parison with Group 4, a significant difference was seen in day of vaginal opening.

Summary. Twenty, 21-day-old female albino rats were subjected to bilateral lesions of the region of the mammillary bodies. Within one day of receiving a repository dose of 0.5 μg of estradiol valerate, all 6 recipients showed vaginal opening with subsequent constant estrus and reproductive organ growth. Four animals receiving sesame oil and 8 receiving no treatment exhibited definite signs of delayed puberty and reproductive organ immaturity. These results indicate the inability of the prepuberal hypothalamus to elaborate or secrete those neurosecretory substances essential for the functional sexual state of the mammal, and the dependence of estrogen secretion upon normal hypothalamic activity as mediated *via* the hypothysis.

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Adaptation of Fluorescent-Microscopy to Determination of Genetic Variations in Mouse Mammary Tumor Agent. (26496)

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It was previously found that fluorescent microscopy could be adapted for studying the antigenic mosaic make-up of tissues containing the mouse mammary tumor agent (MTA) (1). While fluorescent microscopy, as Hughes(2) indicated, has definite limitations in differentiating neoplastic from normal tissues, we have found that this method offers certain advantages over the routine serologic and neutralization tests used for such studies.

"Incidence" and "age of occurrence" of mammary tumors in the mouse, have been shown to be influenced by certain inherited factors(3). Especially implicated have been the hormonal make-up of the animal and the genes responsible for natural protection or susceptibility to the MTA. Certain inbred stocks of mice have been developed that not only have a high incidence of tumors at an early age, but can pass the agent to succeeding generations by nursing. Inbred genetically pure mice have also been selected which are highly resistant to the MTA.

Little is known about the antigenic changes occurring within the MTA when it is passed from one strain of mice to another. In part, this is due to inability to isolate and purify a single entity which might be termed the "agent" and in part due to the time period necessary for neutralization tests. Inhibition or neutralization tests are the only reliable method so far available for determining the effect of antisera on the MTA. Unfortunately, it requires a period of 6 months, in

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the case of positive tests, to 2 years to obtain the results of such studies. Despite these handicaps, some antigenic variations are known to occur when the MTA is transferred to different strains. Bittner and Imagawa (4) described the results of neutralization tests using antisera prepared from MTA extracts. They noticed that antigenic differences were apparent between the mammary tumor agent of a cancerous stock and that of F_1 hybrids having mothers of the same strain and that the antigenic properties of the agent in a transplanted tumor may become altered with continued passage of the tumor in agent-free animals. In collaboration with Hirsch and others, it has been reported(5) that antigenic variations occurred in the MTA depending upon passage of the tumors and the hosts. It was found that antisera produced against the agent in tumors transplanted in mice of the inbred strain neutralized the MTA in extracts of the same tumor grown in either inbred or agent-free F_1 animals. On the other hand, antisera elicited against the tumor which had been transplanted in F_1 hybrids neutralized the agent from similar tumors but did not neutralize the agent in the same tumors carried in inbred animals of the strain of origin. These experimental data could indicate that the antigenic characteristics of the MTA had been influenced by incorporation of normal tissue component(s) from the host.

Technic. To expand the initial findings on the antigenic changes mentioned above it was felt that fluorescent microscopy would offer several advantages: a) microscopic visualization of site of reactivity on the antigen, b) more rapid method for obtaining results and c) a greater specificity in the reaction.

Rabbit or guinea pig antitumor sera were prepared, as previously reported(1), against the Z tumor in Z mice and the A tumor in A mice where a minimum of 3 rabbits or 12 guinea pigs was used for each serum preparation. After exsanguination the sera were pooled and adsorbed with various normal mouse tissues to remove common antibodies. Ammonium sulfate fractionations were run to obtain a higher concentration of gamma globulins and the material was then conjugated with fluorescent dye(s) prior to testing against tumors derived from mice of different strains.

The preparation of isothiocyanate for conjugation to the antisera was carried out by the method of Riggs(6). This dye was found to give maximum contrast when viewed under ultra-violet light. In addition to the use of isothiocyanate alone, the combination technic of Lissamine-Rhodamine RB 200 conjugated to a 1% albumin solution and then mixed (1:20) with the isothiocyanate conjugated antisera, was found effective in eliminating non-specific background fluorescence. Preparation of the Lissamine-Rhodamine RB 200 was undertaken by the technic of Smith and associates(7). Method of preparation and staining of tissue slides was essentially that of Cherry and colleagues(8) using fluorescent-free paraffin mounts and tissues of 3 μ or less in thickness. The material to be studied was fixed in 10% formalin for 24 hours, carried through the usual dehydration cycles, cut, stained with the conjugated antisera, washed with phosphate buffered saline, and mounted under a coverslip with buffered glycerol.

The tissues found most convenient for adsorbing out common or normal tissue antibodies was liver powder; method of preparation was that of Coons and associates(9). Mouse liver powder was obtained from both the strain C and strain Ax mice which did not contain the MTA. The rabbit or guinea pig antisera prepared against the Z tumor in Z mice, and the A tumor in A mice, were then adsorbed with one or the other of these liver powders prior to conjugation with the fluorescent dyes. The treated antisera were tested for specificity and degree of fluorescence against host tumor specimens by the immuno-fluorescent technic described above. Normal mouse tissues, a spontaneously occurring sarcoma, and an adenocarcinoma produced in an agent-free animal were used for controlled antigen studies. Normal guinea pig or rabbit sera were conjugated with the fluorescent dyes and used to control the serum preparations. For each slide stained with a fluorescent dye the next tissue slice from the microtome was stained with hemo-

toxylin and eosin for routine histologic comparison.

To avoid erroneous interpretation of the material and to have a more unbiased appraisal of the technic, "unknown" tumor samples were submitted for evaluation. One of the authors (JJB) selected animals bearing a tumor derived from a known strain and whose history was well pedigreed, as an unknown for the other author to evaluate. When received, the "unknown" tumor was numbered and prepared for fluorescent microscopy in the manner described above. "Unknowns" were compared to known tumors and degrees of fluorescence found using the various tumor antisera was noted. When the experiment was completed the findings were evaluated for specificity.

Results. Early in these studies it was found that while either the rabbit or the guinea pig would respond to the antigenic stimuli of the mouse tumor antigen, that considerable "normal tissue antibodies" were also elicited by the animal. Most of the normal tissue antibodies were removed by adsorption (4 days with 6 hour changes of adsorbing tissue) with normal mouse liver powder and only a small amount of fluorescence in the preparations was then due to normal tissue antibodies. Despite the use of adsorbing powders a slight degree of nonspecific fluorescence was apparent in all of the studies. Best results were obtained when strain C liver powder was used against the anti-Z or anti-A tumor sera to eliminate nonspecific fluorescence. Despite the handicap of a certain amount of non-specific fluorescence, valid results could be obtained. Differences could be denoted in degree of fluorescence when the various antisera were reacted against the tumor material. The results (Table I) agree quite well with the neutralization studies reported by Bittner and associates(5), and indicate that this technic allows a faster, more specific method for studying degree of specificity and reactivity of anti-MTA sera when reacted on heterologous tumors.

Discussion. Fluorescent-antibody studies on antigenic variations of the MTA when it is passed in various host animals offer a rapid, inexpensive, and accurate technic for determining specificity of the reaction. The only reliable method for determining antigenic variations in the MTA from normal tissues is the bio-assay method. Investigations are now in progress to combine this method with the fluorescent antibody technic. Evaluation of results of these experiments will require at least 2 years. Studies are now underway to investigate antigenic changes which take place in MTA when it is carried in normal mouse tissues.

The fluorescent-antibody technic has definite limitations in defining the antigenic mosaics of the mouse mammary tumor agent. The method is limited by the quality, specificity and reactivity of the antisera under investigation. Considerable skill is necessary to distinguish between the natural fluorescence or non-specific fluorescence of the tumor preparation and that arising as a result of specific protein-protein interactions. In not every case is it possible to avoid these pit-falls. Despite these drawbacks, from Table I it becomes apparent that the fluorescent-antibody technic has a high degree of accuracy, and in particular is a method which reduces the time factor for such studies from 2 years to a few days. The conjugated antisera can be stored indefinitely by rapid freezing without appreciably changing the reactivity of the preparation, thus it is possible to develop a battery of conjugated antisera specific for each of the various materials associated with the MTA. Use of the immunofluorescent technic allows a method to be developed for better understanding of the genetic relationships of MTA and tumors containing MTA when correlated with animal studies.

Summary. A fluorescent-antibody system is reported which allows rapid analysis of the minor antigenic differences between mouse mammary tumor agent (MTA) extracts obtained from different sources. This method correlates very well with the bio-assay method reported by Bittner and associates. An analysis of the antigenic component can be made within a few hours after receiving the MTA extract. In the older bio-assay method up to 2 years of observations were necessary for proper evaluation. The disad-

TABLE I. Fluorescent-Antibody Studies on Mouse Tumor Agent (MTA). Summary of effects of rabbit anti-MTA sera adsorbed with normal C strain mouse liver powder, fractionated, and conjugated with isothiocyanate, then reacted against MTA derived from transplanted tumors. Tumors were transplanted in either inbred or hybrid mice for 7 passages.

	A tumor in				Z tumor in				
Conjugated rabbit antisera preparation	A mice	Z mice	AxZbFl mice	AxZbFl/ ZbAxFl mice	A mice	Z mice	AxZbFl mice	AxZbFl/ ZbAxFl mice	Normal mouse tissue, no MTA
Anti-A sera adsorbed with strain C liver powder	4+	NT	2+	3+	NT	±	1+	2+	±
Anti-Z sera adsorbed with strain C liver powder	±	NT	1+	2+	NT	4+	2+	2+	±
Anti-A sera fractionated and adsorbed with strain C liver powder	4+	NT	1+	2+	NT	±	±-1+	1-2+	±
Anti-Z sera fractionated and adsorbed with strain C liver powder	±	NT	±	1+	NT	4+	3+	3+	±
Anti-A sera whole unadsorbed	4+	NT	3-4+	4+	NT	3-4+	4+	4+	3-4+
Anti-Z sera whole unadsorbed	3-4+	NT	3-4+	3+	NT	4+	4+	4+	4+
				Normal control sera					
Normal rabbit sera whole, unadsorbed	1-2+	NT	1-2+	1-2+	NT	1+	1-2+	1-2+	1-2+
Normal rabbit sera adsorbed with strain C liver powder and fractionated	0	NT	±	0	NT	±	±	±	±

NT = no tumor take with transplanted tumor; 4+, maximum fluorescence; 3+, strong fluorescence; 2+, avg fluorescence, not too bright; 1+, some slight fluorescence; ±, very slight fluorescence; 0, no apparent fluorescence.

vantages of the fluorescent-antibody system include, a) interference by non-specific fluorescence, b) limitations imposed by antisera employed, c) removal of normal tissue antibodies, and d) selection of appropriate fluorescent dyes for conjugation to the sera. These disadvantages can be partially overcome by carefully selecting the antigens to be used for preparing antisera, adsorption of antisera with mouse liver powder extracts, concentration of globulin fractions prior to conjugation, and use of sharply contrasting fluorescent dyes.

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