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Quantitation of maternal-fetal IgG transport in the chicken

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Summary. Quantitative and temporal features of maternal-fetal transport of IgG in the chicken have been determined by means of a sensitive and specific radioimmunoassay. The first step in this two-step process is the transport of maternal IgG from the hen plasma across the oolemma into the maturing oocyte in the ovarian follicle. Oocytes sequester IgG throughout most of their maturation from 10 mg pre-vitellogenic oocytes to fully mature 20 g oocytes ready to be shed into the oviduct. IgG uptake is exactly proportional to mass accumulation at every point. Thus, the concentration of IgG in the yolk is invariant throughout maturation of the oocyte (~ 8 mg/ml) and the rate of uptake in this single specialized cell can be as great as 45 mg/day during its last 3 days of growth before ovulation.

The second step, uptake of yolk IgG across the yolk sac and into the fetal circulation, is detectable at the earliest time points tested (Day 7), but accumulation into the embryonic plasma occurs at a relatively low rate (< 100 μg/day) until a dramatic increase in uptake (> 600 μg/day) occurs in the last 3 days before hatching. The hatchling has 1–2 mg/ml of maternal IgG in its plasma. Increase in IgG uptake lags significantly (~ 4 days) behind increase in mass by the developing embryo, suggesting that IgG uptake occurs predominantly in the last few days before hatching. This ensures that the hatchling is endowed with those maternal IgGs which will be its planopy when it enters the hostile world.

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INTRODUCTION

The transfer of maternal immunoglobulins from the hen to the chick is a two-step process that confers passive immunity to the newborn chick. Circulating IgG from the hen is first sequestered in the yolk of maturing oocytes. After fertilization, IgG is transported from the yolk across the yolk sac to the circulation of the developing chick (Brambell, 1970).

Brambell (1970) originally proposed that this transfer of IgG in the chicken, as well as in other species, occurs via a selective, receptor-mediated mechanism. In this model, IgG from the yolk binds to specific receptors on the yolk sac endoderm, is internalized by the yolk sac, transported intact across the cell, and released into the embryonic circulation.

In support of this hypothesis, specific receptors for IgG have been characterized on the chick yolk sac (Linden & Roth, 1978; R. L. Tressler and T. F. Roth, unpublished observations), and maternal antibodies are known to cross the yolk sac intact (Kramer & Cho, 1970; Rose, Orlans & Buttress, 1974). Uptake of maternal immunoglobulins has been demonstrated by immunizing hens with a specific antigen and measuring the titre of antibody subsequently found in both the oocyte and embryonic circulation (Buxton, 1952; Brierley & Hemmings, 1956; Kramer & Cho, 1970). The uptake of specific antibodies into the embryonic

circulation has also been shown by injecting antibodies directly into the yolk sac and measuring the titre present in the embryonic circulation at later times (Brierley & Hemmings, 1956).

As these previous studies measured only the titre of a specific antibody appearing in the embryonic chick, they provide little information on the total amount of IgG present in the developing chick. In addition, differences in the sensitivity of the various assays used to measure IgG have led to some confusion regarding the time during embryogenesis at which IgG uptake occurs.

In order to characterize the processes which function to transport IgG from the hen to her offspring, it is necessary to measure accurately both the time of appearance and the total concentration of IgG in the embryonic chick. We have developed a sensitive radioimmunoassay specific for chicken IgG and, in the study reported here, have used this assay to quantitate the concentration of IgG present in the oocyte and the embryonic chick at different stages of development.

MATERIALS AND METHODS

Fertile eggs from white Leghorn hens were obtained from Truslow Farms (Salisbury, MD) and maintained in a humidified incubator at 39° until the embryos reached the desired age. Blood samples from the embryos were obtained by removing a portion of the shell to expose an artery supplying the yolk sac. The artery was dissected from underlying tissue, placed gently on the shell, and severed with a glass capillary pipet. The sample was collected in the pipet, allowed to clot, and then centrifuged at 1000 ***g*** for 5 min. Serum samples were stored frozen at $-20°$ until assayed.

Oocytes were taken from freshly killed white Leghorn chickens obtained from the Poultry Science Department, University of Maryland, College Park, MD. The oocytes were washed in PBS (Dulbecco & Vogt, 1954) and weighed. Yolk samples were obtained by slicing open the oocyte and gently squeezing the yolk into a small plastic dish. The yolk was stirred to ensure homogeneity (Patterson *et al.*, 1962a) and stored frozen at $-20°$ until assayed.

Yolk samples from embryonic chicks were obtained in a similar fashion from fertile eggs of a known age. Yolk and serum samples from newborn chicks were obtained on the day of hatching.

IgG purification

Chicken IgG (cIgG) was purified from chicken egg yolks by the method of Loeken & Roth (1983), modified by increasing the time of the 100,000 ***g*** centrifugation to 24 h (R. L. Tressler and T. F. Roth, unpublished observations). Purified cIgG was determined to be greater than 95% pure by polyacrylamide gel electrophoresis. Concentrations were determined by optical density readings at 280 nm using a value of $E^{1\%}_{1cm} = 14$ (Linden & Roth, 1978). Chicken IgG was iodinated with ^{125}I (Amersham Corporation, Arlington Heights, IL) using chloramine T (Hunter & Greenwood, 1962). The specific activity of the ^{125}I-IgG was approximately $1{\cdot}5 \times 10^7$ c.p.m./μg. Stock solutions of cIgG were stored at 4°, and used within 6 weeks.

Preparation of bromoacetyl cellulose antibody conjugate

Purified rabbit anti-chicken immunoglobulin G (RAC) was obtained from Cappel Laboratories (Cochranville, PA). This antibody showed no apparent cross-reactivity with other chicken immunoglobulin classes when tested by immunoelectrophoresis. Bromoacetyl cellulose (BAC) was covalently coupled to RAC using the method described by Robbins, Haimovich & Sela (1967). In general, 1·0 g BAC which had been sonicated to a fine suspension was reacted with 4·0 mg rabbit anti-chicken IgG (RAC). The conjugate, hereafter referred to as BAC-RAC, was stored in PBS with 1% NaN_3 at 4°, and remained active for 2 months.

Radioimmunoassay

Serial dilutions of unlabelled cIgG or samples to be assayed (100 μl) plus ^{125}I-cIgG (80,000–100,000 c.p.m./tube, 100 μl) were incubated with 50 μl of BAC-RAC in PBS overnight at 4°. All samples were diluted into assay buffer (PBS, pH 7·4, containing 1% Triton X-100, 10 mg/ml BSA, 10 mM EDTA). The BAC-RAC was diluted so that 50 μl would bind 40–60% of the iodinated IgG in the absence of competing antigen. Following this overnight incubation, a 200 μl sample from each tube was layered over 200 μl of a mixture of 11 parts butyl phalate plus 10 parts bis-2-ethyl hexyl phthalate (Segal & Hurwitz, 1977, both from Eastman Organics) in an Eppendorf microcentrifuge tube and centrifuged at 10,000 r.p.m. for 5 min in a Beckman microcentrifuge. This method was very effective in separating unbound cIgG from cIgG bound to the BAC-RAC conjugate. After centri-

fugation, the aqueous layer above the oil mixture containing unbound cIgG was removed by aspiration. The tube was washed once by adding 200 μl of assay buffer to each tube, after which both the buffer and oil were aspirated. The microfuge tubes containing the pellet were then counted for radioactivity using a Beckman Gamma 4000 counter.

RESULTS

Characterization of radioimmunoassay for chicken IgG

The RIA employed in these studies was a standard competitive binding immunoassay, wherein a trace amount of ^{125}I-cIgG and cIgG in the sample competed for a limited number of rabbit anti-chicken IgG binding sites immobilized on bromoacetyl cellulose (BAC). The performance of the assay is illustrated in Fig. 1. The midpoint of the inhibition curve fell at 1 μg/ml cIgG, with the dynamic range of the assay essentially spanning a range from 0·1 to 10 μg/ml cIgG. Replicate determinations typically agreed within 10 percent. The assay was specific for chicken IgG, in that neither IgG from other species (Fig. 1) nor other homologous yolk proteins such as phosvitin (0·1 mg/ml) or vitellogenin (1 mg/ml) showed any competition for the iodinated chicken IgG (data not shown).

Determination of cIgG concentration in sera from adult chickens

Sera from 21 laying hens were analysed to determine the IgG concentration (Table 1). Their average concentration was about 16 mg/ml and there was a considerable range, which also has been reported by others (Lebacq-Verheyden, Vaerman & Heremons, 1974; Patterson *et al.*, 1962a). This average value is higher than that reported by Lebacq-Verheyden *et al.* (1974) (15·7 mg/ml *vs* 6·9 mg/ml), although the difference is not statistically significant, due to a SEM of $\pm$7·9. It may simply reflect the difference between the laying hens used in this study and non-laying chickens used in the other study. Oestrogenized roos-

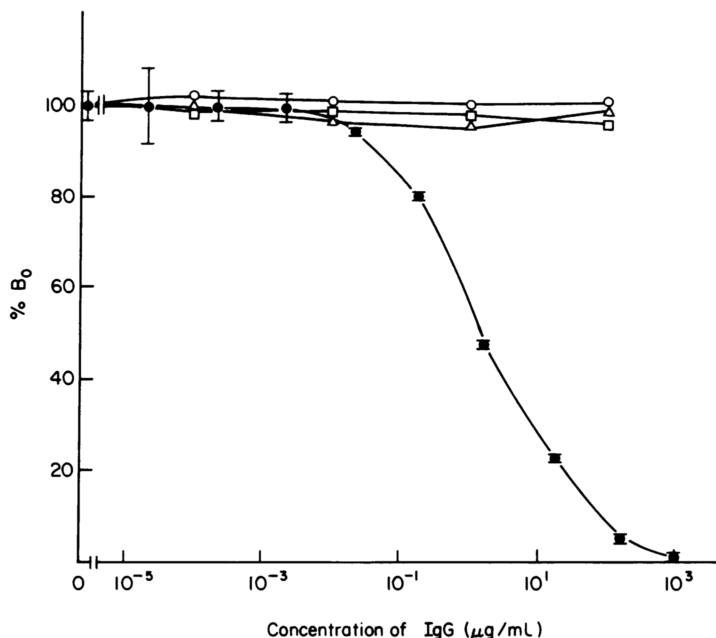

Figure 1. Radioimmunoassay of cIgG. ^{125}I-cIgG and varying concentrations of unlabelled cIgG, human IgG, rabbit IgG or guinea-pig IgM were incubated with a constant amount of insolubilized-rabbit anti-chicken IgG (BAC-RAC). After incubation, the ^{125}I-cIgG bound to BAC-RAC was separated from unbound IgG as described under Materials and Methods. The amount of ^{125}I-cIgG bound at each concentration of competing cIgG (●); human IgG4 (△); human IgG1 (○), human IgG3 (○), guinea-pig IgM (○) or rabbit IgG anti-DNP (dinitrophenol) (□) was calculated as percent of ^{125}I-cIgG bound in the absence of unlabelled cIgG (B$_0$). The error bars represent SEM.

Table 1. Determination of serum IgG concentration in mature fowl

Source	No. tested	Conc. cIgG (mg/ml) ±SEM	Range (mg/ml)
Laying hens	21	15·7±7·9	5·3–43·3
Oestrogenized roosters	2	6·2±1·2	4·5–7·0

Table 2. The concentration of cIgG in oocytes

Growth phase	Oocyte weight (g) and mean (range)	No. tested	Conc. cIgG (mg/ml)
Previtellogenic (−20 days to −5 days)	0·32 (0·05–0·9)	15	9·3±5·2
Exponential growth (−5 days to −2 days)	4·44 (1·0–9·9)	12	7·9±2·3
Linear growth (−2 day to laying)	14·25 (10–20)	8	7·9±2·3

ters have lower IgG levels of 6·2±1·2 mg/ml, which is more consistent with other published values of serum cIgG.

Accumulation of cIgG in maturing oocytes

Within the single ovary of the hen are numerous small (<60 mg) immature oocytes. At approximately daily intervals, single oocytes from this population enter a two-phase maturation process. The first phase is characterized by a relatively slow growth and lasts about 15 days. The second is a 6-day period which includes the rapid sequestration of selected serum proteins, resulting in a 30–50-fold increase in mass. As shown in Table 2, the concentration of cIgG in the yolk is essentially constant throughout the entire maturation of the oocyte, from the smallest oocyte (0·05g) to the largest (20 g) that we obtained.

Mass of the oocyte can be correlated with time before laying by using the oocytes of a single laying hen as an index. As shown in Fig. 2, oocyte mass increases essentially exponentially from Day −8 to Day −2, and then linearly from Day −2 to shortly before entry of the mature oocyte into the oviduct (Day 0). Uptake of cIgG follows this pattern, with maximum rates of uptake occurring around Days −3 and −2, when as much as 45 mg/day is accumulated in the yolk. At maturity, an oocyte will contain 100–200 mg of maternal cIgG.

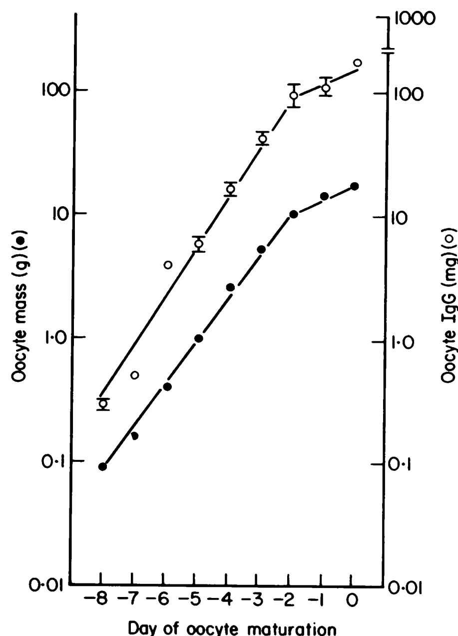

Figure 2. Determination of cIgG concentration in oocytes. Oocytes taken from freshly killed chickens were treated as described under Materials and Methods. Their mass was correlated with days prior to laying (●) using a set of standard oocytes as reference. Total IgG mass (○) was determined using the results of the RIA, recorded weight of the oocyte and the density of yolk (1·1 g/ml). Where error bars are used, they represent the SEM of triplicate determinations.

Transport of cIgG from the yolk to the embryonic circulation

As a means of determining the temporal and quantitative features of cIgG uptake into the circulation of the developing chick, blood samples were drawn from embryos aged 7–21 days (hatching) and assayed for their cIgG content. Depicted in Fig. 3 is the amount of embryonic serum cIgG as a function of age of the embryo. IgG was detectable in the embryo in the earliest samples obtained (Day 7), albeit at low levels (~25 μg/ml). Plasma IgG remains at modest levels (<100 μg/ml) until Day 15, when a substantial increase in the rate of uptake of IgG begins and continues until hatching. Comparison of the mass of the developing embryo (Yosphe-Purer, Fendrich & Davies, 1953) with the mass of cIgG in the plasma (Fig. 3) indicates that accumulation of cIgG is not strictly proportional to increase in mass, but rather lags behind embryo mass until the dramatic increase in rate of transport of IgG which begins between Days 14 and 15. For example, embryo mass reaches 50% hatching weight on Day 16, while IgG in the plasma does not reach half its concentration at hatching until Day 19.

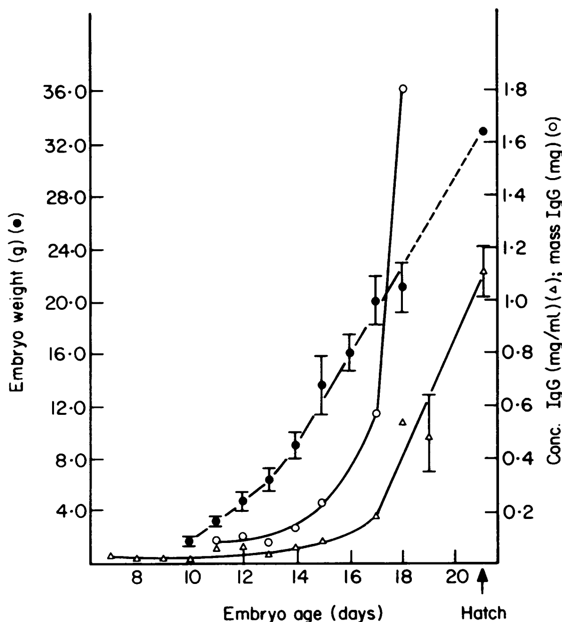

Figure 3. Ontogeny of cIgG embryo serum concentration between 7 days of development and hatching. Serum samples were taken from fertile eggs at the indicated day of development, as described under Materials and Methods. Each point represents the mean of multiple serum samples, as determined by RIA ± SEM. Samples at Day 21 are from newly hatched chicks. Mass of cIgG was determined using the concentration of cIgG multiplied by the plasma volume. Embryo weights and plasma volumes were taken from Yosphe-Purer, Frendrich & Davies (1953), except embryo weight at 21 days, which was a personal communication from the Poultry Science Department at the University of Maryland, College Park Campus.

DISCUSSION

Construction of a sensitive radioimmunoassay provided us with a means for examining both the temporal and quantitative features of cIgG transport from mother hen to developing chick. This assay was comparable in sensitivity and dynamic range to numerous radioimmunoassays for IgG in other species (Nerenberg & Prosad, 1981), and appeared not to be affected by potential interferents like vitellogenin or phosvitin. The principle advantage gained by measuring bulk cIgG was that the results were relatively unaffected by the rather dramatic and rapid changes in titre of specific antibody (Patterson *et al.*, 1962a) which complicated the observations of others (Kramer & Cho, 1970; Brierley & Hemmings, 1956; Buxton, 1952). In addition, absolute values have been determined where only relative values have been obtained before.

Accumulation of cIgG by the maturing oocyte

A few points are immediately apparent about the nature of cIgG accumulation by chicken oocytes. Yolk cIgG is essentially constant from the smallest pre-vitellogenic oocyte to the mature yolk in a freshly laid egg (Table 2). The concentration of yolk cIgG (7·9 mg/ml) is similar to that in maternal serum (15·7 ± 7·9), but always less. Evidently, the mechanism for IgG uptake is active at the earliest stages measured although, as yet, there is no evidence for cIgG-specific receptors on pre-vitellogenic eggs.

Both mass and IgG accumulation increase exponentially, doubling approximately every 18 hr from Day −8 to Day −3, when the growth rate evidently becomes linear and then declines in the last day as the vitelline layer occludes the oocyte surface, thus preventing ligands from reaching the oolemma. This same trend was noted by Patterson *et al.*, (1962a). The dramatic acceleration in rate of accumulation of cIgG uptake between Day −6 before laying (~4 mg/day) and Day −2 (45 mg/day) is exactly proportional to the

increase in mass by the oocyte. If we assume the same parameters for IgG uptake as those set forth for asialo-orosomucoids, selective uptake of 45 mg per day by receptor-mediated endocytosis would require that approximately 1×10^{15} receptors per oocyte bind cIgG, become internalized, release their ligand and recycle every 10 min (Harford & Ashwell, 1981). Expressed another way, this means that if the oocyte were a smooth sphere, about 100 molecules per day are accumulated per nm^2 of oolemma, and that there would be about 1 receptor per nm^2. However, the oocyte surface is many times larger, due to its highly folded surface which is literally bristling with coated pits (J. Heuser & T. F. Roth, unpublished observations; C. Zajak and T. F. Roth, in preparation; Perry, Gilbert & Evans, 1978; Roth, Cutting & Atlas, 1976; Schjeide *et al.*, 1966).

As a consequence of the rapid endocytosis of cIgG by the oocytes, a laying hen loses 100–200 mg cIgG, or about 10–20% of her steady state IgG, into her oocytes every day. The circulating half-life for cIgG in hens is about 36 hr (Patterson *et al.*, 1962b), so she loses an additional 30–40% per day to normal turnover. It seems likely that IgG synthesis rates in hens must be different from those in roosters to accommodate the substantial losses due to oogenesis. Alternatively, hens may compensate by reducing the turnover rate for serum IgG, but available evidence suggests this is not so (Patterson *et al.*, 1962b).

Transport of cIgG from the yolk to the embryonic circulation

Work by other investigators has described several of the properties of transport of yolk IgG across the yolk sac splanchnopleur into the fetal circulation. Transport across the yolk sac is selective for chicken IgG, whereas rabbit, cow and horse anti-sera fail to be transported into the circulating plasma, and pigeon anti-sera are transported poorly (Brierley & Hemmings, 1956). Transport is also not quantitative, in that titres of specific antibody in fetal plasma are generally 80–90% lower than in the yolk from which they are derived. Several investigators have noted that transport of agglutinins occurs well before hatching, and as early as Day 12 of fetal life (Kramer & Cho, 1970; Brierly & Hemmings, 1956).

It has not been established that trans-epithelial transport of IgG into the embryo is accomplished by receptor-mediated endocytosis. However, specific and saturable receptors have been described on the yolk sac (R. L. Tressler and T. F. Roth, unpublished observations; Linden & Roth, 1978), and yolk sac epithelial cells are rich in coated pits and coated vesicles (Mobbs & McMillan, 1981; Lambson, 1970; T. F. Roth, unpublished observations). Taken together with the apparent selectivity for chicken IgG cited above, and with the topological similarity to other tissues where transport of IgG across cells occurs (e.g. neonatal rat ileal epithelium, Rodewald, 1976, 1980, 1982), it is likely that the principal route for passage of maternal IgG to the circulation of the chick occurs by receptor-mediated endocytosis across the yolk sac epithelial cells.

It is unlikely that new synthesis of IgG by the embryo contributes significantly to the levels of embryonic serum IgG. Kincade & Cooper (1971) have shown that IgG present in the circulation of the chick is primarily derived from the yolk. Further, Lawrence *et al.* (1981) have shown that IgG-secreting cells in newborn chicks are not detectable until 6 days after hatching.

The presence of a low level of cIgG in the fetal serum at Day 7 (25 μg/ml) is the earliest reported, to date. The IgG concentration in fetal plasma increases slowly until Day 14, when an apparent acceleration of transport occurs and continues until a high, sustained rate is achieved during Days 19–21. This dramatic increase in rate of IgG uptake into fetal plasma was observed by Buxton (1952) and Marshall & Deutsch (1950). The rate of transport during those last 3 days is approximately 600 μg/day. The resultant concentration of IgG in the serum at hatching of about 1 mg/ml can quantitatively account for the reduced titres in serum antibody, compared to yolk antibody noted by Brierley & Hemmings (1956). They noted that yolk titre exceeded serum titre in the chick by about a factor of eight. Since yolk IgG is typically about 6 mg/ml (Table 2), this is about six-fold more than the concentration of IgG in the hatching chick serum and, thus, could readily explain the lower titre found in the chick serum.

The total amount of cIgG that is present in the chick's serum is approximately 2 mg, assuming a plasma volume of 2 ml. Possibly an equal amount may be in extravascular space, and up to 2 mg more may have been cleared from the embryonic circulation (Patterson *et al.*, 1962b). This accounts for only 6 mg, out of greater than 100 mg initially present in the yolk. The fate of more than 90% of yolk IgG is uncertain. Although the concentration of yolk IgG as late as Day 19 is little different from that in mature, unfertilized

oocytes (Table 2), the yolk volume is considerably reduced. Some of the IgG is presumably phagocytosed with yolk droplets and degraded. Some also may eventually diffuse into the albumin and be ingested internally by the developing chick, but there is no evidence that this IgG ever reaches the plasma intact (Brierley & Hemmings, 1956; Kramer & Cho, 1970).

It is possible that the bulk of the yolk IgG, like nearly all the other yolk macromolecules, serves only a nutritional role. Alternatively, sustained high concentrations of IgG may be essential to ensure high levels of receptor occupancy during the dramatic burst of IgG transport during Days 19–21. As noted in Fig. 3, IgG transport into the serum lags significantly behind the increase in mass by the embryo. The suggestion is, of course, that the mechanism 'recognizes' that IgG uptake is relatively unimportant in the first 2 weeks of embryonic development, but that it is a crucial part of the developmental programme in the last few days immediately before hatching.

The data show that the IgG transport system in the yolk sac operates at a basal level of 25 μg/day until Day 15, when it begins to accelerate to 100 μg/day, and finally reaches about 600 μg/day in the 2–3 days just before hatching. Thus, IgG receptor synthesis or activation occurs principally in the last week of development, and since the rate of transport increases almost 20-fold between Day 14 (30 μg/day) and Day 18 (600 μg/day), it is reasonable to expect significant differences in the expression of IgG receptors on yolk sac from early stages of development (Day 8), compared to later stages (Day 18). R. L. Tressler and T. F. Roth (unpublished observations) have found that two receptor classes are present on Day 18 yolk sac, while only the lower affinity class is present on Day 8 yolk sac. Temporal control of the IgG receptor's activity is an intriguing area for further investigation.

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