Antibody Production in BALB/c Mice Following Injection of Lyophilized Tumor S621 in Freund's Adjuvant.*† (22107)

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Instances of isoimmunization of mice with their own tumors have recently been reviewed by Foley(1) and Snell(2). The criterion of immunization was ability of mice to cause regression of a viable implant of the tumor. Evidence was presented previously(3) for ability of strain BALB/c mice to produce antibody to homologous tumor S621. The criteria of antibody formation were ability of mice sensitized with lyophilized tumor to undergo anaphylactic shock on a second, intravenous injection; and ability of mice previously given tail inoculation of viable tumor, with tail and tumor subsequently removed, to cause regression of a second, subcutaneous injection of viable tumor.

The work reported herein was done to determine whether or not injection of lyophilized tumor in Freund's adjuvant provided a sufficient antigenic stimulus for detection of antibody by 3 technics: tumor regression: *in vitro* anaphylaxis by the Schultz-Dale reaction; and diminution of the number of exudate cells when cells were incubated with antigen and complement.

Experimental. Antibody production by protection against tumor growth. 1953 Experiments—Immunization with lyophilized S621: BALB/c mice from the colony of Dr. Flavia Richardson were used. Approximately equal numbers of both sexes, over 4 months of age, were injected intraabdominally as follows: (a) 20 mice injected with lyophilized S621‡ in Freund's adjuvant prepared as before(3) with the exception that only 50 mg of tumor were contained in the 0.5 ml injected; (b) 33 mice were injected with a preparation of lyophilized S621 identical with (a) except that the tumor in saline suspension was subjected to sonic treatment for 30 minutes in a Raytheon sonic oscillator immediately prior to mixing with adjuvant; and (c) 26 mice were injected with Freund's adjuvant equal in amount to that contained in the S621-adjuvant mixtures. *Injection of viable S621*: Five weeks following injection of lyophilized tumor, mice were inoculated subcutaneously with viable tumor prepared as follows: Fresh, healthy tumor tissue removed aseptically from host, and suspension of single cells prepared by Snell's sieve method(4). After making a cell count, the suspension was diluted with buffered glucose-Ringer's solution(4) to 150000 cells/ml. Each mouse was then injected subcutaneously with 0.1 ml, equivalent to 15000 cells. Twenty-two normal control mice were also injected. This dose was chosen because previous studies indicated that it exceeded, but not greatly, the minimum dose insuring tumor growth. Mice were examined at intervals for appearance of tumors. Experiment was terminated after 3½ months.

1955 Experiments—Mice were BALB/c from the colony of Dr. George Snell. Otherwise, they were comparable in every way to those used in 1953. They were injected with S621 under following conditions: (A) 17 mice were injected intra-abdominally with S621 in Freund's as described previously; (B) 16 mice were injected with the same preparation subcutaneously; (C) 11 mice were injected intraabdominally with 50 mg S621 suspended in 0.5 ml buffered saline; (D) 12 mice were injected with same preparation subcutaneously. Control mice included 11 injected with Freund's adjuvant intraabdominally; and 14 injected subcutaneously.

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‡ A BALB/c fibrosarcoma induced by methylcholanthrene in 1948.

TABLE I. Effect of Prior Injection of Lyophilized S621 in Freund's Adjuvant on Growth of Viable Inoculum.

Material inj.	Date	Route of inj.	Progressively growing tumors; death	Slight, if any initial growth comp. regression	Total* by route	Total*† all routes
S621-Freund's	1953	IA	13/20*	7/20	18/37	32/53
	55	IA	5/17	12/17		
	"	SC	14/16	2/16	14/16	
S621-Freund's sonic	53	IA	28/33	5/33	28/33	28/33
S-621	55	IA	11/11		11/11	23/23
		SC	12/12		12/12	
Freund's	53	IA	25/26‡		36/37	50/51
	55	IA	11/11			
	"	SC	14/14		14/14	
None	53	—	22/22		40/40	40/40
	55	—	18/18			

* $\frac{\text{No. dead of tumors}}{\text{No. inoculated}}$

† No sex difference was ever apparent.

‡ One mouse showed moderate initial growth, followed by complete regression.

Five weeks after injection of lyophilized tissue, immunity of mice was challenged by subcutaneous injection of 30000 cells, prepared as before. Eighteen normal mice were injected as controls. Mice were examined as before, and experiment terminated after similar interval.

Results of the 2 experiments are recorded in Table I. They show that immunization of BALB/c mice with lyophilized tumor S621 in Freund's adjuvant provides sufficient antigenic stimulus to protect 21 of 53 mice from growth of a viable inoculum of tumor. When the immunizing injection was given intraabdominally, over 50% of the mice were immune. Injection of lyophilized S621 in saline did not protect mice against a subsequent injection of viable tumor. Sonic treatment of lyophilized S621 reduced antigenicity, but apparently did not destroy it.

Evidence of antibody production by in vitro anaphylaxis. Fifteen female BALB/c Jax mice were immunized with 50 mg of S621 in Freund's adjuvant as before. After 1 or 2 months, and 3 to 4 weeks before testing, they were given a small booster dose of S621 subcutaneously. Included in these experiments were 6 female survivors from the 1955 experiment previously reported. The technic for testing anaphylactic sensitivity in mouse uteri by the Schultz-Dale technic of *in vitro* anaphylaxis has been described(5). The test antigen was prepared from either lyophilized or fresh tumor. Lyophilized tumor was made to concentration of 50 mg/ml in special Ringer's solution(5), ground in glass, and the supernatant fluid, after centrifugation for 10 minutes at 3500 rpm, was used as antigen. Fresh tumor, which in general proved a little more potent antigen in this test, was prepared in like manner, with a crude attempt to approximate the concentration of lyophilized material. All materials were kept at 0°-4°C, and aseptic technic was used while preparing the antigen. After killing the mice and putting uterine strips in 45-ml water bath, 1.0 or 0.5 ml of antigen was added, and the reaction recorded on a kymograph. After contraction of the tissue, a second addition of antigen was made to test desensitization, and reactivitv of the tissue tested by addition of 10 μg/ml of acetylcholine. Of the 15 BALB/c Jax mice tested, 8 gave positive reactions on at least two strips of uterus, and 7 gave negative reactions. Five of the 6 BALB/c Snell mice which survived the inoculation of viable cells

following immunization, gave positive reactions. Uterine strips from 3 normal BALB/c mice gave no reaction with either fresh or lyophilized antigen.

Evidence of antibody production by in vitro lysis of cells from sensitized mice. To become thoroughly familiar with the technic involved, preliminary work was done using egg white as antigen, as described elsewhere (6). Twenty-seven BALB/c Jax mice were immunized with 50 mg of lyophilized S621 in Freund's adjuvant, and tested 5 to 6 weeks later. Peritoneal exudate cells were obtained as follows: 2 ml of sterile mineral oil was injected intra-abdominally; 16-18 hours later, 2 ml of sterile heparinized serum-Fox solution(6) was injected. Ten minutes later cells were removed by aspiration, and put into chilled siliconed tubes. The cells were washed twice at 0°-4°C with serum-Fox solution, and resuspended in it to a concentration to give a count of 5000-20000 cells/mm^3. The S621 antigen used in the test was the supernatant fluid obtained after 50 mg/ml of lyophilized tumor had been ground in Fox solution and centrifuged 10 minutes at 3500 rpm. The control antigen was normal, lyophilized BALB c liver, prepared in identical manner. Cells from 27 mice were tested individually. In each test, 6 siliconed tubes were set up, and the materials added to each in 0.1-ml amounts. Tubes 1 and 2 contained cells, S621, and complement;§ the controls contained serum-Fox solution instead of complement. The other 3 tubes were comparable except liver antigen was used instead of S621. After all additions had been made to each tube, which was shaken 100 times, a standard cell count made in National Bureau of Standards certified pipettes was counted in a certified counting chamber. The 6 tubes were then incubated for 2 hours at 37°C, after which time they were again shaken and another count made, using for each tube the same pipette. Data were collected for each of the 27 mice, and recorded as the percent difference in number of cells from 0 to 2 hours. For statistical analysis, results of duplicate tubes for each antigen, which showed good agreement in both cases, were averaged. Mean and standard deviation for each group were determined; and the statistical difference between experimental and control groups was determined by the *t* test. Results of this analysis are recorded in Table II.

§ The 1-10000 dilution of commercial lyophilized guinea pig serum was tested many times, and never had any deleterious effect on the cells.

TABLE II. Effect of Antigen and Complement on Peritoneal Exudate Cells from 27 S621 Sensitized BALB/c Jax Mice.

	Mean*	Stand. dev.	Difference†	t
Tests 1 and 2 (S621 antigen)	−11.4	7.71		
S621 control	3.0	7.63	14.4	6.73‡
Tests 3 and 4 (Liver antigen)	.9	7.76	12.3	5.75‡
Liver control	3.1	8.23	14.1	6.55‡

* Avg % difference in No. of cells at 0 time and 2 hr.
† Mean divergence from tests 1 and 2.
‡ Significant beyond .001 level of confidence.

From the results expressed in Table II, it can be seen that peritoneal exudate cells from mice sensitized by injection of tumor S621 in Freund's adjuvant were subject to lysis in the presence of antigen and complement; and that no such lysis occurred when normal tissue antigen from liver was used instead of the tumor.

Discussion. Work reported in a previous paper showed that strain BALB/c mice were capable of forming antibody to their homologous tumor S621. After injection of viable tumor in the tail, mice caused regression of a second implant of viable tumor. Some survivors from this experiment, as well as mice immunized by injection of lyophilized tumor in Freund's adjuvant, or lyophilized tumor alone followed by adrenalectomy, were anaphylactically sensitive to an antigen derived from S621.

Experiments here reported lend further support to the concept of formation of these antibodies. Mice immunized with lyophilized tumor tissue in Freund's adjuvant caused regression of subsequent viable implants; and uteri from female survivors of this experiment, as well as from other similarly immu-

nized mice, were anaphylactically sensitive as tested by the Schultz-Dale technic. The exudate cells of other mice, also sensitized with S621 in Freund's adjuvant, were lysed *in vitro* in the presence of the S621 antigen and complement.

With the exception of the technic of tumor regression, all our methods for detection of antibodies—*in vivo* anaphylaxis, *in vitro* anaphylaxis, and specific immune lysis of cells—are generally accepted immunological methods to detect "hypersensitive" antibodies, which in either immediate or delayed type of hypersensitivity reactions are believed to be in close association with cells. Recent work of Cole and Favour(7) has shown that in hypersensitivity of the guinea pig to tubercle bacilli, antibodies responsible for delayed "tuberculin" type hypersensitivity, as well as those of anaphylactic type, occur in the serum. Repeated attempts to find antibodies in circulation of BALB/c mice sensitized with S621 by various technics—precipitation, complement-fixation, hemagglutination of cells, which have or have not been treated with tannic acid prior to treatment with antigen, and passive protection with serum from immunized mice—have so far failed. Other studies of anaphylaxis in mice(8,9) indicate that a circulating, bivalent antibody is not the antibody concerned in anaphylaxis in the mouse. This leads to the tentative hypothesis that the antibody concerned in immunity to tumor S621 in BALB/c mice is not the conventional bivalent antibody, and even though it may be in circulation, is an antibody in close association with tissue cells.

Stewart(10) has presented a survey of spontaneous regression of various types of malignancies in man, many of which were accompanied by the classical signs of hypersensitivity in the host at time of regression of the tumor.

Immunity to S621 usually is accompanied by a state of hypersensitivity, but hypersensitivity can apparently exist in absence of sufficient immunity to protect the animal from viable cell growth. In a previous paper (3), as well as in present experiments, it is noteworthy that mice immunized with the lyophilized tissue in the absence of the adjuvant were not capable of causing regression of a viable implant of tumor, even though they had antibody demonstrable by the more sensitive anaphylactic technic. It is quite possible that lyophilized tissue alone did not provide sufficient antigenic stimulation for the formation of enough antibody to protect against relatively large challenge inocula.

Summary. 1. Antibodies were demonstrated in BALB/c mice which had received an injection of lyophilized homologous tumor S621 in Freund's adjuvant by the following technics: tumor regression; *in vitro* anaphylaxis using the Schultz-Dale reaction; decrease in the number of exudate cells when cells were incubated with antigen and complement. 2. Mice which recovered from a challenge injection of viable tumor were shown to react when tested by the Schultz-Dale technic.

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