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Growth and CF Antigenicity of Measles Virus in Cells Deriving from Human Heart. (23926)

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Studies on virus susceptibility of cells deriving from adult human heart tissue indicated that measles virus attained comparatively high titers in such cultures(1). Availability of these preparations and the relative paucity of data on antigenicity of inactivated measles virus led us to employ complement-fixation (CF) as a method of antigenic investigation. The contributions of Enders and his group have included data on ability of tissue culture grown measles virus to serve as diagnostic CF antigen(2). Ruckle and Rogers(3) extended this approach to more fully characterize the antibody response. The material which follows amplifies these basic data.

Methods. A. *Tissue Culture*: Source of original tissue was a biopsy specimen from right atrial appendage of human heart removed during commisurotomy. Cultures were prepared by classic chick plasma clot method using minced tissue and basal medium (Eagle) containing 20% human serum. Growth of a fibrocytic type cell was observed after about 72 hours and became progressively extensive. Serial transfers to daughter cultures were performed after trypsinization of the sheet. After 4 months, the predominant cell type had become epithelial-like and has since propagated as such. *Tube cultures*, prepared by seeding about 70,000 cells/tube in 0.5 to 1 ml of above medium, were ready for use at 24 to 48 hours. Washed cultures were then maintained on medium containing 2 to 5% of either horse or chicken serum. 65% Scherer's MS and 30% Medium 199. The balanced salt solution portions of each of the synthetic media were according to the formula of Earle. *Virus*. Edmonston strain of measles virus was obtained from Dr. M. Reissig and had been through 23 human kidney passages, one HeLa cell passage and 4 passages in the Hep-2 cell. Production of initial pools of virus was performed by inoculation of tube cultures of heart cells. When cytopathic effects indicated that almost all cells had been infected (3 to 4 + degeneration), the cultures were frozen and thawed and supernatant fluid of lightly centrifuged preparations (TCF) reinoculated into fresh cultures. *Titrations* were performed by inoculating 2 to 4 culture tubes each with 0.1 ml of given virus dilution prepared in Hanks' BSS, and observing them for cytopathic effects daily for 14 days. Degeneration of fusiform or stellate type at dilution endpoint usually began by 8th day and progressed to completion by 12th to 14th day. *Complement-Fixation.* Crude tissue culture fluid antigens were prepared by inoculating flask cultures containing about 30,000,000 cells with large virus inocula (1 to 10 x 10^6 TCD_{50}). The basal medium in this instance was supplemented with 2% chicken serum.

When complete cell degeneration had occurred, the cultures were frozen at –20°C and following thawing and light centrifugation the supernatant fluid was harvested as crude antigen. Concentrated antigens were prepared by ultracentrifuging crude infected fluids to which was added gelatin (final concentration 0.06%)(4) for 90 minutes at 30,000 rpm in Spinco #30 rotor (78,000G). Upon completion of the run, the supernatant fluids were decanted and the pellets resuspended to 1/10th original volume in Hanks' BSS. The CF method was based on the Kolmer procedure and employed overnight fixation at 4-6°C, and 30 minute incubation at 37°C for the hemolytic system. Tests were performed in tubes employing 0.25 ml each of antigen and serum dilutions and 0.5 ml of complement previously titrated and adjusted to contain 2 full units. Hemolysis was read visually and 2 plus or greater fixation was considered as positive. A control antigen, prepared from non-infected human heart tissue culture, was included in each test. *Preparation of Antisera.* Guinea pigs were inoculated at 3 weekly intervals with 1 ml of infectious measles virus prepared in *monkey kidney tissue cultures.* After 7-10 days, they were bled by cardiac puncture. Such sera, used for control purposes only, did not contain CF antibodies against human heart tissue or chicken serum, both component parts of the CF antigens employed in these studies. Monkey immune serum from a measles convalescent animal was kindly furnished by Dr. J. Melnick.

Results. Optimal harvesting time for infectivity and CF activity. Table I indicates results of 2 experiments to show appearance of infectivity and complement-fixing activity in crude culture fluids. These properties appeared highest at 4 or 5 days after infection, and although further incubation seemed to decrease infectivity, the CF titer remained unchanged when tested against an immune monkey serum. *Effect of inactivation methods on CF antigenicity.* Because a non-infectious antigen would be desirable as a diagnostic reagent for measles, 3 methods of inactivation were investigated for their relative ability to maintain CF activity while rendering the preparation non-infective. (a) *Heat inactivation* was performed by immersing infected fluids in a 56°C water bath and replicate sealed tubes were removed at various times for infectivity and CF tests. (b) *Formalin inactivation.* One part of a 1:400 dilution of formaldehyde, U.S.P. was added dropwise to 9 parts of an infectious measles virus preparation held in ice bath with constant agitation (final concentration 1:4000). The preparation was then incubated at 37°C and sampled for assay at various times. (c) *Ultraviolet light.* Clarified infectious culture fluids were irradiated with the Habel-Sockrider apparatus (5) employing a flow rate of about 7 ml/minute. The irradiation cycles were repeated several times, a sample being removed for assay after each cycle. Tests for inactivation were performed as follows. A 10 ml sample was inoculated into 10 ml of culture medium in 24-36 hour old flask cultures containing about 5 x 10^6 cells. These were incubated at 37°C for one week at which time 10 ml of the fluid was passed into fresh cultures and old cultures refed. Both sets were re-incubated for an additional week and passages repeated 3 times. In all cases where live virus was found, the cytopathic effect was obvious in the initial passage; if doubtful initially, it was noted on first passage into new flasks. It was also of interest to note that if live virus was detected by these rather exhaustive procedures, it was also seen in titration tube cultures receiving undiluted inoculum. Table II presents results of 2 experiments employing inactivation methods described above. Infectivity had en-

TABLE I. Relation of Harvesting Time to Yield of Measles Virus.

Day of harvest	Cytopathic level*	T.C. infectious titer/ml	CF titer
3	1	4.8	<1:2
4	2-3	6.8	"
5	3–4	"	1:2
6	4	"	"
7		"	"
3	1–2	5.8	1:2
4	3–4	7.8	1:4
5	4	6.8	"
6		"	"
7		5.8	"
10		"	"

* 1 = 25% degeneration; 4 = complete degeneration of culture.

TABLE II. Inactivation of Measles Virus by Heat, Formaldehyde or Ultraviolet Light.

Procedure	Exp.	Time	Infectious titer	CF titer
		Min.		
Heating at 56°C	1	0	7.8	1:4
		30	3.8	<1:4
		75	<Und.	ND
		120	"	"
		150	"	<1:4
	2	0	5.8	1:4
		30	1.8	ND
		45	.8	1:2
		60	<Und.	"
		75	"	"
		Days		
Formaldehyde, 1:4000 at 37°C	1	0	6.8	1:8
		3	2.8	"
		5	<Und.	"
		7,9,11,13	"	"
	2	0	4.8	1:4
		3	.8	"
		4	<Und.	"
		5,6,7	"	"
		Exposure cycle		
Ultraviolet irradiation	1	0	6.8	1:8/ 1:8*
		1	1.8	" / 1:4
		2	<Und.	1:4/<1:2
		3	"	" "
	2	0	6.8	1:4
		1	.8	1:2
		2	<Und.	<1:2

* *vs* monkey hyperimmune serum/*vs* human convalescent serum.
ND = not done; Und. = undiluted.

tirely disappeared after 60 minutes at 56°C, after 4 days with 1:4000 formaldehyde at 37°C, and after 2 cycles of ultraviolet irradiation. The CF titer of the TCF was reduced by heat or ultraviolet irradiation but was not affected by formaldehyde even when held at 37°C for a 13-day period.

Stability of frozen-dried measles CF antigen. Because of its potential usefulness in epidemiologic studies of measles infection, the stability of measles CF antigen following freeze drying was determined. Five ml of infected culture supernatants were shell-frozen and lyophilized for 20-22 hours on an Aminco dryer. No external heating was employed. At completion of the run, the ampules were filled with "super-dry" nitrogen and flame sealed. Such preparations retain some infectivity and exhibit considerable CF stability. A comparison of CF antigen content in wet and dried culture fluids stored under various conditions is shown in Table III. After 14 days in the cold or at room temperature there was no loss of CF antigenicity and even after exposure to 37°C for this interval there was considerable residual CF activity. Although longer intervals have not been investigated, these results suggest that measles virus can be stored in the dried state at low temperatures for considerable periods and retain its usefulness as a CF antigen. In contrast, preliminary evidence has indicated that the original titer ($10^{-6.8}$/ml) of wet material does not decrease in 21 days at either 4°C, –20°C, or –70°C. However, after 18 weeks at –70°C, there was considerable loss of titer (10-4).

Effect of sonic vibration on measles virus. Exposure of measles virus in crude tissue culture fluids to sonic oscillation (10 kc/sec at 4-6°C in a Raytheon oscillator driven at 1.25 amps.) resulted in complete loss of infectivity after 2 hours. This was accompanied

TABLE III. Effect of Freeze-Drying on Infectivity and CF Antigenicity of Measles Virus.

Treatment	Exp.	Storage		TCD_{50}/ml	CF titer *vs* 4 units: Rhesus immune serum	CF titer *vs* 4 units: Human convalescent serum
None—Crude TCF		5°C		6.5	1:16	1:4
Freeze-dried	1	5°C	14 days	—*	"	"
		23–25°C	14 "	—	"	—
		37°C	14 "	—	1:8	1:4
None—Crude TCF		5°C		4.8	1:4	"
Freeze-dried	2	"	7 "	2.0	"	"
None—Crude TCF		5°C		5.8	1:8	"
Freeze-dried	3	"	29 "	—	"	—

* — = not done.

by partial loss of CF activity which became evident after 1 hour. It is of interest that a similar exposure did not diminish infectivity of poliovirus or several types of ECHO viruses.

CF reaction between measles antigen and human and animal sera. a. *Concentration of CF antigen*: In the procedure described, supernatant fluid from measles infected human heart cell cultures will fix complement with hyperimmune monkey sera to maximal dilutions of 1:4-1:8. For early diagnosis of measles, where antibody levels may be low, at least 2 full antigen units are desirable. To achieve this consistently, crude infectious culture fluids have been concentrated 10 times by ultracentrifugation. This results in increased serum titers with hyperimmune monkey or convalescent human sera. Two grid

TABLE IV. CF Reactions of Crude and Concentrated Measles Virus with Immune Human and Monkey Sera.

Serum		Infectious antigen dilution end-point	
Type	Dilution	Crude TCF	Conc., 10×
Human acute pool	1: 16	1:2	1: 2
Human convalescent pool	1: 16	1:4	1: 8
	1: 32	"	"
	1: 64	1:2	1: 4
	1:128	"	"
Rhesus immune	1: 16	1:4	1:32
	1: 32	"	1:16
	1: 64	"	"
	1:128	"	"
	1:256	"	1: 8
	1:512	"	"

titrations illustrating this are found in Table IV. In the first experiment a pool of acute (less than 4 days after appearance of illness) and convalescent (14 days or more) human sera* were tested against crude and 10X concentrated infectious antigens. No fixation occurred with acute phase sera at a dilution of 1:16. The convalescent pool had a titer of 1:128 against concentrated and 1:32 against crude antigens. A 4-fold increase in antigen titer, from 1:4 to 1:16, was also observed. Tests of a potent convalescent serum obtained

*We wish to express our thanks to Dr. Robert Ward for providing these sera.

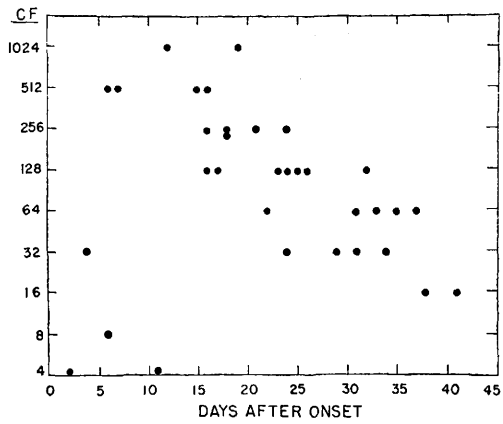

FIG. 1. Distribution of measles CF antibody titers in 33 cases of clinical measles.

from a rhesus monkey following inoculation of Edmonston strain of virus provided additional example of increased CF reactivity which accompanies concentration of antigen.

b. *Development of measles CF antibody in man*: A series of human sera was obtained from patients with clinical measles, from healthy adults with a history of childhood measles, and from a small series of adults who denied having the disease. The CF antibody levels in these sera were determined with concentrated measles antigens. In general our findings paralleled those obtained by Enders *et al.*(2) and Ruckle and Rogers(3) who employed crude tissue culture fluid antigens. Time of appearance of CF antibody in a group of 33 children with clinical measles is shown in Fig. 1 and exemplified in data from serial bleedings on one 6-year-old child (Fig. 2). In practically all of these cases, the interval is based from time of appearance of rash; in 3 instances from onset of fever; in 3 other cases the onset symptoms are not stated. Sera of 15 individuals obtained prior to onset of

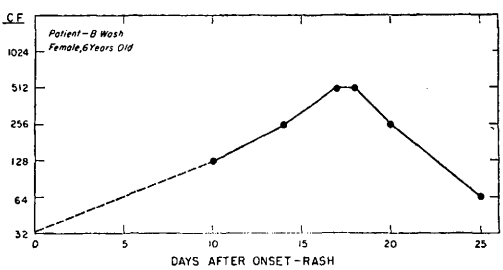

FIG. 2. Development of measles CF antibody following clinical measles.

TABLE V. Persistence of CF Antibody in Adults with History of Childhood Measles, in 14 Sera.

Age, yr	Measles CF antibody With crude TCF*	With conc. virus*
44	1: 4	1: 16
41	1: 8	1: 64
31	1: 4	1: 16
30	1:32	1: 32
30	1: 4	"
28	"	1: 64
28	"	1: 16
27	"	1: 8
27	<1: 4	"
25	1: 8	1: 32
25	1:32	1:256
22	1:16	1:128
?	1: 4	1: 32
?	1: 8	"

* Infectious antigens used.

measles did not react at a dilution of 1:8. Ruckle and Rogers(3) have demonstrated that there is a close correlation between levels of measles neutralizing and CF antibody in man and therefore we have not measured the former antibody in these sera. On the basis of data shown here, it is apparent that appearance of measles CF antibody to readily detectable levels is rapid and prolonged. To establish whether concentrated measles antigens were more sensitive for detection of residual CF antibody, the sera of 14 adults with a history of measles in childhood were collected and titrated against crude and concentrated virus. As will be observed in Table V, low CF levels (1:4-1:32) were obtained using crude TCF; a higher titer was almost invariably noted when 10X concentrated measles antigen was employed. All sera failed to react with a control of non-infected human heart TCF. This demonstration of persistence of measles antibody in adults is more striking when one considers the CF levels in children and adults who have no history of this disease. No serum from 15 children under 6 years of age and 3 adults had a CF titer in excess of 1:8. This suggests that CF antibody determination may provide a simple method for estimating either prevalence of measles in a population group or immunity in an individual.

Discussion. Successful propagation of measles in tissue culture by Enders *et al.* has enabled the application of many conventional technics of virology to the study of this disease. The human heart cell employed in our studies, like the Hep-2 cell as reported by Black, Reissig and Melnick(6), provides a relatively large yield of virus and is capable of propagation in stable serial passage. Benyesh, *et al.*,(7) using ionizing radiation and gradocol membrane filtration have shown that measles virus has a particle size of 100-200 mμ whereas the size of the CF antigen based on irradiation sensitivity is considerably smaller. The resistance of CF antigen to inactivation after infectivity is lost may well be associated with this size differential.

The CF antibody in measles, unlike that in most viral diseases, appears to develop and persist with the same chronology as the neutralizing antibody(3). Since persons without a history of measles do not have CF antibody and since it makes its appearance shortly after onset of illness and rapidly reaches a high titer, the detection of CF antibody provides a simple procedure not only for diagnosis, but also for establishing the immune status of an individual or group. Freeze-dried, non-infectious antigen prepared from crude or concentrated tissue culture fluids may constitute a simple, safe and stable reagent for this purpose.

Summary. The infectivity and CF antigenicity of measles virus grown in a serial passage human heart cell culture have been investigated. Several methods of inactivation are described, as well as the use of crude or concentrated CF antigens for the detection of measles antibody.

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