda. *Under conditions permitting extensive reassociation, measurements of the extent of interspecific reaction and the thermal stability of the hybrid molecules indicated that* O. claytoniana *L. (interrupted fern) shares more DNA homology with* O. cinnamomea *L. (cinnamon fern) than it does with* O. regalis *L. (royal fern). These findings are in conflict with predictions from a recent analysis of living and fossil specimens by numerical techniques. However, they are consistent with the earlier, more traditional, taxonomic assignments.*

Molecular hybridization techniques have been used to explore evolutionary relationships in both plants (*1*) and animals (*2*). In cases where fossil evidence is available, it has been possible to relate relative rates and patterns of DNA change in different groups of organisms (*2*) to an absolute time scale. We describe here a case in which DNA hybridization experiments provide molecular evidence to support one of two possible phylogenetic schemes and help clarify interpretation of the fossil record.

It has recently been suggested (*3*) that lines leading to *Osmunda claytoniana* and *O. cinnamomea* diverged at least 70 million years ago, but that only 1 million years have elapsed since *O. claytoniana* and *O. regalis* last shared a common ancestor. This newly derived phylogeny is based on the most extensive fossil record found among the ferns (*4*) as well as on anatomical features of living representatives. However, *O. claytoniana* and *O. cinnamomea* are extremely similar morphologically while *O. regalis* has a clearly different leaf morphology. Since previous classifications have considered *O. claytoniana* to be more closely related to *O. cinnamomea* than to *O. regalis* (*5*), we used interspecific DNA hybridization as an independent method of assessing genetic relationships. Our results accord more closely with the earlier system of classification.

We extracted and purified DNA from the three *Osmunda* species native to North America; we used only mature, vegetative fronds collected from fertile plants in order to assure proper identification (*6*). The DNA was sheared to a mean fragment length of approximately 400 to 500 nucleotides, concentrated by lyophilization, and dissolved in the reaction buffer (*7, 8*) to a concentration of about 10 mg/ml.

Families of related DNA sequences are a characteristic feature of eukaryotic DNA (*9*), especially in the many vascular plants with large genomes (*10*). These sequences reassociate rapidly in renaturation experiments, the rate of reassociation being greater for more extensively repeated sequence families. Therefore, we compared the reassociation kinetics of DNA from the three *Osmunda* species to determine whether large differences existed in the distribution of repetitive sequences in various kinetic classes. Reassociation was followed optically as a function of C_0t [the product of DNA concentration and time; (*11*)]. As may be seen in Fig. 1, DNA from all three species reassociates with remarkably similar kinetics. In each case, the DNA is highly repetitious, with most fragments reassociating more rapidly than expected for sequences present only once per haploid genome (*12, 13*). There is an essentially continuous distribution of sequences with different repetition frequencies, with no clear separation between repetitive and single-copy DNA. The similarity of reassociation kinetics indicates that there have been no large changes in degree of sequence repetition which would complicate measurements of DNA homology.

In order to measure relationships among the three species, *O. claytoniana* DNA was labeled in vitro with ^{125}I (*14*) and mixed with a 1000-fold excess of unlabeled DNA from *O. claytoniana*, *O. cinnamomea*, or *O. regalis*. At this concentration ratio, the tracer reacts primarily with the unlabeled DNA (*15*). Portions of these mixtures were then denatured by heat and placed at the appropriate temperature for reassociation. Incubation periods of up to 120 hours and unlabeled DNA concentrations of about 10 mg/ml were used to obtain the indicated C_0t values. Reassociated duplexes, both homologous and heterologous (interspecific), were isolated by hydroxyapatite chromatography (*8*) after dilution with 0.12*M* sodium phosphate buffer. The extent of reassociation of unlabeled DNA was the same for all three species and was about 90 percent in all experiments reported.

In all our experiments, the percentage of interspecific duplexes formed at any C_0t is always higher for reactions between ^{125}I-labeled *O. claytoniana* and *O. cinnamomea* than for reactions between ^{125}I-labeled *O. claytoniana* and *O. regalis*. For example, at a C_0t of 1.6×10^4 the reaction of ^{125}I-labeled *O. claytoniana* DNA with *O. cinnamomea* DNA is 86 percent of that of the homologous control, while the corresponding value with unlabeled *O. regalis* DNA is 73 percent under the same conditions.

Precision of base pairing in interspecific duplexes provides another measure of DNA homology, especially where the interspecific reactions are as extensive as they are in the *Osmunda* species. Thermal stability of DNA is reduced in proportion to the percentage of bases that are unpaired; the T_m (midpoint of the thermal dissociation profile) is depressed on the order of 1°C for each 1 percent of mismatched base pairs (*16*).

Thermal stability profiles for native *Osmunda* DNA and reassociated homologous and heterologous duplexes were obtained by thermal elution from hydroxyapatite (*8*). In all three species, reassociated homologous duplexes showed identical profiles with a $T_{m,i}$ (*17*) nearly 8°C lower than that for native DNA of the same fragment length. Similar reductions in thermal stability, resulting from cross-reactions between similar but nonidentical sequences within families of repetitious DNA, have been observed in many organisms (*9, 15*). In the case of the interspecific duplexes, however, an additional reduction in thermal stability is observed (Fig. 2). When compared with the reassociated duplexes of ^{125}I-labeled *O. claytoniana* with *O. claytoniana*, the $T_{m,i}$ of the DNA hybrids of ^{125}I-labeled *O. claytoniana* with *O. cinnamomea* shows an additional reduction of 1.8°C, while the $T_{m,i}$ of ^{125}I-labeled hybrids of *O. claytoniana* with *O. regalis* is reduced by 3.5°C. This indicates less homology of DNA sequence