Propagation and Modification of Infectious Bovine Rhinotracheitis (IBR) Virus in Porcine Kidney Tissue Culture. (23844)

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The propagation of infectious bovine rhinotracheitis (IBR) virus(1,2) and its modification in bovine kidney tissue culture(3), as well as the adaptation of this virus to HeLa cells(4) have been previously reported. However, McKercher *et al.*(5) failed in attempts to isolate a cytopathogenic agent in porcine kidney tissue culture from nasal washings of cattle containing IBR virus. This report shows that IBR virus can be isolated and propagated serially in porcine kidney tissue culture (PKTC) with production of a typical cytopathogenic effect (CPE). Furthermore, such passages can result in a virus which does not produce any disease when inoculated intramuscularly or intranasally into susceptible cattle.

Material and methods. Virus. A 10% suspension of turbinate, larynx and trachea from 2 cows in acute phase of IBR infection was used to demonstrate primary isolation of IBR virus in PKTC. From part of the same respiratory tissues, IBR virus had been previously isolated in bovine kidney tissue culture (BKTC) and designated as Colorado I(2). The 7th passage of this virus in BKTC was also employed to initiate further passages in PKTC. *Tissue culture procedure.* The porcine kidney tissue cultures were prepared by trypsinizing kidney cortex of 1 to 8-week-old pigs, and suspending the cells in nutrient medium consisting of 0.5% lactalbumin hydrolysate and 5% horse serum in Earle's basic salt solution, to which 200 units of penicillin and 200 μg of streptomycin ml was added. This mixture was dispensed in 0.5 ml amounts into culture tubes, and allowed to stand in a stationary position until good cellular growth was observed, following which the tubes were placed in roller drums. In the course of this work, it was noted that more extensive and rapid growth occurred when calf serum was used in the medium. Therefore, 10% calf serum which did not contain IBR antibodies was substituted for the horse serum in the growth medium but before use, the growth medium was replaced with the 5% horse serum medium. The propagation and maintenance of embryonic bovine kidney tissue culture cells has been described(2). *Neutralization tests.* Tests for IBR antibodies in the sera of cattle were performed in calf kidney tissue culture exclusively, as described previously(2,3), with IBR virus propagated in BKTC. *Cattle.* Cattle 4-8 months old, weighing 350-500 lb, regardless of sex or breed, were used for virulence and antigenicity studies. The susceptibility of these cattle to IBR was determined by absence of neutralizing antibodies in the serum. In addition, they were held in isolation for at least 2 weeks prior to inoculation, and daily temperature records as well as other observations were made to insure that the animals were normal before use. After inoculation, in addition to the use of serum-neutralization tests to demonstrate antibodies, some of the animals were challenged intranasally with 1-2 ml of virulent (low passage BKTC fluid) virus as described(3). No response was considered definite unless fever and one or more signs of illness such as increased respiration, anorexia, nasal discharge, hyperemia of the nasal mucosa, serofibrinous exudate covering the nasal mucosa, were observed.

Results. Propagation of virus. A 10% suspension of upper respiratory tissues from acutely ill cattle was centrifuged at 1,000 rpm for 10 minutes and 0.2 ml of the supernatant inoculated into each of several PKTC tubes. Two-three days after inoculation, a cytological change was observed, consisting of granulation and rounding up of the cells. This effect progressed until all cells became involved and finally fell off the wall of the tube. When 50-100% of the cells were affected, tissue culture fluid from the tubes was transferred in 0.2 ml amounts into each of several new tis-

sue culture tubes. A total of 10 serial passages in PKTC was made. The 10th passage material, referred to as 0-10, was titered simultaneously in PKTC and BKTC, using the CPE as an indicator of end point. Ten-fold serial dilutions of the virus were prepared in tissue culture medium without serum, and 0.2 ml amounts inoculated into each of several tissue culture tubes for each dilution. The titer in PKTC was $10^{4.8}$ 50% tissue culture infectious doses ($TCID_{50}$), and $10^{5.3}$ $TCID_{50}$ in BKTC per 0.2 ml. This indicated that the virus had multiplied in the PKTC, since the end point of titration for the 10th passage would represent a dilution of at least $10^{-14.8}$ of the original tissue suspension.

IBR virus was also propagated in PKTC using as seed material virus that had been previously passed 7 times in BKTC. The tubes were inoculated with 0.2 ml of the infected tissue culture fluids, and all subsequent passages were made by transfers of the same amounts of infected fluid. Serial passages of the virus were performed in two ways: 1) transferring the tissue culture fluid when 90-100% of the cells showed a CPE; and 2) transfers at 1-2 day intervals or when approximately 25-50% of the cells were involved. In the first series after 60 passages, terminal dilution titrations were performed to select the virus particles that were present in the greatest number at that time. Ten-fold dilutions of the infected tissue culture fluids were prepared and at least 3 tissue culture tubes were inoculated with each dilution. The fluid from the culture inoculated with the highest dilution which produced a CPE was harvested, and the procedure repeated. Three such terminal dilution transfers were made. Several larger batches of virus were then prepared, using this third terminal dilution virus as seed material. This virus is henceforth referred to as P-60. Titrations of 2 batches of this virus material were performed simultaneously in porcine and bovine kidney tissue culture, with titers of $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ in PKTC, and of $10^{4.8}$ and $10^{6.3}$ in BKTC per 0.2 ml. In the series where transfers were made when only a part of the cells were involved, 100 serial passages were performed, followed by 3 terminal dilution steps. Again 2 batches of infected tissue culture fluid prepared from the third terminal dilution virus (RP-100) had titers of $10^{4.8}$ and $10^{5.8}$ $TCID_{50}$ per 0.2 ml in PKTC, and of $10^{5.8}$ and $10^{6.3}$ per 0.2 ml in BKTC. One lot titered only in BKTC had an end point of $10^{7.5}$. The cytological changes occurring with the use of P-60 as well as RP-100 in BKTC could not be differentiated from those caused by BKTC-propagated IBR virus.

Identification of the virus. Identification of O-10, P-60, and RP-100 virus passage material was performed with known IBR immune sera, utilizing neutralization tests in tissue culture. As controls either normal bovine sera or tissue culture media were employed. Equal quantities of undiluted immune sera or control fluids were mixed with concentrations of virus ranging from approximately 10-3200 $TCID_{50}$. These mixtures were incubated at 37°C for 2 hours and each serum-virus mixture inoculated in 0.2 ml amounts into each of 2-4 culture tubes, using either porcine or bovine kidney tissue culture, or both simultaneously. These cultures were observed regularly, with the final reading approximately 5-7 days after the beginning of the test. It was found that IBR immune sera neutralized each of the 3 virus lines as indicated by the absence of any cytological changes in the cultures while normal serum or tissue culture medium did not prevent the CPE.

Tests in cattle. Further evidence that the cytopathogenic agent propagated in PKTC is IBR was obtained by inoculation of 1-2 ml amounts of infected tissue culture fluid intramuscularly and intranasally in cattle, using each of the 3 virus lines (O-10, P-60, and RP-100). All virus lots were titered simultaneously in porcine and bovine kidney tissue culture prior to inoculation. Serum samples were taken from the animals at the time of inoculation and again 3 weeks later. None of the animals had IBR antibodies prior to inoculation. Some of the animals were challenged intranasally with known virulent virus at the time the second serum sample was taken. The results of these tests (Table I) show that none of the virus lines inoculated intramuscularly produced any significant evi-

TABLE I. Results of Intramuscular and Intranasal Inoculation of Cattle with Pig Kidney Tissue Culture Adapted IBR Virus.

Route of inoc.	Virus	$TCID_{50}$ inoc. (logs)	Clinical response: Fever	Clinical response: Other	Antibody response‖	Challenge response
Intramusc.	P-60	5.5	1/1*	0/1†	1: 3	0/1
	"	4.5	0/1	"	1: 7	"
	RP-100	6.5	"	"	1:19	"
	"	5.5	"	"	1: 3	"
	"	6.0	0/2	0/2	1: 7; 1: 9	NT§
	"	5.0	"	"	1:11; 1: 6	"
	"	4.0	"	"	1: 6; 1: 7	"
	"	3.0	"	"	1: 3; 1: 6	"
	O-10	6.3	0/1	0/1	1: 7	0/1
Intranasal	P-60	7.3	0/1	?‡	trace	0/1
	RP-100	7.3	"	0/1	0	1/1
	"	8.5	0/2	0/2	0; 0	NT
	O-10	6.5	2/2	2/2	1:19; 1:23	"

* Fever of 103.8 for one day. † Numerator = No. responding; denominator = No. tested. ‡ A slight hyperemia and nasal discharge noted for two days. § Not tested. ‖ Tested against 50-200 $TCID_{50}$ of virus.

dence of disease. All inoculated animals produced neutralizing antibodies, even when as little as 1,000 $TCID_{50}$ of RP-100 virus was used, and all those challenged proved to be immune. Following intranasal inoculation, O-10 produced a typical IBR and a good antibody titer. On the other hand, P-60 in one test caused only a slight clinical illness and a trace of antibodies, while RP-100 produced no disease or antibody response.

Discussion. As shown in results when either suspensions of IBR-infected respiratory tissue or low passage BKTC virus was inoculated into PKTC, a cytopathogenic agent was isolated. In one instance this agent was serially transferred for at least 100 passages. It may be noted in titrations of virus batches that the dilution end point was, in most instances, lower in PKTC than in BKTC tubes. This difference does not necessarily indicate a lower sensitivity of the virus for porcine tissue, but was probably due to the deterioration of the porcine kidney cultures before the virus reaction in the bovine cultures was complete, thereby preventing an accurate estimate of the end point with the higher dilutions of virus in PKTC. Evidence that this agent is IBR virus and not some other CPE-producing virus is shown by the fact that the effect is prevented by use of known immune sera, and that cattle inoculated intramuscularly developed antibodies which neutralized standard BKTC-propagated IBR virus. As shown in previous work(2,3), it is only by the intranasal route of inoculation, however, that all signs of illness observed in the field could be reproduced in experimental cattle. Thus, whether any change in the virulence of the virus occurred during passage in PKTC could be evaluated only by this route of inoculation. It appears that a definite change had taken place, because low passage virus (O-10) produced characteristic signs of IBR; P-60 only a questionable response; while as much as 320,000,000 $TCID_{50}$ of RP-100 produced no signs of illness. It is also of interest to note that in spite of this large amount of RP-100 virus inoculated, no antibody production could be demonstrated. The comparison between the results in cattle inoculated intranasally with high PKTC passage material (RP-100) and other animals inoculated with low PKTC material (O-10) demonstrates that passage in PKTC results in a virus population which lost much of its infectivity for the natural host. However, this phenomenon appears not to be dependent entirely on the porcine tissue itself, since it could also be observed to a lesser degree previously with BKTC passage virus. The same virus, Colorado I strain, after more than 40 passages in BKTC, did not produce any disease, and only 2 out of 4 animals developed neutralizing antibodies when inoculated intranasally with 200,000 $TCID_{50}$. On the other hand, 320 $TCID_{50}$ of low passage virulent virus did

cause typical signs of IBR and a definite antibody response(3). In spite of this decrease of infectivity, however, the antigenicity of the virus when inoculated intramuscularly, regardless of passage level or tissue culture employed, was not significantly changed as shown by serum neutralization titers of experimental cattle. Thus, the PKTC-propagated virus could be used as a vaccine with the additional advantage that such a product would be free of cattle viruses which might be present in bovine kidney.

Summary. 1. IBR virus was isolated in porcine kidney tissue culture from infected respiratory tissues of cattle, and produced a typical CPE on these cells. The virus was maintained for 10 passages and, when inoculated intranasally into susceptible cattle, produced signs of illness characteristic of IBR. 2. IBR virus passed previously through 7 passages in BKTC could be successfully propagated in PKTC in 2 separate attempts, and was passed 60 times (P-60) and 100 times (RP-100), respectively, in PKTC. 3. Inoculation of P-60 and RP-100 did not cause disease when inoculated either intranasally or intramuscularly into susceptible animals. Intramuscular inoculation of these viruses resulted in a specific antibody response.

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Received January 22, 1958. P.S.E.B.M., 1958, v97.

Conversion of Progesterone-4-C^{14} to Aldosterone by Perfused Calf Adrenals. (23845)

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Previous studies with isolated calf adrenals have demonstrated production of sodium retaining activity following perfusion with an artificial medium, both with(1) and without (2) added progesterone. The zone containing this activity migrated with cortisone in the toluene-propylene glycol(3) paper chromatographic system and with cortisol in the Bush C system(4), thus resembling aldosterone. Because of low yield of this bio-conversion, chemical identification of the aldosterone has not thus far been practicable. With isotopic technics, we have been able to show definitively that perfused calf adrenals can produce aldosterone from progesterone. Tritiated aldosterone was added to aldosterone-4-C^{14} isolated from a perfusate containing progesterone-4-C^{14}, and the mixture shown to have a constant C^{14} to H^3 ratio through 3 chromatographic systems before, and 2 after, acetylation to aldosterone diacetate.

Methods. Seven left calf adrenals were perfused for 2 hours with one liter of artificial perfusion medium(5) containing 101 μc/40 mg progesterone-4-C^{14}.† The progesterone was added to the medium in 5 ml of propylene glycol. The perfusion fluid was extracted 5 times with 500 ml of dichloromethane, and the wash fluid from the apparatus (800 ml) extracted 3 times with 400 ml dichloromethane. These extracts were dried over sodium sulfate, combined, and evaporated *in*

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† Progesterone-4-C^{14} was obtained from Tracerlab, Boston, Mass., (sp. act. of 1.71 mc/mM). The 20.5 mg obtained was diluted with 19.5 mg of non-radioactive progesterone.