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Cross Plaque Neutralization Tests with Cloned Crimean Hemorrhagic Fever-Congo (CHF-C) and Hazara Viruses[1] (38154)

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(Introduced by J. Casals)
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The antigenic similarity between the virus causing Crimean hemorrhagic fever (CHF) and Congo virus, which was reported by Casals (1) in 1969, has led to designation of the agent as CHF-C virus. Hazara virus, isolated by Begum *et al.* (2) in West Pakistan, is distantly related to CHF-C virus (3), and the two form the newly established CHF-C antigenic group.

This communication reports techniques pertaining to reproducible plaquing of CHF-C (4, 5) and Hazara viruses in the LLC-MK_2 cell line (6), cloning, and cross plaque neutralization (N tests. Quantitation by plaque assay of the CHF-C virus inhibitory substance, as well as its removal by acetone-ether treatment (7), is also described.

Materials and Methods. Mice. Mice derived from the Charles River CD(R)-1 strain were random bred in a barrier colony maintained at this laboratory. Two-day-old mice were used for preparation of virus stocks, and 35–40-day-old mice for preparation of immune reagents.

Viruses. Two strains of CHF-C virus were employed: prototype strain Ug 3010 (8), 10th mouse passage, and strain IbAr 10200 (9), 9th mouse passage. Hazara virus, strain JC 280, was used at the 10th mouse passage.

Parent virus stocks, consisting of 10% suspensions of infected newborn mouse brain tissue, were made in 7.5% bovine albumin in phosphate-buffered saline, pH 7.2. The suspensions were clarified by centrifugation and then held in 1-ml amounts in sealed glass ampules at −65° in an electrically driven freezer.

Cell cultures. The LLC-MK_2 cell line was propagated and maintained as follows: stock cultures were carried in Roux bottles with a growth medium consisting of Eagle's minimal essential medium prepared with Hanks's or Earle's balanced salt solution and 10% fetal bovine serum, supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). For plaquing, 3-oz flint glass prescription bottles were seeded with 8 or 10 ml of LLC-MK_2 cell suspension prepared by dispersing cells of one 2-week-old stock culture in 100, 150, or 200 ml of growth medium. These cultures were incubated at 36° and used 2–4 days after seeding.

Diluent. The diluent consisted of 0.75% bovine albumin in phosphate-buffered saline, pH 7.2.

Titration, plaque-passaging, and preparation of cloned virus stocks. Increasing 10-fold dilutions of virus were inoculated in 0.2-ml amounts into 2 bottles per dilution. Inocula were adsorbed for 1 hr at 36°, after which 10 ml of a freshly prepared nutrient agar overlay was added. The nutrient portion of the overlay (10, 11) was composed of: Earle's balanced salt solution (without $NaHCO_3$) (10x), 18.0 ml; lactalbumin hydrolysate (10%), 3.0 ml; yeast extract (5%), 1.2 ml; fetal bovine serum (inactivated at 56° for 30 min), 3.6 ml; neutral red (0.1%), 3.0 ml; $NaHCO_3$ (7.5%), 5.4 ml; DEAE dextran (2%), 0.9 ml; penicillin–streptomycin solution (10,000 units penicillin and 10,000 μg streptomycin

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per ml), 1.8 ml; fungizone (100x), 1.8 ml; and sterile, distilled, demineralized water to 90.0 ml.

To complete the agar overlay, the nutrient portion (90.0 ml) was mixed with 90.0 ml of 2% ionagar No. 2S (Wilson Diagnostics, Inc., 3 Science Road, Glenwood, Illinois 60425). The overlaid cultures were incubated in an inverted position at 36° for 11 days and thereafter at room temperature (20°–25°). Plaque counts were carried out at intervals for up to 21 days after inoculation. Virus titers are expressed as plaque-forming units (PFU) per ml. Titration endpoints were read 13–21 days after inoculation.

Cloned viruses were isolated by randomly selecting progenies of single plaques (12) and plaque-passaging them 3–4 times. Cloned virus stocks, prepared and stored as described for parent virus stocks, consisted of 10% suspensions of brain tissue from infected newborn mice of the 1st mouse passage after cloning.

Antisera. Mouse hyperimmune antisera for the parent viruses were kindly supplied by Dr. Jordi Casals. Antisera for cloned viruses were prepared in mice given 5 ip inoculations (13) of cloned stock virus suspended in physiological saline and bled out 1 week after the last inoculation. Sera were pooled by virus and stored at −20°. Two pools of normal mouse serum, one collected at the beginning of the experiments and the other after 5 ip inoculations of normal mouse brain suspended in physiological saline, were processed identically.

Removal of CHF-C virus inhibitory substance. Acetone-ether extraction of serum was done as recently described by Casals and Tignor (7).

Plaque N tests. Untreated as well as acetone-ether-treated normal and hyperimmune mouse sera were thawed rapidly at 37° and inactivated for 30 min at 56° before testing. Serial 2-fold dilutions of serum (or diluent for control purposes), beginning at 1:8, were mixed in equal volumes (0.4 ml) with a virus suspension containing an estimated 100–200 PFU in 0.1 ml. After incubation at either 37° for 1 hr or at 4° overnight, the mixtures were inoculated in 0.2-ml amounts into 2 plaque bottles per mixture. Fifty per cent plaque reduction endpoints were determined by probit analysis (14, 15). Tests were terminated either 13–17 days (CHF-C virus) or 13–21 days (Hazara virus) after inoculation.

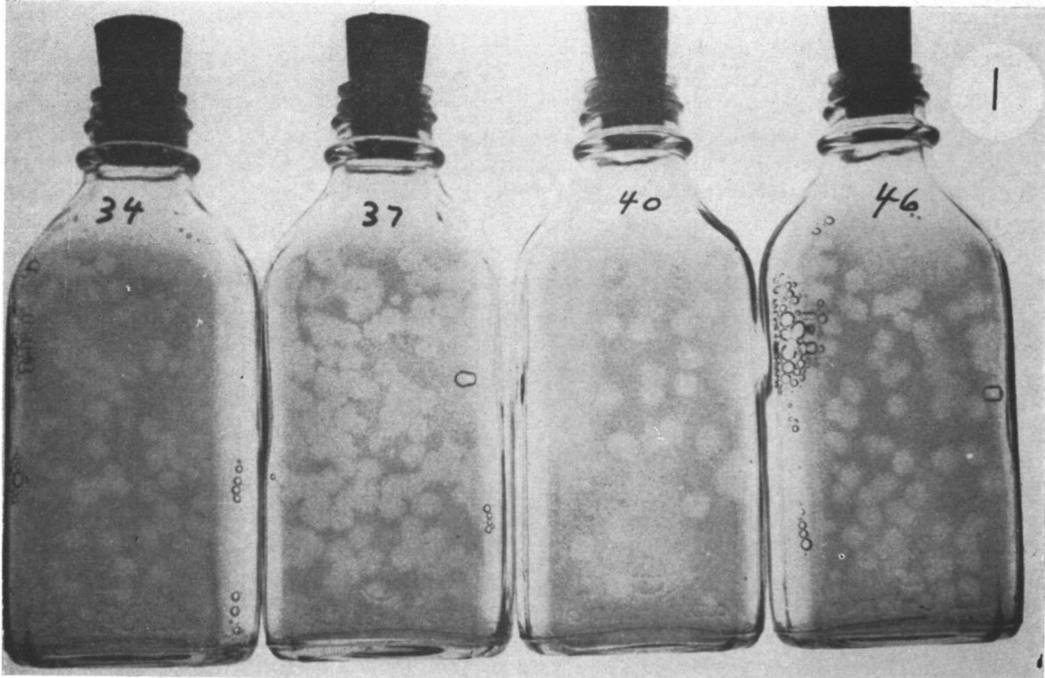
Results. Details pertaining to viral cloning are given in Table I. Plaque-passaging of Hazara virus was readily accomplished at 13–21-day intervals; 20 plaques were examined during 4 plaque passages, with up to 1,000 plaque-producing viral progenies (PPVP) obtained from a single plaque (Fig. 1). The first attempt to clone CHF-C virus (IbAr 10200) failed, but in a second experiment 3 plaque passages were made serially at 8–14-day intervals; 24 plaques were examined, with up to 1,000 PPVP obtained from one plaque (Fig. 2). Plaque-passaging of CHF-C virus (Ug 3010) was not accomplished until the third attempt. Of 81 plaques examined during 3 plaque passages, 27%–53% produced PPVP (Fig. 3); in the first and second plaque passages, only 10–20 PPVP were obtained from one plaque, whereas a maximum of 83 PPVP was formed by one plaque in the third plaque

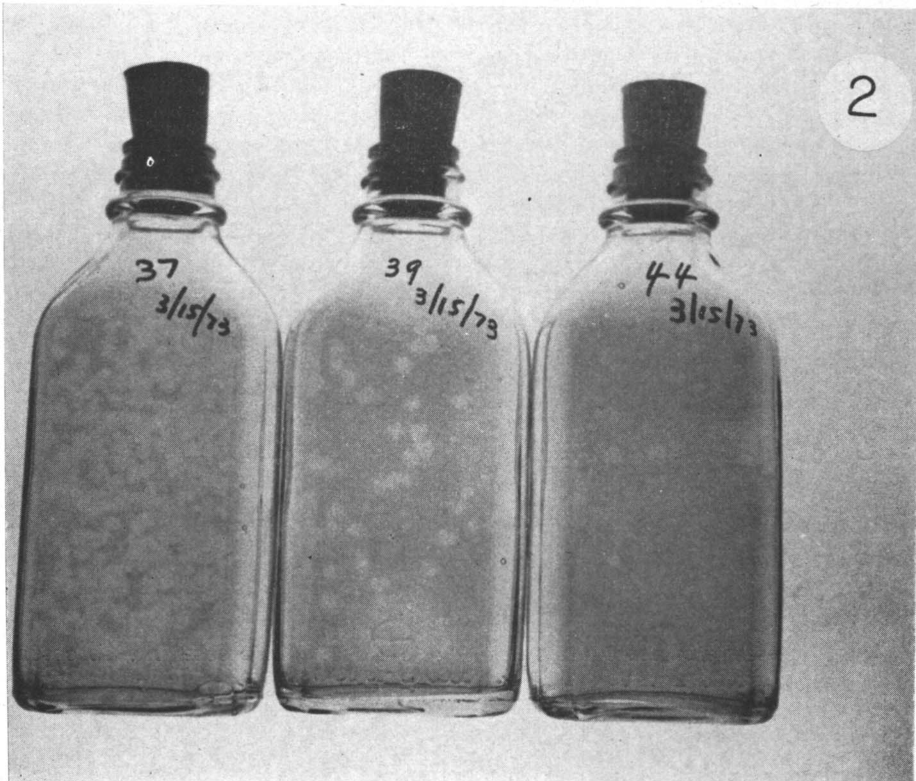
TABLE I. Cloning of CHF-C and Hazara Viruses in LLC-MK_2 Cell Cultures.

Virus	Plaque passage intervals (days)	Maximum no. of PPVP[a] from 1 plaque	Plaque morphology	
			Shape	Diameter (mm)
CHF-C, Ug 3010	11–14	83	Round[b]	1.5–2
IbAr 10200	8–14	1000	Round	2–3
Hazara, JC 280	13–21	1000	Round	4–5

[a] PPVP, plaque-producing viral progenies.

[b] Plaques occasionally triangular or quadrangular.





FIGS. 1–2. Plaques produced in LLC-MK$_2$ cell cultures by (1) cloned Hazara virus and (2) cloned CHF-C virus, strain IbAr 10200.

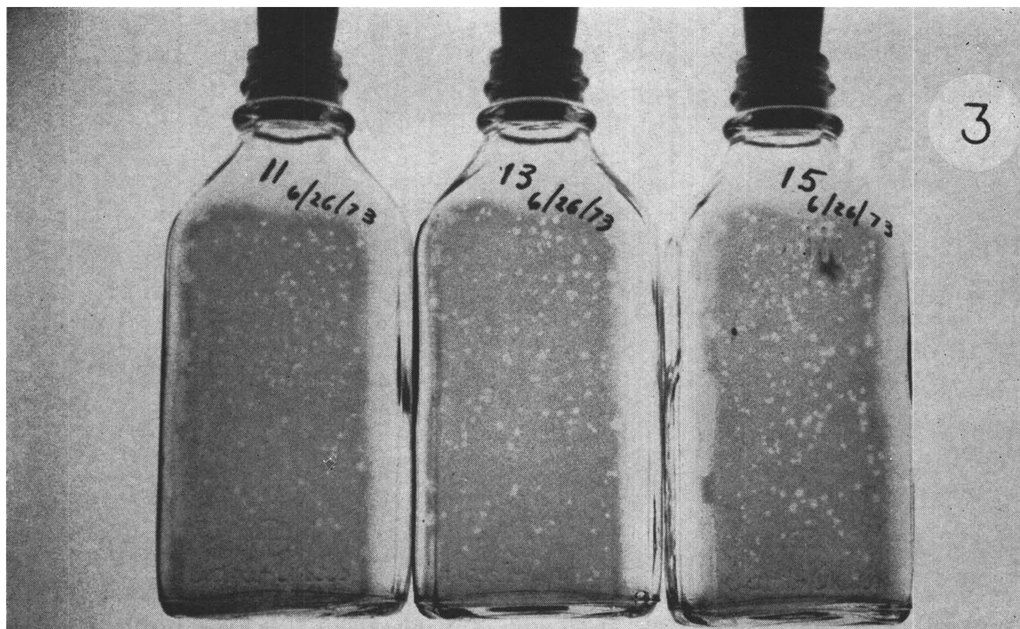


FIG. 3. Plaques produced in LLC-MK_2 cell cultures by cloned CHF-C virus, strain Ug 3010.

passage.

Parent and cloned virus stocks were titrated in duplicate experiments. The reproducibility of titration endpoint determinations is indicated by the data in Table II. There was no striking difference in plaque morphology between parent and cloned strains. Plaques appeared at the end of the first week, after inoculation, but predominantly during the second week and at the beginning of the third. Hazy in appearance at first, they became clear, distinct, and easily countable during incubation of plaque bottles at room temperature. CHF-C virus (Ug 3010) produced the smallest plaques, and Hazara virus the largest (Table I).

TABLE II. Plaque Assays of Parent and Cloned CHF-C and Hazara Virus Stocks in LLC-MK_2 Cell Cultures.

Exp. no.	Stock designation	PFU/ml		
		CHF-C, Ug 3010	CHF-C, IbAr 10200	Hazara
I	Parent	8.0×10^6	2.1×10^7	7.0×10^7
II	Parent	1.4×10^7	1.0×10^7	1.0×10^7
I	Clone	1.0×10^6	9.5×10^6	1.8×10^8
II	Clone	1.0×10^6	6.8×10^6	3.1×10^8

Untreated hyperimmune mouse sera prepared against the parent viruses specifically inhibited plaque formation by cloned viruses (Table III). Quantitation of CHF-C virus inhibitory substance by plaque assay is exemplified by plaque counts obtained with dilutions of 1:8 through 1.64 of normal mouse serum, pool No. 1. Nonspecific inhibition of

TABLE III. Specificity Tests with Cloned CHF-C and Hazara Viruses, Untreated Normal Mouse Serum, and Untreated Hyperimmune Mouse Sera Prepared against the Parent Virus Strains.

Dilution of serum	Plaque counts obtained in series with								
	CHF-C, Ug 3010			CHF-C, IbAr 10200			Hazara		
	N [a]	H [b]	Dil.	N	H	Dil.	N	H	Dil.
1:8	12	0		18	0		67	9	
16	20	1		16	0		91	20	
32	17	0		55	0		82	28	
64	24	1		50	0		95	47	
128	35	1		50	0		85	78	
256	37	0		50	10		93	80	
Diluent			36			50			92

[a] N, normal mouse serum, pool No. 1.

[b] H, hyperimmune mouse serum.

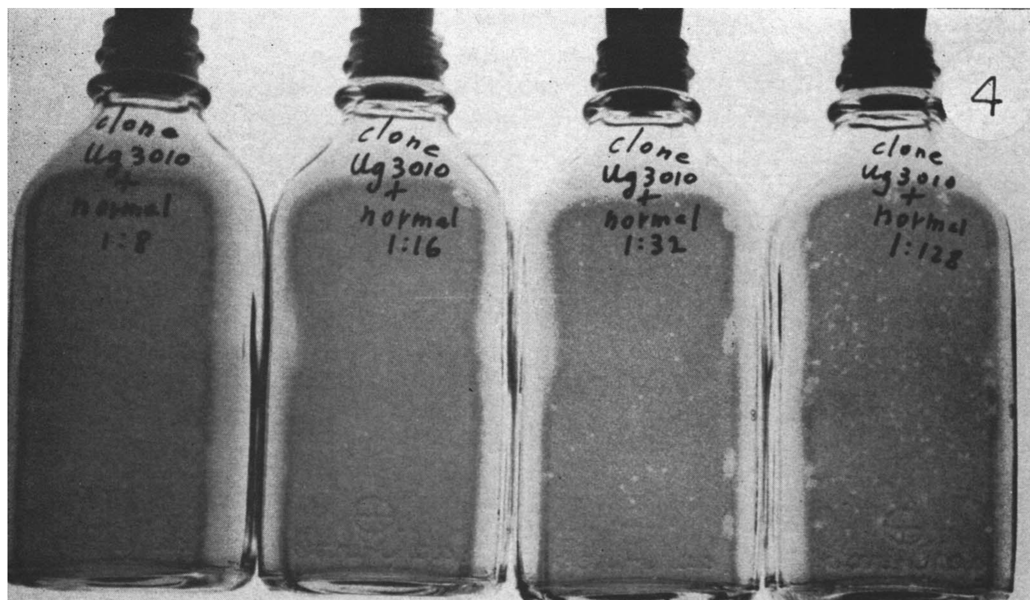


FIG. 4. Effect of CHF-C virus inhibitory substance on cloned CHF-C virus (Ug 3010); normal mouse serum, diluted 1:8 to 1:32, exerted nonspecific plaque reduction of cloned viral progenies.

plaque formation was marked with cloned CHF-C virus (Ug 3010), moderate with cloned CHF-C virus (IbAr 10200), and negligible with Hazara virus. Mixing of plaque-derived viral progenies with normal mouse serum, diluted 1:32, resulted in nonspecific 50% plaque reduction. Figure 4 shows the effect of CHF-C virus inhibitory substance on cloned strain Ug 3010.

TABLE IV. Cross Plaque N Tests with Cloned CHF-C and Hazara Viruses and Their Respective Untreated Hyperimmune Mouse Sera in LLC-MK_2 Cell Cultures.[a]

Virus	Titer of hyperimmune serum			CHF-C virus IS[b] titer, normal mouse serum[c]
	Ug 3010	IbAr 10200	Hazara	
CHF-C, Ug 3010	190	130	140	<130
IbAr 10200	40	260	60	20
Hazara	20	40	640	10

[a] Titers calculated by probit analysis and expressed as reciprocals of serum dilution giving a 50% reduction of plaques.

[b] IS, inhibitory substance.

[c] Normal mouse serum, pool No. 2.

Results of cross plaque N tests with cloned viruses and their respective untreated hyperimmune mouse sera are summarized in Table IV. All antisera inhibited plaque formation by cloned CHF-C virus (Ug 3010). With each of the other cloned viruses, plaque formation was inhibited to high titer by the homologous antiserum and to low titers by the heterologous antisera. Titers of CHF-C virus inhibitory substance in normal mouse serum, pool No. 2, varied from 1:10 to 1:<130.

As shown in Table V, acetone-ether treatment efficiently removed the CHF-C virus inhibitory substance present in mouse serum. Ongoing experiments indicate that specific homologous antibody is not eliminated by acetone-ether extraction. From preliminary results of cross plaque N tests with viral clones and acetone-ether-treated antisera, it is evident that genuine crossing exists between CHF-C virus strains Ug 3010 and IbAr 10200, whereas a CHF-C (Ug 3010) antiserum failed to react with Hazara virus.

Discussion. First class methods are of course always desirable, but are not always delivered on a week-to-week basis throughout the year. The present investigation offers a case in point, for consistently satisfactory results were

TABLE V. Effect of Acetone-Ether (AE) Treatment on the CHF-C Virus Inhibitory Substance Present in Normal Mouse Serum and Hyperimmune Mouse Sera Prepared against Cloned CHF-C and Hazara Viruses.

Virus	Serum (1:8)	Treatment	Plaque counts on days			
			7	8	11	13
CHF-C, Ug 3010	Normal	None	0	0	0	0
		AE	15	18	26	26
	Ug 3010	None	0	0	1	1
		AE	0	0	5	5
	Diluent	None	16	27	32	33
CHF-C, IbAr 10200	Normal	None	2	2	2	2
		AE	16	26	48	49
	IbAr 10200	None	0	0	0	0
		AE	0	0	2	4
	Diluent	None	19	31	45	45
Hazara	Normal	None	0	0	0	0
		AE	0	4	20	25
	Hazara	None	0	0	0	0
		AE	0	0	0	0
	Diluent	None	0	1	17	24

obtained only when densely textured monolayers of the LLC-MK_2 cell line were produced routinely. This was achieved by selecting 2-week-old stock bottles, by reducing the split-ratio from 1:200 to 1:150 or 1:100, and by decreasing the amount of cell suspension per 3-oz flint glass prescription bottle from 10 to 8 ml.

Although repeated cross plaque N tests gave results indicating definite, but not always close, relationships between CHF-C and Hazara viruses, it should be noted that these results were obtained *in vitro* with use of randomly selected, plaque-derived cloned viral progenies and 5-injection mouse sera. Since selection of clones thus involved survival of the fittest PPVP, it is possible that viral progenies of cloned CHF-C virus (Ug 3010), the smallest in plaque size, are less immunogenic than reported in comparison with the larger-plaque progenies of cloned CHF-C (IbAr 10200) and Hazara viruses. Nevertheless, the present results confirm reported observations with complement-fixation and hemagglutination-inhibition tests (7).

For investigators concerned primarily with the study of antigenic differences occurring among strains of the CHF-C group of viruses, the use of wild strains and preferably 1-injection antisera is indicated. Such a project is now feasible, since it has been shown (a) that mouse-brain-grown CHF-C and Hazara viruses regularly produce plaques in LLC-MK_2 cell cultures, and (b) that CHF-C virus inhibitory substance can be removed by acetone-ether treatment of sera, with specific antibody titer not being affected by the method.

A long-term goal of this laboratory is to develop a practical, reproducible, and specific N test for determining the immune status of human and lower animal populations with regard to CHF-C virus (7). The test described here is reproducible and specific under carefully controlled conditions with hyperimmune mouse sera. Its applicability to human and other sera on a larger scale as a routine procedure for surveys will be determined next.

Summary. Plaque-derived clones of Crimean hemorrhagic fever-Congo (CHF-C) and Hazara viruses were randomly selected in LLC-MK_2 cell cultures. Results of cross plaque neutralization tests with the cloned viruses and their respective hyperimmune mouse sera showed a definite and reproducible relationship between the viruses, confirming observations with complement-fixation and hemagglutination-inhibition tests. CHF-C virus inhibitory substance was removed by acetone-ether treatment of sera; specific antibody titer was not affected by the method.

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