16423

A Precipitin Test for Antigens Present in Mouse Tissues Containing the Milk Agent.*

DAVID T. IMAGAWA, ROBERT G. GREEN (DECEASED), AND H. ORIN HALVORSON.
(Introduced by John J. Bittner).

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis, Minn.

One of the causes of mouse mammary carcinoma was shown by Bittner[1] to be an agent transmissible through the milk of nursing females to their young. Investigations on the nature of this agent show that its size is within the realm of viruses, as demonstrated by Visscher, Green, Bittner, Ball, and Siedentopf.[2] The antigenic character of the milk agent was described by Andervont and Bryan,[3] who demonstrated the presence of specific neutralizing antibodies in the sera of rabbits inoculated with extracts of tumor tissues, while Green, Moosey, and Bittner[4] showed that highly active specific antisera, which would neutralize the agent, could be produced in rabbits and rats.

The first successful attempt to identify the milk agent by an *in vitro* serological method was described by Gerodilova and Shabad,[5] who used a complement fixation test, and more recently by Bennison,[6] who used similar methods.

In our studies on the antigenic nature of the milk agent, we have been able to demonstrate that when rabbits are injected with centrifugates of mouse tissues containing the agent, sera are produced which specifically precipitate ultramicroscopic elements derived from cancer tissues and other agent-bearing tissues of mice. The test to be subsequently described will therefore be referred to as a precipitin test and the serum antibodies as precipitins.

Materials and Methods. Adenocarcinomas of the mouse mammary glands, which contain relatively large amounts of milk agent, were selected as a source of antigen. The cancerous tissues were first ground in a mortar and diluted approximately 1-10 by weight in physiological saline. The suspensions were then passed through a homogenizer to further break up the tissue cells. This homogenate was centrifuged at 2,000 R.P.M. for 30 minutes at 4° C. The sediment, consisting of unbroken cells and large particles, was discarded, while the supernatant fluid was again centrifuged at 15,000 R.P.M. in an angle centrifuge for one hour at 4° C. The resulting pellet of centrifugate was suspended in saline so that 1 cc contained the centrifugate derived from one g of tumor tissue. Rabbits received this suspension as antigen in five intramuscular injections of 1 cc at 5 day intervals. Two weeks after the final injection sera were collected and utilized as immune sera.

The antigens used in the precipitin test were prepared similarly to those used for rabbit inoculation, except that the final suspension contained the centrifugate derived from 1 g of tissue in 3 cc of diluent. The centrifugate was carefully put into suspension by mechanical agitation and cleared by recentrifuging at 1,000 R.P.M. for 30 minutes.

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and The Graduate School of the University of Minnesota.

1 Bittner, J. J., *Science*, 1936, **84**, 162.

2 Visscher, M. B., Green, R. G., Bittner, J. J., Ball, Z. B., and Siedentopf, H. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 94.

3 Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

4 Green, R. G., Moosey, M. M., and Bittner, J. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 115.

5 Gerodilova, V. V., and Shabad, L. M., *Bull. Eksp. Biol. i. Med.*, 1946, **22**, 9.

6 Bennison, B. E., Abstract, Fourth International Cancer Research Congress, September 2-7, 1947, St. Louis, Mo.

The actual quantity of antigen used in the precipitin tests was determined accurately by the micro-Kjeldahl technic. Analyses were conducted by using a steam distillation apparatus and the ammonia was collected in 2% boric acid solution containing methyl red.

Antigens were prepared from mouse cancer tissue, non-lactating mouse mammary gland containing the agent, non-lactating mammary gland without the agent, pooled spleen, heart, liver and lung containing the agent, and similar tissues without the agent. Sera were collected from rabbits immunized against mouse cancer antigen and antigens derived from non-lactating mammary glands with and without the agent.

The precipitin tests were carried out in the following manner. Antigen and antiserum were separately diluted in serial 2-fold dilutions. One-half cc of antigen dilution was added to ½ cc of antiserum dilution in Wassermann-type tubes. The antigen-antibody mixtures were then placed in an incubator at 37.5° C for half an hour. The tubes were then stored at 4° C for 18 hours. At the end of this time the settled granular precipitates were observed and read as 1+, 2+, 3+, or 4+, depending on the degree of precipitation. 4+ was the maximum precipitate observed, while the minimum definite precipitate was recorded as 1+.

Precipitin Tests. Antiserum prepared with mouse cancer antigen was tested against antigens derived from mouse cancer tissue, non-lactating mammary glands with and without the agent, and pooled spleen, heart, liver, and lung with and without the agent. In no case did precipitation occur when normal rabbit serum was mixed with various antigens. The results, presented in Table I show that antiserum against mammary cancer antigens will precipitate in high serum dilutions normal mammary gland tissue and pooled organ tissues containing the agent, while the same antiserum will precipitate normal mammary gland tissue and pooled organ tissues without the agent only in low serum dilutions. These precipitations occurring in low serum dilutions are best explained on a basis of normal tissue occurring within the masses of cancer tissue.

TABLE I.

Precipitin Reactions of Specific Sera Against Milk Agent Derived from Different Tissues.

Antigen	Antiserum	Serum dilutions										
		U	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
*Mouse cancer	Mouse cancer	++++	++	++	+++	++	+	+	+	+	±	
* " "	Gland without agent	++	+++	+	++	±	+	+	+	±		
* " "	Gland with agent	+++	++	++	++	++	++	++	++	±		
*Gland with agent	Mouse cancer	+++	++	+++	+	+	++	+				
*Gland without agent	" "	+	+++	++		++	±					
†Tissue with agent	" "	++	++	++	++		±					
†Tissue without agent	" "	++	++	++	±							

* 1:45 dilution.
† 1:60 dilution.

It is not to be expected that serological reactions involving antigenic preparations made from cancerous tissues, such as those reported here, would be entirely devoid of cross reactions with normal mouse tissue. A mass of cancer tissue would appear to contain many normal tissue elements, such as blood vessels stimulated to grow into the cancer cell mass and normal tissue enclosed by the cancerous growth and not yet degenerated. A serum made by injecting antigens derived from such a mixture of elements would be expected to contain antibodies against both the cancer cell and the normal tissue derivatives.

Antisera prepared with non-lactating glands containing the agent and without the agent were then tested against antigens derived from mouse cancer tissue. As shown in Table I, antiserum against normal mammary gland tissue abundant with the milk agent will precipitate mammary cancer antigen in high serum dilutions. Opposite results are observed when the milk agent is absent in the normal mammary gland tissue. These results indicate that the specific antigen abundant in mouse mammary cancer tissues can also be observed in non-lactating mammary gland and organ tissues containing the agent and is absent in mammary glands and organ tissues lacking the agent.

The concentration of all the antigens used in this work is expressed in terms of the weight of the original tissue. For example, a dilution of 1-60 means that the centrifuged pellet obtained from 1 g of tissue is diluted in 60 cc of saline. It is evident from this that the concentration of the specific antigen will not be the same in similar dilutions from different tissue preparations unless the concentration of the antigen is the same in all these tissues. This is not likely to be the case.

If the antigens were pure, the concentration could be controlled by running nitrogen determinations and diluting the various preparations to the same organic nitrogen concentration. The control of the organic nitrogen concentration will not suffice in this case, because the various preparations may contain different percentages of organic nitrogen from sources other than the specific antigen.

It is a well-known fact that the amount or degree of precipitation following an antibody-antigen reaction is dependent upon the relative concentrations of antibody and antigen.

TABLE II.
Precipitin Reactions of Mouse Mammary Cancer Antiserum Against Tissues with and without the Milk Agent.

A.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/3	++	+	+	+	+
1/6	+	+	+	+	+
1/12	+	+	+	+	—
1/24	+	+	+	—	—
1/48	+	+	+	—	—
1/96	+	+	+	—	—
1/192	+	+	+	+	—
1/384	+	+	+	—	—
1/768	+	+	+	—	—

Antigen: Mouse Mammary Cancer, 1/60 dilution = .018 mg N/cc.
Serum: Mouse Mammary Cancer Antiserum.

B.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/3	+	+	+	+	+
1/6	+	+	+	+	—
1/12	+	+	+	+	+
1/24	+	+	+	—	—
1/48	+	+	+	—	—
1/96	+	+	—	—	—
1/192	+	—	+	—	—
1/384	—	+	+	—	—
1/768	+	+	—	—	—

Antigen: Mammary Gland Tissue with agent. 1/60 dilution = .009 mg N/cc.
Serum: Same as A.

C.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/3	+	+	+	—	—
1/6	+	+	+	—	—
1/12	+	+	—	—	—
1/24	+	+	—	—	—
1/48	—	—	—	—	—
1/96	—	—	—	—	—
1/192	—	—	—	—	—
1/384	—	—	—	—	—
1/768	—	—	—	—	—

Antigen: Mammary Gland Tissue without agent. 1/60 dilution = .007 mg N/cc.
Serum: Same as A.

In fact, if the ratio of antigen to antibody, or the reciprocal ratio of antibody to antigen is too great, the result may be no precipitation. It is for this reason that as long as the absolute concentration of antigen is unknown, 2 antigen preparations cannot be compared by trying each against several dilutions of the same serum. Thus, the results given on Table I may not be conclusive.

To overcome this objection, varying dilutions of the antigen were tried against varying dilutions of the antibody. The results of a titration of this kind are presented in Table II-A, II-B and II-C. Antigen controls in dilutions of 1-60 and greater were negative. Serum controls in all dilutions were also negative. As in previous experiments, normal rabbit serum did not react with any of the antigen. It is evident from a study of Table II that the zones of precipitation are similar and greater when the mouse mammary cancer antiserum is tested against mouse mammary cancer or mammary gland tissues with the agent (Table II-A and II-B) than when the same serum is tested against mammary gland tissues without the agent (Table II-C). This would indicate that there is a common antigen present in mouse mammary cancer and mammary gland tissues containing the agent and which is not present in the antigen prepared with the mammary gland tissues without the agent.

Adsorption Tests. The results so far indicate that in sera of rabbits injected with mouse mammary cancer centrifugates, antibodies against both the cancer cell and normal tissue derivatives are present. In order to broaden the observations, tests were carried out to eliminate the normal tissue antibodies by precipitin-adsorption reactions. When antigen derived from normal mammary gland tissues without the agent is added to the mouse cancer antiserum, a moderate precipitate will result after incubation (0.279 mg N of antigen to 1 cc of serum). Centrifugation at 3,000 R.P.M. for 30 minutes at 4° C will clear the antiserum, and the adsorbed serum will give only a very slight positive precipitation with normal mammary gland tissues without the agent but will still give a positive precipitation with tissues containing the agent.

The results of such an experiment are given in Table III-D, III-E and III-F. Comparing Table III-F with Table II-C, it is evident that practically all the antibodies against the normal tissue antigen, exclusive of the milk agent, had been removed from the adsorbed mouse mammary cancer antiserum. Table

TABLE III.
Precipitin Reactions of Adsorbed Serum Against Tissues with and without the Milk Agent.

D.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/6	+	+	+	+	—
1/12	+	+	+	+	—
1/24	+	+	+	+	—
1/48	+	+	+	—	—
1/96	+	+	+	—	—
1/192	+	+	—	—	—
1/384	+	+	+	—	—
1/768	+	+	—	—	—

Antigen: Mouse Mammary Cancer, 1/60 dilution = .016 mg N/cc.
Serum: Mouse Mammary Cancer Antiserum adsorbed with mammary gland tissue without milk agent.

E.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/6	+	+	+	+	—
1/12	+	+	+	—	—
1/24	+	+	+	—	—
1/48	+	+	+	—	—
1/96	+	+	—	—	—
1/192	+	+	+	—	—
1/384	+	+	+	—	—
1/768	+	—	—	—	—

Antigen: Mammary Gland Tissue with agent. 1/60 dilution = .007 mg N/cc.
Serum: Same as D.

F.

Serum dilutions	Antigen dilutions 1/60	1/120	1/240	1/480	1/960
1/6	+	±	—	—	—
1/12	+	—	—	—	—
1/24	—	—	—	—	—
1/48	—	—	—	—	—
1/96	—	—	—	—	—
1/192	—	—	—	—	—
1/384	—	—	—	—	—
1/768	—	—	—	—	—

Antigen: Mammary Gland Tissue without agent. 1/60 dilution = .007 mg N/cc.
Serum: Same as D.

III-D and Table III-E showed similar precipitation zone indicating that the removal of normal tissue antibodies did not remove the antibodies necessary to precipitate the common antigen or antigens found in mouse mammary cancer and normal mammary gland tissues containing the agent.

Summary. The data presented in this report describe a precipitin test for mouse tissues containing the milk agent. Antiserum produced in rabbits against mouse mammary cancer reacts in high titre with tissues containing the cancer agent and in low titre with tissues lacking the agent. Antiserum prepared against normal mammary gland tissues abundant with milk agent will precipitate mammary cancer antigen in high serum dilutions. Antiserum prepared against normal mammary gland tissues lacking the agent will precipitate mammary cancer antigen in low serum dilutions. Precipitin-adsorption reactions show that the removal of antibodies against mammary gland tissues without the agent in mouse mammary cancer antiserum will not remove antibodies which react with the common antigen or antigens found in mouse mammary cancer and mouse mammary gland tissues containing the milk agent.

The authors are indebted to Dr. J. J. Bittner of the University of Minnesota for generously supplying the mice and advising as to the strains of mice that should be used in this investigation.

16424

The Effect of Phrenicotomy on Gastrointestinal Mechanisms.*

NELSON C. JEFFERSON AND H. NECHELES.

From the Department of Gastro-Intestinal Research, Medical Research Institute of Michael Reese Hospital and the Department of Surgery, Provident Hospital, Chicago, Ill.

A number of observations point to a functional relationship between left diaphragm and stomach. Sauerbruch[1] has described a gastro-cardiac symptom complex after exeresis of the left phrenic nerve, consisting of cardiovascular and gastro-intestinal disturbances, caused by changes in the gastro-intestinal tract. One factor was considered to be the pressure exerted by the stomach on the left cup of the diaphragm, resulting in displacement of the heart and large vessels. Bradycardia and premature systoles were ascribed to vagal stimulation, but occasionally, tachycardia and vertigo were observed. Frequently anxiety, epigastric distress, lack of appetite, pallor, nausea, vomiting and eructations occurred,[2] which were relieved by removing the subdiaphragmatic accumulation of air.[3] The latter did not seem to be due so much to swallowing of air, as to inability to belch it up. This symptom complex has been named "Roemheld's Syndrome."

Joannides[3] found that he could induce prompt contractions of the stomach by having the patient take deep breaths. This he ascribed to be due in part to the intermittent increase and decrease of intragastric pressure, induced by the contractions of the diaphragm. Joannides stated that cardiospasm may be an exaggeration of the normal contractions of the diaphragmatic pillars, and that partial contraction and relaxation of the diaphragm plays a role in belching.

Rickers[4] reported 2 cases of left side phrenic exeresis for tuberculosis, which developed the gastro-cardiac symptom complex.

* Aided by a grant from the Isaac and Kate Meyer Fund. The Department is in part supported by the Michael Reese Research Foundation.

1 Quoted by Roemheld, L., *Münch. Med. Wchnschr.*, 1928, **75**, 1872.

2 Noack, R., *Beitr. z. Klin. d. Tuberk.*, 1933, **82**, 397.

3 Joannides, M., *Arch. Int. Med.*, 1929, **44**, 856.

4 Rickers, L., *Beitr. z. Klin. d. Tuberk.*, 1933, **83**, 175.