AM. ZOOLOGIST, 3:235-243 (1963).

THE LABILE CHORIOALLANTOIC MEMBRANE: CHANGES EFFECTED BY VIRUSES AND INHIBITORS

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I propose to consider further the thesis advanced two years ago (Ebert and Wilt, 1960) that animal viruses can be used to detect differences in cell-specific properties and to explore some of the problems of the origin of such differences. Specifically, I will describe some of the properties of an interferon-like inhibitor produced by the chorioallantoic membrane, *in vitro,* attempting to relate it on the one hand to the emergence of immunologic mechanisms in the chick embryo, and on the other to the morphologic changes effected in the labile chorioallantois by Rous sarcoma virus and by mixtures of that virus and cardiac microsomes.

The demonstration by Rous and Murphy (1911) of the growth of the Rous sarcoma on the chorioallantois of the chick embryo and the observation of Woodruff and Goodpasture (1931) that fowl pox virus can be cultured in similar fashion focused attention on the membrane as an uncommonly favorable site for the cultivation of viruses. The practical aspects of the growth of rickettsiae and viruses in the chick embryo have been reviewed repeatedly (references in Ebert and Wilt, 1960); in addition, the usefulness of the chorioallantoic membrane as a site for differentiation and growth of grafts of embryonic and adult tissues is so well known that further review would be redundant (Willier, 1924; Hamburger, 1960). Moreover, it is important to make clear that in dwelling on these earlier observations we would be doing ourselves a disservice, for they stress the "neutrality" of the membrane as a site for the propagation of cells and viruses. Far from being neutral, as the burgeoning literature of developmental biology, immunology, and virology now attests, the cells of the chorioallantois are remarkably reactive to stimuli (DeLanney, Ebert, Coffman and Mun, 1962; Moscona, 1960).

In recent years, my co-workers, especially DeLanney and Mun, and I have paid increasing attention to the properties of this labile membrane. Evidence has been advanced that the character of the membrane may be altered by a variety of specific and nonspecific stimuli. For example, cardiac muscle is formed in the chorioallantoic mesenchyme following inoculation of a mixture of cardiac microsomes and Rous sarcoma virus (Ebert, 1959). Less convincing, but nevertheless suggestive evidence is available for the production of amorphous melanin following inoculation of a mixture of a microsomal fraction from the pigmented layer of the retina and Rous sarcoma virus, and for the differentiation of granulocytes in the allantoic endoderm following administration of a splenic ribonucleoprotein fraction. Except for the latter, inconclusive findings, I have not observed specific, directed changes in the chorioallantois without the intervention of the virus. My experience thus far runs contrary to that of van Haeften (1958), and Ranzi, Gavarosi, and Citterio (1961), while agreeing with the observations of Wilt and Stolz (1962).

Finally, grafts of spleen and, to a lesser extent, other tissues, or the inoculation of suspensions of spleen cells, result in the appearance in the membrane of a variety of responding cells, including giant cells, which wall off the homologous cells in cystic masses. Thus, as argued by DeLanney and Ebert (1959, see also DeLan-

In addition to co-workers cited in the body of the article, the following persons contributed directly to the conduct of the work: T. Garnett, N. J. Sype, and B. Trimmier. A few preliminary, nevertheless helpful, experiments were conducted by G. Schabtach.

ney et al., 1962), and as Burnet (1962) now agrees, such lesions on the chorioallantois are largely derived from the host. Mun, Tardent, Errico, Ebert, DeLanney, and Argyris (1962) offer evidence, derived from tritium-thymidine labeling studies, which demonstrates the mobilization, adjacent to chorioallantoic grafts of adult spleen, of cells of the host embryo. Whether they migrate in from the embryo, or are mobilized in the membrane *in situ* or both, is not yet clear.

THE CULTIVATION IN FLUID MEDIUM OF THE LABILE CHORIOALLANTOIS

It is evident that to continue these lines of research effectively, a technique for the cultivation of the chorioallantoic membrane *in vitro* is required. The perfection of an organ culture technique should permit studies of the controlled modification of pieces of the membrane as well as resolution of the question of the origin of cells reaching homologous grafts of adult spleen.

However, Moscona (1959) reported that when small fragments of the chorioallantoic membrane of eight-day chick embryos are cultivated on a plasma clot by Fell's watch glass technique, the chorionic epithelium stretched on small squares of rayon-acetate net showed a keratogenic metaplasia. Earlier, Delson (1958) described a method for the cultivation of the membrane supported by filter paper, without such metaplasia.[1] Her observations have now been extended by my coworker, Takata, some of whose findings have been reported (in Ebert, 1962a).

In the course of the latter study, several hundred large fragments of the chorioallantoic membrane were taken from eight-day chick embryos. Each fragment, supported on filter paper, was placed on the surface of the medium to be tested (in an embryological watch glass) and cultured in a humid chamber for five to seven days at 37.5°C. The explants were transferred to fresh media every second day.

The filter paper (No. 5243-C, Arthur H. Thomas Co.) used for supporting the membrane was cut into rectangles 2.3 × 2.0 cm and cut out leaving a frame 3 mm wide. They were treated with 1% HCl for one hour, washed in three changes of distilled water, extracted a second time for one hour in 95% ethanol, rinsed in several changes of glass distilled water, dried in an oven (80-90°C), and sterilized. The experimental media were prepared from sterile stock solutions; Hanks' balanced salt solution, containing 3 g/liter of glucose and buffered with tris or bicarbonate at pH 7.5-7.8, served as the basic suspending fluid. Several components were tested singly and in combination, including calf serum, horse serum, nine-day embryo extract, and Puck's medium N-16 (synthetic medium without protein).

After cultivation, many explants were fixed in Bouin's or in Zenker's fluids, stained with Weigert's iron hematoxylin and eosin, and examined as whole mounts. Other explants were sectioned at 5, 6, or 8 μ and stained with Weigert's iron hematoxylin and eosin or Biebrich's scarlet for microscopic observation.

The criteria for determining effectiveness of each medium were: degree of maintenance of mesenchyme, ectodermal and endodermal layers, blood cells, and cell proliferation. As tabulated previously (Ebert, 1962a), in several combinations maintenance of the explanted membrane was achieved (see Ebert, 1962a, plate 5); mitotic figures often were observed. Keratogenic metaplasia was not observed in a flattened cell layer of ectodermal epithelium. A number of media tested were effective for maintenance of mesenchyme and endodermal layer.

THE INFECTION OF THE CHORIOALLANTOIC MEMBRANE *in vitro* WITH THE ROUS SARCOMA VIRUS

Takata (in Ebert, 1963) demonstrated that the chorioallantois could be infected with Rous sarcoma virus *in vitro* and de-

[1] Cited with permission of C. P. Dagg and with thanks to M. V. Edds, Jr. and R. J. Goss for assistance and information.

scribed some of the cytopathology of the infected membrane. In these experiments, as in those that follow, the stock of virus employed was a strain (Mun-II) of the Rous no. 1 chicken sarcoma, obtained originally from F. B. Bang and T. Borsos, maintained in this laboratory for several years by serial passage of crude or partially purified virus on the chorioallantois *in ovo* (Ebert, 1959), and stored at −70°C for about one year. All dilutions were made in the same medium as that employed in the subsequent cultivation of the membrane. Three different media were compared: (1) 65% Hanks' solution, 20% horse serum, 15% embryo extract; (2) 80% Hanks' solution, 20% horse serum; and (3) 50% Hanks' solution, 10% calf serum, 40% Puck's medium N-16. Fragments of chorioallantois supported on filter paper were rinsed in Hanks' solution and cultured in medium containing a standard dilution of virus (usually 1 ml of a 10^{-7} dilution of strain Mun-II). After exposure for either 24 or 48 hours, they were transferred to fresh culture medium without virus, the medium then being changed every second day. After incubation at saturation-humidity for 6 days at 37.5°C, they were fixed in Bouin's fluid and either mounted whole or sectioned at 5 μ, and stained with Weigert's iron hematoxylin. Pocks or lesions were counted microscopically in the whole mounts (Figs. 1-4).

All of the media employed permitted some conversion of ectodermal and mesodermal cells of the membrane into cells having the altered morphology characteristic of Rous sarcoma. In the lesions in thickened membranes, round basophilic cells and fibroblast-like, fibromyxosarcoma cells were found. In some cultures, almost complete necrosis of ectodermal cells was observed. Keratogenic metaplasia was not common. In the initial studies the frequency of production of pocks or lesions varied widely from 40 to 70% from experiment to experiment, without any clear relation to the nature of the medium employed. Since the morphological alteration of chick and turkey embryo fibroblasts by virus has been shown to depend on the physiological state of the cells at the time of infection, i.e., morphological response can be regulated by choice of medium and by infection at various times after preconditioning (Temin, 1960; Prince, 1962), it seemed essential that the causes of variation be explored. In a more extensive series of membranes cultured in medium (3), the over-all frequency of morphologic alteration was 37%. Temin (1960) reported that cells infected with Rous sarcoma virus may display no visible morphologic alteration. As Prince (1962) emphasizes, further evidence of infection without morphologic alteration is provided by his earlier (1960) finding that only a small fraction of virus-producing cell clones can be distinguished from uninfected clones, and by Rubin's (1960) observation that fetal calf serum can suppress the appearance of foci of morphologically altered cells following infection of monolayers with small amounts of virus, but does not change the proportion of infected cells. In the current study, however, infection and morphologic alteration, as judged by the appearance of lesions within six days (i.e., four or five days after removal from medium containing virus), appear to be synonymous, for the recovery of crude virus for assay *in ovo* three, four, or five days after initial exposure to virus revealed evidence of infection in only 41% of cultures, a figure which does not differ significantly from the 37% with clearly recognizable lesions (cf Rabin, 1962).

It was necessary, therefore, to seek an explanation for the low frequency of infection and morphologic alteration. The embryos employed from the outset of the study had been from a variety of White Leghorn fowls (Elder Farms) in which the percentage of nonreactors had been 17.4 (Ebert, 1959). It seemed only remotely possible that there had been a substantial increase in resistance in this variety since it was last tested; nevertheless, in view of the compelling evidence for the influence of the genotype of the chick embryo on tumor production by Rous sarcoma virus

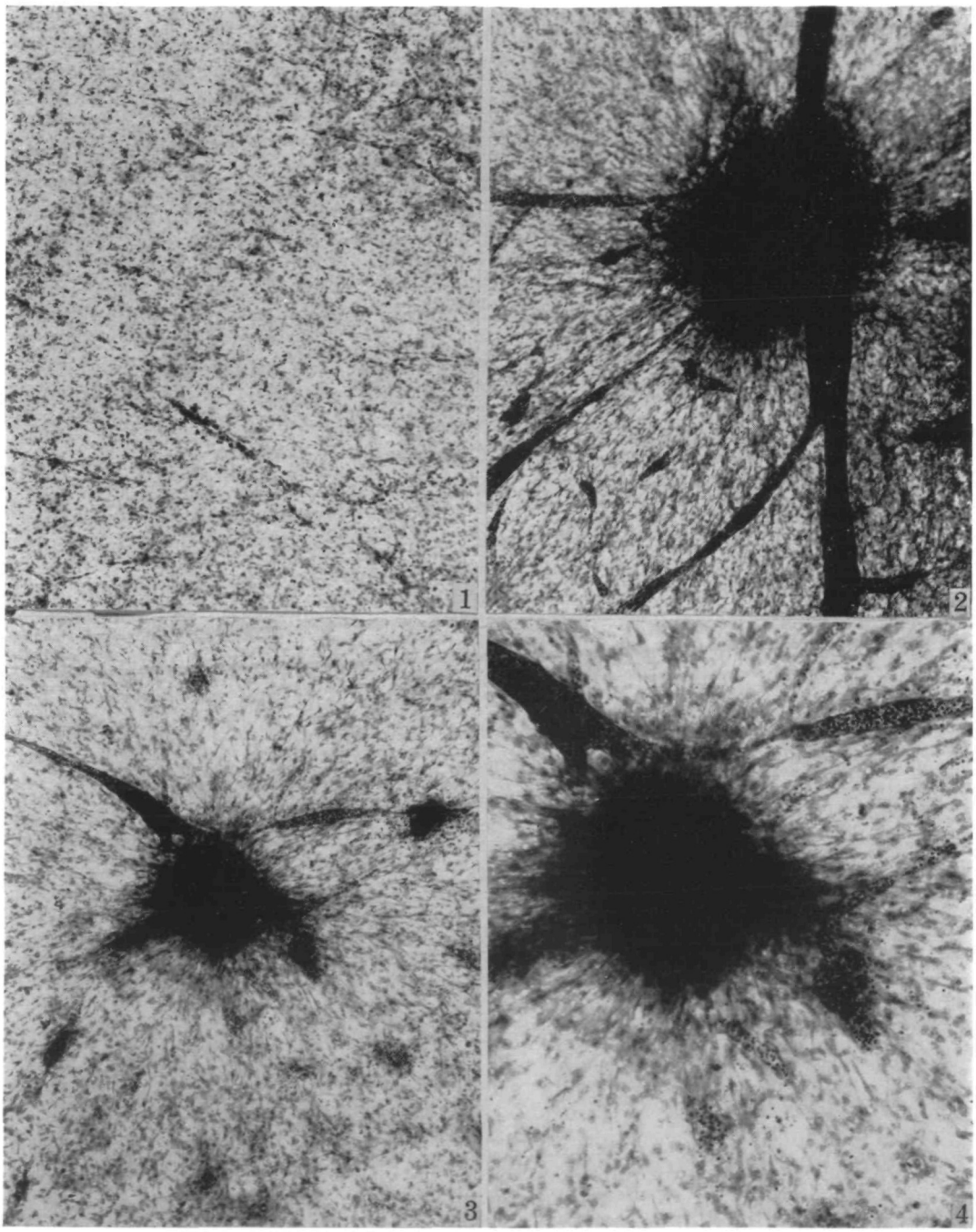

FIG. 1. Chorioallantoic membrane cultured for 2 days in 50% Hanks' solution, 10% calf serum and 40% Puck's N-16, after exposure for 24 hours to medium containing Rous sarcoma virus, 10^{-7}. Whole mount, iron hematoxylin and eosin. X75.

FIG. 2. Lesion produced on the chorioallantoic membrane, *in vitro*, 6 days after exposure to Rous sarcoma virus for 48 hours (Takata). 65% Hanks' solution, 20% horse serum, 15% embryo extract. Whole mount, iron hematoxylin and eosin. X75.

FIG. 3. Lesion produced on the chorioallantois *in vitro*, 6 days after infection with Rous sarcoma virus (Takata). 50% Hanks' solution, 10% calf serum, 40% Puck's N-16. Whole mount, iron hematoxylin and eosin. X75.

FIG. 4. As in Fig. 3, X150.

TABLE 1. *Tumor response of chorioallantoic membranes cultured from chick embryos from different sources.*

Source of membrane	No. explanted	No. showing lesions	Mean No. of lesions per membrane
California Gray x White Leghorn	60	21	27
White Leghorn (Elder Farms)	59	22	59
White Leghorn (Elder Farms) maternal antibody present	49	16	31
White Leghorn Rous-antibody free	44	15	19

(Bower, 1962; Dougherty and Simons, 1962) a further series of comparisons was made. Membranes from four types of donor were explanted and inoculated: (1) a cross of California Gray × White Leghorn in which the percentage of nonreactors had been set earlier (Ebert, 1959) at 34; (2) White Leghorn (Elder Farms); (3) selected White Leghorn (Elder Farms) in which maternal antibody to Rous sarcoma virus was known to be present; and (4) a single comb White Leghorn variety, obtained from F. B. Bang, in which maternal antibody to the virus was known to be absent. The culture medium was again held constant. Significant differences in frequency of alteration were not observed (Table 1).

Neither the addition of 10% tryptose broth nor infection of cells after varying periods of starvation markedly favored the production of morphological alteration, except that the latter treatment enhanced the possibility of keratogenic metaplasia (cf Prince, 1962). Therefore, in an effort to increase the frequency of morphologic alteration, experiments with conditioned media were initiated. Surprisingly, such media had an effect opposite to that expected.

INHIBITING EFFECT OF MEDIA CONDITIONED BY GROWTH OF MEMBRANES PREVIOUSLY EXPOSED TO VIRUS

Cultures were grown in the presence of virus for 24 hours and removed to fresh medium without virus for an additional 48 hours. At the time the medium was next changed, the old medium, so "conditioned," was saved and used in combination with fresh virus and varying amounts of fresh medium as the primary infecting medium and as the medium for subsequent transfers of new cultures. As summarized in Table 2, morphologic alteration was suppressed rather than enhanced. These first experiments suggested that an interferon-like inhibitor had been produced. Interferon, the inhibitor of virus production first described by Isaacs and Lindenmann (1957) following exposure of chick embryos to inactivated influenza virus, has recently been demonstrated in chick embryo cells exposed to Rous sarcoma virus *in vitro* (Bader, 1962). Accordingly, the following experiments were undertaken (see Ebert, 1963). (1) Active Rous sarcoma virus was replaced by heat-inactivated virus, the resultant "conditioned" medium again being employed;

TABLE 2. *Production of inhibitor by chorioallantoic membrane* in vitro *following infection with Rous Sarcoma virus.*

Source of membrane	No. explanted	Medium; ratio fresh/conditioned	No. showing lesions	Mean No. of lesions per membrane
California Gray x White Leghorn	30	0/100	0	0
White Leghorn (Elder Farms)	29	50/ 50	0	0
White Leghorn (Elder Farms)	60	75/ 25	0	0
White Leghorn (Elder Farms)	57	90/ 10	2	3.5
California Gray x White Leghorn	59	95/ 5	9	11
California Gray x White Leghorn	30	99/ 1	8	24
White Leghorn (Elder Farms)	30	95/ 5*	4	19
White Leghorn (Elder Farms)	29	95/ 5†	11	37

* Heated 56°C 30 min. † Heated 85°C 15 min.

an inhibitor was recovered. (2) After incubation of infected cultures for two days following the primary transfer, the fluids to be tested for inhibitory activity were centrifuged at 42,000 $\times$ g once for one hour or three times for one hour. The inhibitor remained in the supernatant fluid. (3) Fluids retained inhibitory activity after centrifugation at 100,000 $\times$ g for two hours. (4) The further characterization of the inhibitor is incomplete, but the following properties may be stated: it is nondialyzable; it is not affected significantly by changes in pH from 2 to 10; and it is stable at 56°C, retaining full activity after heating at that temperature for 30 minutes. Activity is lost progressively at higher temperatures, the inhibitory capacity being totally inactivated at 85°C. It is inactivated by trypsin. Pepsin, which is more effective according to Bader (1962), has not been used. Deoxyribonuclease and ribonuclease do not alter its activity.

I am not prepared to discuss the mechanism of action of the inhibitor at length. However, conclusions drawn from the following experiments appear to be meaningful. First, the inhibitor does not act on the Rous sarcoma virus directly. Preincubation of inhibitor and virus in varying concentrations from one to three hours at 20°, 25°, and 37°C does not result in a significant diminution of effectiveness when compared with virus samples diluted with normal medium and incubated for the same periods.

On the other hand, addition of the inhibitor to the medium at the time membranes are transferred from the infecting medium to one without virus, i.e., 24 hours after inoculation, produces no effect. Much variation in results of further experiments along these lines makes a firm conclusion impossible, but the weight of evidence thus far implies that the inhibitor loses effectiveness progressively when added at intervals after infection. Thus far, inhibition has been most pronounced when the inhibiting fluid has been added shortly before or at the same time as the addition of virus (cf Bader, 1962).

INTERFERON

Interferon was described by Isaacs and Lindenmann (1957) as a nonviral substance formed by the interaction of inactivated influenza virus and living cells. When transferred to normal cells it renders them resistant to infection with the same and other viruses. Isaacs (1962) has emphasized recently that the mounting evidence permits the conclusion that interferon plays an important role in recovery from virus infections. He argues that antibody may play a less important role in recovery than hitherto believed, citing the recovery of cells from virus infections *in vitro* in the absence of specific antibody, and the recovery from virus infections of patients with hypogammaglobulinaemia. Cells chronically infected with virus *in vitro* produce interferon (Ho and Enders, 1959), and the amount of interferon allowed to accumulate in them seems to determine whether the chronic infection is maintained (Glasgow and Habel, 1962).

A series of experiments reviewed by DeSomer (1962) points to the conclusion that interferon, which is believed to be a protein having a molecular weight of about 60,000, affects virus multiplication. It does not inactivate virus or viral RNA directly, nor does it prevent the penetration of viruses. Virus multiplication appears to be blocked after penetration and before the formation of precursor RNA and viral protein. Moreover, the intruding nucleic acid is not degraded; it remains intact, and is not damaged by cellular nucleases.

It appears likely that the inhibitor produced by the chorioallantois exposed to active or heat-inactivated Rous sarcoma virus *in vitro* is an interferon. The inhibitor blocks virus reproduction and the formation of lesions. It differs from other inhibitors of Rous sarcoma virus which appear to act directly upon the virus (e.g., Carr, 1944; Groupé and Rauscher, 1959). Its characteristics, from the experiments advanced so far, are compatible with the

view that an interferon has been produced. There is no evidence in this study that the inhibitor is the resistance-inducing factor (RIF) described by Rubin and since shown to be one of the avian leukosis viruses, possibly lymphomatosis virus (Friesen and Rubin, 1961). In fact, as Dougherty and Simons (1962) point out, there is no evidence that infection of an embryo with RIF induces resistance to infection of the chorioallantois.

Yet the question remains—if the interferon produced by the membrane *in vitro* is meaningful, i.e., if it has a bearing on the resistance of the membrane to morphologic alteration by virus, why is it not produced, or why is it ineffective, *in ovo*?

Relation to the development of immune mechanisms

On other occasions I have discussed the development of immune mechanisms (Ebert and DeLanney, 1960; Ebert and Wilt, 1960; Ebert, 1962b). It will suffice, at this time, to emphasize that although there is no compelling evidence of the production of antibodies or the capacity to mount a homograft reaction in the chick embryo, there is an increasing body of evidence (DeLanney, Ebert, Coffman and Mun, 1962; Mun, Tardent, Errico, Ebert, DeLanney and Argyris, 1962; see also Burnet, 1962) that the embryo is capable of mounting a response to antigenic stimuli in the chorioallantoic membrane, a reaction, as Burnet put it, like the foreign body reaction. Whether such reactions can be demonstrated *in vitro* remains to be established.

The chick embryo does develop the capacity to resist viruses. If interferon plays a role in recovery from infection, one might expect to find a correlation between the age of the embryo and mortality following virus infection. Isaacs and Baron (1960) observed that young chick embryos differ from older embryos in being less sensitive to the antiviral action of interferon and in producing less interferon on stimulation with ultraviolet-irradiated influenza viruses. The interferon mechanism begins to function at about eight days of incubation. Baron and Isaacs (1961) showed that the mortality rate of embryos exposed to each of four different viruses decreased as the age of the embryo increased, lethality being high at four and seven days, lower at 10 and 13 days. Moreover, between the seventh and tenth days there was a rapid development of sensitivity to the antiviral action of interferon.

Relation to morphologic alteration following infection

Bader (1962) points out that the demonstration of an interferon in chick embryo cells infected with the Rous sarcoma virus *in vitro* is an initial step in the examination of the possible role of the inhibitor in determining the characteristics of the tumors induced, and in the development of "noninfectious" tumors and regressing tumors.

Ebert and Wilt (1960) examined available data on the effects of tumor viruses during progressive stages of development, emphasizing the changing spectrum of effects of the Rous sarcoma virus in the developing chick embryo. In doing so they drew largely on the observations of Duran-Reynals, (summarized 1953) and Borsos and Bang (1957). In younger embryos the virus produced hemorrhagic lesions; in older embryos, tumors. Duran-Reynals argued that the change was a consequence of increasing resistance to the virus. Ebert and Wilt (1960) concluded, obviously with insufficient foresight, "Yet it is difficult to concur that the difference . . . can be related to increasing resistance without invoking a 'special class' of resistance to tumors. We have no evidence of the operation of immune mechanisms, either cell-mediated or humoral, at this point of development. One must seek other sources of variation in the host to account for this progressive change." Is interferon the developing defense mechanism that Duran-Reynals projected? Are the sarcoma cells those which have escaped from the control of interferon (cf Isaacs and Baron, 1960)?

Relation to congenital defects

One final question of interest to developmental biologists may be considered. Isaacs and Baron (1960) have conjectured that the production of congenital defects by rubella virus may be related to the absence of interferon in the early embryo or to its insensitivity to interferon. At later stages the interferon mechanism limits the viral infection. It seems unlikely that the lack of interferon in the embryo may be meaningful in this connection. First, it seems improbable that maternal interferon would not be available to the embryo; and second, and of greater consequence, is the observation that infection of the mother with other viruses does not produce defects. As I have argued elsewhere (Ebert, 1962c), evidence has yet to be produced for infection of the embryo by rubella as a cause of defects. The possibility remains that the syndrome results indirectly, as an "autoimmune disease," as a consequence of the reaction of maternal antibodies with antigens held in common by rapidly developing cells and rubella virus.

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