through which electrical contact was made, and was approximately 50 μm above the embryo surface. The embryo was not touched by the electrode. The electrodes were glass micropipettes with tip diameters of 10 to 15 μm, filled with either Ringer solution alone (controls) or Ringer solution plus $10^{-3}M$ cyclic AMP solution (experimentals). The tips were sealed with molten agar to prevent loss of electrolyte by passive flow. A current of 40 na was passed through chlorinated silver wires placed in the electrodes. A return path was provided by a chlorinated wire in the agar supporting each embryo. Current was individually monitored with an electrometer (*13*). After incubation at 38°C for 18 hours, the final state of each preparation was photographed and the embryos were fixed and stained for subsequent histological examination. Thus a pair of "before and after" photographs was obtained for each embryo and scored for effects of stimulation.

The results for 298 embryos are shown in Table 1. The top figure of each pair represents the total sample; the lower figure represents the score obtained when preparations with electrodes which had lost electrical contact overnight, or in which the embryo had broken up or detached from the vitelline membrane sufficiently to prevent scoring, were removed from the sample. Each preparation was scored for bending of the embryonic axis toward the electrode and for attraction of cells to the electrode; both effects were confirmed by histological examination and were thus not destroyed by fixation. Significant differences ($P < .01$) were found between experimentals and controls by the two criteria. The number of rejected embryos was similar for experimentals (70) and for controls (61). Both experimentals and controls showed delays of 1 or 2 stages (up to 5 hours) in development as a result of the experimental procedure, but there was no difference between experimentals and controls in this respect.

In addition, we made time-lapse films which confirm these observations and show that development was continuous, although biased, during the experiments. Figure 1C shows a photograph of an intermediate stage of such a filmed embryo stimulated with a microelectrode containing $10^{-3}M$ cyclic AMP released continuously by a current of 10 na. The electrode was switched off after 5 hours of filming and on again after a delay of 5 hours. The axis progressed anteriorly while the electrode was off but laterally while it was on, leading to the distinctively S-shaped primitive streak. We found that effects of stimulation were lost at electrode concentrations below approximately $10^{-8}M$ for axis bending and $10^{-9}M$ for cell attraction.

Table 1. The results of stimulation of early embryos with Ringer solution (controls) and Ringer solution plus $10^{-3}M$ cyclic AMP (experimentals). The top figure in each category shows the fraction of the total number of controls (150) and experimentals (148) scored as responding. The bottom figure in each category shows the fraction after failures were rejected from the sample, as described in text.

Effect	Fraction responding	
	Controls	Experimentals
Axis bending		
Entire sample	0.05	0.39
Failures deleted	0.08	0.76
Cell attraction		
Entire sample	0.08	0.31
Failures deleted	0.11	0.43

We tried similar experiments with cyclic guanosine, inosine, and uridine monophosphates; adenosine 5′-monophosphate; adenosine; serotonin; and L-glutamine. None showed comparable effects. We also incubated embryos with cyclic AMP phosphodiesterase, which slowed and at very high activities stopped development, and with acetylcholinesterase, which had no effect.

These experiments thus show that extracellular cyclic AMP signals can divert the axis of early chick embryos and can attract cells on the ventral surface. They imply that a systematic search for a cyclic AMP signaling mechanism within the undisturbed embryo might be profitable; we have undertaken this (*7*). To this end it has already been shown (*14*) that there are regional differences in cyclic AMP and phosphodiesterase content in chick embryos at stages 5 and 6. One interpretation of these findings and our own results is that an extracellular cyclic AMP signal may be used in the control of early chick development. If this were so, cyclic AMP might correspond to the heat-stable (animalizing) morphogen postulated by others and its phosphodiesterase to the heat-labile (vegetalizing) antagonist also postulated (*15*). Such an identification would resolve many of the inconsistencies arising from grafts of inducing regions into host embryos, but much more knowledge is needed before this interpretation can become more than a mere speculation.

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Rous Sarcomas in Chickens: Enhanced Growth Coexisting with Concomitant Immunity

Abstract. *Chickens bearing Rous sarcoma virus–induced tumors in one wing did not develop new tumors when subsequently inoculated with Rous sarcoma virus in the other wing. However, the second inoculation of Rous sarcoma virus caused accelerated growth of the established tumors. This phenomenon was found to be bursa-dependent. Paradoxically, established tumors in bursectomized chickens grew at a diminished rate if the chickens were reinoculated with Rous sarcoma virus.*

Animals bearing large progressively growing tumors are resistant to the development of new tumors following challenge with syngeneic or autochthonous tumor cells of the same type (*1*). This resistance to tumor growth has been attributed to an acquired immunity (concomitant immunity) to tumor antigens, and has been postulated to be a determining factor in controlling metastases

(*1*). Recent studies by Gyles *et al.* (*2*) show that genetically susceptible chickens possessing Rous sarcoma virus (RSV)-induced tumors may be resistant to the induction of new tumors by RSV. Similar findings with rats bearing Rous sarcomas have been reported (*3*).

In this report we confirm the observation of Gyles *et al.* (*2*) and present findings which show that (i) a second inoculation of RSV, performed to demonstrate concomitant immunity, results in a marked increase in growth rate of tumors induced by RSV 9 days earlier; (ii) the increased growth rate of previously induced tumors can be demonstrated only in chickens with an intact bursa of Fabricius even though bursectomy does not influence the expression of concomitant immunity; and (iii) previously induced tumors grow more slowly in bursectomized birds given a second inoculation of RSV 9 days after the first.

For these studies, chickens from an inbred line susceptible to the induction of Rous sarcoma (line G-B1) were inoculated into the wing web with Schmidt-Ruppin RSV (subgroup B). Either 1800 or 3600 focus forming units (FFU) of virus in 0.2 ml of tris-buffered diluent were used. The initial inoculation of RSV was made into the left wing web and challenge was made into the opposite wing web at the same time (day 0) or at specific intervals thereafter. In one experiment, newly hatched chicks were surgically bursectomized and given cyclophosphamide (4 mg per bird) on the same day and 2 days later. Cyclophosphamide was administered because surgical bursectomy alone is not completely effective in lowering levels of immunoglobulins or in suppressing antibody formation (*4*). Marked hypogammaglobulinemia was documented in these birds by means of Ouchterlony tests 5 weeks after bursectomy. Tumor development was assessed at 2- to 3-day intervals by measurement with calipers. Scores of tumor size were based on area of wing web involvement according to the formula for an ellipse.

Table 1. The proportion of G-B1 chickens developing tumors in left and right wings after initial RSV inoculation in the left wing web. Surviving birds were monitored for 54 days after the first RSV inoculation.

Number of birds	Interval between RSV inoculations (days)	Proportion of birds that developed tumors in	
		Left wing	Right wing
	Experiment 1		
3	0	3/3	3/3
3	8	3/3	0/3
3	18	3/3	0/3
3*			3/3
	Experiment 2		
5	0	5/5	5/5
5	8	5/5	0/5
5	18	5/5	0/5
5*			5/5

*Age-matched control birds that received an initial RSV inoculation in the right wing only on the same day that the two experimental groups of birds received the second RSV inoculation on day 18.

To determine the effects of prior inoculation of RSV on the ability of a subsequent challenge with the same virus to induce tumors, we performed two separate experiments (Table 1). Birds were 8 or 4 weeks old at the start of the first and second experiments, respectively. Four groups of age-matched birds were used for each experiment. Groups 1 through 3 received RSV in the left wing at day 0 (3600 FFU per recipient); group 1 also received 1800 FFU in the right wing at the same time; groups 2 and 3 received 1800 FFU in the right wing 8 and 18 days later, respectively. A fourth group, which served as the control, received the initial and only inoculation of 1800 FFU in the right wing at the same time that group 3 received the second inoculation (day 18). These experiments (experiments 1 and 2, Table 1) clearly demonstrated that RSV inoculation in one wing, which resulted in progressively growing tumors by 14 days after inoculation, induced a state of resistance to development of new tumors by RSV inoculation into the other wing 8 or 18 days later. Since virus challenge was given to birds which were 8 or 18 days older than those which received the two inoculations on day 0, age related resistance to Rous sarcoma induction (*5*) was a possible explanation for the resistance observed. This was ruled out because all birds in the control groups which received the one and only inoculation of RSV at the day-18 interval developed progressively growing tumors 14 days later.

To study the influence of the bursa of Fabricius on the expression of concomitant immunity we performed an additional study. Six groups of 5-week-old chicks (five or six birds per group) were used; three groups had been bursectomized and treated with cyclophosphamide, and three nonbursectomized groups had been treated in an identical fashion with cyclophosphamide. The latter groups were given cyclophosphamide to determine whether the dose and administration schedule of this drug was immunosuppressive for T cells, which are known to play a role in resistance to Rous sarcomas (*6*). The RSV inoculations (1800 FFU per site) were given in the left wing on day 0 to two bursectomized and two nonbursectomized groups. The RSV inoculations of the right wings (1800 FFU per bird) were either made on the same day or after an interval of 9 days. Control bursectomized and nonbursectomized birds were given an initial and only inoculation of RSV (1800 FFU per bird) in the right wing on day 9. As before (Table 1), prior RSV inoculation in one wing caused resistance to tumor induction by RSV inoculated 9 days later (Table 2). This resistance occurred to the same extent in bursectomized and nonbursectomized recipients. The two control groups given RSV on day 9 only

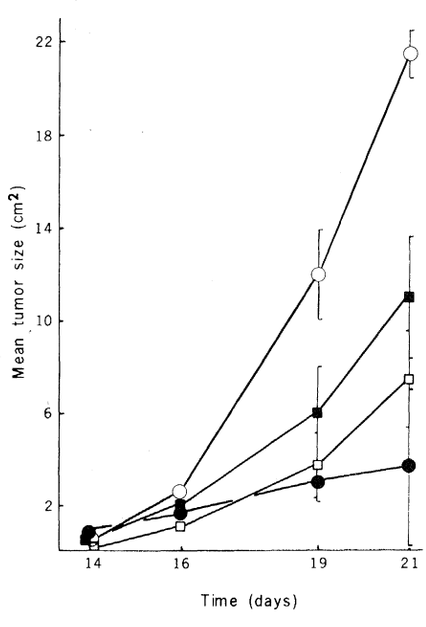

Fig. 1. Growth rate of left wing web tumors of chickens inoculated with RSV at 5 weeks of age (day 0). Birds were bursectomized (■) or nonbursectomized (□) and also inoculated with RSV in the right wing web on day 0, or they were bursectomized (●) or nonbursectomized (○) and also inoculated with RSV in the right wing web on day 9 (these were the same birds as those used in the experiment in Table 2). Standard errors of the means are indicated by vertical lines. Among the nonbursectomized birds, the left wing web tumors were significantly larger when RSV was given in the opposite wing web on day 9 ($P < .001$). The differences in the size of the left wing web tumors of bursectomized birds inoculated with RSV in the right wing web at day 0 or at day 9 were also statistically significant at day 21 of observation ($P < .05$). Comparisons of means were made by Hartley's method, as described by Snedecor (*16*), and Student's *t*-test. The high level of mortality occurring after day 21 of observation among nonbursectomized birds given virus 9 days apart (○) precluded later meaningful comparisons.

were equally susceptible to RSV-induced sarcomas; that is, all birds developed progressively growing tumors by day 14 after virus inoculation. Thus concomitant immunity was fully expressed in chickens with severe hypogammaglobulinemia which followed bursectomy. Since all nonbursectomized birds treated with cyclophosphamide and given virus on day 0 and again on day 9 were resistant to the induction of new tumors, it appears that, if cyclophosphamide had impaired the T cell function of these birds, their T cells had recovered from the effects of the drug 5 weeks after treatment.

From the observation of tumor size among birds in experiments 1 and 2 (Table 1), there was a suggestion that growth of the initially induced tumor was increased in the recipients given a second inoculation of virus 8 or 18 days after the first. This new finding was confirmed in the experiment presented in Table 2 where we found that the rate of growth of the Rous sarcomas induced on day 0 was significantly increased among nonbursectomized birds given a second inoculation of virus on day 9 (Fig. 1). On the other hand, mean tumor size was lowest among bursectomized birds which had received virus on day 0 and again on day 9. Bursectomy appeared to have an opposite effect on the rate of tumor growth among birds which received virus inoculations on day 0 only; that is, tumors grew more rapidly in bursectomized than in nonbursectomized birds. These bursectomized birds also had significantly larger tumors than bursectomized birds given virus 9 days apart (*7*).

One paradox of our results is that the reinoculation of RSV, while failing to induce a second tumor, causes increased growth rate of a preexisting tumor. Enhancement of a primary tumor allograft in mice following rejection of a second graft of the same tumor has been reported by Kaliss and Bryant (*8*). Our observation that increased growth of the first tumor does not occur in bursectomized birds after a second inoculation of RSV suggests that antibody mediates the increased growth rate (*9*). Although our results do not rule out immunological enhancement of tumor growth, based on blocking of cellular immunity by antibody, this possibility appears incompatible with the finding that birds which did develop more rapidly growing tumors were at the same time completely resistant to the induction of new tumors by RSV. In order to reconcile the possibility that concomitant immunity can occur with antibody-mediated enhanced tumor growth, it is necessary to assume either or both of the following: (i) the antibodies that block cellular immunity against a tumor induced 9 days earlier do not block for a new tumor or (ii) nascent tumor cells induced by the second virus inoculation are uniformly sensitive to the inhibiting effects of antibody and sensitized cells induced by the tumor established 9 days earlier. However, the demonstration in mice (*10*) that small numbers of immunogenic tumor cells may grow more rapidly in previously immunized hosts than in nonpreimmunized hosts suggests that antibody-mediated enhancement of a preexisting tumor should be accompanied by enhanced growth of a subsequently induced tumor.

Table 2. The proportion of bursectomized and nonbursectomized G-B1 chickens developing tumors in left and right wings after initial RSV inoculation in the left wing web. Surviving birds were monitored for 33 days after the first RSV inoculation.

Bursectomy regime	Number of birds	Interval between RSV inoculations (days)	Proportion of birds that developed tumors in	
			Left wing	Right wing
BX*	5	0	5/5	5/5
Non-BX	5	0	5/5	5/5
BX	5	9	5/5	0/5
Non-BX	5	9	5/5	0/5
BX	5†			5/5
Non-BX	6†			6/6

*Birds were surgically bursectomized (BX) at hatching and given cyclophosphamide (4 mg per bird) on the same day and 2 days later. Nonbursectomized birds were treated with cyclophosphamide in the same manner. †Age-matched control birds that received an initial RSV inoculation in the right wing only on the same day that the two experimental groups of birds received the second RSV inoculation on day 9.

The increased growth of the first tumor may, on the other hand, be a consequence of adaptation of the tumor cells. Cells transformed after the first RSV inoculation begin proliferating prior to the full development of the immune response. The relative weakness of the earliest immune response may exert selective pressures that favor tumor cell variants that are not inhibited but which may be stimulated to divide (*11*). These variants would possess heritable alterations (*12*). This mechanism of tumor adaptation is compatible with the concept advanced earlier by Kaliss and Bryant (*8*). Cells transformed by RSV inoculated on day 9 would begin dividing in the milieu of an already established and hence stronger immune response. As a consequence, the chance of emergence of these variant sublines may be diminished. The second inoculation of RSV may, however, result in an enhanced immunostimulation of the established tumor.

Slower growth of tumors induced on day 0 in bursectomized birds given an inoculation of RSV 9 days later may be caused by a mechanism unrelated to the absence of antibody. The existence of a subpopulation of bursa-dependent suppressor T cells within the thymus of young chickens (*13*) may account for the increased effective antitumor immunity among these birds (*14*).

In contrast to birds given two inoculations of RSV on different days, bursectomized birds inoculated with RSV only on day 0 developed larger tumors than nonbursectomized birds treated in the same manner (*7*). Either direct immunostimulation of tumor growth or, alternatively, the lack of bursa-dependent suppressor T cells which possibly limit proliferation of tumor cells (*15*) might account for the observations in these birds.

Our results strongly suggest that although concomitant immunity may limit the establishment and growth of metastases (*1*) it may also be a cause of more rapid growth of solid tumors that shed tumor cells into the circulation. The system used in our study may serve as a model for developing immunological approaches to cancer treatment by defining the conditions which can either accelerate or retard the growth of established tumors.

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Autoantibodies to Zona Pellucida: A Possible Cause for Infertility in Women

Abstract. *Human and pig ovaries were tested by agar gel diffusion and found to contain several cross-reacting (common) antigens. At least one common antigen was located in the zona pellucida as determined by indirect immunofluorescence. Serum samples from 22 infertile women were tested on pig eggs by immunofluorescence, and six of these samples produced strong and nine produced moderate reactions with the zona pellucida. The autoantibodies may be responsible for infertility in these women.*

Mature mammalian eggs in the ovary or oviduct are enclosed by a noncellular, gelatinous-like layer known as the zona pellucida. Because of its position, anything passing into or out of the egg, including spermatozoa, must pass through this layer. Thus, a number of roles are ascribed to the zona, including sperm recognition, attachment, and penetration; block to polyspermy; protection of the egg and embryo; movement of the embryo in the reproductive tract; support of blastomeres; and osmotic regulation (*1*). The importance of these suggested roles make the zona pellucida an attractive target for manipulation in regulating fertility.

An immunological approach to the regulation of fertility has attracted much attention recently with the emphasis being on antibodies to hormones and spermatozoa (*2*). Antibodies produced against the zona pellucida alter the zona surface in such a way that receptor sites are no longer available to spermatozoa, so that attachment and penetration through the zona, and consequently fertilization, are inhibited (*3*). In addition to blocking fertilization, antibodies to the zona agglutinate zona-coated eggs; alter the light-scattering properties of the zona by forming a precipitate on the zona surface; block zona digestion by enzymes usually highly effective in dissolving the zona; and, finally, in the case of fertilized eggs prevent the embryos' escape from the zona (*4*), thereby inhibiting implantation (*5*). Since the zona is accessible to immunoglobulins in both the ovary and the reproductive tract (*6*), there are at least two focal points for attack in attempts to regulate fertility, the first being prior to sperm contact while the egg is in the ovary or oviduct, the second being after fertilization while the egg is in the oviduct or uterus but before it has undergone implantation.

The ovary appears to be a good source for large quantities of zona pellucida antigen or antigens, and a number of workers agree that the zona contains one of the strongest ovarian antigens (*3, 7*). It has been estimated that the human ovary may have as many as a million eggs at the time of birth, and a high proportion of these become zona-coated during maturation. Most eggs eventually become atretic and are absorbed while still in the ovary. Less than 1 percent of the eggs are ovulated during the reproductive life of the individual, and although most of these pass into the reproductive tract, they are probably absorbed in this location.

In view of the zona's strong antigenicity and the large amount of it that is synthesized and absorbed, autoimmune properties might be expected of the zona because it forms rather late in ontogenesis, during the diplotene stage of meiosis and after the time at which tolerance to the antigen might have developed. The breakdown and absorption of the zona in the ovary and reproductive tract could continually expose the zona antigen to the immune system and thus result in autosensitization. Autoantibodies are currently suspected as being responsible for a variety of immunologically related diseases. Indeed, auto- and isoantibodies to spermatozoa are believed to be responsible for some cases of infertility in both men and women (*8*). For these reasons we decided to examine serums for possible autoantibodies to zona pellucida in a group of women who were infertile for unknown reasons. The rationale was that autoantibodies to zona might react with the zona to produce infertility by preventing sperm-egg interaction at fertilization or by preventing the embryos' escape from the zona at implantation.

In order to test for autoantibodies to the zona pellucida in women, a large number of eggs were needed for exposure to the serums. The difficulty of obtaining enough human eggs to perform the tests led us to examine the zona pellucida of several mammals for antigens which might cross-react with the human zona. The pig turned out to be the animal of choice since large numbers of zona-coated eggs can easily be obtained by

Table 1. Antigens in pig and human ovaries and pig eggs. The results are expressed as the maximum numbers of precipitin bands formed in agar gel double diffusion tests. Whether the common antigens between pig and human ovaries are identical cannot be determined from the present experiments.

Antiserums	Antigens		
	Pig ovary	Human ovary	Pig eggs*
Antiserum to pig ovary			
Unabsorbed	8 to 12	4 to 6	2
Absorbed with pig kidney and spleen	4	2	2
Antiserum to human ovary			
Unabsorbed	2 to 4	9 to 12	2
Absorbed with pig kidney and spleen	2	3	2

*Saline homogenates of zona-coated eggs were tested against antiserums. Approximately 200 eggs were used per well in diffusion tests.