8. Vantsis, J. T., Wildy, P., Virology, 1962, v17, 225.
9. Nii, S., Kamahora, J., Biken's J., 1961, v4, 255.
10. Merchant, D. J., Kahn, R. H., Murphy, W. H., Handbook of Cell and Organ Culture, 2nd ed., Burgess Publishing Co., Minneapolis, 1964.
11. Cooper, P. D., Adv. Virus Res., 1961, v8, 319.
12. Rapp, F., Seligman, S. J., Jaross, L. B., Gordon, I., Proc. Soc. Exp. Biol. & Med., 1959, v101, 289.
13. Schulze, I. T., Schlesinger, R. W., Virology, 1963, v19, 40.
14. McNair Scott, T. F., Tokumaru, T., Bact. Rev., 1964, v28, 458.
15. DeMaeyer, E., Schonne, E., Virology, 1964, v24, 13.
16. Cooper, P. D., ibid., 1961, v13, 153.

Received July 25, 1967. P.S.E.B.M., 1967, v126.

Use of Alveolar Macrophages for Cultivation of Canine Distemper Virus.* (32509)

MAX J. G. APPEL AND OSWALD R. JONES (Introduced by James A. Baker)
Veterinary Virus Reserach Institute, New York State Veterinary College, Cornell University, Ithaca, N. Y.

Lack of methods to demonstrate and assay virulent canine distemper virus (CDV) *in vitro* has hampered studies of the disease that it causes. Adaptation of CDV in eggs and in tissue culture required serial transfers(1,2, 3,4). Once adapted, CDV strains have been propagated readily in a variety of primary cell culture systems and in continuous cell lines with typical cellular changes(5), but an assay method for virulent virus remained elusive. Cultivation of CDV from infected dogs has succeeded in ferret kidney cell cultures, but demonstration of virus required staining procedures(6). This report describes a method for demonstration and assay of virulent CDV that makes use of dog lung macrophages.

Materials and methods. Tissue culture. Puppies 2 to 6 weeks of age were used for dog lung cell (DL) culture. A puppy was given deep anesthesia and then exsanguinated by severing brachial arteries. Lungs were removed under sterile conditions, minced with scissors and washed in phosphate buffered saline (PBS) at pH 7.2. Lung pieces were stirred in Medium 199 (M 199) plus 0.5% lactalbumin hydrolysate for one-half to 1 hour at room temperature. The suspension was filtered through cheesecloth and filtrate was centrifuged 2 minutes at 1000 rpm. Sedimented cells were resuspended 1:150 by volume in M 199 plus 0.5% lactabumin and either 20% newborn lamb serum or 20% rabbit serum that had been heated at 56°C for 30 minutes. Bovine serum, dog serum, horse serum, pig serum and guinea pig serum did not always yield good virus growth and none were used for these studies. An antibiotic mixture was added that consisted of 500 units of penicillin, 100 μg of streptomycin, 100 units of mycostatin and 2 μg of fungizone per ml. Final pH was approximately 7.1. The suspension contained approximately 8×10^5 cells per ml. Into each Leighton tube with coverslip, 1 ml of cell suspension was placed and incubated in a stationary rack at 36°C. A lung yielded approximately 100 to 150 tubes. These cultures consisted of epitheloid, fibroblastic-type cells and macrophages. Epitheloid and fibroblastic cells began formation of monolayers after 5 days and could be maintained for several weeks. Macrophages degenerated after cultivation for 2 weeks.

After Myrvik(7) reported that almost pure cultures of macrophages were obtained from washings of bronchial and alveolar surfaces of intact lungs of rabbits, his method was applied to dog lungs in order to study this cellular element present in the minced lung preparations. Lungs were removed from euthanized dogs; the trachea or 1 bronchus at the bifurcation was then tied with a hemostat and PBS was injected with syringe and needle until the lungs were distended. After slight

* Supported by Grants from Whitehall Foundation and USPHS Res. Grant AI07512-01 from Nat. Inst. of Allergy & Infect. Dis.

massage, washings were withdrawn with a syringe and this was repeated twice, with care to maintain sterility. Washings were filtered through cheesecloth, centrifuged for 10 minutes at 2000 rpm and the sedimented cells then were handled in a manner similar to that described for minced lung cultures. A lung usually yielded 15 to 30 tubes. Similar cultures were prepared from distemper infected dogs.

Several hours after cells were placed in culture tubes, they appeared as single round cells attached to the glass (Fig. 1). In contrast to minced lung cell cultures, cells obtained from washings did not form monolayers and did not live longer than 12 to 16 days. When cultures were inoculated with opsonized staphylococci, these organisms were engulfed after incubation for 1 hour at 36°C. When stained with Shorr's stain, cells appeared as a round mononuclear type with eccentric nuclei. These findings suggest that these cells were macrophages.

Virus studies. These studies made use of 2 strains of virulent CDV, the Snyder Hill strain and a strain designated St. Joseph. From infected dogs, conjunctival, nasal, oral, rectal, and vaginal specimens were obtained with swabs. The swabs were placed in M 199 plus antibiotics and either tested immediately or stored at —70°C. For their inoculation, specimens were centrifuged, supernatant fluids were removed and were tested undiluted except those from rectal swabs which were filtered through a 450 mμ Millipore® filter. In addition, heparinized blood specimens were taken from the jugular vein and, following centrifugation, the plasma and the leukocyte fractions were tested. Also, cerebrospinal fluid was withdrawn from the cisternia magna of anesthesized dogs with distemper encephalitis. Then into each of 4 DL cultures that had been freshly prepared, 0.1 ml of a specimen was inoculated. Inoculated cultures were placed at 36°C. Beginning 1 day later and daily thereafter, inoculated tubes and others left uninoculated were examined for cellular changes.

To prove that DL cultures were infected with distemper virus, cultures were stained with Shorr's stain for inclusion bodies, and

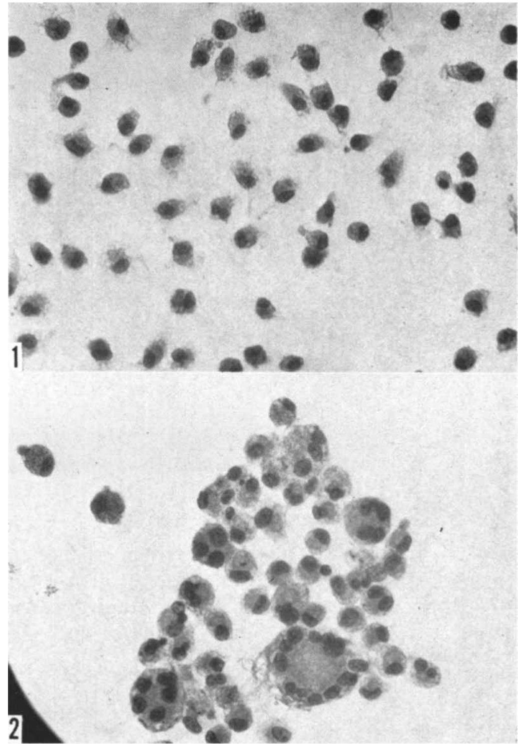

FIG. 1. Dog lung macrophages in tissue culture. (Shorr's stain 250×).
FIG. 2. Multinucleated giant cell formation in DL cultures 2 days after CDV inoculation. (Shorr's stain, 250×).

were tested for viral antigen by fluorescent antibody method. In addition, fluids from 4 tubes that had been inoculated with a 10^{-3} splenic emulsion of St. Joseph strain were pooled and then transferred for 2 additional serial passages, using tubes that each time had been inoculated with a 10^{-3} dilution. After the last transfer, each of 2 susceptible dogs was inoculated intravenously with 1 ml of a 10^{-3} dilution that represented a dilution of 10^{-12} of starting material. Inoculated dogs were observed for signs of distemper. Also, 2 ferrets were inoculated intraperitoneally with fluids after 38 serial passages. As a further test that virus was cultivated, a serum shown to contain antibodies for CDV in egg neutralization tests was mixed with 100 tissue culture infectious doses of St. Joseph strain that had been transferred for 10 serial passages in DL cultures. Cellular changes in these tubes were compared with a portion of the same virus suspension that had been mixed with a

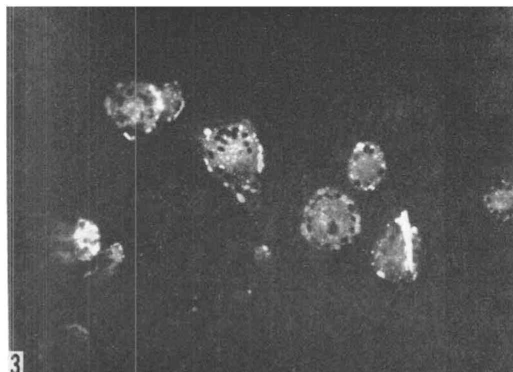

FIG. 3. Multinucleated giant cell formation in DL cultures 2 days after CDV inoculation. (Fluorescent antibody stained, 100×).

serum that did not contain CDV antibodies.

For assay of virus content, a 20% splenic emulsion from an infected dog was prepared in M 199. After centrifugation at 2000 rpm for 10 minutes, supernatant fluid was removed and 10-fold dilutions were prepared in M 199 that ranged from 10^{-1} to 10^{-7}. Then each dilution was tested comparatively in dogs and in DL cultures. A dog was given 1 ml intravenously of a dilution and each of 4 cell cultures was inoculated with 0.2 ml. Dogs were examined daily, clinical signs recorded and antibody studies made on those that recovered. Tissue culture tubes were examined daily for cellular changes. Titrations of each virus strain were repeated twice and endpoints were calculated by the Sperman-Karber method. Log titers were adjusted to content of virus per ml.

Results. Multinucleated giant cells (Fig. 2, 3) were seen in both DL cultures and lung macrophage cultures that had been inoculated with conjunctival, nasal, oral, rectal, and vaginal specimens, with plasma and leucocytes from infected dogs and with cerebrospinal fluid from dogs that showed encephalitis. Giant cells appeared 2 to 6 days after inoculation with infected material but formed 1 to 48 hours in cultures prepared from an infected dog. These cells showed characteristic, clear, circular edges in which the cytoplasmic structure of single small cells initially observed had disappeared. After 1 to 2 weeks, circular giant cells disappeared and irregular shaped syncytia were seen in the monolayer similar to those produced by the Rockborn strain in dog kidney cell cultures. Uninoculated tubes occasionally showed clumps which could be mistaken for giant cells on initial inspection. Closer observation showed cell walls intact. Syncytia in uninfected cultures were not seen. Also, a fusion of 3 or 4 cells was observed occasionally in stained preparations but these did not resemble giant cells produced by CDV.

When stained with Shorr's stain, the nuclei of most giant cells had a clockface arrangement (Fig. 2). Intracytoplasmic and intranuclear inclusion bodies were seen. Viral antigen was demonstrated in giant cells by fluorescent antibody (Fig. 3). No viral antigen was found in cell aggregations in uninoculated tubes.

After 3 serial transfers of virulent virus with 10^{-3} dilutions, fluids from cultures that showed giant cells produced distemper when inoculated intravenously into susceptible dogs. Also, ferrets inoculated with DL tissue culture fluid after 38 serial passages died from distemper. Fluids from cultures that represented 10 serial passages that had been mixed with distemper antibodies failed to produce giant cells but a portion of the same fluids mixed with serum that did not contain antibodies produced giant cells.

Giant cell formation in DL cultures and titration in dogs showed approximately the same endpoints, that is, $10^{5.9}$ compared to 10^{6} for St. Joseph strain and $10^{4.7}$ compared to 10^{5} for Snyder Hill strain.

Discussion. In studies on pathogenesis of CDV in dogs(8), the observation was made that CDV was removed from lungs of dogs after infection by aerosolization and appeared in large mononuclear cells in adjacent lymph nodes. Presumably, these cells were macrophages that represented initial infection by CDV. It was not unexpected, therefore, that CDV infected macrophages *in vitro*. Giant cell formation that permitted ready detection and assay of virus was fortunate.

Other viruses have been propagated in macrophages either collected from peritoneal cavity or in buffy coat cultures(9,10,11,12, 13,14). More macrophages, however, were available from lungs and, when trypsinization was avoided, good cultures were obtained.

Other dog viruses, infectious canine hepatitis, canine herpesvirus and reovirus did not produce giant cells in DL cultures. The conclusion was made, therefore, that giant cell formation was induced by CDV and the method for assay of virulent virus in dog tissues had been demonstrated. Rapidity of giant cell formation in cultures prepared from infected dogs indicated that this process may occur *in vivo* and giant cells have been reported associated with distemper.

Summary. Cell cultures prepared from dog lungs without trypsinization contained viable lung macrophages. When such cultures were inoculated with CDV, giant cells were seen 2 to 6 days after inoculation. When virulent CDV was titrated in DL cell cultures and in dogs, comparable endpoints were obtained. Giant cell formation in lung macrophage cultures, therefore, permitted demonstration and assay of virulent CDV.

1. Haig, D. A., Onderstepoort, J. Vet. Sci. Animal Ind., 1948, v23, 149.
2. Cabasso, V., Cox, H. R., Proc. Soc. Exp. Biol. & Med., 1949, v71, 246.
3. Rockborn, G., Arch. ges. Virusforsch., 1958, v8, 485.
4. Bittle, J. L., York, C. J., Newberne, J. W., Cornell Vet., 1961, v51, 359.
5. Bussell, R. H., Karzon, D. T., Arch. ges. Virusforsch., 1965, v17, 163, 183.
6. Vantsis, J. T., Vet. Rec., 1959, v71, 99.
7. Myrvik, Q. N., Leake, E. S., Fariss, B., J. Immunol., 1961, v86, 128.
8. Appel, M., Ph.D. Thesis, Cornell University, 1967.
9. Barski, G., Science, 1957, v125, 448.
10. Franklin, R. M., Virology, 1958, v6, 81.
11. Malmquist, W. A., Hay, D., Am. J. Vet. Res., 1960, v21, 104.
12. Loan, R. W., Gustafson, D. P., ibid., 1961, v22, 741.
13. Mallucci, L., Virology, 1965, v25, 30.
14. Seamer, J., Arch. ges. Virusforsch., 1965, v17, 654.

Received July 20, 1967. P.S.E.B.M., 1967, v126.

Experimental Canine Endotoxin Shock: Failure to Correlate Outcome With Persistent Endotoxemia.* (32510)

WESLEY W. SPINK AND BOLESLAW STARZECKI†
Department of Medicine, University of Minnesota Medical School, Minneapolis

Although studies on experimental canine endotoxin shock have yielded valuable information on the pathogenesis of this form of shock there is a lack of knowledge on the distribution of endotoxin in the tissues and body fluids during the course of shock. The present investigation was concerned with correlating the persistence of endotoxemia with recovery or death. Observations in man have suggested that the detection of endotoxin in the circulating blood can have diagnostic and prognostic values(1,2).

It was previously observed upon rapidly injecting a bolus of endotoxin intravenously into adult dogs that a precipitous drop in systemic blood pressure occurred within 30 to 60 seconds(3). Within the following 60 minutes partial recovery of the blood pressure occurred and hemodynamic stability often lasted up to several hours before death ensued. A fatal outcome was related to renal failure and metabolic acidosis. Since no information was available on the duration of endotoxemia in the dog serial blood determinations for endotoxin were carried out using a bioassay, postulated as specific for endotoxin.

Materials and methods. Adult mongrel dogs were anesthetized with 30 mg/kg sodium pentobarbital and maintained lightly anesthetized during the course of the experiments. A catheter was placed in the lower aorta of each of 10 dogs through the femoral artery, and coupled to a transducer for continuous monitoring and recording of blood pressures. All injections were made into a catheter in-

* This work was supported by a research grant (AI 04415-06) from USPHS.

† Postdoctoral Trainee, USPHS Training Grant 5 T1 AI 194-05.