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Neutralization and Hemagglutination-Inhibition Tests with Crimean Hemorrhagic Fever-Congo Virus[1] (37933)

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Seroepidemiological surveys with Crimean hemorrhagic fever (CHF) virus have generally been carried out by complement fixation or agar gel diffusion and precipitation tests (1). The seldom used neutralization (N) test has been found difficult to interpret owing to nonspecific antiviral activity of the sera (2).

Studies in this laboratory have shown that CHF and Congo viruses are antigenically alike, hence the designation CHF-C virus, and are related to Hazara virus (3–5); these agents constitute the CHF-C group. Detection of possible antigenic subtypes of CHF-C virus, as well as determination of the immune status of a population, requires an accurate N test. Methods for eliminating a nonspecific antiviral activity of sera and for conducting reproducible N tests in mice are described in this paper. Successful attempts to develop a specific hemagglutinating antigen also are described.

Materials and Methods. Viruses. Four strains of CHF-C virus were used: IbAr 10200, from Nigeria (6); Ug 3010, from Zaire (7); Drozdov, from the USSR (1); and JD 206, from Pakistan (4). Hazara virus was available only as strain JC 280, from Pakistan (5). The strains were put through 3 or 4 terminal-dilution intracerebral (ic) passages in newborn mice; these dilutions represented dilutions 10^{-5} or 10^{-6} of infected brain tissue. After the last terminal-dilution passage, stocks were prepared as 10% suspensions of infected newborn mouse brain tissue in a diluent consisting of 7.5% bovine plasma albumin (bpa) in phosphate-buffered physiological saline, pH 7.2. The suspensions, in 0.5-ml amounts, were stored in sealed glass ampules at −70°.

Mice. All mice were derived from the Charles River CD(R)-1 strain and were random bred in a barrier colony maintained at this laboratory.

Immune mouse sera. Antisera for the CHF-C group of viruses had been prepared 8–9 months prior to the tests, and antisera for arboviruses unrelated to that group had been prepared over a period of 3–4 years prior to the tests. The immunization schedule carried out was as follows, with freshly harvested brain tissue from infected newborn mice resuspended in physiological saline being used at all times. On Day 1, groups of 30 mice, 35–40 days old, were inoculated intraperitoneally (ip) with 0.3 ml of dilution 10^{-2} of infected brain tissue; on Day 22–25, a second injection, consisting of 0.3 ml of virus dilution 10^{-1}, was given ip. The mice were bled by cardiac puncture under ether anesthesia 7 days after the second injection, and the bloods were pooled; 8–10 ml of serum was obtained and stored at −20°. About 80–90% of mice survived this bleeding. On Day 50, the surviving mice were given a third injection of virus, similar to the second, and again bled 7 days later.

Human sera. Fifty-one of the human sera used were from persons not known to have experienced clinical infection with CHF-C virus. Twenty of these donors were lifetime

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or long-time residents of the northeastern USA, of whom 3 had resided for several years in either Brazil or East Africa; 22 were native, lifetime residents of Nigeria; and 9 were Caucasians who had resided in Nigeria for several years. The remaining 3 sera came from a patient convalescent from CHF, and from 2 persons bled 10–12 years after suffering Congo fever in Entebbe, Uganda.

N tests. The human sera used for the N test had been stored at −20° for periods of up to 3 years (occasionally longer) and had undergone thawing several times; they were otherwise untreated. Mouse antisera were used either without treatment or after acetone–ether extraction. The extraction procedure was carried out as follows: To 1 vol of serum (1 or 2 ml) was added 4 vol of distilled water, which was then poured dropwise into a volume of acetone equal to 20 times that of the serum–water mixture. The resulting mixture was shaken by hand for 1–2 min and then centrifuged in the cold at 2,000 rpm for 5 min. The supernatant was decanted, and the sediment, after addition of 40–50 ml of ethyl ether, was shaken by hand and centrifuged as before. The supernatant was then aspirated off, and the wet sediment was spread out over the inside of the centrifuge bottle and dried in a vacuum jar for 30–45 min. The dried residue was then resuspended in a volume of distilled water equal to that of the original serum and centrifuged at 6,000 rpm for 30 min in an angle-head centrifuge. The clear supernatant was the acetone–ether-treated serum.

The N test was done by ic inoculation of 2-day-old mice. In the routine finally adopted, a fresh passage of CHF-C virus was made 4 days before the test was due. On the day of the test, a 10% suspension of the infected brain tissue was centrifuged at 6,000 rpm for 30 min in an angle-head centrifuge; with use of a solution of 0.75% bpa in phosphate-buffered saline, the supernatant was further diluted to 1:50 and, by increasing tenfold dilutions, to 2×10^{-8}. Mixtures of equal volumes of virus dilutions and undiluted serum were incubated at 37° for 1 hr before use. Each test included a control titration in which diluent was substituted for serum. The mixtures were held in an ice-water bath while the inoculation proceeded.

Hemagglutination (HA) and HI tests. HA antigens were prepared from brains of infected 2-day-old mice by the method of Ardoin *et al.* (8). In essence, a sucrose-acetone antigen was subjected, before final centrifugation, to four 2-min periods of sonication, separated by 1-min pauses. A Branson Sonifier[3] was used, operating at 20 kcycles and 40–50 W output. The antigens were lyophilized and stored at −20°.

Sera for the HI test were treated either by kaolin adsorption or acetone extraction, followed by adsorption with goose erythrocytes (9).

The HA and HI tests were carried out basically as described (9), in plastic microtest plates with a total volume of reagents of 0.1 ml (4 drops) in each well. The following modifications were found necessary and were routinely adopted: The concentration of goose erythrocytes was adjusted to an optical density of 0.375, as determined in a Coleman Junior spectrophotometer at a wave length of 490 nm; this is approximately equivalent to a 0.2% suspension of packed cells and is half the concentration used in the standard test. After addition of the cells, and with all possible speed, the plates were sealed with 1-in.-wide strips of Scotch tape, shaken vigorously by hand for 4–5 sec, and placed in a walk-in warm room at 37°. At 45–60 min after addition of the cells, the test was read in the warm room, using a mirror viewer with as little disturbance as possible. With erythrocytes obtained 1 or 2 days before use, the sedimented patterns, if undisturbed, remained stable for several hours; otherwise, they tended to slip, and reading became difficult. Sedimented patterns from Hazara virus were stable and hence easy to read; in the case of CHF-C virus, those from strains IbAr 10200 and Ug 3010 were fairly stable, but those from strains Drozdov and JD 206 showed a rapid tendency to slip.

[3] Distributed by Heat Systems Company, Melville, New York 11749.

TABLE I. Results of N Tests (Newborn Mice, IC) with CHF-C Virus, Strain IbAr 10200, and Untreated Sera: Nonspecific Antiviral Activity of Mouse, but not Human, Sera.

Test No.	Virus passage	Dex LD_{50} titer of virus in diluent	Difference between dex titers of virus in diluent and serum	
			Human sera	Mouse sera
1	6	5.8	0.1, 0.4	0.6, 1.2, 2.2
2	6	6.1	$\leqslant$0.4, $\leqslant$0.1, 0.0, 0.1, 0.1, 0.1, 0.1, 0.2	Not done
3	6	6.1	0.1, 0.4, 0.4, 0.5, 0.6, 0.6, 0.7, 0.7, 0.8	0.7, 1.5, 1.8, 1.9, 2.2, $\geqslant$2.4, $\geqslant$2.5, $\geqslant$2.5
4	7	6.5	0.5, 0.6, 0.6, 0.8, 0.9, 0.9	Not done
5	7	6.7	$\leqslant$0.2, $\leqslant$0.2, $\leqslant$0.2, $\leqslant$0.2, 0.3, 0.3, 0.3, 0.3, 0.5, 0.7	1.2[a], 1.2, 1.7, 2.0, 2.1, 2.2, 2.7, 2.8, 3.2, 3.3
6	7	6.7	0.1	1.6[a], 2.4
7	7	6.0	$\leqslant$0.4, $\leqslant$0.3, $\leqslant$0.2, $\leqslant$0.1, $\leqslant$0.1, 0.0, 0.1, 0.1, 0.1, 0.1, 0.1, 0.1, 0.3, 0.4, 0.4, $\geqslant$3.5[b], 2.1[c], 0.4[c]	Not done

[a] Serum samples from normal, unvaccinated mice. All other mouse sera were prepared with viruses unrelated to CHF-C virus; see text.

[b] Convalescent serum from patient with CHF.

[c] Sera from patients bled 10–12 years after Congo virus infection.

Complement-fixation (CF) tests. These were done as described elsewhere (10).

Results. N test. The results in Table I illustrate the marked nonspecific antiviral activity of untreated mouse sera against CHF-C virus (IbAr 10200) and the absence of such activity from untreated human sera. The activity is expressed in dex ($=\log_{10}$) (11) as the difference in virus titers.

Of the 51 sera from persons not known to have experienced clinical infection with CHF-C virus, 37 depressed the titer of the virus by $\leqslant$0.4 dex, and the other 14 gave a reduction of 0.5–0.9 dex. The 3 convalescent sera reduced the titer by $\geqslant$3.5, 2.1, and 0.4 dex, respectively.

These differences are in marked contrast to those observed with the 23 mouse sera. The latter sera comprised 2 from normal, unvaccinated mice and 21 prepared with the following viruses: Bandia, Banzi, Central European tick-borne (2 sera), Colorado tick fever, dengue type 3 (2 sera), eastern equine encephalitis, Hughes, Japanese encephalitis, Kao Shuan, Kemerovo, Nyamanini, Punta Salinas (2 sera), Quaranfil (3 sera), Royal Farm, Tyuleniy, and Wanowrie. As shown in Table I, the reduction in titer was <0.9 dex with 2 sera, 1.0–1.4 dex with 3 sera, 1.5–1.9 dex with 5 sera, and $\geqslant$2 dex with 13 sera.

The question of whether acetone–ether extraction of mouse sera would remove their nonspecific antiviral activity was investigated by comparing, in single tests, untreated and treated samples of 14 different sera (Table II). The 11 immune sera were prepared with the following viruses not of the CHF-C group: Bandia, Central European tick-borne, Colorado tick fever, dengue types 2 and 3, epizootic hemorrhagic disease of deer, Hooper 1361,[4] Huacho, Inkoo, Japanese encephalitis, and Mayaro. Of the 3 different samples of normal mouse serum, 1 (used in tests 8 and 10) had been stored for 8–10 months, 1 for 1 day, and 1 for 5 days.

All untreated samples of immune sera depressed the titer of CHF-C (IbAr 10200) virus—in 7 cases by >2 dex. The untreated sample of the normal serum stored for 8–10 months had a marked depressing effect,

[4] Hooper 1361 is an unpublished virus isolated in Malaysia by Dr. Albert Rudnick. Mention of it here is not intended to constitute priority.

TABLE II. Results of N Tests with CHF-C Virus, Strain IbAr 10200, and Mouse Immune Sera for Viruses Not of the CHF-C Group, Used after Acetone–Ether (AE) Extraction: Removal of Nonspecific Antiviral Activity.

Test No.	Dex LD_{50} titer of virus in diluent	Treatment of serum	Difference between dex titers of virus in diluent and serum	
			Immune sera	Normal serum
8[a]	5.1	None	2.2	$\geqslant$2.5
		AE	0.4	0.3
9	6.6	None	2.2, 2.5, 2.8	0.1
		AE	0.3, 0.5, 0.2	0.2
10	5.7	None	$\geqslant$3.0, 1.7, 1.4	$\geqslant$3.2
		AE	2.0, 0.4, 1.0	0.7
11	6.1	None	3.4, 2.6, 1.6, 1.5	0.9
		AE	1.2, 0.2, 0.1, 0.2	0.5

[a] This test was done with strain JD 206 of CHF-C virus.

$\geqslant$2.5 dex and $\geqslant$3.2 dex; however, the untreated samples of the 1-day-old and 5-day-old normal sera had either no effect (0.1 dex) or minimal effect (0.9 dex). Acetone–ether treatment eliminated the nonspecific effect of the sera in nearly all instances. Before treatment, the inhibitory action was $<$1 dex in 2 instances, 1–2 dex in 4 instances, and $>$2 dex in 9 instances; after treatment, the inhibitory action was $<$1 dex in 12 instances and 1–2 dex in 3 instances.

Table III shows that acetone–ether treatment did not affect the capacity of CHF-C

TABLE III. Results of N Tests with CHF-C Virus, Strain IbAr 10200, and Mouse Immune Sera for CHF-C Group Viruses Used after Acetone–Ether (AE) Extraction: Persistence of Specific Antibody.

Test No.	Serum	Treatment	Virus titer (dex)	Difference from diluent (dex)
12	Diluent		5.7	—
	Normal mouse	None	$\leqslant$2.5	$\geqslant$3.2
		AE	5.0	0.7
	CHF-C, IbAr 10200	None	$\leqslant$2.5	$\geqslant$3.2
		AE	$\leqslant$2.5	$\geqslant$3.2
13	Diluent		6.4	—
	Normal mouse	None	3.0	3.4
		AE	6.2	0.2
	Dengue type 2	None	3.6	2.8
		AE	6.0	0.4
	CHF-C, IbAr 10200	None	1.9	4.5
		AE	2.0	4.4
	CHF-C, Ug 3010	None	2.0	4.4
		AE	4.4	2.5
	Hazara	None	5.9	0.5
		AE	5.7	0.7

TABLE IV. Capacity of CHF-C Virus Antigens to Agglutinate Goose Erythrocytes As Influenced by Type of Antigen Preparation, Type of Test, and pH.

Virus strain	Type of antigen	Type of test[a]	Reciprocal of antigen titer at pH								
			6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8
IbAr 10200	Standard	Standard	0[b]	0	2	2	4	4	4	8	4
		Modified	2	8	16	16	32	32	32	32	32
	Sonicated	Modified	4	16	32	64	128	128	128	128	128
Ug 3010	Standard	Modified	0	4	16	32	64	64	128	—	64
	Sonicated	Modified	8	64	256	256	256	256	128	—	128

[a] See text.

[b] 0, no agglutination at dilution 1:2, lowest used.

immune mouse sera to neutralize strain IbAr 10200 of the virus. Two different samples of IbAr 10200 serum neutralized the virus equally as well after treatment as before; and a Ug 3010 serum had a neutralizing index of 2.5 dex after treatment. In contrast, the control sera (2 normal and a dengue type 2 immune) lost their strong neutralizing activity after treatment. A Hazara immune serum did not cross-react with CHF-C virus.

HA and HI tests. The influence on CHF-C virus antigens of sonication, pH, and method of performing the test is illustrated in Table IV. An HA antigen for strain IbAr 10200, prepared and tested in the standard manner, i.e., with a 0.4% cell suspension and without vigorous shaking of the plates, would be of dubious value for use in the HI test; the contrast in the titers obtained with the modified and the standard procedures, and the effect of sonication are apparent. The high pH required for optimum titer can also be seen. Unlike most other arboviruses, which, when active, agglutinate cells at pH 6–7, the CHF-C virus strains tested have an optimum range between pH 7.0 and 7.6.

Strains IbAr 10200 and Ug 3010 have consistently yielded antigens similar to those illustrated in Table IV, with titers (in the optimum pH zone) between 1:32 and 1:256; on the other hand, strains Drozdov and JD 206 have given antigens with low titers, between 1:8 and 1:16, and unstable patterns. Antigens for Hazara virus have varied in titer between 1:16 and 1:256, with an optimal pH range of 6.7 to 7.0 and stable patterns.

Table V gives the results of 2 HI tests with different lots of antigens, and illustrates the marked cross-reactions between CHF-C and Hazara viruses. The same samples of sera and antigens showed less crossing by CF test. Normal sera or sera from mice immunized with viruses not related to the present group have consistently failed to inhibit agglutination by CHF-C group viruses at the lowest dilutions used, 1:10 and 1:20.

Discussion. The N test has not been used extensively for serological surveys with CHF-C virus owing to a nonspecific antiviral activity of sera and to the low neutralizing capacity of sera from persons recovered from CHF (2). The nonspecific activity of mouse sera has been amply documented in our work; additional studies in this laboratory have demonstrated similar activity for CHF-C virus in sera from 8 other vertebrate species tested. No systematic effort has been made in this laboratory to determine whether other viruses are similarly affected; should such prove to be the case, acetone–ether treatment of sera will be tested.

It appears possible that, with the methods described, the N and HI tests can now be used for serological work with CHF-C virus. Whether these 2 tests will give the same information as either CF or agar gel diffusion and precipitation can be established only through comparative studies. The present limited results show that when the same

TABLE V. Results of HI and CF Tests with CHF-C Group Viruses.

Test No.	Serum	Antigen, 8 units: CHF-C: IbAr 10200 HI	IbAr 10200 CF	Ug 3010 HI	Ug 3010 CF	Hazara HI	Hazara CF
1	Man, convalescent CHF	80[a]	—	—	—	40	—
	Man, convalescent Congo fever	20	—	—	—	0	—
	Mouse, immunized, CHF-C, JD 206	160	—	—	—	20	—
	Mouse, immunized, Hazara (serum A)	640	—	—	—	1280	—
	Sheep, presumed natural exposure to CHF-C or a related virus	640	—	—	—	80	—
2	Mouse, immunized, CHF-C, IbAr 10200	640	256	640	256	80	16
	Mouse, immunized, CHF-C, Ug 3010	160	256	640	256	20	16
	Mouse, immunized, Hazara (serum B)	160	8	160	8	320	128

[a] Reciprocal of serum titer; 0, no inhibition at serum dilution 1:10, lowest used.

immune sera were used, there was considerable crossing between CHF-C and Hazara viruses by HI, much less by CF, and none by N tests.

Zavodova *et al.* (2) found that prolonged storage of the sera increased the frequency of nonspecific neutralization, and our limited observations with mouse sera point in the same direction. On the other hand, all human sera used in our tests had been stored for long periods, yet they showed no nonspecificity. At any rate, it is unrealistic to assume that serological surveys or antigenic comparisons can only be done using freshly drawn sera; therefore, a simple procedure, such as acetone–ether extraction, for eliminating nonspecific neutralizing activity seems desirable.

Summary. The neutralization test by intracerebral inoculation of newborn mice can be satisfactorily carried out with at least 1 strain of Crimean hemorrhagic fever-Congo (CHF-C) virus; human sera showed no nonspecific antiviral activity and that of mouse sera was removed by acetone–ether extraction. An antigen usable in hemagglutination-inhibition (HI) tests has been developed with at least 2 strains of CHF-C virus. The cross-reactivity between CHF-C and Hazara viruses has been confirmed in the HI test.

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