[23] Smith, L. H., M. Sullivan, and C. M. Huguley, *J. Clin. Invest.*, **40**, 656 (1961).
[24] Pontecorvo, G., and E. Kafer, *Advances in Genet.*, **9**, 71 (1958).
[25] Grumbach, M. M., P. A. Marks, and A. Morishima, *Lancet*, **1**, 1330 (1962).
[26] Yanofsky, C., D. R. Helinski, and B. D. Maling, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 11.

EFFECT OF VIRUSES ON EARLY MAMMALIAN DEVELOPMENT, I. ACTION OF MENGO ENCEPHALITIS VIRUS ON MOUSE OVA CULTIVATED IN VITRO

BY RALPH B. L. GWATKIN

KING RANCH LABORATORY OF REPRODUCTIVE PHYSIOLOGY, SCHOOL OF VETERINARY MEDICINE, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

Communicated by Warren H. Lewis, July 1, 1963

The possible effect of viruses on mammalian eggs has never been explored. The closest studies that have been reported deal with relatively late embryonic stages.[1] This is unfortunate, since a knowledge of the interaction of mammalian eggs and viruses would be valuable for at least three reasons. First, such knowledge would clarify the known relationship between viruses and congenital defects.[2] Secondly, it would elucidate the origin of innate resistance to virus infection, such as is known in certain strains of mice.[3] Thirdly, it would show us whether the specific cellular receptors required by certain viruses for attachment to the host cell are present on the surface of the vitellus, or whether they are formed later in development.

This communication reports the effect of Mengo encephalitis virus on mouse eggs at the 2-cell stage. The virus was found to pass through the zona pellucida surrounding the egg and to block further development *in vitro*.

Materials and Methods.—Randomly bred 6- to 8-week-old Swiss mice were superovulated by the intraperitoneal injection of 5 I.U. of pregnant mare serum gonadotrophin (Gestyl, Organon), followed 43 hr later by 5 I.U. of human chorionic gonadotrophin (Pregnyl, Organon). A mature male was placed with each female at the time of the second injection. This treatment results in ovulation and mating about 12 hr afterwards. Females with vaginal plugs (usually 70–90% of the animals) were killed 34–36 hr later, that is, 10–12 hr after the expected time of the first cleavage division.[4] The 2-cell eggs were flushed from the Fallopian tubes using a syringe with a blunted no. 30 needle. The medium used for flushing out the eggs and also for culturing them was developed by Dr. R. L. Brinster and is to be published elsewhere.[5] This medium consists of modified Krebs-Ringer balanced salt solution, supplemented with sodium lactate and crystalline bovine plasma albumin.

To determine whether the zona pellucida was a barrier to viral entry it was removed with *Streptomyces griseus* protease ("Pronase," Calbiochem Co.).[6] Ova were exposed 5–10 min at room temperature to 0.25% "Pronase" in phosphate buffered saline, containing 1.0% polyvinylpyrrolidone (PVP). The PVP was added to protect the naked blastomeres and to prevent their attachment to the glass. Further details of the action of "Pronase," and other enzymes, on the zona pellucida of the mouse egg are described elsewhere.[7]

The eggs, either naked or with their zonae intact, were placed in drops of the lactate-albumin medium, with or without virus or antiserum. The drops were submerged in mineral oil in a 60 mm Petri dish to permit gas-exchange while preventing evaporation. The culture dishes were incubated at 37°C in an atmosphere of 5% CO_2 in air.

The 37A (heat-stable mutant) of Mengo encephalitis virus, isolated by Brownstein and Graham,[8] was used. The virus was assayed by the plaque technique decribed by these authors.

Purified virus was prepared by the method of Homma and Graham.[9] In this method nonviral RNA synthesis is blocked in infected L cells with actinomycin D. After lysis the culture is desalted by pouring it through a column of Amberlite mixed-bed ion-exchange resin (MB-1 resin). Zinc hydroxide gel is then added to the column effluent to adsorb and concentrate the virus. Virus is then released from the gel with sodium EDTA and further purified by treatment with trypsin and by two cycles of differential centrifugation. The antiserum used was prepared against the virus of encephalomyocarditis (EMC), which is nearly indistinguishable immunologically from Mengo virus. The rabbit was given two injections of EMC virus grown in L cells. The first consisted of a 100-fold dilution of L-cell lysate and the second of partially purified virus, so that the anti-mouse antibody content was slight. The neutralizing activity of the antiserum was measured at 37° using virus suspensions containing approximately 10^7 PFU/ml. Under these conditions 200-fold diluted serum neutralized 99% of the virus in 10 min.

Results.—Table 1 shows the effect of the virus (L cell lysate) on the development of 2-cell eggs *in vitro*. A variable proportion (22–92%) of eggs with an intact zona developed into blastocysts in the 3-day period of the experiments. Figure 1 shows a group of ova, several of which have developed to the blastocyst stage. These results fully confirm those obtained by Brinster.[5] When the zona pellucida was

TABLE 1

EFFECT OF MENGO ENCEPHALITIS VIRUS* ON THE DEVELOPMENT OF MOUSE OVA *in vitro*

Virus concentration (PFU/ml)	Proportion of 2-Cell Eggs Which Formed Blastocysts in 3 Days					
	Expt. 1		Expt. 2		Expt. 3	
	Zona intact	Zona removed	Zona intact	Zona removed	Zona intact	Zona removed
0	5/23	8/21	12/27	9/25	12/13	6/16
2.8×10^5	...	...	7/24	1/26	10/14	5/15
2.8×10^6	...	...	3/25	0/23	5/15	3/15
2.8×10^7	0/34	0/49	0/46	0/20	0/25	0/13

* Lysate of L strain cells infected with strain 37A (heat-resistant virus mutant).

removed prior to cultivation, blastocysts were still formed. However, in two experiments the proportion doing so was less than when the zona pellucida was present. It was noted that the blastomeres of naked eggs tended to separate at the 4-cell stage. Some of these isolated blastomeres failed to develop further, or formed miniature blastocysts. Occasionally giant blastocysts developed, presumably by the fusion of naked eggs. These effects are shown in Figure 2.

Virus at a concentration of 2.8×10^7 PFU per ml arrested development completely whether the zona was removed from the eggs or left intact. At a concentration of 2.8×10^6 PFU per ml the proportion of eggs which formed blastocysts was reduced, and those eggs which failed to develop became necrotic. At a concentration of 2.8×10^5 PFU per ml the inhibitory and necrotic action was less, but still demonstrable. The effect of Mengo virus on ova with and without their zonae pellucidae is shown in Figures 3 and 4, respectively.

Table 2 shows the results obtained when purified virus was used and the ova observed over a longer period of time. After 3 days the results are the same as those observed with the crude lysate in the previous experiments. However, necrosis of eggs and blastocysts continued with time so that by the 5th day *in vitro* 3×10^5 PFU per ml reduced all the eggs to a necrotic, shriveled condition (Fig. 5). In the absence of virus, blastocysts "hatched" from their zonae pellucidae by the 3rd–4th day *in vitro* and by the 5th day were considerably expanded (Fig. 6). By the 7th day a virus concentration as low as 3×10^3 PFU per ml had the same effect. Since the average drop was about 0.03 ml, this concentration corresponds to 5 PFU per egg.

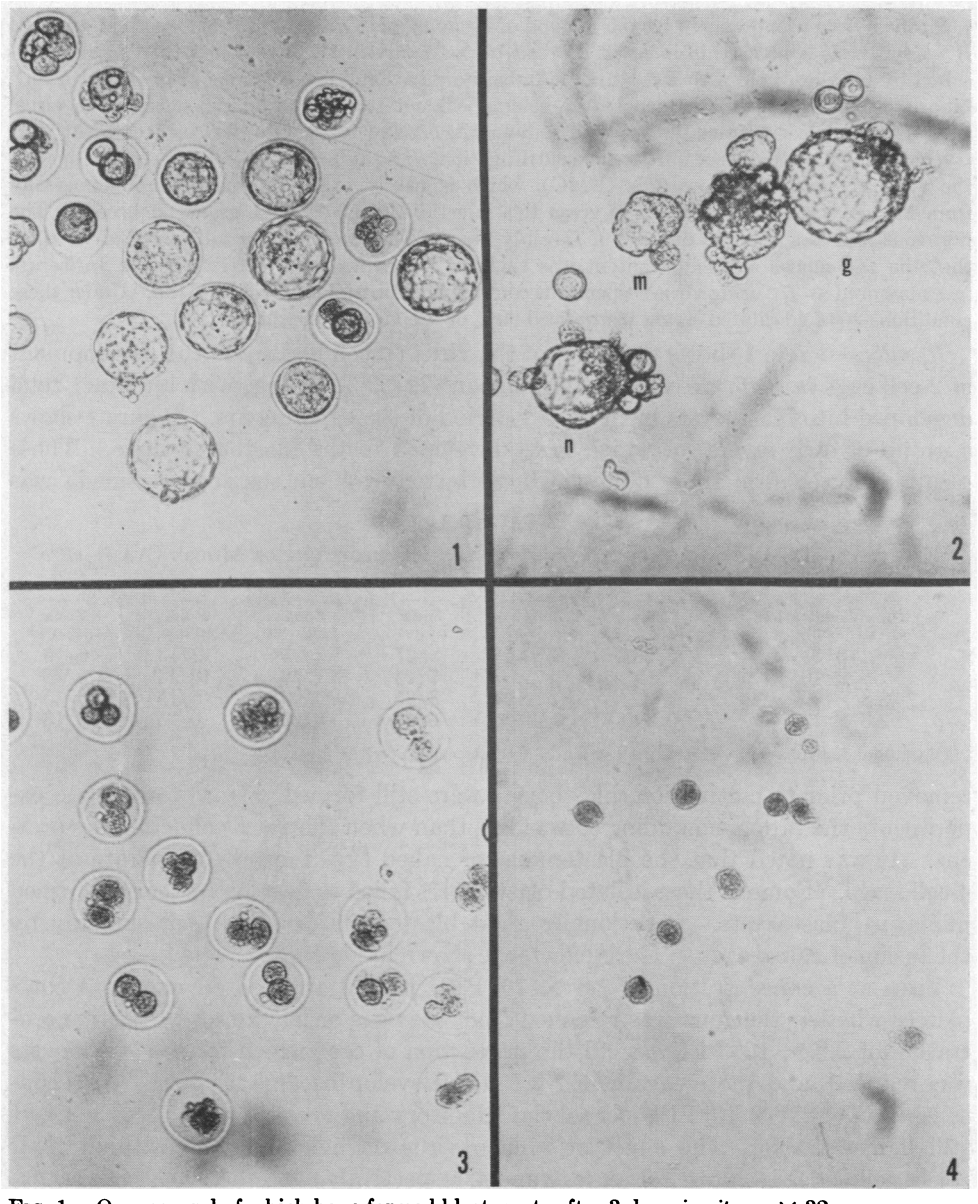

FIG. 1.—Ova, several of which have formed blastocysts after 3 days *in vitro*. × 32.

FIG. 2.—Stages formed from 2-cell ova after removal of the zona pellucida: n, blastocyst of normal size; m, miniature blastocyst; g, giant blastocyst. *In vitro* 3 days. × 32.

FIG. 3.—Ova with intact zonae pellucidae exposed to Mengo encephalitis virus (2.8×10^7 PFU/ml). Further development was blocked and the ova appeared necrotic. *In vitro* 3 days. × 32.

FIG. 4.—Necrotic, shriveled blastomeres from 2-cell eggs without zonae pellucidae, exposed to Mengo encephalitis virus (2.8×10^7 PFU/ml). *In vitro* 3 days. × 32.

The fact that these virus preparations were purified and active at dilutions up to 10^{-6} is strong evidence that the virus itself was responsible for blocking development and producing necrosis of the blastomeres. To confirm this, antiserum was combined with the virus 10 min prior to adding the eggs. Experimental details

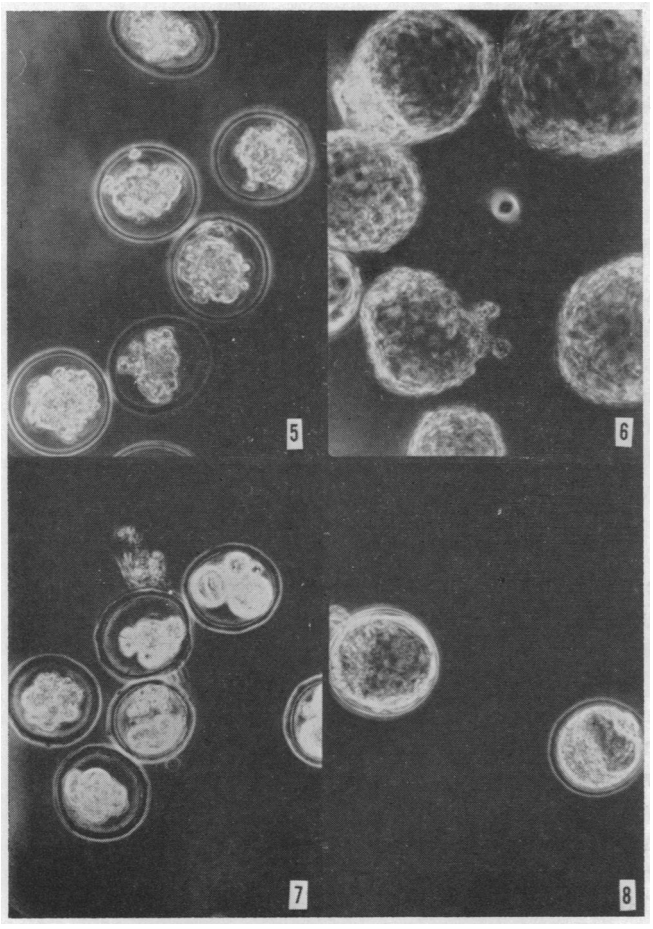

FIG. 5.—Ova exposed at the 2-cell stage to purified Mengo encephalitis virus (3×10^5 PFU/ml). Photograph taken after 5 days *in vitro*. × 50.

FIG. 6.—Blastocysts after 5 days *in vitro*. Hatching from the zona pellucida occurred on the 3rd or 4th day, after which the blastocysts expanded considerably. × 50

FIG. 7.—Ova *in vitro* 3 days after initial exposure to purified Mengo encephalitis virus (3×10^6 PFU/ml). × 50.

FIG. 8.—Blastocysts formed from same batch of ova as shown in Fig. 7, when antiserum was combined with virus. × 50.

TABLE 2

EFFECT OF PURIFIED MENGO ENCEPHALITIS VIRUS* ON THE DEVELOPMENT OF MOUSE OVA† *in vitro*

Virus concentration (PFU/ml)	Days in Culture: 3		5		7	
	Blastocysts	Necrotic cells	Blastocysts	Necrotic cells	Blastocysts	Necrotic cells
0	15	1	17	3	17	3
30	14	0	15	5	15	8
3000	14	3	11	9	0	20
3×10^5	8	8	0	20	0	
3×10^7	0	20	0		0	

* Virus purified by method of Homma and Graham (*J. Cell. Comp. Physiol.*, in press).
† For each treatment 20 eggs were used.

and results are given in Table 3. The antiserum completely prevented the inhibitory action of the virus on development and also prevented necrosis. Figure 7 shows the appearance after 3 days *in vitro* of 2-cell ova exposed to virus. The ova remained undeveloped and became necrotic in appearance. Figure 8 shows blastocysts which formed when antiserum was combined with the virus before adding the ova.

TABLE 3

NEUTRALIZATION OF THE INHIBITORY ACTION OF MENGO ENCEPHALITIS VIRUS ON THE DEVELOPMENT OF MOUSE OVA *in vitro* BY ANTISERUM TO THE VIRUS

Conditions	Number of Morulas and Blastocysts Formed from 15 2-Cell Eggs in 3 Days	
	Morulas	Blastocysts
Medium only	0	9
Virus (3×10^6 PFU/ml*)	0	0
Antiserum (10^{-2})	0	7
Virus and antiserum	1	10

* Purified by method of Homma and Graham (*J. Cell. Comp. Physiol.*, in press).

Discussion.—It is apparent from these results that the zona pellucida is not a complete barrier to virus infection. The only study of zona permeability was made by Austin and Lovelock,[10] who reported that compounds of molecular weight 1,200 or less, but not heparin, which has a molecular weight of 16,000, passed through the zona pellucida of rabbit and rat eggs. Their results suggested that the zona pellucida would act as a barrier to infection of the ovum by viruses. Our results indicate otherwise. However, Mengo encephalitis virus is small, 27–28 mμ,[11] and it will be of interest to determine whether large viruses will also pass through the zona.

While the function of the zona pellucida as a barrier to infection with viruses is in question, our results clearly indicated that the zona was needed to prevent loss of blastomeres or egg fusion under the *in vitro* conditions which were employed. It seems likely that loss of blastomeres and egg fusion could occur within the Fallopian tube if the zona pellucida were not present. Experiments are in progress to establish whether this is in fact true.

The fact that 2-cell ova without their zonae pellucidae developed *in vitro* to the blastocyst stage shows that under these conditions the zona is not required to preserve a microenvironment about the ovum necessary for development. Again, however, whether this is true *in vivo* requires further study. The next phase of this research will be to establish whether Mengo virus is capable of multiplication within the mouse ovum and, if so, whether the yield of virus corresponds to that given by a somatic cell. This problem is being studied by adding virus to ova in microdroplets under mineral oil. After adsorption the virus is washed off and at subsequent intervals the drops are frozen and thawed repeatedly to disrupt the ova and release the virus particles, which are then titrated by a plaque method.

One of the ultimate goals of these studies is to determine in detail the relationship between virus infections and specific congenital defects. The virus spectrum of mouse ova is being determined, and those viruses which do not destroy the ova will be studied further by exposing eggs to virus and then transplanting these ova to the uteri of hormonally prepared foster mothers.

The author wishes to thank Dr. J. D. Biggers and Dr. A. F. Graham for helpful advice and discussion. Dr. A. F. Graham also kindly provided the purified Mengo virus. The gonadotrophic hormones were supplied by Dr. W. J. Tindall of Organon Laboratories, Ltd., London, England. Dr. C. R. Fuerst supplied the antiserum. Mrs. Pamela Yates and Mr. Dorsey Williams contributed their technical skills. This research was supported in part by grant GB-617 from the National Science Foundation.

[1] Ebert, J. D., and F. H. Wilt, *Quart. Rev. Biol.*, **35**, 261 (1960).

[2] Warkany, J., and H. Kalter, *New Engl. J. Med.*, **265**, 993, 1046 (1961).

[3] Goodman, G. T., and H. Koprowski, these PROCEEDINGS, **48**, 160 (1962).

[4] Edwards, R. G., and A. H. Gates, *J. Endocrinol.*, **18**, 292 (1959).
[5] Brinster, R. L., in preparation.
[6] Mintz, B., *Science*, **138**, 594 (1962).
[7] Gwatkin, R. B. L., *J. Reprod. Fertility*, in press.
[8] Brownstein, B., and A. F. Graham, *Virology*, **14**, 303 (1961).
[9] Homma, M., and A. F. Graham, *J. Cell. Comp. Physiol.*, in press.
[10] Austin, C. R., and J. E. Lovelock, *Exptl. Cell Res.*, **15**, 260 (1958).
[11] Dales, S., and R. M. Franklin, *J. Cell Biol.*, **14**, 281 (1962).

ERRATA

In the article entitled "Incorporation of Parental DNA into Genetic Recombinants of *E. coli*" by Obaid H. Siddiqi, which appeared in the May issue of volume 49 (1963), the last line of paragraph 3 on page 589 should read "it is S^{s}"; and in the legend to Figure 1 on page 591 "Hfr DNA" should appear as "F^{-} DNA."

In the article entitled "Synthetic Polynucleotides and the Amino Acid Code, IX" by Albert J. Wahba, Robert S. Miller, Carlos Basilio, Robert S. Gardner, Peter Lengyel, and Joseph F. Speyer, which appeared in the June issue of volume 49 (1963), pages 880–885, *Lys* should be substituted for *Leu* in the amino acids listed in Table 6 under the heading "Triplet composition 2A1U."

In the article entitled "Fluorogenic Substrates for β-D-Galactosidases and Phosphatases Derived from Fluorescein (3,6-Dihydroxyfluoran) and Its Monomethyl Ether" by Boris Rotman, John A. Zderic, and Marvene Edelstein, which appeared on pages 1–6 of volume 50 (1963), the following data should be inserted on page 4 between the two paragraphs of the section entitled "Fluorescein-3-O-methyl-6-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside) (IIg):"

Analysis calculated for $C_{35}H_{32}O_{14}$: C, 62.1; H, 4.8; O, 33.1. Found: C, 62.5; H, 4.8; O, 32.7.