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Further Studies on Abnormal Serum Proteins in Tumor-Bearing Hosts.* (29665)

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Previous studies on abnormal serum proteins in mice bearing certain transplanted or spontaneous tumors have demonstrated the existence in the host animals of minute amounts of antigenc serum components which were absent in normal control animals of the same strain(1,2). These observations were based on a combination of immunological and biochemical techniques, which led to the production of rabbit antisera against the abnormal serum components. However, the antisera thus obtained reacted also with a number of normal murine serum proteins, and interpretation of the results appeared ambiguous at times.

A new procedure for selective and quantitative removal of certain antibodies from antisera has recently been developed(3). By use of this technique it has now become possible to prepare rabbit antisera *specific* for abnormal components which occur in minute amounts in the serum of tumor-bearing mice; such antisera do not react at all with serum from normal control animals(4).

The sequence of techniques described here may be applied also to other diseases or conditions and may also be employed for detection of abnormal proteins at other sites.

Methods and materials. Mouse serum. Female C3H/He mice with spontaneous mammary adenocarcinoma of one to two cm diameter served as tumor-bearing animals. They were retired breeders from the Jackson Laboratory in Bar Harbor, Maine. Animals with necrotic tumors were rejected. Male littermates were used as normal control animals unless stated otherwise. Mice were bled as described earlier(5), except that no citrate was used and serum was collected.

Murine serum α-globulin fractions. Pooled serum from tumor-bearing mice, in portions of 20 ml, was fractionated by zone electrophoresis on a carrier medium consisting of 3% cornstarch gel with 3% additional potato amylose as described earlier(2,6). An average of 20 protein fractions was obtained from each batch. These fractions were characterized by paper electrophoresis and by plotting the total protein content of each fraction, measured according to Lowry *et al*(7), as a function of the distance of the fraction from the origin. In general, three-fourths of the total α_2-globulin was distributed over 4 to 5 fractions; these also contained variable amounts of α_1- and β-globulins. There was an equal number of fractions encompassing 75% of the α_1-globulin; they contained variable amounts of albumin and α_2-globulin. These α_1- and α_2-globulin fractions partially overlapped each other.

Immunization of rabbits. Young female rabbits weighing 5 to 6 lb received subcutaneous injections of α-globulin (including β-globulin or albumin impurities) in Freund's adjuvant mixture as described by Cohn(8). The initial immunization consisted of one mg of protein per pound of rabbit. Starting 6 weeks after the initial injection, booster in-

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jections of half the initial amount of protein were administered at 2-week intervals for 4 to 6 months. After the first 3 immunizations (one initial and two booster injections), a different α-globulin fraction was given on 2 consecutive occasions; rabbits receiving α_2-globulin at first were now given an α_1-globulin fraction and *vice versa*. As a rule, the second fraction administered to each rabbit was 2 to 3 fraction numbers distant from the fraction given at first to the rabbit. After 2 immunizations with the second fraction, each rabbit received again 2 consecutive injections with the fraction given initially. This schedule of alternation between 2 injections of α_2-globulin and 2 of α_1-globulin fractions was continued further. Such "cross immunization" resulted in superior sensitization of the rabbits against the abnormal mouse serum component. Periods of one or several weeks of rest without injections were intercalated occasionally. Rabbits were bled from the ear vein 2 weeks after the third injection and thereafter always 2 weeks after every second booster injection.

Treatment of antisera with normal antigens. Protein from whole serum of normal control mice was transformed into a water-insoluble form by entrapping it into the lattices of highly cross-linked polyacrylamide. This was achieved by polymerizing the synthetic monomer, methylenebisacrylamide, in the presence of serum protein in aqueous solution as previously described in Procedure I(3). One ml of rabbit antiserum against α-globulin from tumor-bearing mice was then treated with a suspension of washed, insoluble protein derived from 60 mg of normal murine serum protein (about 1.2 ml mouse serum). The procedure described previously (3) was followed, except that the insoluble polymer, remaining after the "treated" antiserum had been separated by centrifugation, was washed by stirring it up 3 times with one ml of distilled water. The two phases were subsequently separated by centrifugation. The washings were added to the main portion of "treated" antiserum, and the combined solutions were concentrated by lyophilization to establish the protein concentration of the original rabbit antiserum before "treatment." In this way, the recovery of specific antibody could be improved to 70 or 80%.

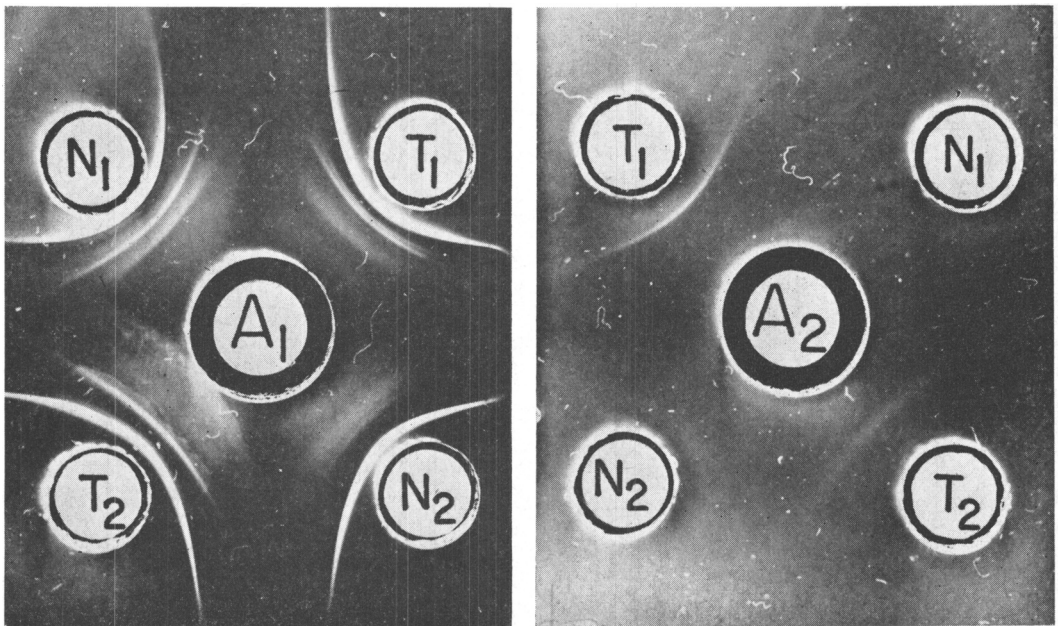
Precipitin tests. The specific antibody-antigen interactions were observed by the Ouchterlony agar plate diffusion technique (9) as modified by Korngold(10).

Results. Immunization of rabbits with α-globulin fractions from the serum of tumor-bearing mice resulted in development of at least 5 different rabbit antibodies against mouse serum proteins, as seen from the number of precipitin lines in the agar plate diffusion test of Fig. 1a. The same number of precipitin lines appeared on the agar plate independently of whether serum from tumor-bearing mice or from normal control animals was used as the antigen. After the rabbit antiserum had been treated with insoluble serum protein from normal control mice, all antibodies against normal murine serum proteins had been removed, according to the complete absence of precipitin lines with normal mouse serum as the antigen (Fig. 1b, wells N1 and N2). When serum from tumor-bearing mice was applied on the plates as the antigen, however, a single and sharp precipitin line appeared with the same treated rabbit antiserum (Fig. 1b, wells T1 and T2), indicating the presence in the serum of tumor-bearing mice of an antigenic material which is absent in normal animals. This component is therefore referred to subsequently as an abnormal component.

Abnormal murine serum proteins were observed with antisera from a number of different rabbits; the same findings were also obtained with murine serum α-globulin fractions from different batches of fractionation, with different pools of serum from C3H mice bearing spontaneous mammary tumors, and after treatment of the rabbit antisera with insoluble protein from different pools of serum from normal C3H mice.

The appearance of the precipitin line of the abnormal component was highly dependent on the mouse serum concentration used in the agar test. This line was always observed at 1:16 and 1:32 dilutions of the

mouse serum (*i.e.* at about 3.2 and 1.6 mg protein per ml), sometimes at 1:8 and 1:64 dilutions, and rarely at lower or higher dilutions (Table I). To exclude the possibility that a corresponding precipitin reaction also exists in normal serum in a narrow range of



a b

FIG. 1a, 1b. Center well A1 contained untreated rabbit antiserum (rabbit 35) after 3 immunizations with an α_2-globulin fraction from tumor-bearing C3H mice, followed by 2 immunizations with an α_1-globulin fraction, then by one with the initial α_2-globulin fraction. Center well A2 contained the same rabbit antiserum as A1 but *after* treatment with insoluble protein from serum of normal C3H mice. Outside wells N1 and N2 contained pooled sera from normal C3H mice at 1:32 and 1:8 dilutions, respectively. T1 and T2 contained pooled sera from tumor-bearing C3H mice at 1:32 and 1:8 dilutions, respectively.

TABLE I. Reaction of "Treated" Rabbit Antisera Against Abnormal Murine Serum Protein with Various Materials from C3H Mice at Different Concentrations.

	Precipitin reaction*						
	Tumor-bearing mice				Normal mice		
Protein concentration in agar test (mg/ml)	Serum	Liver extract†	Lysed RBC‡	Tumor extract†	Serum	Liver extract†	Lysed RBC‡
100				—			
50	—§			+++	—§	—	
25	—	+++	—	+++	—	—	—
12.5	—	—	—	++	—	—	—
6.3	+	—	—	—	—	—	—
3.2	+++	—	—	—	—	—	—
1.6	+++	—	—		—	—	—
.8	+		—		—		—
.4	—				—		
.2	—				—		
.1	—				—		

* Precipitin reactions were graded as follows: +++ Strong precipitin reaction observed in almost all instances. ++ Strong precipitin reaction observed sometimes. + Precipitin reaction weak and observed only in a few instances. — No precipitin reaction observed.

† Cells were dispersed by means of a cytosieve, washed repeatedly with saline and then disintegrated by successive treatments in a Virtis homogenizer and in an ultrasound disintegrator.

‡ Red blood cells.

§ Undiluted serum.

TABLE II. Influence of Repeated Immunizations and "Cross Immunizations" of a Rabbit (No. 36) on Development of Antibody Against Abnormal Mouse Serum Protein.

Protein fraction injected			
Principal components	No. of times injected	Date of bleeding of rabbit	Immune reaction against abnormal protein on agar plate*
α_2 (α_1)	3	2/27/63	+
α_1 (A)	2	4/ 4/63	(+)
α_2 (α_1)	1	6/ 5/63	+++
α_1 (A)	1	10/ 8/63	+++
α_1 (A)	1	11/29/63	+++

* Evaluation of intensity of precipitin line.

dilution and has escaped detection, normal mouse serum was tested over a wide range of concentrations from undiluted serum to a final 1:512 dilution in a logarithmic progression of 1:2. No precipitin line was observed at any of these concentrations (Table I).

Abnormal protein was found in almost all fractions of the serum from tumor-bearing mice which contained α_1- or α_2-globulin, but not in any of the other protein fractions (Fig. 2).

When rabbits were immunized repeatedly with a single fraction containing the abnormal protein, an antibody against the latter was observed in only a few instances and this antibody was always present at a very low titer (low intensity of the precipitin line on the agar plates). The immune response of the rabbits against the abnormal mouse protein could be greatly enhanced by a schedule of injections in which murine α_2- and α_1-globulin fractions, both containing the abnormal component, were administered alternately to the rabbits (Table II). Best results were obtained when the 2 fractions injected were apart from each other by 2 to 3 fraction numbers (equal in length to about one-fourth to one-sixth of the distance of migration of the albumin). Eventually, the rabbits became sensitized against the abnormal mouse serum protein to such a degree that the corresponding precipitin line became clearly visible on the agar plates even when "untreated" rabbit antiserum was used.

Tests of the antibody-antigen interactions between *individual* mouse sera and "treated" rabbit antisera exhibited a distinctly positive reaction with each of 20 sera from tumor-bearing female C3H retired breeders (Table III). This reaction consisted in all instances in the appearance of a single and sharp precipitin line in the agar plate diffusion technique.

Because all the tumor-bearing animals of the host-tumor system under investigation were of necessity female, the choice of the normal control animals as to age and sex was important. For the purpose of removing antibodies against normal mouse serum proteins from the rabbit antisera, pooled serum from male retired breeders was used in all instances. Consequently, no reaction was found between the rabbit antisera thus treated and any of 20 individual mouse sera from male C3H retired breeders (Table III). Likewise, no positive reactions were observed between the same treated rabbit antisera and any of 15 individual sera from normal young male C3H mice (3 to 4 months old). However, about 20% of the sera from normal individual young female C3H mice (3 to 4 months old) seemed to exhibit a positive precipitin reaction. This reaction was much weaker than that with tumor-bearing animals; in one case it consisted in multiple precipitin lines on the agar plates.

All of 8 individual sera from young healthy pregnant female C3H mice exhibited positive reactions with the rabbit antiserum (Table III).

Whereas washed and lysed red blood cells from both tumor-bearing and normal mice

TABLE III. Reaction of Individual Sera from C3H Mice with "Treated" Antiserum from Rabbits Immunized with Serum α-Globulin Fractions from C3H Mice Bearing Spontaneous Mammary Adenocarcinoma.

Donor mice			No. of individual mice studied	Positive reactions	
Condition	Sex	Age (mo)		No.	%
SMT*†	♀♀	>10	20	20	100
Normal†‡	♀♀	>10	5	2	40
" †	♂♂	>10	20	0	0
"	♂♂	3–4	15	0	0
"	♀♀	3–4	21	4	20
Pregnant	♀♀	3–4	8	8	100

* Spontaneous mammary tumor.
† Retired breeders.
‡ No visible signs of tumor at autopsy.

gave no precipitin reactions, extracts from carefully washed liver and tumor cells exhibited positive reactions at very high concentrations of the extracts (Table I). At the same high protein concentrations, the tumor cell extract gave a positive precipitin reaction also with a rabbit antiserum against whole serum protein from normal C3H mice. This suggests that the washing was insufficient to remove small amounts of serum adhering to the tumor cells, and possibly also to the liver cells. The observation of abnormal protein in the extracts of tumor and liver cells at these high concentrations is not significant, therefore. No precipitin reaction was observed when an extract of liver cells from normal mice was allowed to react with "treated" antiserum.

Discussion. Our data clearly show that the serum of female C3H mice, bearing spontaneous mammary adenocarcinoma, contains an antigen which is absent from the serum of normal C3H male littermates. This antigen in the serum of tumor-bearing mice is referred to, therefore, as an *abnormal* antigen. From its behavior as an α-globulin upon electrophoresis, this antigen is likely to be a protein. Since an antibody against this abnormal protein can only be obtained after a great number of immunizations of the rabbits, and then only when a special schedule of immunization is observed and when a partially purified antigen is used, it must be assumed that the abnormal protein occurs in no more than trace amounts in the host serum.

The methodology used in this work for detection of minute quantities of unknown abnormal serum proteins of undefined physiological and biochemical functions is based on 3 principal features.

1. It is well known that little or no antibody is formed against many antigenic components, especially when they occur in very small amounts. Adler(11) interpreted this phenomenon as being due to a competition between the various antigens for antibody-forming sites. For this reason, purified fractions of the abnormal murine serum proteins were used in the present work instead of whole mouse serum to immunize the rabbits. In this way, about 90% of the normal mouse serum proteins were removed from the antigen before its injection into rabbits and, consequently, the antigenicity of the remaining minor component was considerably increased.

2. To enhance further the sensitization of rabbits specifically against the abnormal component, a schedule of alternate immunizations with 2 different murine serum protein fractions was developed. Both of these fractions contained the abnormal component, but their composition of normal mouse serum proteins was slightly different. Sensitization of the rabbits by this method of "cross immunization" was, thus, focused on those antigens which were administered to them with every single injection, *i.e.* especially the abnormal component.

3. The rabbit antisera thus obtained still predominantly contained antibodies against normal mouse serum proteins, and a separate precipitin line corresponding to the abnormal component could not be detected in the agar-plate-diffusion tests, except in a few instances where many repeated "cross immunizations" were administered to the rabbits, *e.g.* in the last 2 bleedings of the experiment shown in Table II or in a few especially responsive rabbits(2). A new procedure using water-insoluble antigens for absorption of antibodies against normal murine serum proteins was developed. This method has proved to be highly successful in quantitative removal of all antibodies against normal mouse serum proteins, without affecting the antibodies specific for the abnormal component, and it has permitted the demonstration of the presence of antibodies against abnormal components even when the unabsorbed antiserum did not disclose the presence of such an antibody (Fig. 1).

The existence of abnormal antigens in various malignant *tissues* has been described by several laboratories(12-19). However, only a few reports on abnormal *serum* proteins in tumor-bearing hosts have appeared; these include data from our own laboratory(1,2,4,20, 21) and recent observations by Bogden *et al* (22).

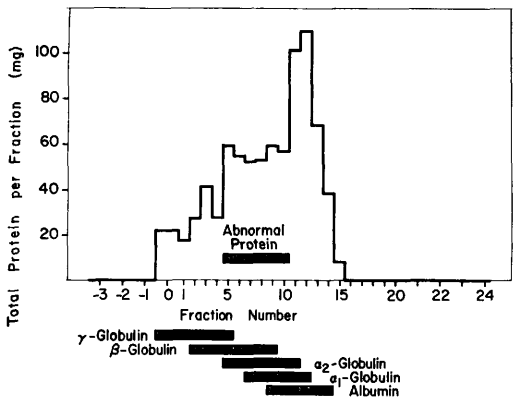


FIG. 2. Fractionation of serum from C3H mice bearing spontaneous adenocarcinoma, by use of zone electrophoresis on a supporting medium of cornstarch gel containing additional potato amylose. Abscissa: fraction numbers; fraction 0 corresponds to origin of migration; since the width of the fractions varied from 0.5 to 1 cm, each fraction was plotted at its actual distance in cm from the origin. Ordinate: amount of protein per fraction in mg. The 5 major protein components were located by paper electrophoresis, zones totaling 85 to 90% of each component are indicated at bottom. Presence of abnormal protein was determined by agar plate diffusion technique, and its location is indicated above abscissa.

There are no indications as to the site of origin of the abnormal serum protein. It appears, however, that it arises neither in the tumor tissue nor in the liver since extracts of these tissues were found to be practically free from the abnormal component. The demonstration of abnormal protein at very high concentrations of these extracts (Table I), *i.e.* its presence in concentrations considerably below those in which it occurs in serum, must be assumed to be due to incomplete removal of blood from the cells before homogenization. Uterus, ovaries or mammary glands might be likely sites of origin of the abnormal serum protein, in particular the latter since the abnormal component occurs in a host bearing a mammary tumor.

Whereas the abnormal protein has never been found in a single male animal, about 20% of the normal young females and all of the pregnant animals exhibited a positive reaction. It is possible that the abnormal serum protein, being a part of the overall process of malignancy, frequently precedes the appearance of a detectable tumor growth. On the other hand, it cannot be ruled out that the "abnormal" serum component may also occur in normal females but not in males, that the amount of it in the serum of normal females would usually be below the threshold of perceptibility by the method used, and that its concentration in the serum would increase markedly during tumor growth, pregnancy and possibly also other conditions.

It is unknown whether or not the abnormal antigen has any relationship to the Milk Agent discovered by Bittner(23). This milk factor was found by Andervont(24) in C3H mice with spontaneous mammary adenocarcinoma, as well as in other strains of mice. It is a filterable and heat labile virus(24,25) which possesses definite antigenic properties (26).

Summary. The presence of an abnormal serum protein in C3H mice bearing spontaneous mammary adenocarcinoma was confirmed. A rabbit antiserum specific for this abnormal murine serum protein was prepared, and the specific antiserum obtained did not react at all with the serum of normal male C3H littermates. The procedure for its preparation was based on 3 essential features: 1. Immunization of the rabbits with purified antigen; 2. development of a schedule of "cross immunization" consisting of alternate injections with 2 slightly different murine serum protein fractions; and 3. quantitative absorption of all antibodies against normal murine serum proteins by the use of water-insoluble antigens.

The abnormal serum component is present in all tumor-bearing animals but only in minute amounts; it was also found in pregnant females and in about 20% of normal females, but never in males; it is assumed to be a forerunner of tumor growth. It migrates on electrophoresis as an α-globulin and only insignificant amounts of it were found in extracts of washed tumor or liver cells of the hosts. It is not known whether it is related to Bittner's Milk Agent.

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Reduction of Biliverdin-C^{14} to Bilirubin-C^{14} *in vivo*.* (29666)

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Bilirubin is the major end-product of heme catabolism. The intermediates in this degradative process are not well defined, but it has long been assumed that biliverdin is the immediate precursor of bilirubin(1). The structural relationships between heme, biliverdin, and bilirubin support this concept, and enzymatic conversion of biliverdin to bilirubin has been performed *in vitro*(2,3). Surprisingly few attempts have been made to demonstrate experimentally that mechanisms permitting conversion of biliverdin to bilirubin do, in fact, function *in vivo*(4,5). In view of recent reports describing an enzyme system which facilitates conversion of hemoglobin-haptoglobin to a biliverdin precursor(6), characterization of the physiologic relationship between biliverdin and bilirubin has become increasingly important. For this purpose, C^{14}-labeled biliverdin was prepared from bilirubin-C^{14}, and investigations of its metabolism and excretion were performed.

Materials and methods. Bilirubin-C^{14}(7) dispersed in methanol was refluxed for 30 minutes in the presence of ferric chloride and hydrochloric acid. The reaction mixture was neutralized, and the green pigment was extracted successively into ethyl ether, 1 N hydrochloric acid, and chloroform. The final solution was washed with water and dried by filtration through chloroform-moistened paper(8). Biliverdin-C^{14} was crystallized by displacement of chloroform with methanol; recrystallization was accomplished by dissolving the primary crystals in hot methanol and permitting the solution to cool at —5°C. The crystals were stored in the dark *in vacuo* until immediately prior to use.

In order to establish purity of the bili-

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