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Detection in chick embryo of fetoproteins not recognized by the dam's immune system and of soluble alloantigens. Presumptive teratogenic and abortogenic capacity of their specific IgY

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Abstract

Background: The aim of this work was to detect antigens, non-self to the dam, potentially present in chick embryo prior to organogenesis with a view to establishing the consequences of their neutralization on chick development. To this end, hens were immunized with the extract from embryos incubated for 53 h. Their eggs were either used to isolate immunoglobulins for dot and blot tests or incubated for variable lengths of time.

Results: Immunoblot tests, using adsorbed primary and secondary antibodies against paternal serum, revealed the presence of at least four antigens of 32, 34, 70 and 200 kDa that can be classified as soluble alloantigens. The same antibodies against chick embryo extracts (between 53 h and 9) showed at least five aged antigens of 34, 52, 90, 200 and 250 kDa, not detected in cock serum, that can thus be considered as soluble, foreign to the immunized hens and transitory antigens. The abnormalities observed included arrested development and fetal death, as well as minor functional damage in the few chicks that were born alive. The ratio of abnormal to normal embryos was 2.85 in the experimental group and 0.43 in the control group. With regard to congenital anomalies it must be said that of the 81 eggs incubated only four chicks were born alive, and of these, only one had a healthy birth and subsequent growth. The other three showed a transitory ataxia and one of them presented adult lumbar scoliosis and asymmetric pelvis.

Conclusions: The problem of recurrent spontaneous abortions is revisited in the light of these results. Some recent data suggest that soluble alloantigens may be candidates for a new etiological entity in recurrent spontaneous abortions. They can also be the cause of some congenital anomalies. The soluble, foreign, transitory antigens may have a similar effect although there is no supportive data in the literature.

Background

Chick embryo antigens were successfully neutralized with specific antibodies for the first time in the 1950s. Tests

were then conducted with a view to determining what happened when a given protein or protein group ceased to serve its physiological function. Since then, a variety of

antisera for pure or mixed adult and embryo antigens have been tested. Anti-embryo antibodies rendered specific by adsorption with adult antigens and additionally purified immunoglobulins have also been used for this purpose. Some studies have been carried out *in vitro* and others *in ovo*. Either the growth of specific tissues or organs was altered, or complete body development was prevented and death caused in various ways [1–13]. Specific sera against differentiation antigens in chick embryo (*e.g.* α -fetoprotein) have also been used in this context. Some *in ovo* experiments involved injection of its heterologous antiserum from rabbit into the yolk sac. Neutralizing α -fetoprotein was found to give rise to congenital malformations, arrested development and fetal death [14]. Also, incorporating α -fetoprotein antiserum to the culture medium was found to disrupt histological development of the retina [15].

Previous experiments revealed the presence of seven transitory antigens (TrAg) in allantoic fluid, four of which were also encountered in chick embryo liver [16], and of three TrAg in the chorioallantoic membrane of 13-day chicks [17], in addition to α -fetoprotein in both cases. This aroused the author's interest in searching for antigens in the early embryo, which might be present prior to organogenesis. Should any be found, this would allow one to determine the involvement of these proteins in such a crucial developmental stage. To this end, chick embryos incubated for 53 h – which corresponds to stages 14–15 in Hamilton and Hamburger's Table [18] – and soluble embryonic antigens were examined. Because heterologous antisera were avoided for the reasons stated under Discussion, the study was conducted by immunizing hens with embryo extracts. By definition, inoculated hens only produced antibodies against those proteins with which their specific immature B cells (sIgM⁺ IgD⁻ B lymphocytes) never came into contact with high avidity during their central clonal negative selection and therefore these specific B cells were not removed [19]. This approach focuses on the findings of two groups of highly attractive and yet scarcely studied proteins, namely: (a) soluble alloantigens (sAlloAg), which are permanent antigens of paternal origin, and (b) soluble, foreign to the dam and transitory antigens (sF-TrAg). These last fetoproteins are coded by genes that are only expressed during the embryonic period [20] or some weeks after hatch preceding maturity of the embryo's immune system and therefore they are non-self to the adult. Both groups fitted the designation "soluble, fetal, dam-foreign antigens" (sFF-Ag). It is known that gene conversion and Ig diversification are ongoing in the bursa through sexual maturity of the bird. Since gene conversion randomly creates new specificities of Ig, new Ag specific clones are being produced throughout of the first 16 weeks or so after hatch [21]. If then the specific sIgM⁺ IgD⁻ B lymphocyte appears for a

given sF-TrAg when already its synthesis has been stopped or already is not present in the chick after hatch, then this event will have the same consequences – according to the position of this article – as for the case of an sF-TrAg that was only present during the embryonic period.

Hen IgG antibodies are known to pass from blood to yolk, and so, when the egg is incubated, IgY are transferred from the yolk sac to the embryo via the bloodstream. Therefore, if these specific IgY react with their respective antigens, they will do so *in ovo*, in a natural physiological medium, as molecules synthesized by the dam itself. Theoretically, this reaction can suppress the functions of these proteins, particularly of enzymes. In broad terms, this consequence has been experimentally confirmed (*e.g.* in [2,5] and [14]). However, this cannot always be the case for various reasons, including the inability of the IgY molecule to access the cell compartment holding the corresponding protein, its inability to block its active site -if it is an enzyme- even if it can reach it or that of the amount of antibody supplied by the yolk sac to neutralize a large enough amount of antigen produced by the embryo to disrupt its development. On the other hand, if neutralization is efficient enough, some abnormality in embryofetal development will occur and whether or not such a morphological or functional abnormality is compatible with life can be determined.

Because of the many questions involved, this work was addressed as a "preliminary experiment" intended to provide experimental answers and determine the type and number of sFF-Ag involved. Only if the immunization were pathogenic would reporting the results make sense; were this the case, it would warrant planning additional, more detailed studies involving the use of purified samples of the antigens found and immunizing as many hens series in order to identify the specific developmental alterations caused in each or even to conduct a study on a mammal with a hemochorial placenta, similar to that in humans, and its specific sFF-Ag.

In this work, four sAlloAg and five sF-TrAg in 53-h chick embryo were detected. Some (or all) of them may cause various embryonic development alterations including arrested development and fetal death, as well as congenital abnormalities in a few cases.

Results

Figure 1 shows the breed and gender of the birds used and the procedure followed to obtain the antigenic material, the embryonated eggs from the immunized hens, and the IgY from the yolk to detect antigens through dot- and Western immunoblot.

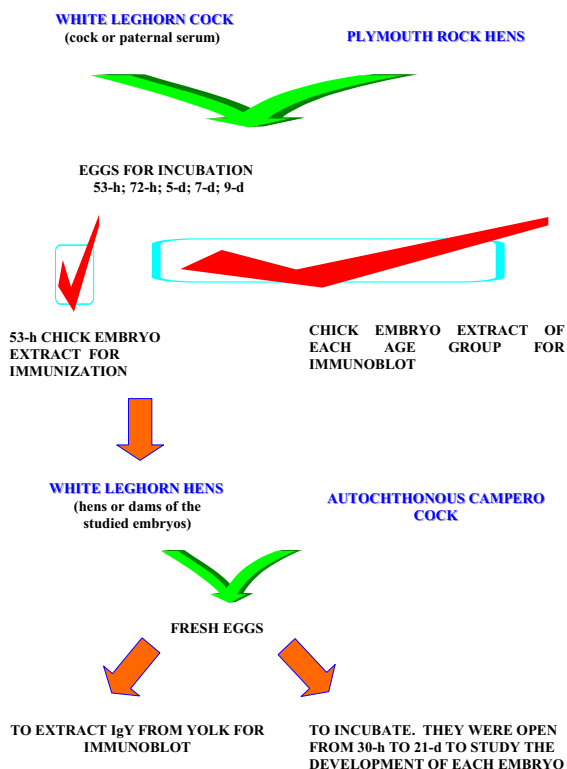


Figure 1
Sequence of steps taken in this study.

Dot-blot was used for controlling the purity of the primary and secondary antibodies and for optimizing these reagents. Anti-ceE IgY gave positive results against White Leghorn (W-L) and Plymouth Rock (P-R) hens' sera and goat serum. Anti-control IgY gave positive results against cock, W-L and P-R hens' sera. "Normal" IgY gave positive results against cock serum. The anti-ceE IgY detected 12 "contaminant" antigens in cock serum when was tried with the immuno-blot test (Figure 2). Dot-blot were repeated but with successive adsorptions of the primary and secondary labeled reagents until the corresponding negative reactions were obtained (results not shown, but see Figure 3).

With these fully adsorbed reagents, the Western immunoblots were carried out (Figures 3 and 4). In this way one can see that the adsorbed anti-ceE IgY does not detect any antigen in sera from W-L and P-R hens but at least four sAlloAg are found in W-L cock serum of *circa* 200, 70, 34 and 32 kDa (Figure 3). On the other hand, five bands (sF-TrAg) of *circa* 250, 200, 90, 52 and 34 kDa are at least patents among the ceE from different ages (53 h, 72 h, 5 d, 7 d and up at least 9 d) (Figure 4).

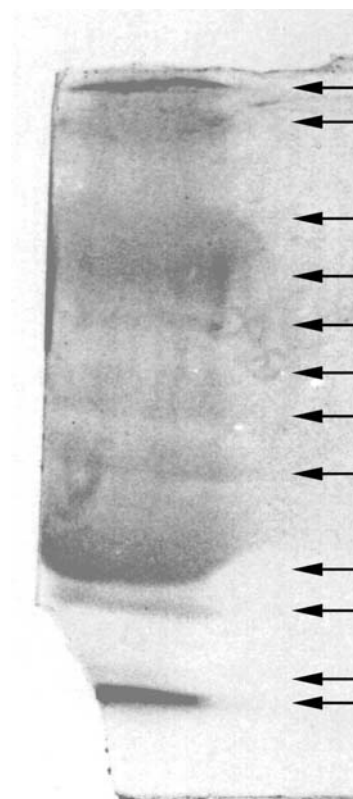


Figure 2
This immunoblot shows 12 bands produced by native anti-ceE IgY against cock serum, and native anti-chicken IgG conjugate. The corresponding antigens were removed through adsorption of the primary antibody with hens' sera and the secondary antibody with hen's liver in order to visualize the sFF-Ag.

Of the 81 embryos studied, 74.1% exhibited some abnormality, which, in many cases, led to death; by contrast, only 30.3% of the 66 controls developed some abnormality. The ratio of total to abnormal embryos was 1.35 for the experimental group and 3.3 for the control group. That of abnormal embryos to normal embryos was 2.85 for the former group and 0.43 for the latter (Table 1).

There are 147 cases as a whole categorized as we see in the Table 2. The response between the two groups considered (immunized and control) is compared. The Fischer' exact test, unilateral, made for table 2 x 2 shows a limit probability of $p < 0.001$. Therefore, it may be considered that the proportion of abnormal chicken from immunized hens is significantly higher than the respective proportion in the control group (Table 3).

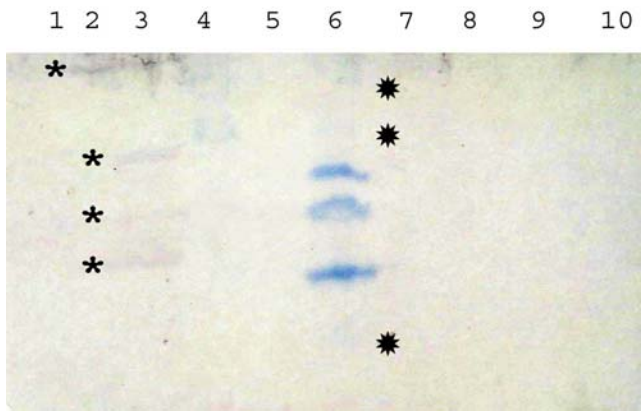


Figure 3

Western immunoblot from SDS-PAGE with gradient 4–15% acrylamide-bis. Paths 1–3: W-L cock serum diluted 1:4, 1:2 and 1:1, respectively. 4: prestained MW standards high range. 5: nothing. 6: prestained MW standards low range (the three asterisks point out those prestained MW that are weak). 7–8: W-L hen serum diluted 1:2 and 1:1, respectively. 9–10: P-R hen serum diluted 1:2 and 1:1, respectively. The primary antibody was anti-ceE IgY adsorbed with sera from W-L hen and P-R hen, and the secondary antibody was adsorbed with liver extract from W-L hen. Four sAlloAg from W-L cock serum are present in the 53-hour-old chick embryo with *circa* 200, 70, 34, and 32 kDa. This adsorbed anti-ceE IgY does not detect any antigen in sera from W-L and P-R hens (paths 7–10).

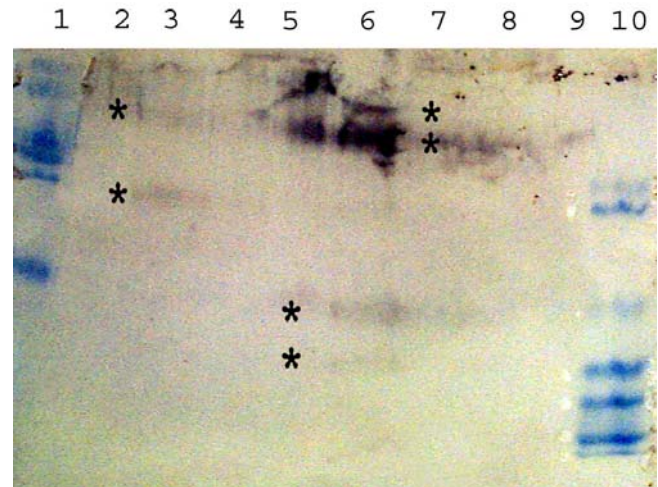


Figure 4

Western immunoblot from SDS-PAGE carried out simultaneously with that of the Figure 3 and with the same primary and secondary antibodies. Paths 1 and 10: prestained MW standards high and low range, respectively. Paths 2–5: four different samples from ceE 53 hours. Paths 5–9: ceE 72 hours and 5–7–9 days, respectively. Five sF-TrAg with *circa* 250, 200, 90, 52 and 34 kDa are present in 53-hour-old chick embryo inasmuch as all sF-TrAg present in the different age samples was detected by the antibodies induced by the chick embryo 53 h-old extract. The six stars point out the five bands.

Figures 5,6,7,8,9 show selected annotated examples of arrested development, malformations and fetal death. In some cases, the abnormality was a mere delay of 24 h or a little longer, with no other dysmorphology when viewed under a magnifier. Some of the eggs opened at the theoretical time of hatching showed already dead chicks and exhibited a greater or lesser lack of yolk resorption. One that looked normally developed to the naked eye was still breathing at the time but died immediately. Three that were alive and exhibited some residual yolk were left in one half-shell and placed in the incubator to complete their maturation but died within 18 h. Some eggs were allowed to stand for 6–24 h after 21 days of incubation in the hope that they would complete their development and the chicks would start hatching. This was not the case and the chicks were found dead when the eggs were opened. A detailed study of the delays and the macroscopic and microscopic dysmorphologies observed is currently under way. Of the four chicks that succeeded, only one was born healthy and grew with no apparent abnormality. The other three were born with transitory neurological disorders, ataxia and an inability to peck at food and drink; one died after 12 days through digestive prob-

lems not associated to the experiment, whereas the others reached adulthood healthy although one had a bony protuberance in the joint between the femur and pelvis. The radiographs and autopsy revealed the presence of lumbar scoliosis and an asymmetric pelvis that caused the trochanter from the femur to protrude. All these and other signs and symptoms observed also will be published in more detail as congenital anomalies.

Discussion

When a serological technique in dot- and Western immunoblot is used, the risk exists that the primary and/or the secondary antibody may be contributing to spurious background bands. In our case these contaminant antibodies were detected in dot-blot using cock and goat sera as antigens, and native anti-ceE IgY, anti-control IgY and "normal" IgY, and as secondary antibody native anti-chicken IgG conjugate. All this indicated that the false positive reactions found with the anti-ceE was neither caused by the embryonic material employed in the immunization (since they occurred also with the anti-control IgY), nor caused by the Freund adjuvant because the "normal" IgY also gave a false positive. The false positive reac-

Table 1: Normal and abnormal embryonated eggs from immunized and control hens

Source	Total N (%)	Normal		Abnormal N (%)			Ratio	
		N (%)	? ^a	Living	Dead	Total	T/An ^b	An/N ^c
Immunized hens	81 (100)	21 (25.9)	27 (33.3)	11 (13.2)	22 (27.1)	60 (74.1)	1.35	2.85
Control hens	66 (100)	46 (69.7)	5 (7.5)	2 (3.0)	13 (19.7)	20 (30.3)	3.3	0.43

^a?, abnormal but uncertain as to whether the embryo was dead or alive. ^b Total/Abnormal ^c Abnormal/Normal

Table 2: State – Response cross-tabulation

Count	Response		Total
	Normal	Abnormal	
State			
- Immunized	21	60	81
- Control	46	20	66
Total	67	80	147

Table 3: Chi-Square tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearse Chi-Square	28.090	1	0.000	0.000	0.000	
Continuity Correction	26.353	1	0.000			
Likelihood Ratio	28.955	1	0.000	0.000	0.000	
Fischer's Exact Test				0.000	0.000	
Linear-by-Linear Association	27.899	1	0.000	0.000	0.000	0.000
No. of Valid Cases	147					

tion observed in the goat serum has not been studied, but was probably caused by a cross-reaction among goat serum proteins and rabbit antibodies against contaminant proteins from avian serum in the preparation of the anti-chicken IgG (conjugate). Its adsorption with liver extract from hen was useful since it yielded firstly a voluminous pellet and needed after to be adsorbed with Sepharose-CNBr-liver extract in order to obtain negative results. An exhaustive adsorption with W-L and P-R hens' sera for the primary reagents (anti-ceE IgY and anti-control IgY) was also made up to get negative results. Using native reagents, various non-sFF-Ag are detected in cock serum through primary antibody anti-ceE IgY (Figure 2). The nature and the significance of these "contaminant" antibodies lies beyond the goal of the current study, where only fetoproteins not present in dam's serum are studied.

However, if some of them are autoantibodies (as they probably are), it will be necessary to make them a subject of further study in the future in view of their importance in medicine.

The classification made, that some fetoproteins found are sAlloAg and other sF-TrAg is based on the argument that they are found in cock serum and in ceE respectively, irrespective that in the two immunoblot tests there are two bands with the same relative mobility (34 and 200 kDa), given the lesser amount of serum proteins (and of sAlloAg) in ceE than in cock serum. Both immunoblot tests were developed with the same primary and secondary antibody, both were developed in the same running and each band of each membrane measured in regard to its own high and low standard MW markers. However,

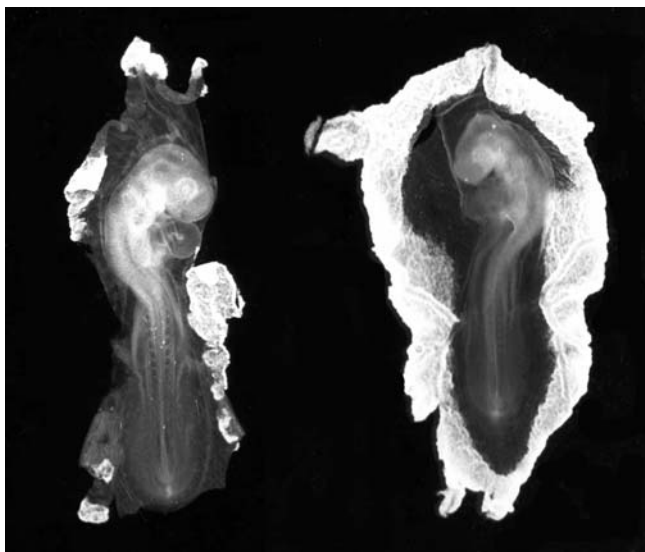


Figure 5
Presumptive teratological consequences of the soluble, fetal and dam-foreign antigens (sFF-Ag) being neutralized by their specific IgY. Both embryos were 53-h old. The one on the left (the control) exhibited normal development: with twenty-two pairs of somites, optic cup, lens and choroids fissure, 1st, and 2nd pharyngeal pouch and the arches 1 and 2, otic vesicle, normal looping of the heart (atrium, ventricle and *truncus arteriosus*), etc. On the right, the embryo shows a more reduced body size, and all the abovementioned anatomical structures are much less noticeable here. What is most noticeable is the malformed development of the brain, pharynx and heart.

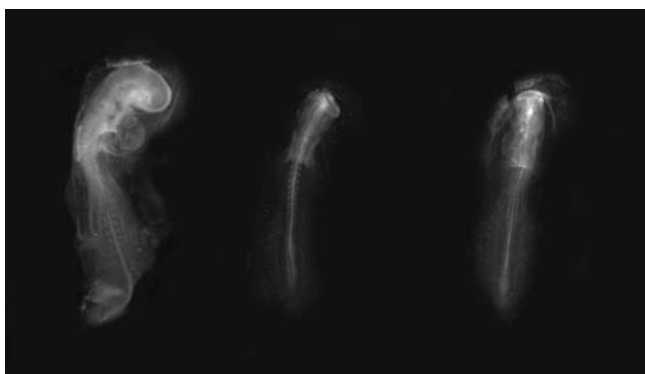


Figure 6
The three embryos were 53-h old. The one on the left was the control. Those in the middle and on the right exhibited noticeably arrested development.



Figure 7
The three embryos were 53-h old (the control is not shown). The one on the right exhibited 8 pairs of somites and delayed development of heart and head, another in the middle had a head corresponding to stage II in the Table of Hamburger-Hamilton but the rest of the body was even more delayed and that on the left had a square cephalic end as its most salient morphologic abnormality.

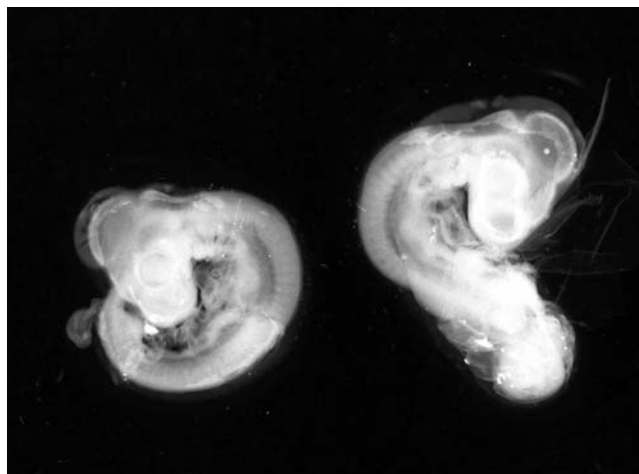


Figure 8
Two 3.5 days-old chick embryos showing few differences in their external aspect. The embryo control is on the right. On the left the embryo shows mainly a minor delay in its development.

because the ceE contains paternal serum proteins (sAlloAg) only when one gets a potent anti-ceE IgY and a concentrate ceE or better a purified sFF-Ag sample from ceE, can one definitively confirm these results. In fact, one sample of that primary antibody would be adsorbed only with hen serum and another sample would be doubly



Figure 9
A 21 day-old chick embryo found dead 9 hours after the predicted hour of hatching without external anomalies.

adsorbed with hen and cock sera. Then the results obtained in each membrane will be able to be compared. Using thus this doubly adsorbed primary antibody against ceE, the bands can only be sF-TrAg. When the primary antibody only adsorbed with hen serum reacts against cock serum the present bands can only concern to sAlloAg. In accordance with this reasoning the antigens of 34 and 200 kDa present in both cock serum and ceE (Figure 3 and 4) should be considered, in my personal opinion, provisionally in a case as sAlloAg and in other as sF-TrAg.

The chick embryo extract used contained some proteins the synthesis of which is dictated either by paternal genes (soluble alloantigens) or by embryonic genes [20] that ceased to be expressed before the immune system had matured (soluble foreign transitory antigens) before or after hatch. So both protein classes are foreign antigens to the hens, if they come into contact with their immune system during the experiment, or -one may speculate- in a natural manner in human and some rodent species if they cross the placenta. This common feature allowed them to be used to immunize hens, and the antibodies thus induced to expose their presumptive teratogenic and/or abortogenic action in a completely physiological manner. Finally, the antigenic analysis conducted via immunoblot tests identified the proteins most likely to be the origin of the pathological effects observed from minimal information about the sAlloAg (*viz.* their molecular weight and soluble nature in cock serum). From these data it follows that the sAlloAg were detected because the chick embryo was included in the *area vasculosa* up to the *sinus terminalis* with the incipient serum proteins (ceE) that were therefore involved in the immunization process.

Because serum proteins can migrate into the allantoic cavity, some of the TrAg detected in allantoic fluid from chick embryos might also be sAlloAg [16]. This antigenic analysis also allowed the detection of sF-TrAg, not present in cock serum, the synthesis of which stops before its specific immature lymphocyte appears and they are already not present -which is obvious as it induces the production of antibodies by the hens- before or after hatch. The fact that some sF-TrAg were not detected in all embryos' extracts (paths 2-9, Figure 4) does not necessarily mean that it ceases to be synthesized at that age as its detection may be hindered by a diluting effect arising from the growth in body volume with age, by small variations in the preparation procedure of each sample and even by the individual "history" of each Eppendorf tube. For all these reasons, they must be purified, used at higher concentrations, raised higher concentration of antibody in order to obtain information other than its molecular weight, soluble nature and the fact that they are foreign to the dam.

IgY against some or all sFF-Ag were found to induce presumably a number of dysmorphisms and/or functional alterations incompatible with life; also, all but one of the few chicks that were born alive had a congenital transitory neurological disease. In a separate study, we shall examine in detail the relationship between the date at which development was arrested and the date at which morphological abnormalities on the macroscopic and microscopic level appeared in relation to the duration of immunization at the time the corresponding egg was laid, as well as any similarities between abnormalities in the batch of eggs that were laid only a few days apart but were incubated simultaneously. There was a significant difference between the total number of embryos from immunized and control hens and that of abnormal embryos in the two groups. This was also the case with the ratios of abnormal to normal embryos in each group (Table 1).

Two previous studies were approached in a similar way to the present one. One examined pre-differentiation stages in the chick embryo. To this end, hamsters were immunized with homogenates from 24-h, 72-h and 6-d chick embryos. The greatest number of abnormalities and deaths was caused by the antiserum induced with the homogenate from 72-h embryos, followed by those from 24-h and 6-day embryos [2]. The other study was aimed at determining whether the early chick embryo is antigenically different from that of the adult and at examining the effect of its anti-embryo antibodies on the development of the homologous embryo. To this end, rabbits were injected an extract from embryos incubated for 72 h and the antiserum inoculated to embryonated eggs incubated for 72 h. Abnormalities were observed within 12 h and deaths within 5 min to 8 h after inoculation [5]. In judg-

ing the results of these pioneering experiments, one should take into account that both the hamsters and the rabbits immunized with material from chick embryo produced antibodies against the wide variety of antigens contained in it – permanent antigens included. All these antibody species were subsequently inoculated into the embryonated egg, which was assumed to be able to neutralize the corresponding antigen classes. The only anti-permanent antigens antibodies detected in our experiment were anti-sAlloAg. Hence our respective results cannot be compared.

Regarding our immunization procedure, we should note the disadvantages of using heterologous sera if the antigens involved are foreign to the hen used to examine the action of the corresponding antibodies in their embryonated eggs. Fresh normal serum from some animals is toxic to the chick embryo. In fact, rabbit serum has been found to contain a thermolabile substance that is embryo-toxic; this requires heating the anti-embryo serum before inoculation, which reduces its ability to induce unspecific abnormalities in relation to unheated serum [5]. In addition to rabbit sera, dog, pig and frog sera are also highly embryo-toxic. On the other hand, guinea pig, duck and mouse sera proved inactive, and cow, rat and goat sera exhibited a teratogenic activity level between the two extremes [22]. Human serum is also very embryo-toxic [23]. When a heterologous anti-chick embryo serum must be made specific against one (e.g. α -fetoprotein [14]) or several transitory antigens by removing anti-permanent antigen antibodies by adsorption with adult serum, with a view to examining the effects of neutralizing their respective antigens *in ovo*, one has the disadvantage that the embryonated egg is inoculated not only with the proteins in the heterologous antiserum, but also with those in the hen or cock serum used for adsorption. This can be avoided by purifying immunoglobulins, which include specific antibodies, by precipitation with ammonium sulphate [9,12,13] or by other methods; this is labor intensive and causes the loss of specific IgG. Finally, the embryonated egg must be manipulated in all cases.

What remains of this discussion is devoted to establishing apparent or hypothetical relationships between these results and the problem of recurrent spontaneous abortion (RSA) in humans. If some sAlloAg or an sF-TrAg reaches the mother, it will induce the production of antibodies that may come into contact with the fetus and cause a congenital disease or abortion.

There have been many attempts at identifying the causes of RSA without success. A high proportion of RSA of unknown origin has been ascribed to alloimmune factors; however, because of the lack of clinical or laboratory tests to confirm classical alloimmune RSA diagnoses, they are

considered "exclusion" or "unexplained" diagnoses [24]. Moreover, the therapeutic counter-test involving immunization with paternal white cells or immunoglobulins, or of one third-party, in order to induce the production of blocking antibodies against the paternal membrane alloantigens in the mother, has failed to meet the initial expectations [24–26]. The embryofetal pathology of classical alloantigens associated to the cell membrane has been known for long and appears to have been virtually exhausted as its concepts and techniques cannot account for so many cases of RSA.

Some recent data suggest that sAlloAg may be candidates for a new etiological entity in embryofetal immunopathology. In most of the following references, soluble MHC antigens (sMHC) detected in maternal plasma were assumed to originate from the mother. In some cases where their presence coincided with RSA, one may still suspect a fetal origin- and this may be the case with the results of this work. If, on the other hand, the assumption that fetal sHLA never cross the placental barrier [27] holds, then extrapolating our results to humans would be unwarranted. Virtually all class I MHC antigens can be solubilized and be present in organic fluids [28]. One metalloprotease is able to solubilize its heavy chain (HC) [29,30]; this HLA-HC, and the whole HLA molecule (HLA/2m), has been detected in healthy individuals and during graft rejection [31]. Recently, truncated soluble class I RT1.Aa MHC antigens were found to induce alloimmunity [32]. Women with RSA have been found to have increased class I sHLA levels the origin of which has been related to that of the increased levels encountered in patients with acute rejection of transplanted organs [33]. HLA antigens of fetal origin are known to be present in the maternal circulation throughout pregnancy beginning at 8 weeks. As early as the 8th week, a woman can have alloantibodies that form complexes with soluble HLA alloantigens. In some women, the production of anti-anti-HLA antibodies is apparent already in the first trimester; in others, it takes somewhat longer. Antibody specificity analyses have revealed that some healthy primiparous women develop antibodies reactive with self-HLA antigens. Although allo- and autoantibodies responses appear to be modulated by soluble HLA antigens, cyclic changes in alloantibodies levels and the selective response of the mother to some, but not all, paternal HLA antigens are more consistent with the production of anti-idiotypic antibodies [34]. Therefore, one can assume that pregnant women respond to their fetal alloantigens with alloantibodies, and that these are suppressed by soluble HLA and by their anti-idiotypic antibodies. If this is so, many cases of RSA may have involved some failure in this defense mechanism, and non-neutralized maternal antibodies may have crossed the placenta and suppressed the biological function of paternal alloantigens in the fetus, thereby

Table 4: Molecular weights (kDa) of the class I and II soluble MHC antigen species detected in rodents and humans. Comparison with four molecular species of similar MW detected in paternal serum using the immunoblot test with anti-chick embryo antibodies, adsorbed with hen serum.

sHLA/2-m class I	beta 2-m	sHLA-HC class Ia	sHLA-HC class Ib	sHLA-HC class Ic	sHLA class II	Reference and animal species ^d
	11	44		33		47 H
		43	39			48 H
		44	40	37-35		49 H
200						50 H
		44	39-36	34		51 H
	12		39	33		52 H
				36		30 H
			39 non-RTI.A associated with β -2 m	36 RTI.A ^a		53 R
		43	39	35	70	54 H
						35 M
200			34	32	70	TW ^e A

^aThe heaviest species resulted from cell shedding and included a transmembrane segment and cytoplasmic tail ^bThe intermediate species resulted from alternate splicing transcript and contained no transmembrane piece ^cThe lightest species contained neither a transmembrane piece nor a cytoplasmic tail ^dH = human, R = rat, M = mouse, A = avian, TW = this work ^eThe author does not prejudice here which MHC class or group the sAlloAg detected in this work belong to

leading to death (abortion) or to the development of congenital diseases. Why then were anti-sFF-Ag IgG in the immunized hens not blocked by their anti-idiotypic antibodies? Perhaps, under natural conditions, birds undergo no antibody induction by sFF-Ag from their offspring.

γ -Interferon facilitates the release of soluble class II MHC antigens present in the cytoplasm of trophoblastic cells. Thus, a purified protein of 70 kDa was found to increase the abortion rate in mice upon inoculation [35]. Moreover, the DQA1*0201/DQB1*0201 haplotype is more frequently encountered in husbands of women with RSA than in control couples. Thus, in a group of seven husbands of women with RSA who were heterozygotic for this haplotype and shared no DQA1*0201 allele with their wives, the haplotype was transferred to 6 of the 7 aborted fetuses. Although the number of cases was fairly small, one cannot exclude HLA-DQ antigens as inducers of these abortions [36].

Based on the above and on the results of this work, it would be interesting to systematically ascertain the maternal or fetal origin of the sAlloAg found, at more or less increased levels, in serum from mothers with RSA and to determine whether they belong not only to classical class I or class II, but also to class III. Surprisingly, there is little reported association of class III sMHC antigens to RSA. A decade ago two articles were published which demonstrated a statistically significant increase of serum complement C4 [37] and of complement C4 "null" alleles [38,39] in women with RSA. Based on the large number of proteins that are coded by class III MHC – most of which, in addition, are polymorphic – [40], some proteins might

correspond to some sAlloAg found in chick embryos (*e.g.* C4 factor of complement is a β_{1E} -globulin with 209 kDa, near the sAlloAg 200 kDa (see Table 4, which shows the data for the four avian sAlloAg with a MW identical with or close to previously reported ones, irrespective of any other criteria).

The lack of reported instances of soluble transitory antigens foreign to their dam (sF-TrAg) provides no basis for discussing the presumptive involvement of these fetoproteins in the embryofetal pathologies described here, which probably occur in humans as well.

Finally, the presence of these interesting fetoproteins, sFF-Ag, in the early chick embryo gives us reason to think that some of them may play an important physiological role in the organogenesis.

Conclusions

The chick embryo is an extremely convenient animal model for identifying the underlying immune mechanisms of the abortions and malformations caused by anti-sFF-Ag antibodies as the physiological separation between an embryo and its dam is anything but small; also, stopping embryonic development and obtaining large amounts of antibodies from the egg yolk is very easy. However, the results obtained for the avian model cannot be extrapolated as such, without further analysis, to rodents or humans. Experiments involving a mammal with a hemochorial placenta and the use of purified sAlloAg and sF-TrAg, from that specific animal are required to identify those antibodies with presumptive teratogenic and/or abortogenic action before the results of this work

can be extrapolated to women. Also, analyses of women with RSA or malformed children, once a non-immune or a classical membrane alloantigen-related etiology has been rejected, should be conducted in search for anti-sFF-Ag antibodies in maternal serum, whether by use of serological techniques or by inoculation into pregnant mice [40]. The information thus obtained will be of assistance in determining, to some extent, the cause and, with greater detail, some of the effects of disrupting the maternal homeostasis that protects the fetus. This in turn will provide solid support for future preventive or therapeutic treatments intended to reduce the currently high proportion of spontaneous abortions and congenital diseases of unknown origin.

Methods

Fertilized eggs

The eggs, from Plymouth Rock hens fertilized by White Leghorn cocks, were obtained from a commercial farm and incubated at 38°C and 60% relative humidity in a laboratory incubator for 53 h in order to prepare antigen extracts.

Antigen extracts

Each 53-h embryo (*viz.* at stage 14–15 in the classification of Hamilton and Hamburger [18]) was withdrawn by cutting the membrane along the edge of the *sinus terminalis*, washed with cold PBS, drained and placed in an Eppendorf tube containing 0.5 ml of lysis buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) that was immersed in an ice bath. Overall 20–24 embryos from each batch, which produced a volume close to 1 ml, were used. Such a volume was supplied with a protective cocktail of the following composition: 0.1 mM pepstatin A, 3 mM dithiothreitol, 2.5 mM iodoacetamide, 1 mM PMFS, 5 mM phenanthroline, 3 mM EDTA and 0.01% merthiolate. After 2 h at 4°C, the homogenate was aspirated and expelled using an insulin syringe equipped with a thin (16/5) needle. The non-viscous sample was filtered through glass wool in another Eppendorf tube and centrifuged at 14 000 g for 10 min in an Eppendorf centrifuge located in a chamber at 4°C. The resulting supernatant constituted the chick embryo extract (ceE) and was used to immunize hens and analyze for sFF-Ag by dot-immunoblot, SDS-PAGE and Western immunoblot test. The same or a similar suitable procedure was used to obtain ceE from embryos incubated for 72 h and 5, 7 or 9 days. Liver extract from W-L hen was prepared also by this procedure.

The concentration of proteins in each sample (Figure 4) and other features were: Path 2: dialyzed ceE 53 h with stacking buffer (Tris-ClH pH 6.8, 0.5 M), protein concentration 94.4 mg/ml; Path 3: ceE 53 h made only with the

embryos without membrane, 89.2 mg/ml; Path 4: ceE 53 h without special treatment, protein concentration 56.3 mg/ml; Path 5: ceE 53 h without special treatment, 91.7 mg/ml; Path 6–9: ceE 72 h, ceE 5 d, ceE 7 d and ceE 9 d were each mixed with sample buffer 1:1 when they were obtained, protein concentration 86.8, 41.6, 46.3 and 39.4 respectively.

Immunized and control hens

Each ceE batch obtained (from 20–24 embryos) was used to prepare one immunizing dose that was emulsified with a double volume of Freund adjuvant (complete in the first injection and incomplete in the rest). Two White Leghorn (W-L) hens were given an injection of one dose each intramuscularly in two aliquots (one on each side of the keel). Injections were given in three different series with the following sequences: (A) days 1, 15, 29, 38, 59, 66 and 70 (followed by 75-day rest); (B) days 1, 40, 49, 66, 94, 147, 178 and 196 (followed by 60-day rest); and (C) days 1, 21, 36 and 47. Simultaneously, two control W-L hens were given injections where the ceE was replaced with PBS.

Embryos from immunized and control hens

An autochthonous Campero cock fertilized the immunized and control hens. Their eggs were incubated in the laboratory for different lengths of time, from 30 h to hatching, depending on their intended use. Embryos were stored in formaldehyde buffered at pH 7.3 for subsequent histological analysis. A few of them were photographed as soon as they were obtained.

Obtainment of IgY

IgY was extracted from the yolk [41] of some eggs at immunization stages B and C with ceE 53 h (anti-ceE IgY) and from the control hens' yolk (anti-control IgY). For a punctual objective were also obtained IgY from yolk of untreated Plymouth Rock hens ("normal" IgY).

Obtainment of sera

Sera from W-L cock, W-L control hen, untreated P-R hen and goat (the latter from an abattoir) were obtained after standard procedure and inactivated at 56°C for 30 min and 0.01% Merthiolate was added.

Dot-blot

Dot-blot for antibody capture assay was carried out onto nitrocellulose membrane (0.45 µm, Bio-Rad). Antigens (ceE, cock and goat sera), primary (anti-ceE IgY, anti-control IgY, and "normal" IgY) and secondary anti-chicken IgG (developed in rabbits, labeled with horseradish peroxidase, Sigma, lot 86H4824) reagents were used in 0.5 µl volume. The substrate used was diaminobenzidine (DAB, 3,3',4,4'-tetraaminobiphenyl) with NiCl₂ as an enhancer [43].

Adsorption of spurious antibodies

The primary reagents, anti-ceE IgY and anti-control IgY, were used native and adsorbed with a tenth part of hens' sera (of which half was from W-L hen and half from P-R). The labeled secondary reagent was used native and adsorbed with a tenth part of liver extract from a W-L hen. The three antibody reagents with their quoted antigens were incubated for 2 hours at room temperature in an end-over-end mixer and spun, each one showing a conspicuous pellet. However, these anti-ceE IgY and anti-control IgY, together with the anti-chicken IgG conjugate so adsorbed, still gave false positive results in dot-blot against the antigens W-L cock, W-L hen, P-R hen sera and goat serum. Obviously always there was a positive reaction between cock serum and anti-ceE IgY. This served as a positive control and for optimizing the dilutions of the reagents. Then anti-ceE IgY and anti-control IgY were mixed with Sepharose CNBr coupled to hens' sera (half from W-L hen and the other half from P-R one) and the anti-chicken IgG conjugate was mixed with Sepharose CNBr coupled to liver extract from W-L hen [44]. After 18 hours of incubation to +4°C in an end-over-end mixer, all of them were spun and the supernatants used later. After other controls, it was checked that with this treatment all the spurious antibodies had been removed and the false positive results were thus avoided. Then these reagents could be used in Western immuno-blot. When they are used no adsorbed they will name "native".

SDS-PAGE and immunoblot tests

The protein components of the ceE and cock serum (and W-L and P-R hens sera, as control) were separated using the SDS-PAGE technique without reducing agents [43,45]. The SDS-PAGE technique was applied by using 5–10% (Figure 2) and 4–15% (Figure 3 and 4) gradient of acrylamide-bis. Western immuno-blot was carried out with the same membrane type and reagents as in dot-blot, unless otherwise indicated. The membrane transfer technique was used to detect sFF-Ag with anti-ceE IgY and anti-chicken IgG conjugate to the dilutions previously already optimized. Mini-Protean cell III and the pertinent transfer complements (Bio-Rad) were used for this purpose following the manufacturer's recommendations. However, the transfer buffer for proteins between 20,000 and 400,000 Da used was as follows: Tris 50 mM, Glycine 380 mM, SDS 0.1% (wt/vol), methanol 20%, distilled water up to 1000 ml [43]. Prestained standards molecular weight markers (Bio-Rad), low (control 94504; 113, 92, 52.9, 35.4, 29 and 21.5 kDa, respectively) and high (control 95686; 207, 117, 95 and 49 kDa, respectively) range were employed.

Statistical analysis

A comparison of proportions has been made in a table 2 × 2 with a SPSS package, version 11 [44].

Approval of the bioethics committee

The author has obtained approval for his research with a cock, hens and avian embryos from the bioethics committee of the Cordoba University, since the experiments are in compliance with the current legislation about animal experimentation (Directiva 86/609/CEE y R.D. 223/1988).

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