Fluorescent Antibody Reactions Against the Mouse Mammary Tumor Agent.* (26317)

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Investigators(1-4) have shown that it is very difficult to prepare antisera against the mouse mammary tumor agent (MTA) and determine its reactivity by routine methods of titration. The method of titration normally used consists of a bio-assay technic in which antitumor sera is mixed and incubated with tissue extracts containing the MTA and given as a challenge to susceptible animals. A period ranging from 6 months to 2 years is necessary to determine the efficiency of the antisera to neutralize the agent. This preliminary investigation reports our findings with a fluorescent antibody system(5,7,8,9) which shows promise of drastically cutting to a few days the time period necessary for obtaining the results of the assay.

Technic. We have prepared antibodies against the Z(C_3H) tumor(6), which contains the agent, by injecting cellular suspensions of the tumor into animals of different species. A minimum of 5 rabbits and 12 guinea pigs being utilized for each serum preparation, wherein the animals received 6 doses of 3×10^8 cells/ml every third day, rested 10 to 12 days and exsanguinated. Pooled rabbit or guinea pig sera was used throughout the investigation. In these experiments the antigen consisted of Z tumor carried in Z strain mice, with normal Z mouse tissue serving as controls. Antisera to normal mouse tissue were prepared and tested in a similar fashion and this material, with normal rabbit guinea pig sera, served as further controls.

Thirty ml of anti-Z tumor sera were adsorbed with liver powder(7) prepared from non-tumored C strain mice which do not possess the MTA; This material with 30 ml of unadsorbed anti-Z tumor sera, was used for routine serologic, inhibition, and fluorescent-dye conjugation experiments. These two (adsorbed, fractionated, and whole sera) were both prepared from the same pools of anti-Z tumor sera. For agglutination tests a cellular suspension of the tumor of approximately 1×10^5 cells per ml was prepared from fresh tisue, while a cell-free homogenate of approximately the same number of original cells per ml was used for precipitin and complement-fixation tests.

Various fluorescent dyes were synthesized in the laboratory prior to use in these studies. Isothiocyanate was prepared by the method of Riggs(8) and Lissamine Rhodamine RB-200 isothiocyanate by the method of Chadwick *et al.*(9), as modified by Smith and associates(10). In addition, isothiocyanate and Lissamine Rhodamine RB-200 isothiocyanate samples were generously supplied by Major Chauncey W. Smith. No significant difference could be determined between the dyes made in this laboratory and those supplied by Major Smith. The method of conjugation was either that of Smith *et al.*(10, 11) when non-adsorbed whole sera were used, or that of Cherry, *et al.*(13) when adsorbed, ammonium sulphate fractionated sera were prepared.

Inhibition tests to determine ability of the antisera to inhibit tumor growth, prior to conjugation of the sera with fluorescent dyes, were set up according to the method of McCredie, Brown, and Cole(12). The technic was modified to the extent that 30 ml of the antisera (either guinea pig or rabbit) were mixed with freshly prepared tumor cells and incubated for one hour at 37°C. One ml of the preparation was inoculated s.c. into 15 Z mice. Normal rabbit sera and normal guinea pig sera were respectively incubated with Z tumor cells in a similar fashion as a

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TABLE I. Inhibition Tests on Z Tumor Transplants in Z Mice.*

Material inj./mouse	Tested	No. of animals Developing tumor	No. of animals Not showing tumor
1 ml of 1 × 10^8 tumor cells + no antisera aged 30 min. at 37°C (Control)	15	15	0
1 ml of 1 × 10^8 tumor cells + normal guinea pig sera incubated 30 min. at 37°C (Control)	15	14	1
1 ml of 1 × 10^8 tumor cells + adsorbed anti-Z sera incubated 30 min. at 37°C	15	0	15
1 ml of 1 × 10^8 tumor cells + anti-Z g.p. sera (unadsorbed) incubated 30 min. at 37°C	15	3	12
1 ml of 1 × 10^8 tumor cells + adsorbed, fractionated anti-Z rabbit sera incubated 30 min. at 37°C	10	0	15
1 ml of 1 × 10^8 tumor cells + normal rabbit sera (fractionated and adsorbed) incubated 30 min. at 37°C (Control)	9	8	1

* All mice were of same inbred strain and approximate age. All animals were saved for 2 mo. Avg control animal developed tumors within 17 days. Equal volumes of antisera and cells were used for neutralizations.

control. In addition 15 Z mice were inoculated with the same preparation of tumor cells without prior serum treatment, but with the same degree of aging, *i.e.* 30 minutes after preparation of the cellular suspension. In each test or control series 15 mice/test were used, all from the same inbred stock and approximately the same age. All animals were held for a period of 2 months before concluding this aspect of the investigation.

For histologic work, slides were prepared from 10% formalin-fixed Z tumor by the method of Cherry *et al.*(13). The best results with the fluorescent stains were obtained when the tissue slices were 3 μ or less in thickness. At this depth maximum differentiation of tissue could be made. For each slide prepared for fluorescent staining, a slide from the next cut on the microtome was prepared for routine H & E histologic observation.

Results. The results of *in vitro* agglutination, precipitation and complement-fixation tests were inconclusive and no determination of titer could be made by these methods. In the case of agglutination tests, results with the antitumor sera ranged from negative to a titer of 1:5120 depending on the aliquot of the same antigen and sera tested. The observations with the other two technics were similarly inconclusive and it was difficult to determine when examining the test tubes whether or not antibody was present. Controls in which normal mouse tissue cells were used gave similar results despite the fact that some of the sera tested was adsorbed with normal mouse tissue preparations to remove common antibodies. When the antigens used in these tests were reacted against normal rabbit or guinea pig sera, results were again inconclusive.

Results of the inhibition tests, were, however, more promising (Table I). It should be noted that these tests utilize viable tumor cells for transplantation of the tumor whereby the tumor usually develops within 2 to 3 weeks in the new host. In bio-assaying the mouse mammary tumor agent(6) a suspension of normal or cancerous tissue containing the agent is used for the challenge. In this case, a minimum of 6 months to 2 years is necessary to determine whether or not the agent will have been neutralized by the antisera.

When conjugated anti-Z rabbit or guinea pig sera were used for *in vitro* testing the results were more pronounced. The use of Lissamine Rhodamine RB 200 conjugated to normal albumin and mixed with the antisera conjugated to isothiocyanate essentially reduced the nonspecific fluorescence of the tissue substance. Use of carefully adsorbed and fractionated (ammonium sulfate) anti-Z sera gave reproducable results when tested against various slides of Z tumor. In general, specific staining by fractionated and adsorbed anti-Z

sera coupled with Lissamine Rhodamine RB 200 isothiocyanate was enhanced due to the contrasting reddish-orange background. When the conjugated antisera were adsorbed and concentrated by the method of Cherry *et al.* (13) and then reacted with normal mouse tissue no specific fluorescence could be distinguished. This was true with both the Lissamine Rhodamine RB 200 isothiocyanate and the isothiocyanate conjugated normal rabbit or guinea pig sera. When the conjugated anti-Z tumor sera were tested against a mouse sarcoma and an adenocarcinoma of different origin from the Z tumor (*i.e.,* did not involve the MTA and different mouse strains were involved) no specific fluorescence was noted. Such cancers, similar to normal tissue controls in regard to degree of fluorescence, were always observed whenever Z tumor was tested. A direct correlation was found between use of fluorescein-labeled antisera and the inhibition tests described. When the conjugated antisera were reacted with normal tissues no specific fluorescence was observed. However, it was found that when unadsorbed anti-Z sera was used that antibodies common to normal tissue as well as to the Z tumor were present, making the slides difficult to interpret. This difficulty could be overcome by judicious adsorption with normal mouse liver powder.

Discussion. Preliminary tests show that the fluorescent antibody technic can be adapted to study of the mouse mammary tumor and the mouse mammary tumor agent. This method offers a more reliable technic for *in vitro* testing of antisera specific against the MTA or mouse mammary tumor containing the agent than our present serologic tests. The best methods of fluorescent staining were found to be either use of isothiocyanate or the Lissamine Rhodamine isothiocyanate technic of Smith and his colleagues (10). Direct correlation was found between ability of the sera to inhibit transplant of Z tumor in Z mice and to show specific fluorescence when conjugated anti-Z sera was used against homologous tissue. The same sera which would inhibit tumor growth, *i.e.,* neutralize, would also show specific fluorescence when conjugated to the appropriate dye. Tests are now underway to determine the specificity of the fluorescent antibody technic as compared to bio-assay of nontumor material containing the MTA.

Interpretation of fluorescein-stained slides is not necessarily a simple technic. As Hughes(14) has mentioned in his work on differentiating neoplastic and non-neoplastic tissues, similar results can be obtained with fluorescein conjugated normal rabbit globulin as with fluorescein labeled protein complexes. Any interpretation of results obtained by the fluorescent technic requires caution and carefully controlled staining when evaluating changes attributed to an antigen-antibody complex. Smith *et al.*(8) point out, "as the LR-labeled normal sera react under these circumstances, it is presumed to be a protein-protein physicochemical interaction, depending on factors other than serological. This system now makes it possible not only to better visualize the sites of antigen deposits but also to detect smaller bacteria and viral aggregates in formalin-fixed tisues and tissue cultures." We have found this to be generally true but careful choices must be made in selecting adsorbing tissue, in preparation of the tumor antigen, and especially in interpretation of the slides.

Summary. A method of assaying the potency of sera prepared against the mouse mammary tumor agent using fluorescent dye labeled antisera is described. Better correlation between this method and serum neutralization tests, with transplanted Z tumor in Z strain mice, were found than in routine serologic testing. Agglutination, precipitin, and complement-fixation tests were found to be completely unreliable when testing anti-Z tumor sera. Use of Lissamine-Rhodamine RB-200 and isothiocyanate as described by Smith *et al.*(8) eliminated most nonspecific fluorescence and gave a better analysis of the reactive sites. Similar results were found using antisera which had been adsorbed with normal tissue and the globulins fractionated by ammonium sulfate and then conjugated with isothiocyanate. Little or no reaction was observed when either of these materials was tested against normal tissues or tumors

not related to the inbred strain Z.

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Deleterious Effects of High Fat Diets on Survival Time of X-Irradiated Mice.* (26318)

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Considerable data are available indicating that sensitivity to total body x-irradiation is enhanced in animals fed diets deficient in fat. Cheng *et al.*(1,2) reported that the average survival time of rats fed a fat-free diet was significantly less following multiple sublethal doses of total body x-irradiation than that of animals fed a similar diet supplemented with cottonseed oil or methyl linoleate. Similar findings have been reported for the mouse(3). It has also been observed that whereas a diet containing 10% cottonseed oil resulted in longer survival following multiple sublethal doses of total body x-irradiation than occurred in mice fed a diet deficient in fat, higher levels of fat (*i.e.*, 20% or 30% cottonseed oil in the ration) decreased survival time to that of mice on the fat-free diet.† These studies were confirmed and extended in the present investigation.

Procedure. The basal fat-free ration employed in these studies consisted of cerelose, 69%; casein,‡ 24%; salt mixture,§ 5%; cellulose,‖ 2%; and the following vitamins per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; Vit. B_{12}, 150 μg; 2-methyl-1, 4-naphthoquinone, 5 mg; choline chloride, 2 g; Vit. A, 5000 U.S.P. units; Vit. D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of cerelose. Tests were conducted with mice fed the basal fat-free ration or the basal fat-free ration supplemented with 2%, 10%, 20% or 30% of the following fats: cottonseed oil, margarine fat¶ or butter fat,¶

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† Ershoff, B. H., unpublished data.

‡ Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, O.

§ Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Inc., Chagrin Falls, O.

‖ Solka Floc, Brown and Co., Boston, Mass.

¶ Margarine fat and butter fat were prepared by the procedure of Deuel *et al*(5). Margarine fat was prepared from Nucoa (Best Foods, Inc., New York, N. Y.); butter fat from butter (Arden Farms Co., Los Angeles, Calif.).