Studies With Human Coronaviruses
II. Some Properties of Strains 229E and OC43 (36224)

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The human coronaviruses fall into two major groups: those which were originally isolated in organ cultures of human embryonic trachea (HET) ("obligate organ culture" group) and those which were recovered in human embryonic monolayer tissue cultures. The latter consists of strain 229E (1) and 13 closely related strains (1, 2) and the former of nine strains (3–6) including two (OC38 and OC43) which are known to be identical, and one (LP) which is closely related to strain 229E (7–10). Despite efforts (3–6, 11, 12) to adapt members of the obligate organ culture coronavirus group to tissue culture, only strains B814, (the first described coronavirus of human origin), LP, EVS, OC43 (and OC38) have been successfully grown in conventional tissue culture cells (6, 11, 12).

Although strain OC43 shows a typical coronavirus structure in the electron microscope, the virus has several biological characteristics other than its serological reactions, which distinguish it from the other human coronaviruses.

1. Virus grown in HET culture causes an encephalitis when inoculated intracerebrally in mice (7).

2. Virus grown in HET culture can be adapted to growth in monkey kidney tissue culture cells (12).

3. The mouse-brain and the tissue culture grown virus agglutinate rat and mouse red blood cells without prior chemical activation (13).

4. Although the external cell membrane is not involved in the formation of the coronaviruses (14–16), cells infected with OC43 hemadsorb rat and mouse red blood cells (17).

The purpose of this present study was to investigate some of the physical and biological properties of tissue culture adapted OC43 and compare them with those of 229E.

Materials and Methods. Viruses. Coronavirus 229E was obtained from Dr. D. Hamre of the University of Chicago, Illinois and tissue culture pools used in this study were derived from this strain. OC43 virus adapted to grow in human diploid cell strain (HDCS) WI38 cultures was available in this laboratory (17) and both viruses were grown in HDCS WI38 cultures maintained on a mixture of half Eagle's Minimal Essential Medium in Earle's BSS and half Medium 199, containing 2% fetal calf serum, or 0.2% bovine plasma albumin (Armour fraction V), 100 U/ml penicillin and 100 μg/ml streptomycin.

Virus assay. 229E and OC43 were assayed in HDCS WI38 cells: 229E by CPE and OC43 by hemadsorption. Hemadsorption assays were done as described elsewhere (18).

Influenza A2/Hong Kong/1968 was grown in human embryonic kidney and calf kidney monolayer cultures (one and twelve passages, respectively), and was assayed by plaque formation in calf kidney monolayers in 6 cm petri dishes with an agarose overlay.

Results. Inactivation of OC43 and 229E by heat, acid pH and ultraviolet light.

The initial work on the inactivation of OC43 and 229E was done with virus seeds prepared in the maintenance medium described in Materials and Methods, and containing 2% fetal calf serum. Only after these

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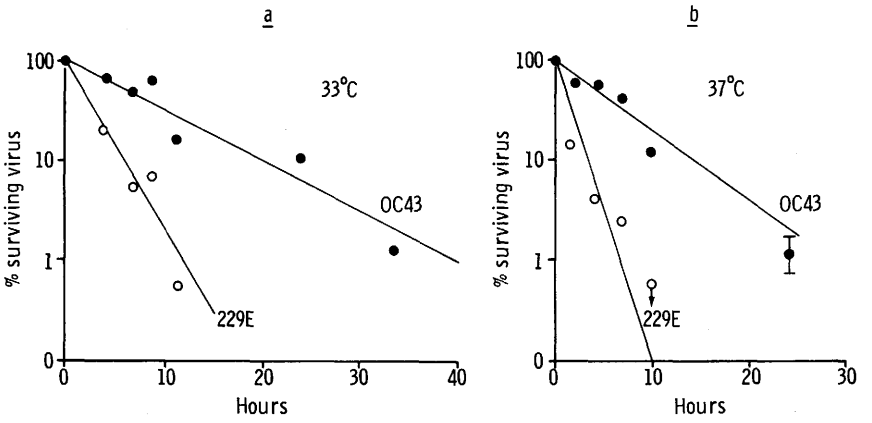

FIG. 1. The thermal inactivation of OC43and 229E in the presence of 2% fetal calf serum: (a) at 33°; (b) at 37°.

initial experiments were done did it become apparent that the presence of serum had a differential effect on the behavior of the two viruses.

Thermal inactivation curves were done for each virus at 33° and 37° in maintenance medium containing 2% fetal calf serum at pH 7.4, and the results are shown in Figs. 1A and 1B. OC43 appeared to be more stable than 229E at both temperatures.

The inactivation of both viruses was measured at 33° and at pH 3.65 in the presence of 2% fetal calf serum and the results are shown in Fig. 2 and Table I. Again, OC43 appeared to be more stable than 229E at low pH.

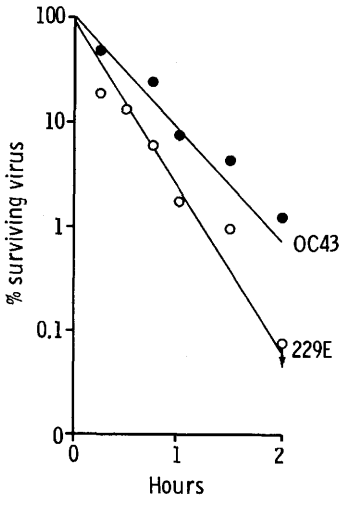

FIG. 2. The inactivation of OC43 and 229E in the presence of 2% calf serum at pH 3.65 and 33°.

In order to estimate the target size of OC43 and 229E, their inactivation by ultraviolet irradiation was studied, and compared with the inactivation of influenza virus. Each virus was diluted 1/10 in maintenance medium containing 2% fetal calf serum, and 4 ml placed in a 6 cm plastic petri dish. The fluids were inactivated 18 in. from a 60 W germicidal ultraviolet tube. The intensity of irradiation reaching the dish was not measured. The results are shown in Fig. 3A. The convex curves obtained for the coronaviruses were in contrast to the conventional "one-hit" curve shown by influenza virus. The coronavirus inactivation curves indicated that the inactivation of an infectious unit was a "multi-hit" process and tended towards a "one-hit" process only after a reduction of approximately $10^{-2.5}$ in the infectivity of each virus. The simplest interpretation of these curves was that the coronaviruses were aggregated into clumps. To test this possibility, suspensions of both OC43 and 229E were sonicated in medium containing 2% fetal calf serum for up to 120 sec in an attempt to disperse any virus clumps. Sonicated and control samples were assayed for infectivity, but sonicated samples showed no increase in infectivity over the controls. Second, both viruses were incubated overnight in medium containing 2% calf serum and 0.1 *M* $MgCl_2$ in the hope that this might enhance the

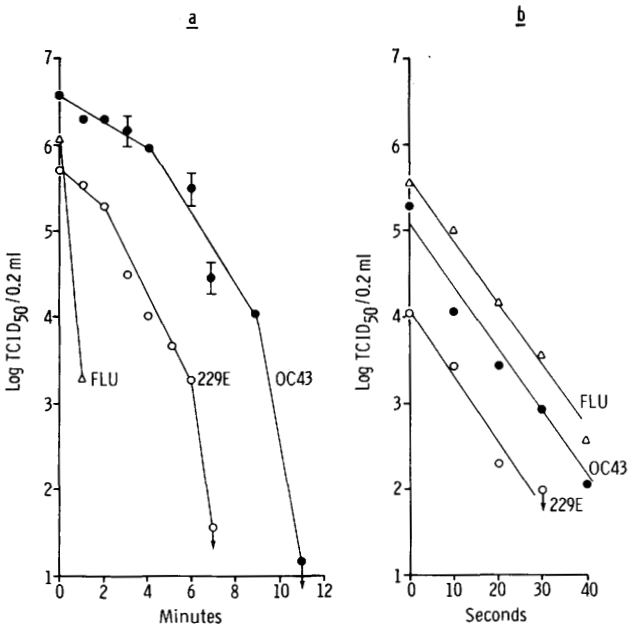

FIG. 3. The inactivation of infectious influenza virus (Δ), OC43 (●), and 229E (o) by ultraviolet irradiation in the presence of (a) 2% fetal calf serum, (b) 0.2% bovine plasma albumin.

clumping effect (19), and so lead to a fall in infectivity, but it did not. However, when a suspension of OC43 in medium with 2% calf serum was centrifuged at 30,000 rpm for 30 min the UV inactivation rate of the virus remaining in the supernatant was almost four times greater than the initial inactivation rate of unspun virus, which suggests that the original seed contained clumps of virus. Suspensions of spun OC43 and 229E were examined in the electron microscope with negative staining, and clumps were often seen (Fig. 4).

It was thought that the clumping of the coronaviruses might be caused by the presence of fetal calf serum in the medium during the preparation of virus seeds (20, 21). Seeds of OC43 and 229E were therefore made which contained 0.2% (w/v) bovine plasma albumin (BPA), rather than serum, and the UV inactivation and thermal stability of these were studied.

Figure 3B shows that the UV inactivation rate for influenza A2, 229E, and OC43 viruses were similar in medium containing BPA in contrast to the results obtained in medium containing fetal calf serum (Fig. 3A). Similarly, the thermal inactivation of both viruses at 37° was similar in BPA medium (Fig. 5) which was in contrast to the earlier results obtained with medium containing calf

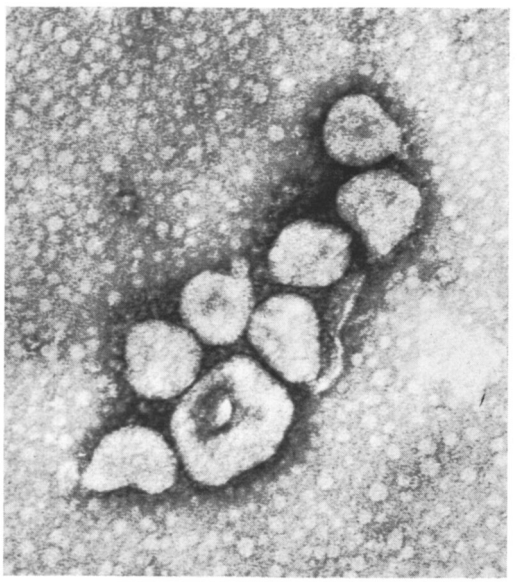

FIG. 4. OC43 virus grown in W138 cells in the presence of 2% fetal calf serum. The virus particles are aggregated.

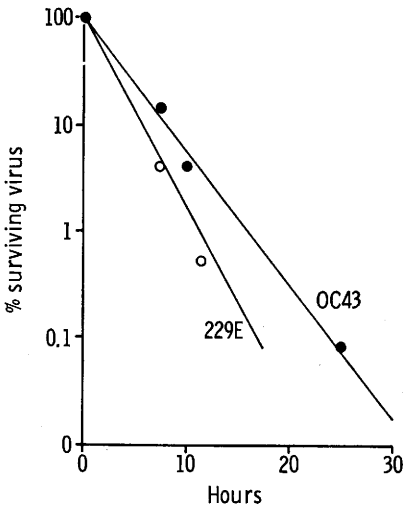

FIG. 5. The thermal inactivation of OC43 and 229E in the presence of 0.2% bovine plasma albumin at 37°.

serum (Fig. 1B). The reduction in both the OC43 thermal inactivation rate, and the OC43 and 229E UV inactivation rates in medium containing serum (Table I) suggested that when OC43 and 229E viruses were grown in HDCS WI38 cultures in the presence of fetal calf serum, they were released into the culture medium in aggregates, but if 0.2% BPA was substituted for serum in the growth medium, they were released as single particles. We attempted to demonstrate the formation of virus clumps after release *in vitro* by mixing virus which had been grown in the presence of 0.2% BPA with 2% calf serum. We examined preparations of 229E virus in the electron microscope but could see only very few virus aggregates. Also treating serum-free viruses with 2% serum did not reduce the infectivity titer.

Growth of OC43 and 229E at various temperatures. Tube cultures of HDCS WI38 cultures on maintenance medium containing 2% fetal calf serum were inoculated with approximately 10^6 $TCID_{50}$ of either OC43 or 229E viruses. After incubation on a roller drum at room temperature (22°) for 2 hr to allow virus absorption, tubes were washed, fresh medium added, and groups of five tubes incubated on a roller drum at temperatures of 32, 33, 34, 35, 36 or 37°. Fluids were harvested daily for 8 days and virus yields assayed in HDCS WI38 cultures (Figs. 6A and 6B). OC43 virus grew well from 32–34° although virus growth was delayed at 32°. At 35°, virus growth was reduced by 90% and at 36° no virus growth was detected. 229E grew best at 32–33° and was progressively inhibited at 34, 35, and 36°. At 37° no virus growth was detectable.

Discussion. The foregoing experiments have shown that in the absence of calf serum OC43 and 229E have similar thermal inactivation rates at 37°, the same UV inactivation rate and, in the presence of serum, their shutoff temperatures differ by only 1°. Thus, although OC43 is unrelated serologically to 229E and exhibits features which are unique among the human coronaviruses, *viz.*, ease of adaption to monkey kidney cells, pathogenicity for mice, hemagglutination, and hemadsorption; nevertheless, it shares with 229E a number of physical and biological features in addition to those used for taxonomic purposes (22).

There are a number of studies which suggest that the optimum growth temperature of a virus is an important factor in determining the site at which the virus grows in an

TABLE I. The Inactivation Rates of Infectious OC43 and 229E in Medium Containing 2% Fetal Calf Serum or 0.2% Bovine Plasma Albumin (BPA).

Inactivating conditions	Units	OC43		229E	
		Calf serum	BPA	Calf serum	BPA
33° pH 7.4	$\log/_{hr}$	0.025	—	0.17	—
37° pH 7.4	$\log/_{hr}$	0.07	0.13	0.30	0.18
33° pH 3.65	$\log/_{hr}$	1.08	—	1.98	—
UV	$\log/_{min}$	0.005 (initial)	0.11	0.005 (initial)	0.08

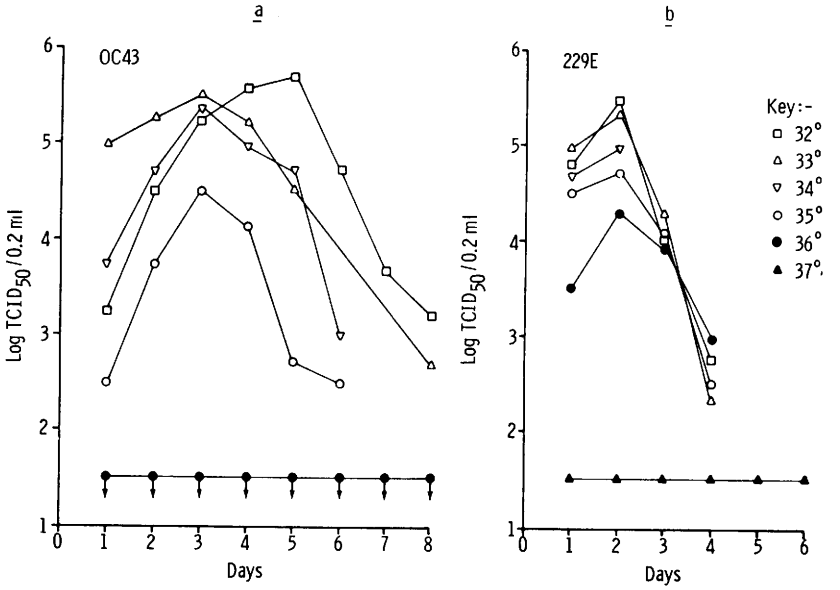

FIG. 6. Growth of OC43 and 229E in W138 cells at temperatures between 32 and 37°.

infected animal or human (23–26) and also the severity of the disease it caused (27–29). The low shutoff temperature of OC43 and 229E viruses may therefore be a determining feature in their characteristic role as pathogens of the upper respiratory tract of man (30). But both viruses used in this study had been passaged many times at 33° in tissue culture, which may well have artificially restricted the range of temperature at which they will grow and until the optimum growth temperature of freshly isolated viruses is determined, then the significance of their low shutoff temperatures must remain theoretical.

The close parallelism of the UV inactivation curves of OC43, 229E and influenza A2 suggests that the target volume of these three viruses is the same. Since the coronaviruses and influenza virus virions are approximately the same size, it is probable that the molecular weight of the coronavirus genome is about the same as that of influenza virus; that is, about 2×10^6 daltons (31, 32). However, this estimate will only be valid if the internal organization of the virion is similar to that of influenza, and as yet, there is not information on this point.

Although we have evidence that OC43 and 229E viruses grown in the presence of calf serum are aggregated into clumps, we were not able to aggregate serum-free viruses by the addition of calf serum. This suggests that the viruses are released from the infected cell in clumps rather than being released singly, and then aggregated by the calf serum in the medium, OC43 (18) and 229E (14, 15, 18, 33) are produced in vesicles in the cytoplasm which are often seen to contain an electron-dense matrix in which viruses are embedded, and perhaps it is this matrix which binds the virus particles together in the presence of serum.

Summary. When the human coronaviruses, OC43 and 229E, are grown in the absence of calf serum, they have similar thermal inactivation rates and similar UV inactivation rates. When they are grown in the presence of serum, the thermal inactivation rate of OC43, and the UV inactivation rate of both OC43 and 229E are reduced, and evidence suggests this is because the viruses are in aggregates. The UV inactivation rate of serum-free OC43 and 229E is very similar to that of influenza A2 suggesting that the genomes are of comparable size. The shutoff

temperatures for OC43 and 229E are 35–36° and 36–37°, respectively.

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