The Effects of Chorioallantoic Grafts on the developing Chick Embryo

II. Studies of Adult Antigens in the Duodenum and Spleen

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WITH ONE PLATE

INTRODUCTION

There is abundant evidence that the eggs and developing embryos of the chick possess antigenically active materials; that during development changes occur in the antigenic pattern; and that many of these antigens are similar to certain adult antigens. An extensive review and summary of the early literature on the origin of adult antigens in the developing embryo has been made by Needham (1931), Cooper (1946), and Schechtman (1947). Consistent results have been obtained only in recent years by the use of more refined techniques and have been reviewed by Woerdeman (1953), Tyler (1955, 1957), Brachet (1957), and Ebert (1958).

Burke, Sullivan, Petersen, & Weed (1944) prepared antisera against saline extracts of adult organs (brain, testis, ovary, kidney, liver, and lens) of the chicken. They observed that adult organ-specific antigens in the chick embryo appeared subsequent to differentiation and development of the organ, e.g. lens at 146 hours, erythrocytes at 100 hours, kidney at 220 hours, and brain, testis, and ovary at 260 hours. On the other hand, Schechtman (1948) and Ebert (1950) maintained that antigens of the brain, heart, and spleen were identifiable in the early chick blastoderm. Furthermore, adult lens antigens were identified in the embryo at 58 hours by Ten Cate & Van Doorenmaalen (1950) and at the time of lens formation by Flickinger, Levi, & Smith (1955). Recently, Van Doorenmaalen (1958), using the fluorescent antiserum technique, was able to demonstrate specific localization of the marginal zone of the lens epithelium on the 5th day of development. This whole problem of immunochemical study of the development of the lens has been thoroughly reviewed by Ebert (1958).

Schechtman (1948) was the first to identify an organ antigen in the early chick

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embryo, i.e. at the primitive-streak, early neurula, and 4- to 5-somite stages. The ontogeny of tissue antigens in the developing chicken spleen was demonstrated by Ebert (1952). He prepared antisera against saline extracts of 18-day embryonic chick spleen and tested it with saline extracts of 12- and 18-day embryonic spleen. It was observed that the number of antigens increased from three to six between the 12th and 18th day of incubation. Absorption of anti-18-day spleen sera with 12-day embryonic spleen reduced antigenic reactions from three to zero with 12-day spleen antigens and decreased antigenic reactions with 18-day preparations from six to three.

Proteins of the blood-serum of chicks have been identified in the egg-yolk and in extracts of various ages of chick embryos by immunochemical methods (Schechtman, 1955). Antigenic activity of the yolk was reduced by absorption with anti-adult serum, anti-albumin, and anti-γ-globulin (Schechtman, 1947; Nace & Schechtman, 1948). Similar results were obtained by Schechtman & Hoffman (1952) using anti-αβ-globulin serum. Nace (1953) observed the ontogeny of blood proteins in the course of development. He was able to demonstrate that serum of chick embryos contained a vitelloid albumin present from the 3rd day, a non-vitelloid albumin by the 5th day, a vitelloid γ-globulin by the 9th day, and a non-vitelloid γ-globulin by the 12th day; a non-vitelloid αβ-globulin was present from the 6th day.

From the above literature it appears that it may be possible to detect an ontogeny of adult antigens in the formation of the duodenum which might be correlated with previous studies of morphological and chemical differentiation as carried out by Moog (1950), Richardson, Berkowitz, & Moog (1955), and Van Alten & Fennell (1957). It was also believed that this would be fundamental to a study of the effect of chorioallantoic grafts on the antigenic pattern of the duodenum. The details of this study were given by Van Alten & Fennell (1959).

MATERIALS AND METHODS

A modification of the agar diffusion technique of Ouchterlony (1949), which has recently been reviewed by Oudin (1952), was used in the study of adult antigen formation in the embryonic duodenum (11 to 21 days of incubation). The geometry of the plates has been described by Fox & Yoon (1958).

Pooled adult White Leghorn chicken duodena and large numbers of embryonic chick duodena of 11 to 21 days of incubation were used as a source of antigens, the pancreas having been removed. When antigenic preparations were needed for immunization or for serological tests, the adult tissue was homogenized in a Waring blender for 10 minutes, followed by a second homogenization with a glass homogenizer for 30 minutes at 4°C in saline (0.15 M NaCl + 0.005 M phosphate, pH 7.4), in a ratio of 1 g. wet weight of tissue per 5 ml. of saline. The homogenate was centrifuged at 4°C for 30 minutes at 500 g. Antigens of embryonic chick duodena of 11 to 21 days were prepared in a similar
manner except that all homogenizing was done with an all-glass homogenizer. Protein determination of the antigens was made by the biuret method of Lowry, Rosebrough, Farr, & Randall (1951). The antigen prepared from adult duodenum was found to contain 19 mg. of protein per ml. and the antigens prepared from embryonic duodena contained about 6 mg. of protein per ml. in each preparation.

Antisera were prepared against adult chicken duodenum and spleen by injecting the pooled antigen into three male rabbits. Five intravenous injections were given during a 10-day period, one every other day. The first two injections were 0.5 ml. of antigen each; the third was 1 ml., the fourth 1.5 ml., and the fifth 2 ml. The rabbits were rested for 2 weeks following the injections and then given an anamnestic injection of 2 ml. of antigen intravenously. Seven days later the rabbits were bled and a precipitin titration was made. If the titre was at least 1:64 the rabbit was bled on the following day and the sera were collected; 1:5,000 merthiolate was added, complement inactivated by heating at 56° C. for 30 minutes and the sera frozen until used. If the titre was not 1:64, the rabbit was given another 2-ml. injection of antigen on the following day and the serum titrated after another 8 days. This procedure was carried out until a sufficient titre was obtained.

RESULTS

The appearance of adult duodenal antigens in the developing embryonic duodenum of the chick

Antisera against saline extracts of adult chicken duodenum were tested with saline extracts of adult and 11- to 21-day embryonic chick duodena by a modification of the methods of Ouchterlony (1949) and Bjorklund (1952).

Plate, fig. A shows a diagram of an Ouchterlony plate obtained with anti-adult duodenum serum. This plate consisted of agar with four wells; the lower well contained the antiserum while the other three wells contained saline extracts of 12-, 13-, and 14-day embryonic duodenum. In the plate a series of lines of precipitate have developed where there was optimum concentration of antigens and antiserum, and these have been numbered 1, 2, and 3 in the diagram.

The results of these experiments show that there were changes in the number of antigens present in the duodenum of the chick embryo between 11 and 21 days of incubation. From the 11th to the 13th day only two lines were formed. On the 14th day a third line was present (line 3, Plate, figs. A, B). Line 4 appeared on the 15th day and was also found on the 16th and 17th days (Plate, figs. C, D). However, on the 18th day only lines 1, 2, and 3 were present and line 4 was absent (Text-fig. 1). Lines 1, 2, and 3 remained up to the 21st day, and a fourth line was also present at this stage, but it is not possible from the data to ascertain if this line was identical to line 4 in 15-day duodena.

The distribution of line 4 has been confirmed by repetition of experiments and
also by means of the Bjorklund inhibition technique. When the latter technique was used results were obtained which are shown in Text-fig. 2. In this plate six doses of 0.15 M saline extract of 18-day embryonic duodenum were put into the antiserum well prior to putting the antigens and antiserum into their respective wells. It was shown in Text-fig. 1 that 18-day duodenal antigen reacted with antiserum to form lines 1, 2, and 3. Following the inhibition procedure lines 1, 2, and 3 were absent but line 4 developed adjacent to the 15-day well (Text-fig. 2).

The presence of line 4 and also the line adjacent to the 21-day well suggests that these antigens may be qualitatively distinct from those found in the 18-day duodenum.

The results presented in Text-fig. 3 were obtained in essentially the same manner as those in Text-fig. 2, with the exception that adult chicken serum was used as the inhibiting antigen. The position of lines 1, 2, and 3 adjacent to 20- and 21-day wells is comparable to those in Text-fig. 1. This suggests that lines 1, 2, and 3 are not serum antigens. The absence of the line adjacent to the 21-day well, as illustrated in Text-fig. 2, suggests that this antigen has reactive groupings in common with serum.

Table 1 summarizes the distribution of duodenal antigens during the period from 11 to 21 days. The inhibition analyses are also summarized. It has been demonstrated that (1) the pattern of adult duodenal antigens found in the developing duodenum changes during development, and (2) at least three of the antigens found in the embryonic duodenum do not appear to be present in adult serum.
### Table 1

**A summary of the results obtained with the serum-agar precipitin test**

Adult duodenum antiserum was tested with saline extract of adult and 11- to 21-day chick embryonic duodena

<table>
<thead>
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<th>Antiserum</th>
<th>Antigen</th>
<th>No. lines</th>
<th>Inhibition</th>
<th>No. lines</th>
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<tr>
<td>&quot;</td>
<td>12-day embryo duodenum</td>
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<tr>
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<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>14-day embryo duodenum</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>15-day embryo duodenum</td>
<td>4</td>
<td>18-day embryo duodenum</td>
<td>1</td>
</tr>
<tr>
<td>&quot;</td>
<td>16-day embryo duodenum</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>17-day embryo duodenum</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
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<td>Adult serum</td>
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<td>&quot;</td>
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</table>

**The effect of chorioallantoic transplants of adult chicken duodenum and spleen on the development of adult duodenal and splenic antigens in the duodenum and spleen of 17-day embryos**

Antisera against saline extracts of pooled adult chicken duodena or against adult chicken spleen were tested with saline extracts of (1) normal 17-day embryonic chick duodenum or spleen; (2) 17-day embryonic duodenum or spleen which was stimulated by CAM (chorioallantoic membrane) grafts of adult chicken duodenum; (3) 17-day embryonic duodenum or spleen which was stimulated by CAM grafts of adult chicken spleen. Immunochemical methods similar to those mentioned in the preceding section were used.

The antigenic pattern of the developing duodenum following CAM grafts of adult chicken duodenum or spleen is shown in Plate, figs. E, F. In this figure the antiserum well contained anti-adult chicken duodenum and the other wells contained antigenic mixtures prepared from 17-day embryonic duodenum which had been stimulated by grafts of adult spleen (Spleen Duo), antigenic mixtures prepared from 17-day embryonic duodenum which had been stimulated by grafts of adult duodenum (Duo-Duo) and antigenic mixtures prepared from control 17-day embryonic duodenum (17-Duo). It may be seen that opposite wells 17-Duo, Duo-Duo, and Spleen Duo, lines 1 to 4 are present. This demonstrates the presence of an antigenic component of identical specificity in all three preparations. It will be further noted that line 5 is only opposite wells Duo-Duo and Spleen Duo. On the other hand, lines 6 and 7 are present only opposite well Duo-Duo.
Text-fig. 4 shows an Ouchterlony plate obtained with anti-adult chicken duodenum serum and tested with antigenic mixtures prepared from adult duodenum (Adult Duo), antigenic mixtures prepared from 17-day embryonic duodenum which had been stimulated by adult duodenum grafts (Duo-Duo), and antigenic mixtures prepared from control 17-day embryonic duodenum (17-Duo). It was evident (see Text-fig. 4; Plate, figs. E, F) that grafting of adult duodenum to the CAM of 9-day chick embryos caused an increase in antigenic components 4 to 7 in the 17-day embryonic chick duodenum. It was also observed that if adult chicken spleen was transplanted to the CAM, one extra antigenic component was found, but this one was also found if duodenum was used on the CAM.

It has been shown by Van Alten & Fennell (1959) that the spleen of the embryo undergoes a marked increase in size subsequent to grafting adult duodenum or adult spleen to the CAM. Text-fig. 5 shows an Ouchterlony plate obtained with anti-adult chicken spleen in the antiserum well and saline soluble antigenic mixtures prepared from control 17-day embryonic spleen (17-day Spleen), antigenic mixtures prepared from 17-day embryonic spleen stimulated by adult spleen grafts (Spleen Spleen) and antigenic mixtures prepared from 17-day embryonic spleen stimulated by adult duodenum grafts (Duo-Spleen) in the other 3 wells. Lines 1, 2, and 4 can be seen opposite well 17-day Spleen, and from other plates it has been confirmed that line 4 consists of two antigenic components which are seen as lines 3 and 4 opposite the Duo-Spleen well. Only two lines can be seen opposite well Spleen Spleen, but these have also been shown to consist of two lines each; it can therefore be concluded that lines 1, 2, 3, and 4 are all present. Lines 3 and 4 are clearly seen opposite well Duo-Spleen. However, lines 1 and 2 form one line as they did opposite well Spleen Spleen. It is clear then that lines 1 through 4 are opposite each of the antigen wells and are continuous, and that they therefore demonstrate antigenic components of identical specificity. However, because the lines are together opposite some of the wells and divided when opposite others, this shows that there are quantitative differences in the various spleens. Line 5, on the other hand, is present only opposite well Duo-Spleen, thus clearly demonstrating a qualitative difference.
From the above results it can be seen that grafting of adult chicken duodenum to the CAM of 9-day chick embryos causes both quantitative and qualitative antigenic differences in the duodenum and spleen of 17-day embryos. On the other hand, grafting of adult spleen to the CAM produces both qualitative and quantitative changes in the antigenic picture of 17-day duodenum, but only quantitative changes were observed in the host spleens.

**DISCUSSION**

It was observed in this study that antigens of the adult chicken duodenum can be identified in the 11-day embryonic duodenum. However, from the 11th to the 21st day of incubation there is an increase in the number of antigens in the developing duodenum; thus, two antigens were present in 11-, 12-, and 13-day embryos; three antigens in 14-day embryos; four antigens in 15-, 16- and 17-day embryos; three antigens in 18-, 19-, and 20-day embryos; and four antigens in 21-day embryos. Ebert (1952) identified an increase in the number of antigens in the development of the chick spleen. He observed three antigens in spleens of 12-day embryos and six in spleens of 18-day embryos when he used 18-day embryonic spleen antiserum.

The appearance of an antigen on the 15th day and its disappearance on the 18th day is rather striking. A somewhat similar situation has been reported by Telfer & Williams (1953) in the *Cecropia* silk worm. They identified five pupal blood antigens present throughout metamorphosis, with a sixth appearing at the fifth instar. The latter antigen persisted during the pupal stage and disappeared in the adult. In the case reported in this study the antigen observed on the 15th day was also found in the adult because antiserum was prepared against adult duodenum. It must be kept in mind that at the present time it is not possible to prove the complete absence of an antigen on the basis of its absence in agar plates.

Several explanations for the appearance and disappearance of an antigen during the course of development can be advanced. Tyler (1957) suggested that many of the large molecular substances of an organism may have similar determinant groups and therefore may react with the same antiserum. Thus, it might be that during the 15th, 16th, and 17th day the fourth antigen which was present may not have been the actual adult antigen, but rather one which had similar determinant groups and thus reacted with the antiserum. This disappearance, then, could be ascribed to an alteration of determinant groups in the course of development, so that a reaction with the antiserum no longer occurred. However, a more satisfactory explanation would seem to be one related to changes which might occur in protein configuration during morphogenesis.

During morphogenesis there may be a structural change in antigenic macromolecules. Fox (1958) suggested that an antigen may be transformed from a complete antigen into an incomplete antigen. An incomplete antigen is one
which has specificity for antibody formation but which will not react in the precipitin test. A somewhat different change in antigenic structure could occur, i.e. a change from a globular to a fibrous protein. Mazia & Dan (1952) observed that during spindle fibre formation there was a change from globular to fibrous protein. Heidelberger (1956) suggests that antigenic specificity may be due to multiple reactive areas on the surface of antigens. He believes that changes in specificity of native proteins can be accounted for by the unfolding of polypeptide chains which leads to their separation from adjoining chains, and that, as a consequence, the specificity of the antigen is altered.

If Heidelberger's concept correctly explains antigenic specificity it is reasonable to assume that during the course of development of an antigen its polypeptides may undergo foldings and separation. Under these conditions the reactive surface of the molecule is altered and cannot be detected by immunological methods. On the basis of the evidence on hand and the wide divergence of views of antigen reactivity, a final explanation cannot be given as to why line 4 appears in 15-day duodena and disappears in 18-day duodena.

It has been further observed in the course of this study that transplantation of adult chicken organs (duodenum and spleen) to the CAM caused changes in the antigenic pattern of both the duodenum and spleen. This change was most pronounced following grafting of adult duodenum. Adult duodenal grafts increased the number of antigens from 4 to 7 in the 17-day embryonic duodenum and from 4 to 5 in the spleen. The grafting of adult spleen altered the antigenic pattern of the 17-day duodenum (from 4 to 5) but was without effect in the spleen. It must be kept in mind, however, that even these changes in the antigenic picture are the end products of differentiation and not the causal factors.

Ebert (1955) maintained that growth of host tissues following homologous grafts was due to a 'building block' rather than a 'template' or catalytic mechanism (Weiss, 1947). The findings in this investigation tend to support this 'building block' hypothesis, i.e. selective incorporation of tissue-specific antigens from grafts into homologous host tissues. However, this hypothesis does not adequately explain the generalized effects, i.e. growth of heterologous organs. Also, the 'building block' hypothesis does not allow for the possibility that the graft may be exerting an influence which causes an acceleration of antigenic differentiation rather than a direct transfer mechanism. The evidence of this investigation does not rule out the possibility of a catalytic mechanism as advanced by Weiss (1947). This catalytic mechanism could be similar to that of increasing the substrate and accelerating differentiation of a specific enzyme. Recently, Kato & Moog (1958) have shown that after injection of disodium phenylphosphate into the chick on the 14th day of incubation there was an increased alkaline phosphatase activity in the duodenum, liver, mesonephros, and metanephros on the 17th day of incubation.

This study has shown that when adult duodenum or spleen were grafted the increase in the number of antigens was not confined to the homologous organ.
It might be postulated that some non-specific material, common to several organs, might accelerate growth and enhance differentiation of the adult antigenic pattern.

SUMMARY

1. The development of adult duodenal antigens in the embryonic duodenum was studied by means of the Ouchterlonny agar diffusion technique.

2. In 11-, 12-, and 13-day embryonic duodena there were two antigens; by the 14th day three antigens; in the 15-, 16-, and 17-day duodena four antigens; while in 18-, 19-, and 20-day duodena there were only three antigens with a fourth antigen again present in the 21-day duodenum. The disappearance of the fourth antigen found in 15-, 16-, and 17-day duodena was attributed to a change in molecular configuration of the antigen in the process of differentiation.

3. Following grafts of adult duodenum there was an increase of three antigens observed in the host duodenum and an increase of one antigen in the host spleen.

4. When adult spleen was grafted there was no increase in the number of antigens in the host spleen, but an increase of one antigen was observed in the duodenum.

5. The results are discussed in the light of the organ-specific growth stimulation hypothesis, but this is rejected because it cannot account for the generalized effects observed.

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REFERENCES


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