Distribution of Mumps Virus in Tissue Cultures as Determined by Fluorescein-Labeled Antiserum.*† (19329)

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Tissue cultures make possible the study of viruses under conditions which are simpler and easier to control than those prevailing in the intact animal or embryo. The detection of viruses in tissue cultures, on the other hand, necessitates the use of cumbersome and expensive procedures which, in the final analysis, yield only indirect evidence as to what is actually taking place in the cells. This is particularly true of viruses such as mumps which do not form inclusion bodies or cause any visible tissue damage. The development of fluorescent antibody(1), and its successful application to the microscopic localization of mumps virus antigen in the parotid glands and central nervous system of experimentally infected monkeys(2,3), opened a new approach to numerous problems relating to the growth of viruses in tissue cultures.

The present experiments deal with the use of the method in a study of mumps virus in various kinds of chick embryonic tissue infected *in vitro*. The answers sought were: 1) Is the amount of virus produced in tissue cultures sufficient to be detected by the fluorescein labeled antiserum; 2) what is the distribution of the virus in the infected tissues; 3) is it the same or different with different kinds of tissue?

Materials and methods. Tissue cultures of the Maitland type were prepared in a manner similar to that described by Weller and Enders (4). The tissues used were the amniotic and chorioallantoic membranes, whole embryo, and brain of 7-8-day-old chick embryos. The tissues were minced with scissors and centrifuged in graduated test tubes at 1,000 rpm for 2 minutes. The fragments were resuspended with medium, consisting of 1 part ox serum ultrafiltrate‡ to 2 parts of Hanks' balanced salt solution(5) and made up to a 10% suspension by volume. The tissue cultures were set up in 50 ml Erlenmeyer flasks fitted with tight rubber stoppers. The volumes used were 0.5 ml of the 10% tissue suspensions, 3.5 ml of medium and 0.1 ml of a 10^{-2} dilution of the stock virus. The latter consisted of pooled allantoic fluid derived from the 62nd egg passage of the Enders strain of mumps. Duplicate cultures were prepared with each of the tissues and incubated at 35°C. The controls included: 1) Uninoculated cultures; 2) cultures in which the tissues were killed by freezing prior to inoculation; and 3) inoculated cultures held at 4°C. After 7 or 14 days of incubation, duplicate cultures were pooled and centrifuged. The supernatant fluids were pipetted off, tested for the presence of hemagglutinins and inoculated intra-amniotically in 0.1 ml amounts of serial 10-fold dilutions, into groups of 8, 7-8-day-old chick embryos to determine their infectivity. The eggs were incubated for 7 days and tested for the presence of hemagglutinins according to the methods previously described(4,6). The sedimented tissues were resuspended in 1 ml amounts of a 10% solution of gelatin, transferred from the graduated centrifuge tubes to ordinary bacteriological test tubes and placed in the incubator. The tubes were shaken occasionally to insure the penetration of the gelatin around the tissue fragments and after about 1½ hours, centrifuged and chilled in an ice water bath to solidify the gelatin. The gelatin-imbedded tissues were loosened from the tubes by dipping them into hot water, shaken out into chilled Petri dishes and cut into appropriate blocks. The blocks were returned to the respective tubes, being placed on the wall about half way down the tube. The tubes were closed with rubber stoppers and the tissues quick-frozen in an alcohol dry ice mixture and stored at –20°C.

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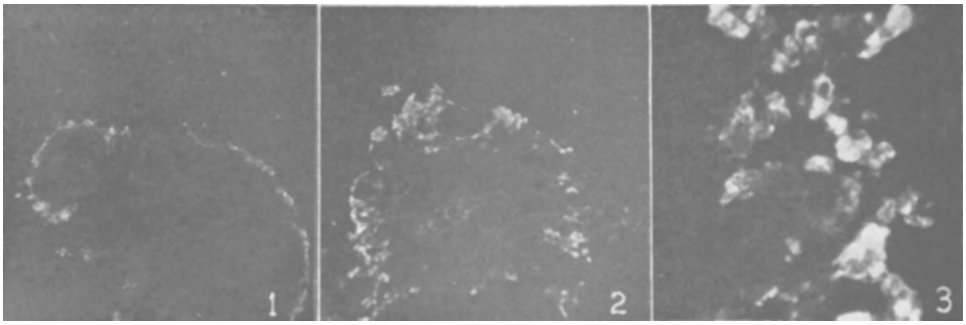

FIG. 1. Surface staining of piece of brain tissue from Maitland type of tissue culture 14 days after inoculation. × 84.

FIG. 2. Staining of cyst-like structures in piece of chorioallantoic membrane from Maitland type of tissue culture 14 days after inoculation. × 84.

FIG. 3. Section of chorioallantoic membrane showing 2 cells in which entire cytoplasm is stained and 2 in which brightly fluorescent granules are arranged around a centrally unstained nuclear shadow. × 360.

The preparation of the frozen sections, the procedure used in their staining and the controls of specificity were carried out according to the methods described by Coons *et al.* (1,7). The fluorescein conjugated antimumps serum used in this experiment was also prepared in the same way and from the same batch of convalescent monkey serum as that employed in (2) and (3) with the exception that it was absorbed twice with chicken liver powder instead of human or mouse liver powder prior to its use as a stain.

Results. Areas of bright yellow-green fluorescent staining were seen only in sections prepared from cultures with living tissues incubated at 35°C. This staining occurred in most though not in all the pieces of tissue examined on a given slide. The specific fluorescent precipitate was deposited over the cells lying at the extreme periphery of the larger tissue fragments but not in the central regions. The stained cells were distributed individually or in clusters in isolated or irregularly scattered patches at the edges of the sections. Occasionally they formed a halo-like ring around the entire edge of the fragment (Fig. 1). Some of the smaller pieces of tissue, which were usually no larger than 3-4 cell layers thick, were stained throughout. Specifically stained cellular debris and granular material were also present. The thin-walled cyst-like structures seen in many of the pieces of amniotic and chorioallantoic membranes (which according to Weller and Enders (4) represent areas of active tissue growth) were frequently outlined by the presence of the fluorescent precipitate (Fig. 2). With this exception very little difference could be detected in the distribution of the virus in cultures with the various kinds of tissue examined. Thus, whether the tissue was amnion, chorioallantois, whole embryo, or brain, the fluorescent precipitate was deposited in an irregular fashion over the surfaces which were directly exposed to the inoculum.

Within the stained cell the detectable virus antigen was found to be located in the cytoplasm but not in the nucleus (Fig. 3). In some of the cells the entire cytoplasm was clearly outlined by the specific staining. In others brightly fluorescent granules of various sizes were seen. These were often arranged in a circular or semicircular fashion around the central unstained nuclear shadow. Due to the embryonic nature of the cultures the identity of the cells which reacted with the conjugate could not be determined. The formation of the fluorescent precipitate was specifically inhibited by preliminary treatment of the sections with unlabeled immune serum.

The multiplication of the virus was established by the appearance of hemagglutinins and by an increase in the infectivity of the culture fluids. However, whereas all the 7-day culture fluids were infective through the highest dilution tested (10^{-6}), the titer of hemagglutinin was 1:16, 1:8, 1:4 and 0 respectively

in the amniotic, chorioallantoic, whole embryo and brain cultures.

No virus could be demonstrated in either the tissue or the supernatant fluid of the uninoculated cultures or of the cultures in which the tissues were frozen prior to inoculation. When infected cultures were stored at 4°C, however, the virus survived through a 10^{-2} dilution of the fluid but could not be demonstrated in the cells.

Discussion. Specific fluorescent immune serum has been used successfully as a histochemical tool in several fields of investigation (1-3, 7-10). Its application to problems dealing with the growth of viruses in tissue cultures has been suggested(11). In the present studies it has been shown that the amount of mumps virus produced in tissue cultures of the Maitland type is sufficient to be detected by fluorescein labeled antiserum. The distribution of the virus was found to be restricted to the cells which came in direct contact with the inoculum irrespective of the kind of tissue used. This would indicate that to increase the yield of the virus in a given culture it would be necessary to increase not the total amount of tissue but its surface area. Since the staining seen in cultures with amnion, chorioallantois, whole embryo mince and brain tissue did not differ significantly, the results suggested that under the conditions used the cells which supported the growth of this agent were either widely distributed or that the virus had no predilection for any particular type of cell. The occasional gaps seen in the peripheral staining of the individual tissue fragments were believed to be attributable to non-viable cells. The presence of the staining in the actively proliferating cyst-like structures seen in cultures of amniotic and chorioallantoic membrane indicated that the virus was capable of infecting cells which had multiplied *in vitro*. Because of the embryonic nature of the cultures, the identity of the cells which precipitated the conjugate could not be determined.

Summary. Fluorescein-labeled antibody has been successfully employed in the localization of mumps virus in cultures of various types of chick embryonic tissues infected *in vitro*. The distribution of the virus was found to be restricted to the cytoplasm of the cells which came in direct contact with the inoculum irrespective of the kind of tissue used in the culture.

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