Identification by Fluorescent Antibody of Developmental Forms of Psittacosis Virus in Tissue Culture.* (21990)

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Bedson(1) has described a developmental cycle for psittacosis elementary bodies in the cytoplasm of host cells. The cytologic studies (1-6) proved that the intermediate developmental forms such as initial bodies and plaques are regularly associated with the propagation of virus and are structural evidence of cellular infection. Morphologic observations have failed to establish whether the large structures are secondary reaction products or multiplying virus. Electron micrography has so far produced only equivocal evidence(7-9). The present study was undertaken with the hope that the immunocytochemical method for intracellular identification of antigen described by Coons and his coworkers(10) might determine the nature of the inclusion bodies.

Materials and methods. Virus: The psittacosis 6 BC strain was generously provided by Dr. Herbert Morgan, University of Rochester School of Medicine, and was maintained by yolk sac passage in embryonated eggs. A 10% yolk sac suspension with an LD_{50} titer of 10^{-8} was used. Albany strain mice were employed. Infectivity tests were performed in young adults (8-12 g). Pregnant mice 60-80 days old were the source of mouse embryos. Groups of 5 mice were inoculated intracerebrally with 0.03 ml of undiluted supernates of lightly centrifuged ground tissue suspension, then observed for 21 days. *Tissue culture*: Pregnant mice, approximately at the end of the period of gestation, were sacrificed by ether anesthesia. Embryonic livers were removed aseptically and minced with detachable knife blades into 1- x 2-mm pieces. Five pieces were planted on a 10- x 20-mm cover glass and 5 were planted on a 10- x 10-mm glass fiber mesh(11) placed in the flat bottom of a Leighton tube (Microbiological Associates, Bethesda, Md.). Approximately 0.1 ml of horse serum was added to the explants. Preparations were incubated at 40°C for 20 minutes, after which nutrient fluid(12) (beef amniotic fluid 85%, beef embryo extract 5%, horse serum 10%, 50 μg/ml of crystalline dihydrostreptomycin sulfate (Merck)) was added. 0.5 ml per tube. Cultures were incubated at 35°C for 5 days prior to use. *Fluorescent-antibody technic*: The indirect method of Weller and Coons(13), and Coons, Leduc, and Connolly(14) was applied. Fluorescein isocyanate was synthesized and conjugated to the pseudoglobulin fraction of antihuman gamma globulin horse serum‡ according to methods described by Coons and Kaplan(10) with minor alterations. For specific combination with intracellular antigen, human antipsittacosis serum (No. V54-2029) was used. The 50% unit titer was 1285 in a complement-fixation test against a phenolized 10% yolk sac antigen. A human serum (No. V54-1766) was the control. Nonspecific staining was eliminated by absorption of the conjugate with whole mouse embryo powder and subsequent dilution with buffered saline (sodium chloride solution, phosphate buffered to pH 7.0). Cover-glass preparations from inoculated and uninoculated groups were removed and treated as described by Weller and Coons(13). Stained cultures were examined for the presence of intracellular antigen under the fluorescence microscope. Its light source was a 1000-watt General Electric water-cooled mercury vapor lamp (type A-H6). The beam was condensed

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by a quartz lens (61.5 mm diameter, 59.5 mm focal length). Infrared and visible light was filtered by passing the beam through 25% $CuSO_4$ in a water-cooled cell in which a Corning glass filter (#5840, ½ standard thickness) was immersed. Slides were viewed through a dark-field microscope with a Wratten 2 B gelatin filter in the ocular. Photomicrographs were taken with a Zeiss Miflex camera and super ortho press film. Exposure for 400X magnification was 3 minutes. Films were tray-developed in DK-50 for 5 minutes at 20°C.

Results. Specificity of fluorescent antibody technic: In preliminary experiments, infected and uninfected explants were exposed for 20 minutes to 1:10 dilutions, respectively, of human antipsittacosis serum and of human serum which did not react in the complement-fixation test with psittacosis antigen. Cultures on cover glasses were washed for 10 minutes with buffered saline, then overlaid for 20 minutes with a 1:10 dilution of fluorescein conjugate. Cultures were again washed for 10 minutes and mounted as described by Weller and Coons(13). Brilliant yellow-green fluorescence was observed in the cytoplasm of infected cells treated with human antipsittacosis serum. Uninfected explants exposed to human antipsittacosis serum and infected explants treated with nonreacting human serum showed blue-gray autofluorescence.

Relation of inoculation dose to specific fluorescence and infective virus: Cultures with good outgrowth were divided into 3 groups inoculated respectively with 0.05 ml of 10^{-1}, 10^{-3}, and 10^{-5} dilutions in nutrient fluid of yolk sac suspension. At varying times (Table I) preparations of each group were treated in the following manner: 1. Cover-glass preparations were washed free of extracellular antigen in 3 changes of buffered saline. Exposure of infected cultures to human antipsittacosis serum was followed by staining with fluorescent antibody. Time factors for the staining procedure were substantially the same for all preparations. 2. Glass fiber patches carrying infected explants were removed from Leighton tubes and washed in 6 changes of buffered saline to minimize contamination by residual antigen, then ground

TABLE I. Comparison of Appearance of Specific Fluorescence and Presence of Infective Virus at Various Time Intervals following Inoculation of Different Doses of Virus.

Dil. of virus inoc.	Neg.* log of LD_{50} titer	Time after inoculation—specific fluorescence†—infective virus‡										Avg time until death (mice), days
		0 min.	10 min.	1 hr	3 hr	5 hr	8 hr	12 hr	16 hr	20 hr	24 hr	48 hr	72 hr	
10^{-1}	5.3	—	±	+	++	+++	+++	+++	++++	++++	++++	++++	++++	3– 6
		4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	
10^{-3}	2.5	—	—	—	—	—	±	+	+	++	++	++	++	5–14
		NT	NT	0/5	0/5	2/5	3/5	5/5	2/5	4/5	3/5	5/5	5/5	
10^{-5}	<1.0	—	—	—	—	—	—	—	—	—	—	±	±	20
		NT	NT	NT	NT	NT	1/5	0/5	0/5	0/5	0/5	0/5	0/5	

* LD_{50} titer of ground tissue and nutrient fluid at 0 time.
† Grading of results explained in text.
‡ Presence of infective virus in washed tissue explants; numerator = No. of dead mice; denominator = No. of mice inoculated.
NT = Not tested.

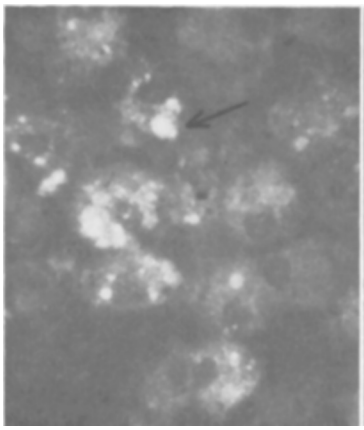

Fig. 1

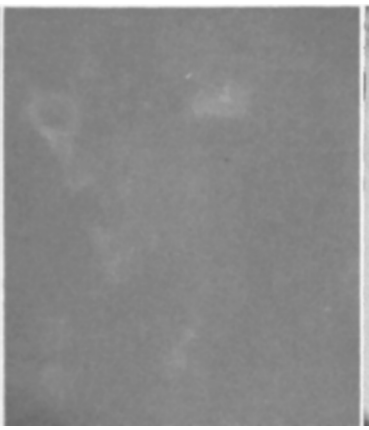

Fig. 2

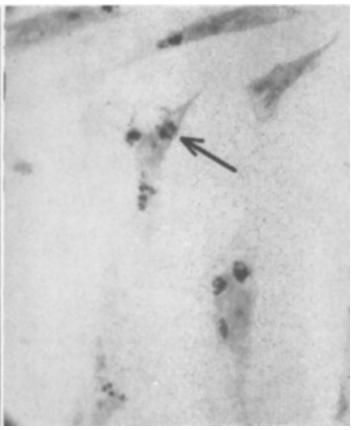

Fig. 3

FIG. 1. Mouse embryo liver cells infected with 10^{-1} dose of virus; stained by fluorescent technic 72 hr after inoculation. Arrow points to plaque. 400×.

FIG. 2. Mouse embryo liver cells infected with 10^{-5} dose of virus; stained by fluorescent technic 72 hr after inoculation. 400×.

FIG. 3. Mouse embryo liver cells infected with 10^{-1} dose of virus; stained by Giemsa's method 72 hr after inoculation. Arrow points to plaque. 400×.

in mortars with alundum and 0.5 ml of nutrient fluid per 5 explants. After light angle centrifugation supernates were tested for infectivity in mice. In 10 different experiments, approximately 260 cover-glass preparations, carrying 5 explants each, were examined for presence of specific fluorescence at different intervals postinoculation. Results were graded from — to +++++, — indicating absence of specific fluorescence; ± a trace; + involvement of 5%; ++ 6-25%; +++ 26-75%; and +++++ the presence of specific fluorescence in more than 75% of the outgrowing cells. With the heaviest virus inoculum (10^{-1}), intensity of specific cytoplasmic fluorescence was moderate early and became marked 16 hours following inoculation and remained intense throughout the experiment (Fig. 1). With a medium (10^{-3}) dose of virus, two distinct features were noted: The appearance of specific fluorescence was delayed and its distribution was focal, involving 5-25% of outgrowing cells. The intensity was weak for the first 16 hours, and became moderate for the rest of the experiment. Negative results were obtained when the 10^{-5} dilution was used (Fig. 2).

Tests for the presence of virus in washed explants indicated that it was present in all groups inoculated with the 10^{-1} dilution. The average time to death decreased in the 10^{-1} and 10^{-3} groups as the experiment progressed, evidence of the multiplication of the virus.

Identification of antigen in developmental forms. In this series of experiments, the morphology of cultures stained with fluorescent antibody was compared with Giemsa§-stained cover-glass preparations. Diffuse fluorescence was observed in the early stages, 1, 3, and 5 hours postinoculation. Stippling, *i.e.*, the appearance of tiny fluorescent granules, distributed evenly throughout the cytoplasm, occurred as early as 8 hours following inoculation. It compared well with purple red staining elementary bodies and blue staining larger initial bodies in the cytoplasm of the cells stained by Giemsa's method. Concurrent with the blue staining vesicles and plaques at 12- and 16-hour intervals, larger fluorescing bodies were observed, sometimes eccentrically located in the cytoplasm. The size of the inclusion bodies in the Giemsa preparations continued to be much the same as in the fluorescent-antibody preparations throughout the growth cycle of the virus in cultures inoculated with the moderate (10^{-3}) and heavy (10^{-1}) doses (Fig. 3). Cultures in-

§ Giemsa stock solution, Merck and Company, Darmstadt, Germany.

oculated with the low (10^{-5}) dose, which showed no specific fluorescence, likewise developed no inclusion bodies detectable by Giemsa stain.

Discussion. By the immunocytochemical criterion described it was shown that antigen was regularly present within the developmental forms of psittacosis virus and that the inclusion bodies can be considered to be virus colonies rather than reaction products of infected cells. Bedson and Gostling(15) have suggested that multiplication of psittacosis virus is initiated as soon as elementary bodies are introduced into a susceptible tissue. This view is substantially supported by our *in vitro* observation that virus can be located within the cytoplasm of a few cells as early as one hour after inoculation. Whether the early intracellular appearance of antigen signifies the presence of infective virus has not been settled, since repeated washing of explants failed to remove traces of residual virus. In groups inoculated with a moderate dose, however, the early stages were characterized by lack of specific fluorescence and negative infectivity tests. With the appearance of intracellular fluorescence, infective virus was also demonstrated.

A large dose of virus infected 75% or more of outgrowing cells within the first 16 hours, whereas a smaller dose produced only a focal distribution of specific fluorescence, leaving 75% or more of outgrowing cells uninfected.

Weiss(5) has described intranuclear granules resembling elementary bodies 7 to 10 days after infection of experimental animals, and considered the possibility that the virus had invaded the nuclei. In our experiments specific fluorescence appeared within the cytoplasm alone during the 72-hour observation period. The various stages of cellular division were not correlated with the detection of specific fluorescence. No attempt was made to examine for extracellular forms of virus(5), since the search for intracellular antigen necessitated frequent washing of cultures to minimize residual virus. The fluorescent-antibody technic proved to be more sensitive than Giemsa's differential stain for detection of virus provided the inoculation dose was large. Giemsa staining failed to disclose with certainty intracellular inclusions at 1- and 3-hour intervals following inoculation, whereas the immunocytochemical stain detected intracellular antigen earlier and in greater quantity throughout the experiment. The difference may have been affected by the difference in method of fixation, formalin(16) being used for the Giemsa preparations, while air drying (1 hour at 37°C) followed by acetone was employed for the fluorescent-antibody studies. Antigen might be lost during the former treatment. Numerous observations of empty vesicles within the cytoplasm of Giemsa-stained cells suggested this might have been the case. A similar loss of antigen may have occurred during the studies of Kurotchkin *et al.*(7) who reported disintegration of large structures following treatment with ether for 5 minutes and reasoned that inclusion bodies represent secondary reaction products, perhaps of lipid nature, rather than virus colonies.

Summary. 1. Developmental forms of psittacosis virus have been studied in mouse embryo liver tissue cultures *in vitro* by the fluorescent-antibody technic. 2. Inoculation of a large dose of virus resulted in the intracellular appearance of antigen in most of the outgrowing cells within 16 hours. A moderate dose of virus resulted in the focal distribution of specific fluorescence. When a small dose of virus was inoculated, no specific fluorescence was detected. 3. The evidence indicates that virus antigen is present in the developmental forms within the cytoplasm of infected cells throughout the various stages that make up the growth cycle of psittacosis virus.

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Protection Against Tourniquet Shock Afforded by Parabiosis.* (21991)

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When rats are placed in parabiosis one of them may thereby be protected against otherwise fatal consequences. It has been shown for example, that survival of one is prolonged after total nephrectomy(1), a lethal dosage of x-rays(2,3) or bilateral adrenalectomy(4), when the co-twin is normal. Similarly the usually severe diabetes following either pancreatectomy(5) or toxic doses of alloxan(6) is either minimized or abolished by the presence of a normal partner. Recently we have reported that when one of a normal pair of parabiotic rats is subjected to transection of the spinal cord just caudad to C7, the operated rat develops erythremia, with increased hemoglobin, erythrocyte count and hematocrit, whereas the intact co-twin concurrently develops a rapidly fatal anemia(7). This study was interpreted to indicate that the capillary hypotension induced on one side of the capillary anastomosis by spinal transection led to a decrease in the amount of blood exchanged from operated to intact, while the amount traversing the vascular bed from intact to operated remained normal. These changes, typical of spontaneous parabiosis intoxication (8), suggest the latter to be due to a shock-like state in one of the partners.

The present study was undertaken to determine whether the disparity in blood transfer could also be induced by subjecting one of a pair to tourniquet shock, or whether the usually lethal effects of this intervention would be modified by the presence of a normal twin.

Materials and methods. The animals used in these studies were females of the Holtzman strain. Thirteen single animals weighing 100-139 g served as controls, and 10 pairs of parabionts, in union for 2 weeks and together weighing 200-280 g, constituted the parabiotic group. Parabiosis was performed essentially by the method of Bunster and Meyer(9). Tourniquets were applied to both hind limbs of the singles and to both hind limbs of one of the partners of each pair. The method was similar to that described by Rosenthal(10) and Stopack(11). A #8 elastic band was looped 5 times around a hollow tube of 16 mm diameter, each hind limb was then drawn through the tube and the elastic ligature slipped off so as to constrict the limb as close to the body as possible. The ligatures were removed 5 hours later. Rectal temperature, erythrocyte count, hematocrit and hemoglobin determinations were taken on all animals prior to ligation, and at 12, 36, and 48 hours after its removal, where survival permitted. Purina Laboratory Chow and tap water were available to the rats at all times.

Results. Of the 13 single rats to which the tourniquets were applied, 5 died within 12 hours of removal of the ligatures and all but one had succumbed by 48 hours thereafter.

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