Specific Organ Defects in Early Chick Embryos Following Inoculation with Influenza A Virus.* (22469)

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Characteristic defects in the development of early chick embryos following inoculation of influenza A virus have been reported by Hamburger and Habel(1) and by Shear *et al.* (2). The defects consisted chiefly of micrencephaly, abnormal axis twists and impairment of growth of the amnion. However, Hamburger(3) and Kung(4) observed, in microscopic studies of the abnormal embryos, that development of the brain tissue itself appeared normal and that the flattening of the encephalon, resembling a micrencephaly on gross examination appeared to result from collapse of the ventricles of the brain. The authors attributed the collapse of the ventricles to a lack of ventricular fluid, lost possibly through areas of local injury. Specific organ defects, such as those described following Newcastle disease virus(5-8), were not reported as characteristic effects of influenza A infection. It is the purpose of this paper to describe conditions under which influenza A produces characteristic gross defects of the lens and auditory vesicles in early chick embryos, and in a few instances, neural tube defects.

Material and methods. Virus preparation. The PR8 strain of influenza A virus, grown from dilute inocula in the allantoic cavity of 12-day chick embryos for 48 hours, served as stock virus suspension. The infectivity titer was determined by inoculating 10-fold dilutions of virus suspension intra-allantoically into 12-day embryos and testing after 4 days for virus growth as indicated by agglutination of chicken erythrocytes by the allantoic fluids. The EID_{50} was calculated by the method of Kärber(9). For inoculation into the early chick embryos the suspensions were diluted in buffered saline (pH 7.2) to the required infectivity titer. *Inoculation of embryos.* Eggs incubated in a forced draft incubator at 98-99°F for 45 to 60 hours were prepared with openings and inoculated under the vitelline membrane, over the blastoderm(6) with 0.05 ml amounts of virus suspension. *Determination of stage of development.* Developmental stages for embryos of each group inoculated were determined, according to the outline of Hamburger and Hamilton(10), by observation of the embryos *in vivo* under 24 power magnification at the time of inoculation. Such *in vivo* observations yielded results, which, although not strictly accurate for the stage of any individual embryo, were sufficiently accurate to estimate the average stage of the group as a whole. *Examination of embryos for abnormalities.* The inoculated embryos were examined daily *in vivo* for abnormalities or at specified intervals were removed from the eggs, fixed, cleared and stained(5) for examination *in toto* under 24 power magnification. *Immune Sera.* One lot of chicken hyperimmune serum, specific for the PR8 strain of influenza A was supplied by Dr. Herald Cox of Lederle Laboratories and another by Dr. H. H. Shear of Veterans Administration Hospital, Long Beach, California. Hemagglutination-inhibition titers of the sera were 1-2048 and 1-1280 respectively. Hyperimmune serum to Newcastle disease virus, used for control injections, was prepared in chickens in this laboratory and had a hemagglutination inhibition titer of 1-2048. All sera were inactivated at 56°C for 30 minutes before use.

Results. Following procedures successfully employed with Newcastle disease virus for producing a high percentage of gross defects in early chick embryos(6) preliminary studies were made in eggs of 48 hours incubation inoculated with $10^{7.2}$ ID_{50} of influenza A virus. Control inoculations consisted of normal allantoic fluid. Of 37 embryos receiving influenza A, 29 were alive at 24 hours after inoculation and 21 of these showed specific de-

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fects of the lens and/or otocyst, and less frequently the neural tube. The defects consisted of retardation, microplasia or complete absence of the organ. All the embryos showing specific organ defects also showed the characteristic twisted axis and/or collapsed encephalon observed by others. Of 37 embryos receiving control inoculations, only one embryo showed any defect, this one exhibiting a mild flattening of the mesencephalon. Fig. 1 shows a normal embryo of 72 hours incubation, and Fig. 2 illustrates the characteristic appearance of a 72 hour embryo inoculated 24 hours previously with influenza A virus. Few embryos survived longer than 24 hours after inoculation of such large doses of virus.

Further experiments showed that different concentrations of virus in embryos of slightly varying developmental stages had a marked effect on the appearance of specific organ defects. Results are summarized in Table I.

Microscopic examination of serial sections of embryos showing specific organ defects, confirmed the observations on the embryos *in toto*. Lens and otocysts were absent or retarded and the cells of such tissues suffered severe damage.

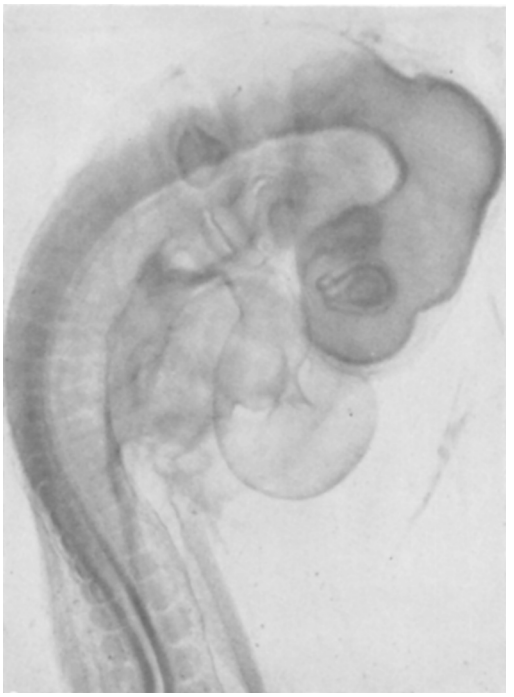

FIG. 1. Appearance of normal embryo of 72 hr incubation.

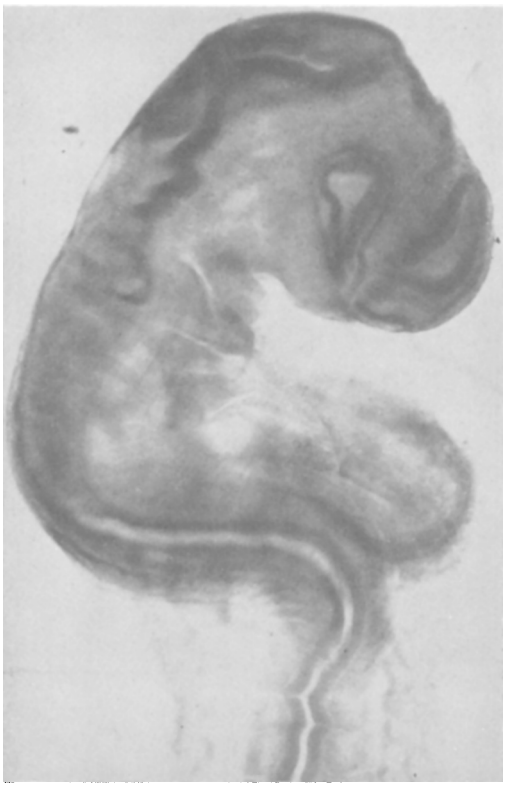

FIG. 2. Characteristic appearance of embryo of 72 hr incubation inoculated 24 hr previously with a high titer of influenza A virus. Note twisted axis, flattened encephalon, absence of lens and retardation of auditory vesicle.

Neutralization of defects with specific immune serum. Since the gross defects of lens and otocyst produced by the influenza virus closely resembled those observed after infection with Newcastle disease virus, a check was made to rule out chance admixture of the two viruses in laboratory usage. Two lots of influenza A hyperimmune serum obtained from separate sources outside our own laboratory gave essentially the same results. Two portions of influenza virus suspension containing $10^{6.7}$ ID_{50} per 0.05 ml were mixed with equal amounts of influenza antiserum in 1-10 dilution and NDV antiserum in 1-10 dilution respectively. After 20 minutes at room temperature each was inoculated in 0.1 ml amounts into respective groups of embryos of 48 hours incubation. As an additional con-

TABLE I. Effectiveness of Different Amounts of Influenza A Virus Inoculated at Slightly Different Developmental Stages in Producing Defects in Early Chick Embryos.

Hr of incubation at inoculation	Mean developmental stage	ID_{50} inoculated	Total embryos inoculated	24 hr after inoculation: Total alive	24 hr after inoculation: Survivors with lens or otocyst defects	24 hr after inoculation: Survivors with axis twists or flattened encephalon	48 hr after inoculation: Total alive	48 hr after inoculation: Survivors with lens or otocyst defects	48 hr after inoculation: Survivors with axis twists or flattened encephalon
48	12.5	$10^{6.7}$	25	15*	15	15			
48	12.5	$10^{4.7}$	25	23	0	3 (mild)	13*	8	12
60	14.2	$10^{6.7}$	29	18*	7	10			
60	14.2	$10^{4.7}$	24	24	0	4 (mild)	19*	4	17

* Harvested at this time.

trol 2 portions of NDV virus suspension containing 10^7 ID_{50} per 0.05 ml were mixed with the influenza A and the NDV antiserum respectively and inoculated into 48 hour embryos of the same incubation setting. Results clearly show that defects of the lens and otocyst as well as the axis twists and flattened encephalon were prevented by the influenza A hyperimmune chicken serum, whereas the NDV hyperimmune chicken serum had no such effect. Moreover, the NDV hyperimmune serum neutralized the teratogenic action of the NDV, whereas the influenza A hyperimmune serum did not.

Discussion. It has been repeatedly observed that the developmental stage of the embryo at the time of exposure is an important factor in determining the type of defect produced by a teratogenic agent. In analyzing teratogenic effects in chick embryos it is necessary to take into account exact developmental stages rather than hours of incubation, since rapidity of development varies markedly depending on such factors as temperature and time of egg storage, differences in incubating conditions, genetic variation, etc. Previous studies with Newcastle disease virus inoculated in early chick embryos at different stages confirmed its similarity to other teratogenic agents in that organs in early stages of differentiation were most susceptible to its teratogenic effects(7). Although small virus inocula resulted in specific tissue damage observable on microscopic examination, gross defects were consistently observed only after inoculation of large amounts of virus. Thus the use of large inocula provides a practical tool for preliminary determination of teratogenic specificity. It is of interest that influenza A, inoculated under similar conditions, showed similar effects to those of NDV upon organs differentiating from the body ectoderm, yet also produced characteristic defects different from those observed with NDV, *i.e.*, the severe axis twists and flattened encephalon. Such marked axis twists and flattened encephalon have occasionally been observed with NDV but not as a consistently characteristic effect. Further studies of the teratogenic effects of influenza A at various stages and in varying dilutions would be of interest.

Summary. The PR8 strain of influenza A virus was inoculated in high titer in chick embryos which were in stages of development ranging from 10 to 15 by Hamburger and Hamilton staging criteria (approximately 48 hours incubation time). Such inoculations produced specific organ defects, particularly noted in the lens and auditory vesicles, in addition to previously reported axis twists and flattening of the encephalon. Embryos inoculated at slightly later stages with more dilute virus also developed axis twists and flattened encephalon, but were much less consistent in their production of defects of the lens and otocysts within the time limits of the experiment. Specific hyperimmune serum to influenza A virus completely prevented all the influenza A-induced teratogenic effects, whereas other hyperimmune serum (Newcastle disease) failed to do so.

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A Broncho-Constrictor Factor in Cigarette Smoke. (22470)

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Although cigarette smoke is of a very complex chemical nature, the pharmacological effects of the smoke have been largely attributed to the nicotine and tar content. Such studies have been well summarized by Wynder and co-authors(1). The present studies are the result of a lack of published information on acute effects of smoke on the pulmonary airway.

Procedure. Cigarettes used were "king-size" popular brands. Six brands were studied. One was available as plain-tip and filter-tip types. Only the filter tip type of one other brand was available. One brand of "denicotinized" cigarette available with and without filter tip was used. Mature stock guinea pigs were etherized and the cervical spinal cord transected. The upper trachea was cannulated with a Y-cannula, connected to positive pressure artificial respirator. The thoracic cavity was opened by mid-longitudinal section of the sternum, and the cut edges were widely separated by self retaining retractors. The animal was then allowed to recover from the ether. A separate animal was used with each cigarette. The order in which animals received smoke from different brands of cigarettes, as well as lengths of the cigarettes during the test puff was randomly mixed. Four animals were used for each brand and type of cigarette, a total of 48 animals. The positive pressure artificial respirator functioned on the principle of commercially available C. F. Palmer apparatus.* A cigarette could be attached to intake of respirator with the glass adaptor tube, so that a delay of 3 to 4 seconds occurred between drawing of smoke and inflation of lungs with the smoke. The special glass Y-shaped tracheal cannula was constructed with a side arm at the level of entrance of the cannula to the trachea. This side arm was connected to a calibrated rubber membrane manometer for recording directly on the kymograph. A record of the tracheal pressure during each cycle of inflation of lungs was thereby obtained. Emptying of lungs was passive and due only to the elasticity of lungs. The functional dead space in the apparatus was measured directly by filling the apparatus with mercury and found to be 65 ml. All cigarettes were smoked to test lengths by mechanical Robot-Smoker so that each minute it took a single 10 ml puff of 2-second duration on the cigarette.

Results. The nature of the control inflation pressure curve obtained on guinea pigs was very uniform between animals. Fig. 1 shows this typical type of control inflation curve and the effect of a single 10 ml respirator puff of cigarette smoke on the nature of the tracheal pressure curve. The different quantitative responses obtained with the various cigarette lengths studied is also presented in the Figure. The predominant effects of cigarette smoke on pulmonary inflation pressure are a more rapid increase in inflation pressure in the middle of the cycle, and a fall in inflation pressure before the end of inflation cycle. This type of effect is interpreted as being the result of a broncho-constrictor

* Ideal Respirator, available through C. F. Palmer, Ltd., London, England.