A Bivalent Live Virus Vaccine Against Canine Distemper (CD) and Infectious Canine Hepatitis (ICH).* (24242)

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Cultivation in tissue culture of ICH virus (1) greatly facilitated its laboratory study, and opened the way for modification of its virulence to the dog. Such modification of the virus was reported, either by continuous serial passage in dog-kidney culture(2), or by propagation in swine-kidney cells(3,4,5). A combined CD-ICH vaccine containing swine-kidney-grown ICH virus recently was reported to be an effective immunizing agent against both diseases(6). This communication reports on attenuation of ICH virus after relatively few passages in swine kidney cultures, and on experiments on susceptible puppies with a bivalent CD-ICH vaccine.

Materials and methods. Virus strains. The CD strain used was a chick-embryo-adapted virus originally isolated in these laboratories from a field case of distemper encephalitis (7). Distemper challenge was carried out with a dog-brain suspension infected with Snyder Hill strain of canine distemper virus (8). The ICH virus used for both initiation of dog-kidney line of passages and challenge of vaccinated animals also was isolated in these laboratories, as reported earlier(9). *ICH virus. Cultivation, titration and serum neutralization.* Serial passages of the virus were made in Povitsky-bottle cultures of either dog- or embryonic-swine-kidney cells grown by the method of Dulbecco and Vogt (10). Growth fluid consisted of Earle's basal medium(11) containing lactalbumin hydrolysate and 15 or 20% normal calf serum. Just before inoculation, culture bottles were renewed with 100 ml of mixture 199(12). Virus titrations and serum neutralizations were carried out in stationary tubes of dog-kidney cells prepared as above. Renewal fluid consisted of 1 ml/tube of mixture 199 alone or of mixture 199 containing 2% horse serum. Titrations were made in 10-fold steps, by inoculating 0.1 ml of each dilution into 2 or 3 tubes. In serum neutralizations, mixtures of 2-fold dilutions of inactivated serum and 100-1000 TCD_{50} of virus also were inoculated in 0.1 ml volumes in 2 or 3 tubes, after incubation for 1 hour in 37°C waterbath. Titration and serum neutralization tubes were examined for 7 days, at the end of which 50% endpoints were calculated by the Reed and Muench formula (13). *CD virus. Cultivation and titration.* Both processes were carried out in 7- or 8-day chick embryos, using inoculation technic described by Gorham(14). For cultivation, a 10% suspension of infected chorio-allantoic membrane (CAM) was inoculated in 0.4 ml amounts/egg, with 0.2 ml dropped on the CAM at base of the natural air sac, and the remaining 0.2 ml introduced into the yolk sac. After 7 days of incubation at 36°C, both CAMs and embryos were harvested, pooled and made into a 30% tissue suspension. For virus titrations, 5-fold serial dilutions of vaccine were inoculated, each into 6 embryos, by dropping 0.2 ml volumes on the CAM. CAMs were examined 7 days later for characteristic lesions, and 50% endpoints were calculated. *Preparation of bivalent vaccine.* One part of undiluted, ICH-infected swine-kidney tissue-culture fluid was mixed with 9 parts of centrifuged (2000 rpm/20 minutes) 30% CD suspension. The mixture was distributed in glass vials in 2.4 ml amounts, and dried from frozen state. Vials were rubber-capped and aluminum-sealed under vacuum. Before its use in experiments, the vaccine was titrated for ICH and CD viruses, and tested on ferrets for potency of the CD component. *Complement fixation (CF).* The technics used, and the method of antigen preparation, were described earlier(15). *Experimental animals.* Litters

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TABLE I. Puppy Virulence of ICH Virus Grown in Dog-Kidney Tissue Culture (Tests Performed 1953-1955).

Inoculation*		Results					
		Clinical ICH			Outcome		
Passage level	No. of puppies	No. typical	No. mild	Incubation	No. dead & time (days)	Inclusions in liver	C.F. antibodies in survivors
2–11	12	12		Usual	11 (3– 5)	11	1
13	1	1		"	1 (3)	1	
20	2	2		Prolonged	2 (14–27)	1	
30	2		2	Usual			2
50	3†						3
52	3†						3
	3‡		3	17–19 days			3

* All animals except those marked (†) and (‡) were inoculated with 0.1-0.15 ml undiluted tissue culture fluid in anterior chamber of right eye.
† Inoculated intraper. with 2 ml undiluted tissue culture fluid.
‡ Cage contacts to animals receiving 52nd passage virus.

of puppies of miscellaneous medium breeds, including mongrels, were secured from private sources. Puppies, with their mothers, were held in isolation from birth until they were weaned. They then were received in the laboratory and kept under careful observation for at least 2 weeks. Immediately preceding its use, each puppy was shown by the CF test to be negative for CD and ICH antibodies.

Results. I. Modification of ICH virus. A. Puppy virulence of ICH virus grown in dog kidney cultures. Over 18 months, each of various passage levels of the virus in dog kidney cultures was inoculated into 2 or 3 puppies. Virus was administered by one of 2 methods: either (a) 0.1-0.15 ml doses of infected undiluted tissue culture fluid were infiltrated into the anterior chamber of the eye of ICH susceptible puppies, a most sensitive route for ICH, or (b) 2 ml doses of undiluted fluid were inoculated intraperitoneally. Virus titers of different passage levels were of approximately the same order, varying between $10^{-5.0}$ and $10^{-6.0}$/0.1 ml. Temperature readings, leucocyte counts and blood sedimentation rate determinations were made daily on all inoculated puppies. Deaths from ICH were confirmed by presence in liver smears of intranuclear inclusions.

Results of these inoculations are summarized in Table I. To the 13th passage level puppies invariably developed within the expected incubation period, an acute form of the disease, with complete opacification of the inoculated eye. All animals but one (9th passage) died 3 to 5 days after inoculation. Beginning with 20th passage, incubation was more prolonged, death was unusually delayed, and intranuclear inclusions were found in only one puppy. By inoculation with the 30th passage only mild symptoms were produced within the usual incubation period, and both animals recovered and developed ICH antibodies.

A definite modification of the virus became noticeable with the 50th and 52nd passages, where no disease symptoms were recorded in any animal, although every one developed antibodies. However, puppies placed as cage contacts with those receiving the 52nd passage did come down with mild but definite ICH symptoms after 17-19 days of contact, but they recovered and acquired immunity to the disease.

B. Passage of ICH virus in swine-kidney tissue culture. The indication that a single puppy passage of the modified virus might cause substantial return of its virulence led to attempts to cultivate it in tissues of an unnatural host. Accordingly the 24th dog-kidney passage of the virus was successfully and serially propagated in embryonic swine-kidney tissue cultures. The 10th swine-kidney passage was subcutaneously inoculated into 3 susceptible litter-mate puppies, each receiving 1 ml of undiluted tissue culture fluid containing approximately 10^6 TCD_{50} of virus. Each puppy was housed in a separate cage, along with an uninoculated litter-mate to serve as

TABLE II. Response of Puppies to Falling Concentrations of Monovalent 14th Passage ICH Virus Grown in Embryonic Swine Kidney.

		Post-inoculation findings					Results of challenge†	
		Clinical						
			Corneal infiltration					
Puppy No.	No. of TCD_{50}*	Fever leukopenia	Dev't	Appearance (days)	Duration (days)	C.F. & SN antibodies at challenge	Clinical	Outcome
424	6.8	No	Yes	9	5	Yes	Normal	
425	Contact	"	No	—	—	No	Typical ICH	Died 6 days; inclusions +
426	5.8	"	"	—	—	Yes	Normal	
427	Contact	"	"	—	—	No	Typical ICH	Died 4 days; inclusions +
428	4.8	"	"	—	—	Yes	Normal	
429	3.8	"	"	—	—	"	"	
430	2.8	"	"	—	—	"	"	
420	1.8	"	"	—	—	"	"	
422	.8	"	"	—	—	"	"	
431 & 432		Challenge controls					Typical ICH	Recovered

* Expressed as log of power 10. Respective dilutions were inoculated in 2 ml volumes subcut.
† Virulent virus was administered subcut. 28 days after 1st inoculation.

contact control. A 5-week follow-up of the 6 animals revealed no disease symptoms whatever, except for a slight corneal cloudiness in both eyes of one inoculated puppy which appeared on the 9th day and cleared completely 5 days later. In this experiment challenge was not attempted, but the 3 inoculated puppies developed neutralizing antibodies in 1 to 2 weeks and CF antibodies in the 2nd to 3rd week. In contrast to experience with 52nd dog-kidney passage virus, cage contacts in this experiment remained normal throughout the 5 weeks of observation, and the only evidence of spread of swine-kidney virus was appearance of neutralizing antibodies in the 3 contacts 4 to 5 weeks after inoculation of their cage mates.

C. Antigenic extinction of swine-kidney-grown ICH virus. In an experiment to determine the puppy titer of swine-kidney virus, and to rule out any possibility of a "zoning effect" regarding its virulence, the 14th passage was used to inoculate a litter of 9 puppies. Each animal was inoculated subcutaneously with 2 ml of either the undiluted tissue culture fluid containing $10^{6.5}$ TCD_{50} of virus per ml or a serial 10-fold dilution thereof, up to 10^{-6}. The actual number of virus TCD_{50} injected ranged from 6 to 6,000,000. Animals were housed in separate cages, and with each of those inoculated with undiluted fluid or the 10^{-1} dilution, an uninoculated litter-mate was placed as contact control. After 28 days of observation the 9 inoculated puppies and 2 susceptible challenge controls were challenged subcutaneously with virulent ICH virus. All animals remained well until challenge, with the only deviation a slight opacification of one cornea in the puppy inoculated with 6,000,000 TCD_{50} (Table II). This corneal reaction started on the 9th day and by the 14th day had completely disappeared. Following challenge, all inoculated animals remained normal, while cage contacts and challenge controls developed typical ICH infection, the former dying in 4 to 6 days, the latter recovering.

D. Response of puppies in intra-ocular injection of swine-kidney-grown ICH virus. Further evidence of modification of the virus by its passage in swine-kidney cultures was obtained from the following experiment. Four susceptible puppies were inoculated by injection into the anterior eye chamber with 0.15 ml of 15th swine-kidney-tissue passage virus containing $10^{5.9}$ TCD_{50}. This route, known to be the most sensitive for ICH, terminates fatally in most cases of street ICH virus. Progress of infection in the 4 animals was fol-

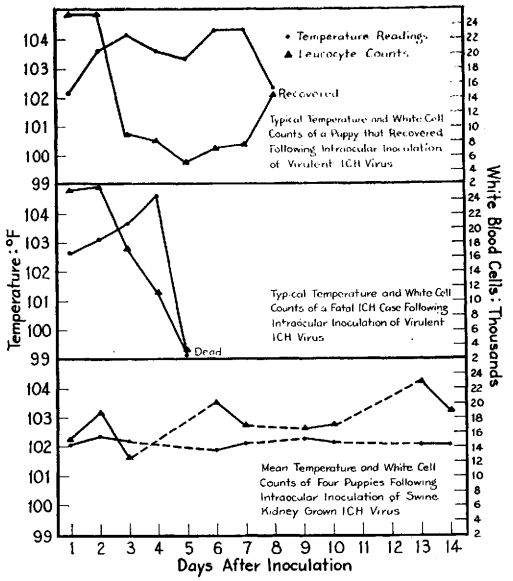

FIG. 1. Comparative response of puppies to intraocular inoculation of virulent and of swine-kidney-grown ICH virus as measured by temperature readings and white blood cell counts.

lowed by temperature readings and leucocyte counts. Beginning 3 to 4 days after inoculation, in all 4 puppies the injected eye became cloudy, and complete unilateral opacification subsequently developed and persisted throughout observation period. However, both temperatures and leucocyte counts remained within normal limits throughout the 20-day observation period, appetites of all animals remained normal, and they appeared to be in perfectly good health. For purposes of comparison, Fig. 1 shows mean temperature values and white cell counts of the 4 puppies for the first 2 weeks after inoculation contrasted with temperatures and cell counts in typical ICH cases, one fatal and one followed by recovery, elicited by intra-ocular administration of virulent ICH virus.

II. Evaluation of a combination of modified ICH and CD viruses. A combined vaccine was prepared, as described under Methods. After lyophilization it was found to contain 200 CED_{50} of CD and $10^{5.7}$ ICH doses/ml.

A. Response to CD component of bivalent vaccine of puppies immune to ICH. In one experiment each of 4 puppies that had survived the ICH test described in Table II was injected subcutaneously with 2 ml of the combined vaccine. Three other puppies that had survived the same test were left uninoculated to serve as cage contact controls. All animals remained normal in every respect for 48 days, when they were challenged intravenously with the Snyder Hill strain of distemper virus. The 4 vaccinated animals remained perfectly normal for one month, when they were put off test. In contrast, 2 of the 3 cage contacts developed typical disease symptoms, and died within 15 and 18 days of challenge, while the 3rd contact remained normal and survived.

B. Response of susceptible puppies to both components of bivalent vaccine, and urinary excretion of ICH virus. In another experiment, of a litter of 6 puppies susceptible to both CD and ICH, 3 were injected subcutaneously with 2 ml of bivalent vaccine and the other 3 were used as cage contact controls (Table III). In addition to usual daily determinations (temperature, leucocyte counts, blood sedimentation rate), every other day for 40 days urine specimens were collected from all 6 animals. For this a rather stressing method was employed: the animal was prevented from breathing by blocking its nostrils and mouth until it urinated (a period of several seconds up to 1 minute), when urine specimen was received in a sterile vessel. All animals remained normal during post-vaccination period, despite the stress of urine collection and daily heart punctures. Small quantities of virus were recovered from undiluted urine of vaccinated animals 18 or more days following injection. Virus recovery persisted in 2 animals for 1 to 3 days, and in the 3rd intermittently for 10 days. A questionable virus detection was made in 1 of the contact animals for a single day (25th after injection of its cage mate), while the other 2 remained negative. Forty-six days after vaccination, the 3 vaccinated animals, their 3 cage contacts, and 2 normal controls were challenged intraperitoneally with virulent ICH virus. The 3 vaccinated animals remained normal. One of the cage contacts developed a typical case of ICH and recovered, and the other 2 showed no disease symptoms. However, both challenge controls developed ICH and died.

TABLE III. Response of Puppies to Combined CD-ICH Vaccine. (Each 2 ml dose contained 400 CED_{50} of CD virus and $10^{6.0}$ TCD_{50} of ICH virus; ICH passage level was 15th in swine-kidney culture.)

Puppy No.	Status	Post-vaccination reactions	ICH virus in urine: Time of appearance (days)	ICH virus in urine: Duration (days)	Results of challenge*: ICH virus	Results of challenge*: CD virus
436	Vaccinated	None	18	2–3	Remained normal	Remained normal
440	Cage contact	"	Questionable 25	1	*Idem*	Sickened—died 26 days
437	Vaccinated	"	18	Intermittent 10	"	Remained normal
438	Cage contact	"	Negative		Typical ICH—recovered	Sickened—died 12 days
439	Vaccinated	"	27	1	Remained normal	Remained normal
441	Cage contact	"	Negative		*Idem*	Sickened—sacrificed 29 days
452	ICH challenge controls				Typical ICH—died 4 days	
453					Typical ICH—died 5 days	
468	CD challenge controls					Sickened—died 19 days
469						Sickened—recovered
470						Sickened—died 29 days
471						Remained normal

* ICH challenge took place 46 days after vaccination and was followed by CD 3 wk later.

Three weeks after ICH challenge the same 6 animals, with 4 normal controls, were challenged intravenously with virulent Snyder Hill distemper virus. The vaccinated animals again remained normal, but all 3 cage contacts developed the disease, 2 dying within 12 or 26 days and the 3rd being sacrificed in extremity on the 29th day. Of the 4 challenge controls, 3 sickened, 1 recovering and the other 2 dying within 19 or 29 days.

Discussion. Our experiments clearly show that virulence of ICH virus was profoundly modified in the course of its serial propagation in dog-kidney cultures and its subsequent continuous cultivation in embryonic swine tissue. When injected peripherally, the virus in its modified form seemed incapable of producing signs of disease in susceptible animals and caused no leucopenia. Although esthetically somewhat objectionable, the occasional cases of corneal opacification were mild and transient, and did not affect the general disposition of the animal. Even intra-ocular inoculation, despite prolonged involvement of the cornea, produced no systemic signs of disease. Following injection of the modified virus, immunity was elicited promptly and afforded solid protection against the virulent virus. It is interesting to note that 6 TCD_{50} of virus elicited as solid a resistance to challenge as 6,000,000 TCD_{50}.

The fact that, for a short time, the virus might be present in urine of vaccinated animals and could spread to intimate contacts does not seem to be of great concern, since no signs of increased virulence were noted even under conditions of severe stress. Spread of the immunizing agent could be detected only through serological tests or challenge, in marked contrast to the experience with the 52nd dog-kidney-passage virus (Table I).

The mixture of modified CD and ICH viruses seems to have no adverse effect on either agent, and in doubly susceptible animals it engenders good protection against both diseases. The bivalent vaccine was not prevented in the case of puppies already immune to ICH from producing an effective immunity to distemper.

Summary. The virulence of ICH virus has

been effectively modified by serial propagation in dog-kidney cultures followed by adaptation to and serial cultivation in swine-embryo tissue. The modified virus is an effective immunizing agent, and produces in the vaccinated animal no adverse reaction except for an occasional case of light and transient opacification of the eye following massive peripheral injection of the virus. The modified ICH virus may spread through the urine of vaccinated animals to intimate contact animals, but although these develop antibodies they show no signs of disease. A combined vaccine prepared by mixing modified CD and ICH viruses elicited good protection against both diseases in doubly susceptible animals, and in puppies already immune to ICH did not interfere with the production of immunity to CD.

1. Cabasso, V. J., Stebbins, M. R., Norton, T. W., Cox, H. R., PROC. SOC. EXP. BIOL. & MED., 1954, v85, 239.

2. Fieldsteel, A. H., Emery, J. B., *ibid.,* 1954, v86, 819.

3. Sarkar, S., Thesis, Cornell Univ., 1957, 17 pp.

4. Fieldsteel, A. H., Yoshihara, G. M., PROC. SOC. EXP. BIOL. & MED., 1957, v95, 683.

5. Emery, J. B., York, C. J., *Science,* 1958, v127, 148.

6. Burgher, J. A., Baker, J. A., Sarkar, S., Marshall, V., Gillespie, J. H., *Cornell Vet.,* 1958, v48, 214.

7. Cabasso, V. J., Cox, H. R., *ibid.,* 1952, v42, 96.

8. Cabasso, V. J., Stebbins, M. R., Cox, H. R., *ibid.,* 1954, v44, 153.

9. Coffin, D. L., Coons, A. H., Cabasso, V. J., *J. Exp. Med.,* 1953, v98, 13.

10. Dulbecco, R., Vogt, M., *ibid.,* 1954, v99, 167.

11. Earle, W. R., *J. Nat. Cancer Inst.,* 1943, v4, 165.

12. Morgan, J. F., Morton, H. J., Parker, R. C., PROC. SOC. EXP. BIOL. & MED., 1950, v73, 1.

13. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

14. Gorham, J. R., *Am. J. Vet. Res.,* 1957, v18, 691.

15. Cabasso, V. J., Johnson, D. W., Stebbins, M. R., Cox, H. R., *ibid.,* 1957, v18, 414.

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Synthesis of Cholesterol by a Strain of Human Uterine Fibroblasts Propagated *in vitro*. (24243)

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It is generally accepted that fibroblasts constitute a high proportion of cells in loose connective tissue(1). The metabolic capabilities of fibroblasts appear to be related in some fashion to their high degree of resistance to the action of antiphlogistic steroids(2). These observations might be related to the fact that fibroblasts appear to play an important role in extrahepatic metabolism of steroids(3,4). It has also been demonstrated that strains of U12 fibroblasts isolated from human myometrium and propagated *in vitro* for many years have the capacity to metabolize cortisol and progesterone to a variety of steroid products(5). These observations raised the question as to whether U12 cells might also be able to synthesize cholesterol and possibly other steroids from low molecular weight precursors such as acetate. The synthesis of cholesterol could not be evaluated on the basis of nutrition since the least fastidious strain of U12 (6) will not proliferate in media devoid of dialyzed horse serum. The data to be presented indicate that strain U12-79 is capable of synthesizing cholesterol as judged by appearance of isotope in this compound when the cells are grown in a medium containing C^{14} acetate.

Experimental. Stock cultures of U12-79 were propagated in a chemically defined solu-

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