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Rapid Focus Reduction Neutralization Test of Influenza A and B Viruses in Microtiter System

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A rapid neutralization test for influenza A and B viruses was developed. In this method, a 96-well tissue culture plate was used for the preparation of cell monolayers and the peroxidase-antiperoxidase staining technique was used for the visualization of foci infected with these viruses. In the presence of trypsin and tragacanth gum, clear foci developed 1 day after infection. A linear relationship between virus dilutions and numbers of foci was observed. When neutralizing antibodies in some test sera were assayed, a good correlation was observed between the titers obtained by the focus method and those obtained by the ordinary plaque method. In addition, many serum specimens were investigated by the neutralization test, and it was demonstrated that the test is useful for serological studies of influenza.

Although the hemagglutination inhibition (HI) test is the most commonly used technique for the measurement of antibody to influenza virus, the neutralization test is superior to the HI test in that it is more specific and usually more sensitive than the HI test (6, 7, 22). Moreover, dissociation of the neutralizing effect and antihemagglutination activity in polyclonal sera (10, 21) or monoclonal antibodies (4, 9) have been reported. Despite the importance of the neutralization test in serological studies of influenza, the test has rarely been used because it is too cumbersome, time-consuming, and expensive. Trials of a microneutralization test that uses microplates (3, 20) have been reported. However, this method requires a long incubation period of approximately 1 week after virus infection. Recently, Harmon et al. (7) reported a rapid neutralizing-antibody assay for influenza B viruses based on an enzyme immunoassay. We tried to develop a rapid neutralization test in a microtiter system for influenza A and B viruses. This paper describes the usefulness of the microneutralization test in serological studies of influenza.

MATERIALS AND METHODS

Virus strains. Six strains of influenza A and B viruses were used. Four of them, A/Yamagata/120/86 (H1N1), A/Fukuoka/C29/85 (H3N2), A/Shisen/2/87 (H3N2), and B/Nagasaki/1/87, which are inactivated-vaccine strains currently in use in Japan, were generously provided by the Research Foundation for Microbial Diseases of Osaka University. Two others, A/Okuda/57 (H2N2) and B/Osaka/152/88, have been kept in our laboratory. A/Okuda/57 (H2N2) was passaged more than 200 times in chorioallantoic membranes of embryonated hen eggs. B/Osaka/152/88 was isolated from patients with influenza by use of Madin-Darby canine kidney (MDCK) cells and passaged several times in the eggs. These egg-passaged viruses were propagated one more time and stored at −70°C for this study.

Cell culture. MDCK cells were maintained at 37°C in a 5% CO_2 atmosphere in Eagle minimal essential medium (MEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml).

Human sera. Sixty serum samples were obtained from students of an elementary school in Osaka City. They were heat inactivated at 56°C for 30 min before use.

HI test. The HI test was performed with receptor-destroying enzyme-treated sera by a standard microtiter assay (1). In the test, ether-treated egg-derived virus antigens which had been prepared at the Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University were used.

Focus reduction neutralization test. In principle, the newly developed neutralization test for influenza A and B viruses was based on our previously described methods (12, 15). Dilutions of the stock influenza viruses and the test sera were carried out with MEM containing 0.2% albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.). Suspensions of MDCK cells (about 10^4 cells per well) were distributed to each well of 96-well flat-bottom plates (Falcon 3072; Becton Dickinson Labware, Oxnard, Calif.) and incubated in a CO_2 incubator at 37°C to make monolayer sheets. On the following day, test sera were serially diluted in fourfold steps in 96-well round-bottom plates (Termo, Tokyo, Japan). Then, each serum dilution and control diluent were combined with an equal volume (25 μl) of influenza virus adjusted to give a final control count of about 30 focus-forming units per well. The serum-virus mixtures were incubated for 1 h at 37°C. After incubation, 25 μl of each mixture was transferred to wells of 96-well flat-bottom plates in which MDCK cells had been seeded on the previous day and adsorbed for 30 min at 37°C. The virus inocula were removed and washed with phosphate-buffered saline. The cells were covered with MEM containing 0.5% tragacanth gum (Wako Chemical Industries, Osaka, Japan) and 5 μg of trypsin (Research Foundation for Microbial Diseases of Osaka University) per

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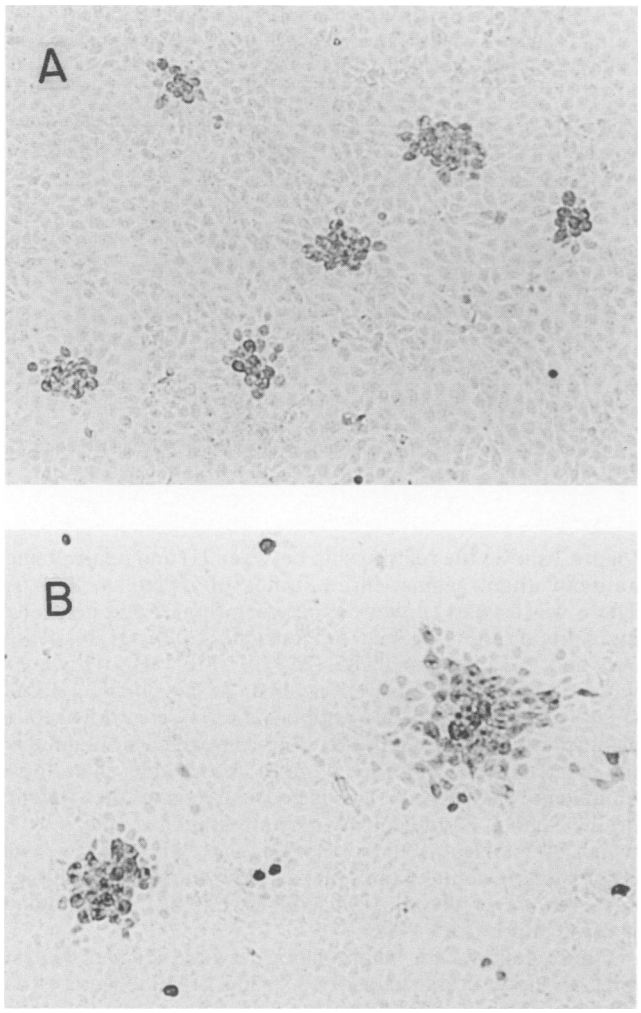

FIG. 1. Foci of influenza viruses stained by the PAP technique. MDCK cells in 96-well microplates were infected with A/Yamagata/120/86 (H1N1) (A) or B/Nagasaki/1/87 (B), and foci were stained by the PAP technique 1 day after infection as described in the text.

ml. Twenty to 24 h after inoculation, the medium was removed, and the cells were washed with phosphate-buffered saline and fixed with absolute ethanol at room temperature for 10 min. Then, the cells were dried with a hair dryer. If the cells were not stained immediately, they were kept at −70°C before they were stained. Focus staining was done by successive treatment of the cells with rabbit anti-influenza A (A/Kumamoto/22/76 [H3N2]) or B (B/Akishima/64) serum (1:1,000), sheep anti-rabbit immunoglobulin G serum (1:500) (Organon Teknika, Malvern, Pa.), and peroxidase-rabbit antiperoxidase (PAP) complex (1:1,000) (Organon Teknika). Each treatment was 40 min long and was followed by a washing with phosphate-buffered saline. The peroxidase reaction was developed for about 5 min by the method of Graham and Karnovsky (5) in which 0.01% H_2O_2 and 0.3 mg of 3, 3'-diaminobenzidine tetrahydrochloride (Wako) per ml in phosphate-buffered saline were used. The cells were then rinsed with tap water and dried. The stained foci were counted under an ordinary light microscope by turning the plates upside down. The neutralizing-antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci to 50% or less of the control value.

Plaque reduction neutralization test. Plaque formation was brought about by the method of Tobita et al. (18), with a few slight modifications. Fourfold serial dilutions of test sera were mixed with an equal volume of influenza virus containing about 100 PFU/0.4 ml. After incubation for 1 h at 37°C, 0.4 ml of the mixtures was inoculated into MDCK cell monolayers in petri dishes (6 cm in diameter). After 30 min of adsorption, the cells were covered with the first overlay medium (MEM supplemented with 1 μl of MEM vitamin solution [100× concentrated; GIBCO Laboratories, Grand Island, N.Y.] per ml, 1 μg of folic acid [Wako] per ml, 1 μg of biotin [Wako] per ml, 0.1% glucose, 100 μg of DEAE-dextran per ml, 10 μg of trypsin per ml, and 0.8% agar [purified agar; Difco Laboratories, Detroit, Mich.]) and incubated in a CO_2 incubator at 35°C for 3 days. The cells were then covered with the second overlay medium (0.006% neutral red in the first overlay medium), and the plaques were counted on the following day. The neutralizing-antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of plaques to 50% or less of the control value.

Microneutralization test that uses hemagglutination for virus detection. The microneutralization test of Frank et al. (3) in which hemagglutination was used to detect influenza A and B viruses was carried out with a few slight modifications.

RESULTS

Optimum conditions for focus counting. Trypsin and tragacanth gum in the overlay medium were essential for the formation of clear foci of the influenza A viruses. In the absence of trypsin, only single stained cells were observed. In the presence of trypsin in concentrations ranging from 2.5 to 10 μg/ml, clear foci were made and no differences in the focus number or focus size were observed. Therefore, in the present study, 5 μg of trypsin per ml was added to the medium. Tragacanth gum was used to localize the foci. The appropriate concentration of the gum in the medium was 0.5%. In the case of the influenza B viruses, clear foci were formed regardless of the existence of trypsin and the gum in the medium.

The appropriate incubation period for focus counting was 20 to 24 h after virus infection. Only single cells were stained 12 h after inoculation. At 48 h, accurate counting was difficult because of the fusion of foci. Virus adsorption was carried out for 30 min because no difference in the focus numbers was observed within the range of adsorption times from 15 to 240 min.

Figure 1 shows typical foci of influenza A (panel A) and B (panel B) viruses in the 96-well tissue culture plates. A cluster of more than four stained cells was designated as one focus.

Quantitative evaluations of the focus method. Figure 2 shows the linear relationships between logarithms of virus dilutions (A/Yamagata/120/86 [H1N1] [panel A], A/Okuda/57 [H2N2] [panel B], A/Fukuoka/C29/85 [H3N2] [panel C], and B/Nagasaki/1/87 [panel D]) and the numbers of foci per well. In one well, at least 50 foci could be accurately counted. Infectivity titers of other virus strains could be assayed quantitatively, too. Therefore, this method is useful as an infectivity assay of influenza A and B viruses.

Infectivity titers of four strains of influenza virus were measured by the plaque and focus methods (Table 1).

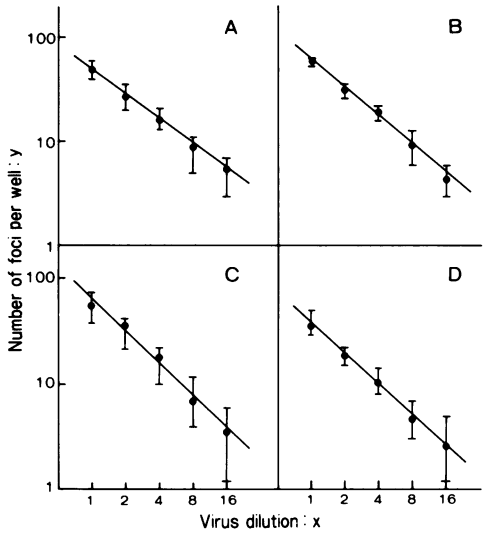

FIG. 2. Linear relationship between virus dilutions and numbers of foci per well. MDCK cells in 96-well microplates were infected with serially diluted A/Yamagata/120/86 (H1N1) (A), A/Okuda/57 (H2N2) (B), A/Fukuoka/C29/85 (H3N2) (C), or B/Nagasaki/1/87 (D), and foci were stained by the PAP technique as described in the text. The regression lines of least squares ($y = -0.243x + 1.929$ [A], $y = -0.277x + 2.057$ [B], $y = -0.310x + 2.110$ [C], and $y = -0.294x + 1.866$ [D]; $y = \log_{10}y$; $x = \log_2x$) are shown.

Although A/Okuda/57 (H2N2) had almost the same titers in the two methods, A/Yamagata/120/86 (H1N1) and B/Nagasaki/1/87 had higher titers in the plaque method than in the focus method. The plaques produced by A/Fukuoka/C29/85 (H3N2) were too small and obscure for counting. To evaluate the focus method for the titration of neutralizing antibodies, we studied serum specimens from six elementary-school students by the plaque and focus reduction methods and by the method of Frank et al. (3) with three strains of influenza virus (Table 2). The difference in the neutralizing-antibody titers measured by the three methods was small, at most a fourfold difference. The results indicate that the ordinary plaque method and the method of Frank et al. can be replaced by the focus method in the neutralization test for influenza A and B viruses.

Application of the micromethod for serological investigations. Sixty serum specimens were collected from students of an elementary school in Osaka City and titrated against influenza A and B viruses by the HI and neutralization tests.

TABLE 1. Comparison of infectivity titers of four influenza virus strains measured by plaque and focus assays

Virus strain	Infectivity titer measured by the following method:	
	Plaque (PFU/ml)	Focus (focus forming units/ml)
A/Yamagata/120/86 (H1N1)	4.8×10^7	6.8×10^6
A/Okuda/57 (H2N2)	4.5×10^6	5.3×10^6
A/Fukuoka/C29/85 (H3N2)	—[a]	7.4×10^6
B/Nagasaki/1/87	1.0×10^8	2.1×10^7

[a] —, Counting of the plaques was impossible because they were too small and obscure.

TABLE 2. Comparison of neutralizing-antibody titers of six serum samples against three influenza virus strains measured by plaque and focus methods and by the method of Frank et al. (3)

Serum sample no.	Neutralizing-antibody titer against:							
	A/Yamagata/120/86			A/Fukuoka/C29/85		B/Nagasaki/1/87		
	P[a]	F[b]	H[c]	F	H	P	F	H
1	2,560	2,560	640	2,560	1,280	640	320	1,280
2	1,280	1,280	640	2,560	2,560	320	640	640
3	640	320	160	80	160	20	40	40
4	320	80	160	640	640	320	320	640
5	80	40	40	1,280	640	640	1,280	640
6	320	320	640	40	40	10	10	10

[a] As measured by the plaque reduction method (P).
[b] As measured by the focus reduction method (F).
[c] As measured by the method of Frank et al. in which hemagglutination was used (H).

Figure 3 shows the relationship between HI and neutralizing-antibody titers against three strains of influenza A virus. There was a good agreement between the HI and neutralizing-antibody titers against A/Yamagata/120/86 (H1N1) (Fig. 3A) and A/Fukuoka/C29/85 (H3N2) (Fig. 3B). However, when they were measured against A/Shisen/2/87 (H3N2) (Fig. 3C), the neutralizing-antibody titers were shown to be significantly lower than the HI titers. The results obtained by the neutralization test seem more reasonable than those obtained by the HI test. This is because most of the students of the school had received current influenza vaccines in which A/Yamagata/120/86 (H1N1) and A/Fukuoka/C29/85 (H3N2) were included over the last few years. They had not, however, been given A/Shisen/2/87 (H3N2), a candidate vaccine strain from 1988.

Figure 4 shows the relationship between HI and neutralizing-antibody titers against two strains of influenza B virus. The neutralizing-antibody titers were slightly higher than the HI titers against B/Nagasaki/1/87 (Fig. 4A), whereas significantly higher titers were shown by the neutralization test than by the HI test against B/Osaka/152/88 (Fig. 4B).

DISCUSSION

Neutralization tests have not been used commonly in virological studies because they require a long time before results can be obtained and because many specimens can not be assayed at the same time. To overcome these difficulties, we developed rapid neutralization tests in a microtiter system for many viruses (2, 8, 12, 13, 15, 17) and demonstrated that the tests were useful for serological studies of these viruses (11, 16, 19). In this study, we developed a microtitration system for influenza A and B viruses based on the method described above (12, 15) and applied the system to a neutralization test for influenza.

In the newly developed neutralization test, residual virus infectivity was obtained by counting foci stained by the PAP technique. The fundamental difference between our focus method and the conventional plaque method is that the former method does not require cell destruction or damage by the virus infection which, however, is essential for the latter method. Therefore, results can be obtained faster by the focus method (1 day after infection) than by the plaque method (more than 3 days after infection). Moreover, the focus method can be applied to viruses such as A/Fukuoka/

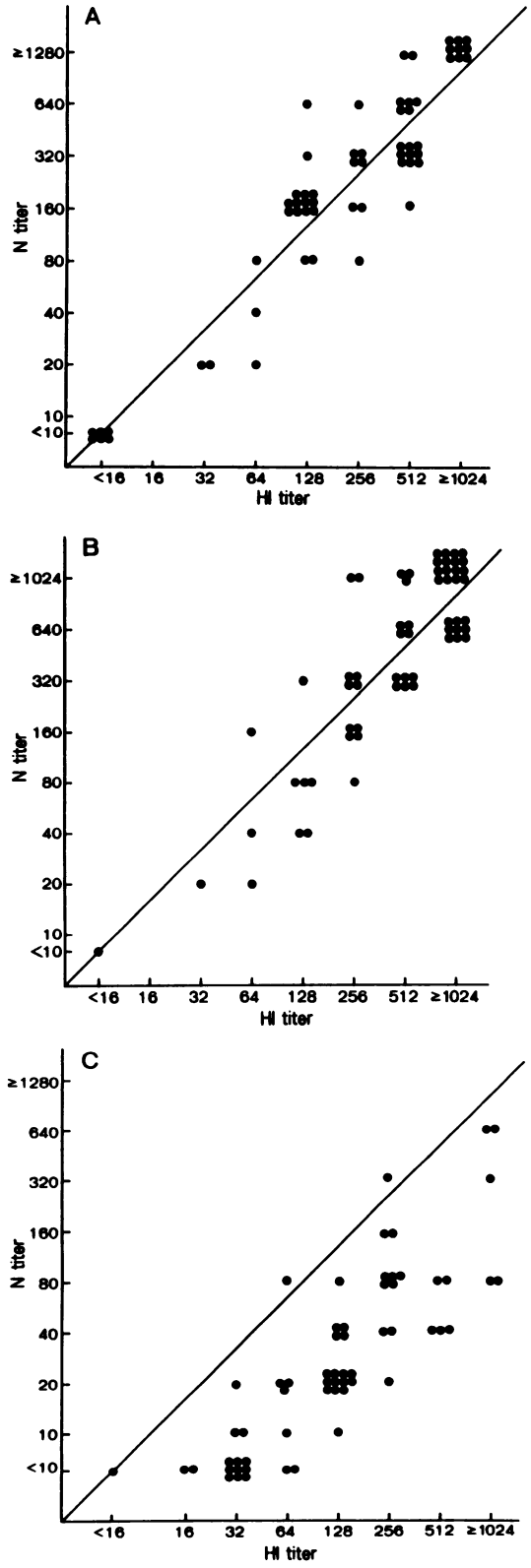

FIG. 3. Relationship between HI and neutralizing-antibody (N) titers against A/Yamagata/120/86 (H1N1) (A), A/Fukuoka/C29/85 (H3N2) (B), and A/Shisen/2/87 (H3N2) (C).

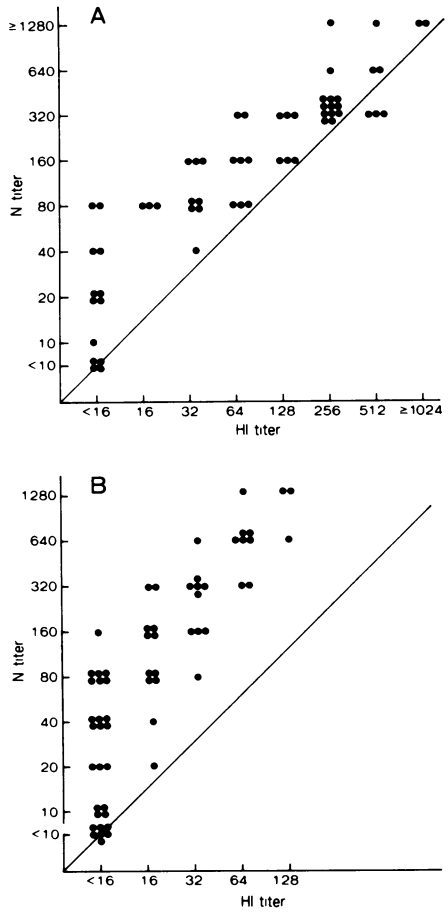

FIG. 4. Relationship between HI and neutralizing-antibody (N) titers against B/Nagasaki/1/87 (A) and B/Osaka/152/88 (B).

C29/85 (H3N2) that have difficulty in producing cytopathic effects or plaques (Table 1). In addition, the focus method has several other advantages over the plaque method. Since the focus method is a micromethod, it requires small amounts of test serum, cells, and medium and little incubator space. Stained foci in the plate can be kept for a long time and can be counted any time. Because 96-well tissue culture plates are used for both cell cultivation and serum dilution, the neutralization test can be carried out systematically in a short time with a multichannel pipette.

In the present study, we used the PAP technique to visualize infected cells because it had been shown to be the most suitable technique in terms of background staining and sensitivity when three immunoperoxidase techniques (direct, indirect, and PAP) were compared (14). The first antibody used in the PAP technique was rabbit anti-influenza A or B serum, which reacted broadly with all the influenza A or B viruses. The cross-reactive antibodies seemed to react against nucleoprotein (NP) and/or matrix (M) proteins in the infected cells, since monoclonal antibodies to NP or M protein of A/Okuda/57 (H2N2) reacted with all the influenza A viruses, whereas those to hemagglutinin reacted only with H2 influenza A viruses (unpublished results).

All the influenza virus strains used in this study and other strains, such as A/PR/8/34 (H1N1), a prototype influenza virus strain passaged many times in eggs, or several newly isolated viruses passaged several times only in MDCK cells,

developed clear foci (Fig. 1). Although differences in focus sizes among these strains were observed, the focus numbers could be counted quantitatively (Fig. 2).

For A/Yamagata/120/86 and B/Nagasaki/1/87, infectivity titers measured by the plaque method were not consistent with those measured by the focus method (Table 1). Moreover, large differences between the sensitivities of virus detection by the method of Frank et al. (3) and the other two methods were observed (data not shown). However, there was good agreement among the neutralizing-antibody titers obtained by the three methods (Table 2). These results indicate that different sensitivities among the three methods in detecting virus multiplication did not affect the neutralizing-antibody titers, since they were determined on the basis of control values.

The serum antibodies in the students were measured by the neutralization and HI tests (Fig. 3 and 4). When they were titrated against the vaccine strains (A/Yamagata/120/86, A/Fukuoka/C29/85, and B/Nagasaki/1/87), differences between the neutralization and HI titers were small. However, when they were titrated against the new strain, A/Shisen/2/87, with which the students had not been vaccinated, or the recent epidemic strain, B/Osaka/152/88, the differences were large. The possibility that noninfectious virus particles consume neutralizing antibodies and thus result in lower actual titers, as suspected in the neutralization tests with A/Shisen/2/87, seems unlikely because the ratio between infectivity and the hemagglutinin titer of A/Shisen/2/87 was not lower than that of other strains. Therefore, these observations seem to indicate that the neutralization test is more specific than the HI test.

When we measured serum antibodies in some adults, positive titers could be detected by the neutralization test but not by the HI test (unpublished results). Other investigators also showed that neutralization tests were more sensitive and useful than HI tests, especially when they were used for the detection of low titers (6, 7, 22). Therefore, the neutralization test seems to have advantages when antibody levels are low, with negligible or negative titers in the HI test.

The usefulness of the microneutralization test was also demonstrated in a recent study in which neutralizing antibodies in nasal wash specimens in children were measured (data not shown). Since local secretory neutralizing antibodies work directly to protect against infection from influenza, an assay of the antibodies is important. Therefore, the neutralization test which we developed can potentially be widely used for antibody titration not only in sera but also in nasal wash specimens.

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