THE ROLE OF A NATURAL ANTIBODY IN THE REJECTION OF MOUSE TUMOR CELLS BY THE CHICK EMBRYO

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Plates 7 and 8

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When cells of the Krebs ascites tumor of mice are injected intravenously into 10 day chick embryos, the tumor cells regularly invade the internal organs of the embryo, where they grow as multiple tumor islands (1), as do certain other heterologous tumors (17).

Beginning about the 17th day of embryonic development, a destructive process begins which is recognized in microscopic section by degeneration of cells in the tumor islands. This is similar to the fate described long ago of certain foreign tumors grown on the chorioallantoic membrane (2, 18).

The present report concerns the mechanism of destruction of the tumor cells. It appears that a serum protein with the properties of a natural antibody to mouse cells appears normally in the blood of chick embryos during the later stages of embryonic development and is responsible for destruction of the tumor. This antibody is a part of the serum gamma globulin, which first appears about this time and increases in concentration to adulthood. The inability of the mouse tumor cells to grow in hatched chicks is believed to be due to the presence of this natural antibody.

Methods

The chickens and eggs used in this investigation were White Leghorn and the mice Swiss albino.

The tumor cells were of the Krebs-2 ascites tumor of mice,[1] which is of hybrid origin. According to Klein and Klein (4) approximately 17 per cent of the cells in the ascites fluid are non-tumorous, but the cell numbers reported in the present work are uncorrected counts of all cells (other than red cells).

For injecting embryos, ascitic fluid was diluted to the correct cell concentration with physiological saline and given in a volume of 0.03 ml. intravenously, as described by Beveridge and Burnet (23).

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[1] Kindly supplied by Dr. Mark Woods, National Institutes of Health, Bethesda.

Eggs were incubated in a forced draft incubator with mean temperature through the on-off cycle of 38.4°.[2]

Agglutination tests for antibody to the tumor cells were carried out as follows: A freshly drawn sample of ascitic fluid was centrifuged and the cells washed 3 times with phosphate buffered saline [0.14 M NaCl + 0.06 M phosphate buffer pH 7.3 (P.B.S.)]. Centrifugations were performed at very low speed, such that any red cells present sedimented on top of the tumor cells and could be aspirated with a fine tipped pipette. The washed tumor cells were suspended to a concentration of 1.5 per cent by volume of packed cells. To each agglutination tube were added 0.25 ml. of diluted serum or protein fraction and 0.05 ml. of cell suspension. The tubes were gently shaken in a Kahn shaker for 30 minutes at room temperature. Marked coarsening of the sediment or pellet formation indicated agglutination.

Serum protein fractionations were carried out by the slow addition while stirring of 30 per cent or 40 per cent sodium sulfate solution to the whole serum, at room temperature. The precipitated protein was suspended and washed once with sodium sulfate of the final concentration, and then dialyzed against a large volume of P.B.S.

Total serum protein was precipitated with an equal volume of 10 per cent trichloracetic acid, washed with 5 per cent trichloracetic acid, and determined as Kjeldahl nitrogen × 6.25.

EXPERIMENTAL

(1) The Fate of the Mouse Tumor Growing in the Chick Embryo Depends on the Number of Cells Introduced.—When about 40,000 cells of the ascites tumor were injected intravenously into 10 or 11 day chick embryos, the tumor cells invaded the embryonic liver, brain, and heart, and could be seen some days later growing in separated tumor islands. Occasionally by the 17th day of incubation, and nearly always by the 18th day, degeneration of tumor cells was seen in microscopic sections (Fig. 1). The persistence at this time of viable tumor cells could be determined in the following way:—

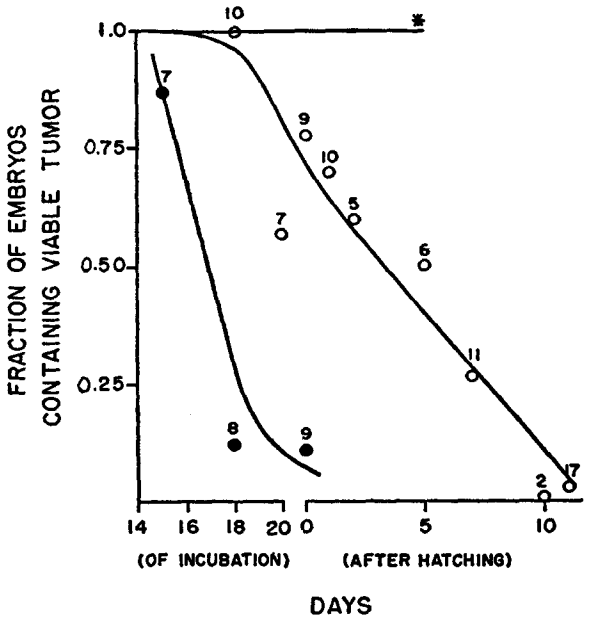
The embryo or chick was killed, and half of its liver was passed through a 30 mesh stainless steel screen. The minced liver was then suspended in physiological saline and divided equally among three 15 gm. mice by intraperitoneal injection. If the ascites tumor developed in at least one of the three mice injected, the embryo was regarded as containing viable tumor cells.

The results of an experiment to determine the relationship between persistence of the cells and the number of cells injected is shown in Text-fig. 1. When a small number of tumor cells was given intravenously on the 11th day of incubation, virtually all the embryos were shown to contain viable cells on the 15th day, but by the 18th day most were cleared of the tumor (lower curve). If a larger number of cells was injected on the 10th or 11th day, viable tumor was still present by the 18th day but was eliminated over the next 2 weeks (middle curve). Virtually none of the chicks succeeded in eliminating

[2] As incubation proceeds, embryonic body temperature rises, reaching 39.5° before hatching. Since the rectal temperature of the mice was only about 36.8°, it is worthy of note that the tumor cells were able to grow at considerably above their physiological temperature. However, increasing the incubator air temperature to 39.5° definitely impaired growth of the mouse tumor.

the largest quantity of tumor given (upper curve); nearly all of them were killed by it either in embryonic life or during the 1st week after hatching.

Chicks which had received approximately 10^5 cells usually hatched with tumor nodules visible in the gross in the liver and heart, but these nodules usually disappeared during the following week. A small proportion of such



TEXT-FIG. 1. Disappearance of viable tumor cells from the livers of chicks injected during embryonic life with tumor cells as follows:

Bottom curve—3.0×10^3 cells—11th day.

Middle curve—4.0×10^4 to 1.0×10^5 cells—10th or 11th day.

Upper curve—6.0×10^5 cells injected 10th day. Asterisk indicates termination of experiment, when only 6 survivors remained of 104 embryos injected. Those birds which survived as late as the 20th day of incubation showed gross tumor nodularity. No viability tests were performed.

Above each point is given the number of embryos or chicks sampled.

chicks failed to eliminate the tumor; some hatched in an obviously weak condition and died of the tumor within a few days, while others appeared healthy at hatching and only days later began to appear ill. Only infrequently were tumor growth and tumor-destroying action so nicely balanced that chick and tumor survived together for any considerable time after hatching. One chick lived 28 days before dying of the tumor, the longest such period observed. Occasionally, the tumor growth in hatched chicks was as extensive as that shown in Fig. 2.

By the time of hatching, chicks which had not received the tumor during

embryonic life were totally resistant to it. Several attempts were made to grow the tumor by injecting large numbers of cells by different routes, but repeated subcutaneous or intraperitoneal injections of 10^8 or more tumor cells were followed by disappearance of all viable tumor cells within about 3 days.

Tumor cells which had grown in a chick embryo through hatching preserved their normal pathogenicity for the mouse and their inability to grow when

TABLE I

Toxicity of Normal Chick Serum to Mouse Ascites Tumor Cells

Cell suspensions added to 0.2 ml. of	No. of cells per mouse						
	1.8×10^5	3.7×10^4	7.0×10^3	1.4×10^3	2.8×10^2	56	11
0.9 per cent NaCl	7	11	15	—	—	—	—
Pooled serum or plasma of:							
Mice	7	11	11	13	15	19	19
16 day chick embryos	7	9	13	13	13	19	19
2 " chicks	7	9	11	13	15	—	27
6 " "	7	9	13	13	17	—	—
11 " "	7	9	11	13	17	19	19
15 " "	7	11	15	17	25	—	21
15 " "immune" chicks*	7	9	15	17	17	—	21
21 day "immune" chicks*	7	11	19	21	—	—	—
4 mo. normal chicks	7	15	—	—	—	—	—

A freshly drawn sample of mouse ascitic fluid was diluted and counted in a hemocytometer chamber. Successive dilutions were then made to appropriate cell concentrations, and four times the indicated number of cells in 0.4 ml. of phosphate buffered saline were added to 0.2 ml. of serum to be tested. The suspensions were incubated at 37° for 3½ hours, with occasional agitation, and the contents of each tube then divided among four 15 gm. mice by intraperitoneal injection.

The numbers in the table indicate the number of days following injection when visible ascites developed in the 1st of the 4 mice of a given group. Those below the step-like line indicate delayed onset of ascites. Absence of a number indicates that none of the mice developed ascites (observed every 2nd day for 31 days).

* Chicks which had received a single injection of washed tumor cells from 0.2 ml. of ascitic fluid 4 days after hatching.

transferred directly to other chicks, indicating no obvious adaptive changes in the tumor for growth in the chick, such as have been shown to occur in mouse tumor cells growing in a mouse embryo of foreign strain (21).

(2) *Tumor Cells Lose Their Viability following Incubation in Chick Serum.*— When a suspension of Krebs tumor cells is injected into mice, the volume of ascitic fluid increases linearly with increase in tumor cell number (3). The time required for visible ascites to develop depends, other experimental conditions being constant, upon the number of viable cells injected. This time was there-

fore used to estimate the number of viable cells in a suspension containing mixed viable and non-viable cells.

When a suspension of the tumor cells was incubated *in vitro* for a few hours in the serum of normal adult chickens, there was considerable loss of viability, as indicated either by complete inability of the tumor cells to produce ascites in the indicator mice, or delay in the onset of the ascites. Since agglutinins for mouse cells (including the ascites tumor) begin to be detectable in the blood of normal chickens after they are a few weeks old (22, 1), it appeared that the loss of tumor viability may have been due to the action of a natural antibody. However, it was found that even the serum or plasma of young chicks in which no agglutinins for the tumor cells could be demonstrated, reduced the viability of a suspension of the tumor cells.[3]

TABLE II

Inability of Embryo Serum to Sustain Cell Viability in Presence of Chick Serum

Cell suspension incubated in	No. of cells per mouse			
	270	90	30	10
0.2 ml. 16 day embryo serum + 0.2 ml. 0.9 per cent NaCl	16	16	18	—
0.2 ml. 15 day chick serum + 0.2 ml. 0.9 per cent NaCl	—	—	—	—
0.2 ml. 15 day chick serum + 0.2 ml. 16 day embryo serum	—	21	—	—

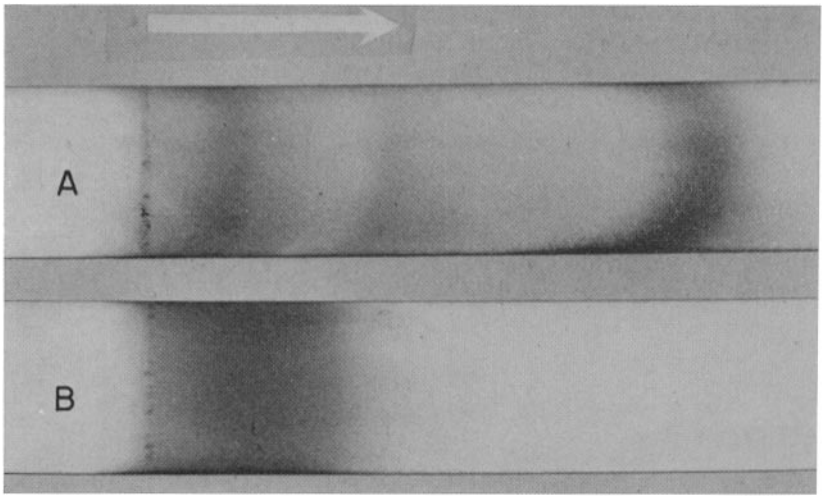
Conditions as for Table I except that incubation was for 7 hours at 37°.

Table I summarizes the experimental results. When tumor cells were incubated in mouse plasma, or 16 day chick embryo plasma, they survived and produced ascites in the indicator mice in a period of time consistent with the number of cells injected. Tumor cells were rendered non-viable by incubation in the sera of chicks as young as 2 days of age, but only when the ratio of cell number to serum volume is small. It appears that the older the chicken from which the serum was obtained, the greater the number of cells affected. The serum from the oldest of the animals examined (4 months) was the only one which produced some visible agglutination of the cell suspension, and it had the greatest effect on the viability of the tumor cells. The injection of immunizing quantities of the tumor cells a few days after hatching did not appreciably affect the lethality of the serum of the birds at the ages of 15 and 21 days.

[3] It is not certain that the cells are rendered actually non-viable during the *in vitro* incubation; it is possible that they become so only upon injection into the mice, where attack by phagocytic cells is possible. However, immune sera are known to kill tissue cells *in vitro* without the assistance of phagocytic cells (13).

(3) Loss of Viability of the Tumor Cells Incubated in Chick Serum Is Due to a Toxic Substance in the Serum.— When the ascites tumor cells are incubated for several hours in isotonic saline, their viability is appreciably reduced, unless the cell concentration is quite high (Table I). This is probably due to failure of the medium to provide nutrients essential for cell survival.

It might be argued that the loss of viability of cells suspended in the serum from chicks is similarly due to absence in the serum of essential nutrients which are present in the blood of chick embryos and of mice. This possibility was



TEXT-FIG. 2. Paper electrophoretic patterns of 2 day old chick serum (*A*) and a concentrated gamma globulin fraction obtained from it (*B*). (Spinco model R cell, barbital buffer $\mu = 0.075$, pH 8.6, 1.5 milliamperes per strip, 3 cm. wide, for 20 hours, room temperature). The protein bands are stained as described in reference 5.

tested by incubating the cells in a mixture containing serum from 16 day embryos (which maintains viability) together with serum from chicks 15 days after hatching. The result is shown in Table II.

As judged by ascites production in indicator mice, the tumor cells survived well at several cell concentrations through a period of incubation in 16 day embryo serum. At the same concentrations cell viability was greatly impaired when the incubation medium contained 15 day chick serum in addition to the embryo serum. Evidently the effect of chick serum on the tumor cells is due to a toxic substance present in the serum, and not to absence of an essential nutrient.

(4) Agglutinins for the Mouse Tumor Belong to the Gamma Globulin Fraction of Chicken Serum and These Agglutinins Are Present in Low Concentration from

Very Early Life.—When chicken serum is subjected to electrophoresis on paper, by the method of Durrum (5), the serum proteins are resolved into three fractions, corresponding approximately in mobility to mammalian albumin, beta globulin, and gamma globulin. These 3 fractions may also be obtained from serum by the addition of sodium sulfate, the gamma globulins being precipitated between 0 and 15 per cent Na_2SO_4, beta globulin between 15 and 26 per cent, albumin remaining in the supernatant (6). The electrophoretic pattern of serum of 2-day chick, and a concentrated gamma globulin fraction obtained from it by salt precipitation, are shown in Text-fig. 2.

A sample of pooled normal serum from 6 to 8 month old chickens was salt-fractionated, and the various fractions tested for ability to agglutinate the ascites cells. Twofold dilutions of the protein solutions were carried out and the concentration of the most dilute protein solution capable of agglutinating the cells determined. The results, shown in Table III, indicate that virtually all the agglutinating power of the serum is contained in the gamma globulin fraction.

TABLE III

Ascites Tumor Agglutinins in Chicken Serum Protein Fractions

Serum fraction	Protein concentration	Agglutination titer (by dilution)	Minimum protein concentration to produce agglutination
	mg./ml.		*mg./ml.*
Whole serum	39.4	1:16	2.5
Gamma globulin	26.2	1:64	0.39
Beta globulin	9.9	1:1	9.9
Albumin	6.1	None	—

Gamma globulin fractions prepared from sera of 15 day chicks, 2 day chicks, and even 20 day embryos produced agglutination of the ascites cells, though only when present in high concentrations. From 3 to 8 mg./ml. of protein per 0.3 ml. test volume were necessary to produce minimal agglutination.

(5) *Agglutinins to Mouse Erythrocytes Similarly Appear in the Chick Serum during Embryonic Life.*—Antibodies to a wide variety of animal red cells are known to occur normally in the blood of the chicken. It has been stated that these antibodies appear for the first time some weeks after hatching, and increase in concentration thereafter (22), but in fact, the earliest time at which such antibodies are found depends upon the sensitivity of the method used for their detection.

It has recently been discovered that the addition of dextran to a red-cell antibody system may greatly increase the degree of agglutination produced by a given antibody concentration (26). The blood of chick embryos of various

ages was therefore examined for the presence of hemagglutinins for mouse red cells, using dextran to increase sensitivity. The degree of hemagglutination of a red cell suspension was estimated quantitatively by determining the fraction of the cells which remains unagglutinated, along the lines described by Filitti-Wurmser and Jacquot-Armand (24), Wilkie and Becker (25), and Karel and Franklin (28). The results are shown in Table IV.

TABLE IV

Agglutination of Mouse Erythrocytes by Embryo Chick Sera

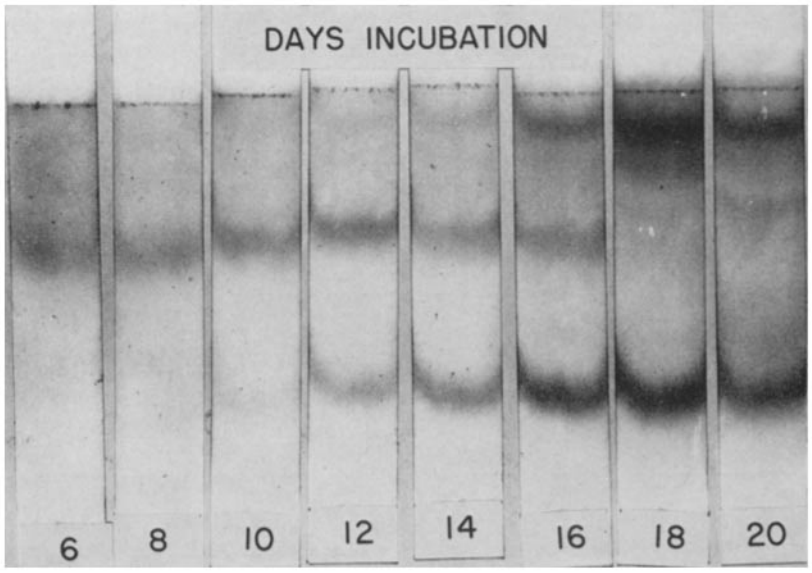
Total No. of erythrocytes/mm.³	2.0×10^8	2.0×10^8	1.0×10^8	5.0×10^8
Final dextran concentration	0	2.4 per cent	2.4 per cent	2.4 per cent
Pooled serum from	Per cent free cells			
11 day embryo	96	87	92	61
15 " "	95	103	90	74
17 " "	89	77*	57	44
19 " "	83	29*	14.7*	17.2
21 " "	90	15.1*	3.9*	5.3
3 " chick	77	10.5*	4.6*	4.8
44 " "	4.2*	3.5*	1.5*	0.33*

All sera to be tested were heated to 56° for 30 minutes. A given number of red cells was added in a volume of 0.01 or 0.02 ml. to 1.0 ml. of solution containing the following components: 6 per cent dextran (Glaxo), 0.4 ml.; P.B.S. 0.5 ml.; and test serum 0.1 ml. (When dextran was omitted, the same volume of P.B.S. was substituted.) The suspensions, in stoppered tubes, were turned end-on-end 10 times per minute at 4° for 3 hours. At the end of this period, an aliquot of the suspension was placed in a hemocytometer chamber and after about 3 minutes, when the cells had settled, the slide was photomicrographed and the free cells counted from the negative. (Free cells were taken arbitrarily to include those touching not more than one other cell). Each aliquot was counted on both sides of the counting chamber; when possible, over 400 free cells were counted. Total cell counts were performed on samples containing serum from embryos of 11 to 15 days' incubation, and averaged. In the table are listed the percentages of cells remaining unagglutinated.

* Fewer than 200 unagglutinated cells counted.

In the absence of dextran, agglutination is produced by the sera of birds only some days after hatching. This is similar to what has been reported for hemagglutinins to other foreign red cells (22). However, in the presence of dextran, agglutinins are readily demonstrable by the 17th day of embryonic life and increase rapidly in concentration thereafter. Such agglutinins could always be removed from the sera by adsorption onto red cells. At higher cell densities, the proportion of free cells is somewhat reduced even in the presence of sera from younger embryos, but this is due simply to the statistically greater chance of a cell coming to rest in contact with others, rather than to agglutination.

(6) *Appearance of Gamma Globulin in the Blood of the Developing Chick Embryo.*—Since the gamma globulin fraction of chick blood seems to contain antimouse cell agglutinins even during embryonic life, it was important to determine the time when the gamma globulins develop as a serum constituent. Sera of chick embryos of various ages were therefore subjected to paper electrophoresis, giving the patterns shown in Text-fig. 3. Beta globulin is the only protein detectable in the serum of the youngest embryos examined (6 days). Subsequently, albumin and gamma globulin make their appearance, and increase in concentration up to the time of hatching. Though it is visible in the



TEXT-FIG. 3. Paper electrophoretic patterns of serum proteins of embryos at various ages Conditions as in Text-fig. 2.

pattern of earlier sera, the gamma globulin does not reach high concentrations until about the 16th day of embryonic life.

These results are consistent with the data of Moore *et al.* (7) who have determined by moving boundary electrophoresis the relative concentrations of the serum proteins at various ages in embryonic life. From the total protein concentration of embryonic sera at these times and the data of Moore *et al.*, the absolute concentrations of their protein fractions were calculated. The results are shown on Table V. It appears that between the 13th and 17th day of incubation the albumin concentration undergoes a moderate increase and the beta globulin a decrease. Fractions 4 and 5, the slowest moving, which apparently travel together on paper as the "gamma" fraction, undergo a combined

increase of ninefold between the 13th and 17th day, and several fold more thereafter. Fraction 2, which is not located as a separate fraction on paper also undergoes a sharp increase in concentration toward the end of the incubation period, but since it is a faster moving fraction than the beta globulins, it is unlikely that it contains agglutinins for the mouse cells. It appears that fraction 4 or 5 or both, containing anti-mouse cell antibodies, reach appreciable concentrations in blood shortly before mouse cells growing in the embryo begin to undergo destruction.

(7) *Chick Gamma Globulin is Shown to Kill the Mouse Tumor Growing in the Chick Embryo.*—

In order to determine whether chick gamma globulin is capable of killing the mouse tumor under the conditions in which the tumor grows in the embryo (*i.e.* as small solid tumors), 10 day embryos were injected with the tumor, at

TABLE V

Age of embryo or chick	Total protein concentration	Concentration of serum protein fractions calculated from D. H. Moore *et al.* (7), *mg./ml.*					
		1 (Albumin)	2	3 (Beta globulin)	4	5	4 + 5 (Gamma globulin)
	mg./ml.						
10 day embryo	6.25	1.55		4.37	.32	0	0.32
13 " "	7.27	4.36		2.54	.36	0	0.36
17 " "	11.9	6.55	1.19	0.95	1.55	1.66	3.21
3 " chick	26.1	9.15	6.53	2.61	6.53	1.30	7.83
"Adult"	54.0*	21.6	13.5	2.70	10.8	5.40	16.2

* From the data of L. W. Brandt *et al.* (6)

various times thereafter given gamma globulin from older chicks, and their livers examined on the 14th and 15th day for the presence of viable cells.

The gamma globulins came from two groups of animals: (*a*) 7 to 10 week old chicks which had received several immunizing injections of ascites cells (immune gamma globulin) and (*b*) normal 15 day old chicks which had never been exposed to mouse cells. The results of this experiment are shown in Table VI.

The livers of embryos which had been injected with the indicated quantities of immune gamma globulin were found to be uniformly free of viable tumor cells. Similar quantities of 15 day chick gamma globulin were very much less effective in killing the tumor cells but were not without effect, as judged from the smaller fraction of embryos containing tumor cells (as compared with the controls), and the fact that those indicator mice which became positive for tumor generally did so later than those of the control embryos.

In another experiment, the effect of gamma globulin from normal adult

chickens was tested for its lethality to tumor cells in embryos using a different criterion for cell death. It was shown earlier that when 10^5 tumor cells are injected into 10 day embryos, nearly all those embryos which survive to the 21st day of incubation contain viable tumor cells. These are sufficiently numerous to produce nodules visible in the gross on the surface of the heart and liver.

A group of 10 day embryos was therefore injected with 10^5 tumor cells. On the 12th day, about half of the survivors were injected with normal hen gamma globulin. On the 21st day, all embryos were examined for the presence of tumor

TABLE VI

Effect of Chick Gamma Globulin on Mouse Ascites Tumor Growing in the Chick Embryo

Treatment of embryo	No. of embryos	
	Treated	Found to contain viable tumor cells
0.9 per cent NaCl 0.15 ml. 11th day	18	10
Immune gamma globulin (7–10 wk. chicks)		
1.90 mg. 10th day	6	0
1.90 " 11th "	18	0
1.90 " 13th "	5	0
0.68 " 11th "	18	0
Normal gamma globulin (15 day chicks)		
0.68 mg. 11th day	17	5
1.13 " 13th "	6	2

All embryos received 6×10^3 ascites cells intravenously on the 10th day of incubation. Intravenous injections of gamma globulin in about 0.15 ml. followed at the indicated times. On the 14th and 15th day, the embryos were killed, the left lobe of the liver was minced, and divided among 3 mice intraperitoneally. If ascites developed in at least one mouse, the embryo is indicated to have contained viable tumor cells.

The control group of embryos produced less than the expected fraction of positives. It is felt that this was due to the use as indicators throughout this experiment of mice having some greater resistance to the tumor than those of previous experiments.[4] However, this does not greatly impair the comparison.

nodules on their hearts and livers. It was found (Table VII) that visible tumor nodules were present in nearly all untreated embryos, but were absent from the embryos injected with the gamma globulin. Most of the organs lacking gross nodules were found on microscopic examination to be totally free of visible tumor cells. It appears that the gamma globulin fraction of normal hen serum uniformly prevented the growth of the tumor.

The lethality of the chicken gamma globulin for the mouse tumor cells is probably dependent on the fact that no other mouse tissue is present in the

[4] This was associated with a change in the source of supply of mice. It was found possible in subsequent experiments to use these mice for the detection of small numbers of tumor cells if they were injected as weanlings weighing about 7 gm.

embryo to compete with the tumor cells for the antibody. It is not very likely that chicken antiserum injected into mice bearing the tumor would be as effective in killing tumor cells. However, certain mouse tumors growing in their native species have been reported by Kidd (27) to be killed by repeated injection of normal guinea pig serum; (see also references 29 and 14).

TABLE VII

Effect of Normal Hen Gamma Globulin on Ascites Tumor Growing in the Chick Embryo

	Untreated		Gamma globulin injected	
	Liver	Heart	Liver	Heart
Embryos living when opened on 21st day	+	+++	0	0
	0	0	0	0
	+	++	0	0
	0	+	0	0
	+	+++	0	0
	+	+	0	0
	++	+++	0	0
	0	0		
	++	+++		
Embryos living on 20th day, but found to be dead when opened on 21st day	+	++	0	0
	0	++	0	0
	+	++	0	±
	++	+++	±	±
	0	+		
	+++	++		
	+	+++		

Forty-eight chick embryos on the 10th day of embryonic life were successfully injected with 1.0×10^5 tumor cells intravenously. On the 12th day, of the 45 survivors, 18 were successfully injected with 0.1 ml. of normal adult hen gamma globulin solution (5.3 mg. of protein), leaving 18 controls uninjected. By the 20th day, 11 gamma globulin-injected and 16 controls remained living. All eggs were opened on the 21st day (some embryos were in the process of hatching) and the surfaces of the liver and heart examined for nodules of tumor.

0 indicates absence of any nodularity; ± indicates doubtful nodularity; +, ++, and +++ indicate degrees of nodularity.

DISCUSSION

The body of the chick embryo provides during the early stages of its incubation a favorable environment for the growth of the Krebs ascites mouse tumor. Later in the incubation period, antibodies to mouse cells normally appear in the blood of the chick embryo, and the tumor cells are then all killed, unless their number is very large.

Apparently, not all foreign tissues growing in the chick embryo begin to undergo degeneration before the time of hatching (16–19). Whether this occurs in a particular case may depend on several factors:—

(*a*) The species from which the tissue is taken. The chick is known to form natural agglutinins to the red cells of a wide variety of species in addition to the mouse but not in equal concentrations to all (22). Probably sensitive enough methods would show that these antibodies too, begin to appear in embryonic life, and that they react with living tissue cells as well as red cells.

(*b*) Whether the tissue is growing within the body of the embryo, on the chorioallantoic membrane, or in the yolk sac.

(*c*) The quantity of foreign tissue involved. The larger the quantity, the more resistant the tissue to a fixed quantity of antibody.

(*d*) The temperature of incubation of the eggs.

It is doubtful that the occasional survival and continued growth of the mouse tumor in the chick after hatching constitutes an example of immune tolerance to foreign cells, for we have no evidence that there is involved a specific suppression of the immune response—one of the criteria of Billingham *et al.* (15) for immune tolerance. The survival of the tumor may simply be due to the fact that the normal rate of antibody production is not sufficient to kill the large number of cells in the already established tumor. While the tumor is present, antibodies may not be found in the circulation, but once the tumor is eliminated it has been shown that the birds become some weeks later, quite normal in their ability to make antibody to the tumor cells (1).

Because of its role in the destruction of foreign cells the origin of embryonic gamma globulin is a matter of interest. There are only two possibilities: (*a*) it may be synthesized by the embryo itself, in which case it would remain to be explained how agglutinins to mouse cells could be formed in the absence of mouse antigens; (*b*) it may be transferred from the serum of the maternal hen to the yolk of the egg and absorbed intact into the embryonic serum. Since the adult hen is known to contain natural antibodies to mouse cells (1, 22), these antibodies would be included in the transferred gamma globulin.

Transmission to the egg of antibodies induced in response to foreign antigens in the hen is a well known phenomenon. An appreciable fraction of the proteins of egg yolk consists of globulins similar electrophoretically to the gamma globulins of hen's serum (8, 9). Antibodies induced in the hen to Newcastle disease virus, diphtheria toxin, and fowl plague are ultimately transmitted *via* yolk to the embryo (10–12). In the case of anti-Newcastle disease virus, Brandley *et al.* demonstrated that the antibodies appeared in embryonic blood only during the last quarter of the incubation period (10). The absence of anti-mouse antibodies in embryonic serum early in the incubation period is not therefore incompatible with a maternal origin of the antibodies which appear later. There are however two reasons for suspecting that the yolk globulins of maternal origin may not be the only source of embryonic anti-mouse cell antibody. The first is that whereas antiviral antibodies passively acquired by embryos decrease quickly in concentration after hatching, and disappear from the blood within a few weeks.(10), anti-mouse cell antibody

increases progressively in concentration from the time of hatching on, suggesting that it is formed by the chick itself from a very early age. The second is that foreign tumors including those of the mouse can be cultured in the embryonic yolk sac early in the incubation period (20) when all the antibodies of maternal origin are presumably still in the yolk. This question deserves further investigation.

SUMMARY

Cells of the Krebs ascites tumor of mice grow well in the body of the chick embryo until about the 17th day of incubation, when degeneration of the tumor can be seen in tissue sections and viable tumor cells begin to disappear from the internal organs of the embryo.

This death of tumor cells follows the appearance in the chick embryo of serum gamma globulins. Among these are antibodies which can agglutinate the tumor cells *in vitro*, and destroy their viability. These antibodies occur in the blood without the introduction of any foreign antigen. Their possible origin is discussed.

Small numbers of mouse tumor cells growing in the chick embryo are completely eliminated shortly after the time when antibodies ordinarily become detectable. When the number of cells present is larger, viable cells persist longer, and at still higher cell numbers, the embryo or chick is unable to eliminate the tumor, and is itself killed by it.

Gamma globulins of older birds injected into young chick embryos bearing growing tumor clear the embryonic organs of viable tumor cells.

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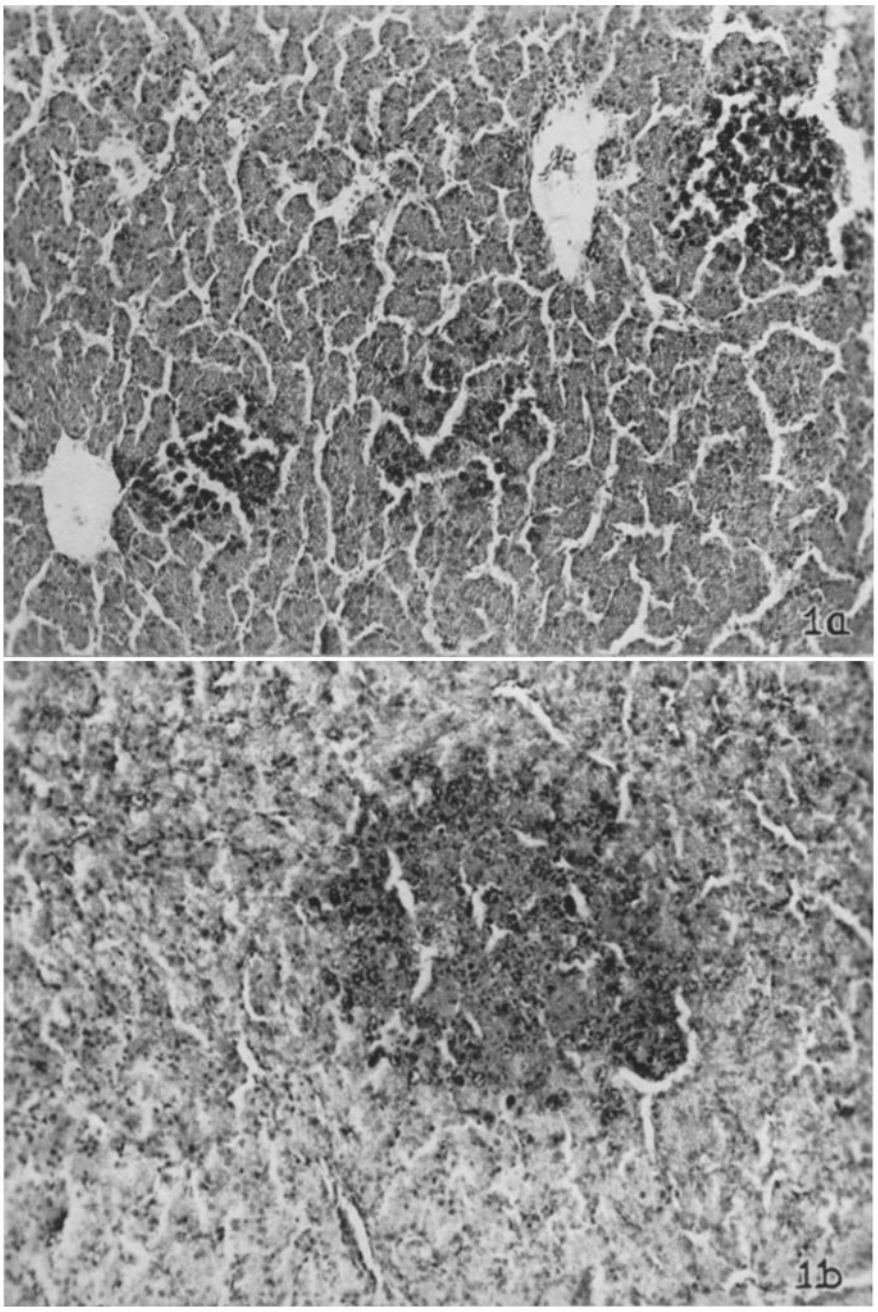
EXPLANATION OF PLATES

PLATE 7

FIGS. 1 *a* and 1 *b*. Photomicrographs of embryonic liver containing islands of mouse tumor cells. Hematoxylin and eosin.

FIG. 1 *a*. 16 day embryo. × 120.

FIG. 1 *b*. 18 day embryo. The tumor cells show degenerative changes. × 144.

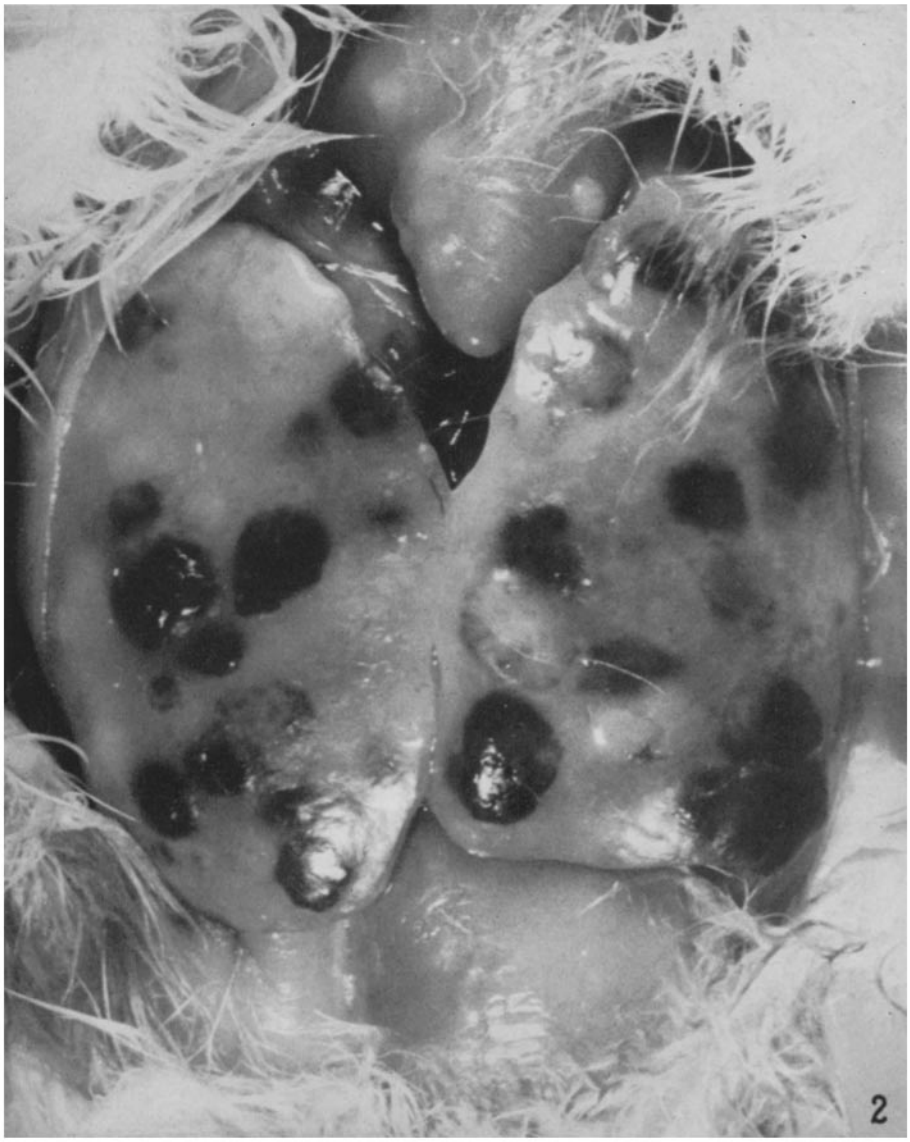


(Green and Lorincz: Natural antibody in tumor rejection)

PLATE 8

FIG. 2. Progressive growth and killing of the hatched chick by the mouse ascites tumor.

Photograph of the opened abdomen of a 6 day old chick which had received 1.0×10^5 tumor cells on the 10th day of embryonic life. The greatly enlarged liver and heart are covered with tumor nodules. Microscopic examination confirmed that the bulk of the liver mass consisted of tumor cells. Approximately 2 $\times$ actual size.



(Green and Lorincz: Natural antibody in tumor rejection)