IDENTIFICATION OF GROUP A COXSACKIEVIRUSES BY IMMUNE ADHERENCE HEMAGGLUTINATION

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SUMMARY: We investigated to find whether the immune adherence hemagglutination (IAHA) test could be used for identification of group A coxsackieviruses (Cox. A). By using homogenate of suckling mouse torsos infected with each of nine prototype viruses (Cox. A 2, 3, 4, 5, 6, 8, 9, 10 and 16) and 46 isolates as antigens and hyperimmune mouse ascitic fluids to the prototype viruses, we compared IAHA with complement fixation (CF) for serotyping of these viruses. The results of identification tests by IAHA were the same as those by combined use of CF and neutralization tests on all the 46 strains. By CF alone, however, six of 46 strains were not identified because of lower antigen titers; IAHA antigen titers were generally higher by 16-fold or more than CF tests. Furthermore, IAHA had a higher type-specificity than CF; a weak cross-reaction was found by IAHA only between Cox. A 3 and Cox. A 8. Nonspecific reactions encountered in IAHA were reduced more readily by kaolin than fluorocarbon treatment of the torso homogenates. From these results, we conclude that IAHA is an alternative method to CF and neutralization for serotyping of Cox. A viruses.

INTRODUCTION

Coxsackieviruses (Cox.) are classified into two groups (A and B) according to their growth characteristics in mice. All six serotypes of Cox. B grow well also in cultured cells of human and monkey kidney origin. Many serotypes of Cox. A, however, grow so poorly in cell cultures that they have to be cultivated in infant mice (Behabehani, 1967). Therefore, the procedures to isolate Cox. A viruses from clinical materials and to identify their serotypes are as follows: for virus isolation, clinical materials are inoculated to infant mice, which are observed for paralysis; for determination of serotypes, neutralization (NT) in mice is carried out, or complement fixation (CF) with use of the torso homogenate of infected mice as antigen is performed.

As an alternative technique to NT and CF, we investigated the feasibility of the immune adherence hemagglutination (IAHA) method for identification of Cox. A viruses; IAHA is known to be more sensitive than CF for detecting virus antigens (Ito and Tagaya, 1966; Mayumi et al., 1971; Inouye et al., 1980).

This paper describes our findings showing that IAHA is an appropriate technique for that purpose.

MATERIALS AND METHODS

Viruses: Prototype strains of Cox. A 2, 3, 4, 5, 6, 8, 9, 10 and 16 were provided by Drs. T. Komatsu and A. Hagiwara (Department of Enteroviruses, National Institute of Health, Tokyo). We used also 46 strains isolated in our laboratory in suckling ICR mice from stool or throat-swab specimens obtained from children with various illnesses (Nishio *et al.*, 1978).

The torsos of paralysed mice inoculated with each of the prototype strains of Cox. A viruses or with clinical materials were added to about 10 volumes of phosphate buffer saline (PBS), and then triturated in a homogenizer (Nihon Seiki, Co., Tokyo) for 30 sec. After clarification by centrifugation at 10,000 rpm for 20 min, the supernatant was treated once with an equal volume of fluorocarbon (Daikin Industry Co., Osaka) and the aqueous phase was used as antigen for IAHA and CF. Some of the supernatants were treated once with an equal volume of 25% kaolin (Fisher Scientific Co., USA) in PBS.

Immune ascitic fluids: Hyperimmune ascitic fluids to prototype viruses were produced in adult mice. The torso homogenates were treated twice with fluorocarbon, and then mixed with polyethyleneglycol 6000 to a final concentration of 8% to concentrate virus antigens (Yamamoto *et al.*, 1970). The resultant precipitates were each resuspended in 0.05 volumes of saline for use as immunogens. The concentrated virus suspension was mixed with an equal volumes of Freund's complete adjuvant, and one-ml volumes were inoculated subcutaneously. Four weeks later, 1.0-ml of the immunogen without adjuvant was given into the intraperitoneal cavity as a booster injection. Additional boosters were given once or twice at one-week intervals. Then Ehrlich's ascite tumor cells were intraperitoneally inoculated (10^6 cells/mouse). Ascites harvested were used as a source of antibody.

IAHA and CF: The methods described by Inouye *et al.* (1981) were employed. Briefly, the IAHA test was carried out in the following way. Veronal-buffered saline containing 0.1% bovine serum albumin, 0.002% gelatin and 0.02% NaN_3 was used as a diluent throughout the test. Antigens and antibodies, in 25- μl volumes each, were mixed and inoculated in wells of U-bottom polystyrene microplates at room temperature for 1 hr. Then, 25- μl of fresh guinea pig serum diluted 1:100 was added. The plates were covered and incubated at 37°C for 40 min. Then, 25- μl of dithiothreitol (3 mg/ml) and 50- μl of 0.4% human erythrocytes were added. Hemagglutination pattern was read 2 hr or more after room temperature incubation of the plates.

The CF test was carried out in microplates with 5 CH50 units of complement.

RESULTS

Comparison between IAHA and CF in Homologous Reactions

Checkerboard titrations by IAHA and CF were carried out with homologous antigens and antibodies of nine prototype viruses. The antigen titers are shown in Table I; IAHA antigen titers were 16- to 32-fold higher than CF antigen titers. The antibody titers at antigen concentrations of 4-units are shown in Table II; IAHA antibody titers were 4- to 16-fold higher than CF antibody titers.

TABLE I

Comparison between antigen titers by IAHA and CF

Antigen	Antigen titer by	
	IAHA	CF
Cox. A 2	128	8
Cox. A 3	512	32
Cox. A 4	64	2
Cox. A 5	1024	32
Cox. A 6	128	8
Cox. A 8	256	16
Cox. A 9	64	4
Cox. A 10	128	8
Cox. A 16	64	4

TABLE II

Comparison between antibody titers by IAHA and CF

Immune ascites	Antibody titer by	
	IAHA	CF
Anti-Cox. A 2	2560	320
Anti-Cox. A 3	640	80
Anti-Cox. A 4	640	40
Anti-Cox. A 5	2560	640
Anti-Cox. A 6	640	160
Anti-Cox. A 8	640	160
Anti-Cox. A 9	1280	320
Anti-Cox. A 10	1280	160
Anti-Cox. A 16	640	160

TABLE III

IAHA antibody titration with homologous and heterologous antigens

Ascites	IAHA antibody titer against					
	Cox. A 2	Cox. A 3	Cox. A 4	Cox. A 5	Cox. A 6	Cox. A 8
Anti-Cox. A 2	2560	<20	<20	<20	<20	<20
Anti-Cox. A 3	<40	640	<40	<40	<40	<40
Anti-Cox. A 4	<40	<40	640	<40	<40	<40
Anti-Cox. A 5	<20	<20	<20	2560	<20	<20
Anti-Cox. A 6	<40	<40	<40	<40	640	<40
Anti-Cox. A 8	<10	40	<10	<10	<10	640
Anti-Cox. A 9	<20	<20	<20	<20	<20	<20
Anti-Cox. A 10	<40	<40	<40	<40	<40	<40
Anti-Cox. A 16	<40	<40	<40	<40	<40	<40

IAHA Antibody Titrations with Heterologous Antigens

IAHA antibody titers of prototype immune ascites were determined with use of 4-unit antigens of homologous and heterologous types. Table III shows that all the ascites were highly type-specific. Only anti-Cox. A 8 ascite gave a slight cross-reaction with Cox. A 3 antigens.

Next, we studied further on this cross-reaction using three ascites preparations each of anti-Cox. A 3 and anti-Cox. A 8. Table IV shows that, by IAHA, all the anti-Cox. A 3 ascites did not cross-react with Cox. A 8 antigen, while two out of three anti-Cox. A 8 ascites reacted with Cox. A 3 antigen, indicating a one-way cross-reaction. By CF, the anti-Cox. A 8 ascites were more cross-reactive with Cox. A 3 antigen; the difference between antibody titers against Cox. A 3 and Cox. A 8 was smaller by CF than by IAHA. This indicates that,

TABLE IV

Cross-reaction between Cox. A 3 and Cox. A 8

Ascites	Lot No.	Number of booster injections	Antibody titer against			
			by IAHA		by CF	
			Cox. A 3	Cox. A 8	Cox. A 3	Cox. A 8
Anti-Cox. A 3	1	1	320	<10	40	<5
	4	2	80	<10	20	<5
	7	3	640	<40	80	5
Anti-Cox. A 8	6	3	40	640	20	80
	12	3	80	1280	20	160
	14	3	<10	640	5	80

Cox. A 9	Cox. A 10	Cox. A 16
<20	<20	<20
<40	<40	<40
<40	<40	<40
<20	<20	<20
<40	<40	<40
<10	<10	<10
<u>1280</u>	<20	<20
<40	<u>1280</u>	<40
<40	<40	<u>640</u>

in addition to its higher sensitivity, IAHA has higher type-specific reactivity than CF.

Identification of Cox. A Isolates by IAHA and CF

We previously isolated 46 Cox. A strains, which were identified by CF and NT (Cox. A 2, 5 strains; A 3, 3 strains; A 4, 8 strains; A 5, 8 strains; A 6, 6 strains; A 8, 3 strains; A 10, 7 strains; A 16, 6 strains) (Nishio et al., 1978). All these strains were again serotyped by IAHA and CF.

The results by IAHA on all 46 strains were the same as those by combined use of CF and NT. However, by CF alone, six of the 46 strains were not identified because of their low CF antigen titers. Table V shows the results on representative 14 strains.

In the IAHA tests, 34 of 46 torso specimens gave low-titered nonspecific hemagglutination in the antigen control wells containing no antibody (hemagglutination titer 1:2, 21 specimens; 1:4, 10 specimens; 1:8, 1 specimen; 1:16, 2 specimens). We found that this nonspecific hemagglutination tended to be observed in the torso homogenates made from older suckling mice (over one-week-old).

Comparison of Kaolin to Fluorocarbon Treatments for Removal of Nonspecific Hemagglutination

We used to pretreat the torso homogenates with fluorocarbon for IAHA and CF testing. As an alternative to fluorocarbon treatment, we tested kaolin treatment for removal of nonspecific hemagglutination.

Table VI shows that the nonspecific reactions were more readily removed by treatment with kaolin than with fluorocarbon. Also, specific IAHA antigen

TABLE V

Identification of Cox. A isolates by IAHA and CF

Strains	Number of passages in mice	IAHA antigen titers determined with immune ascites to							
		Cox. A2	A3	A4	A5	A6	A8	A10	A16
1444/80	1	512	<4	<4	<4	<4	<4	<4	<4
1146/79	2	1024	<8	<8	<8	<8	<8	<8	<8
1202/80	2	<4	16	<4	<4	<4	<4	<4	<4
1107/79	1	<2	<2	128	<2	<2	<2	<2	<2
1123/80	2	<8	<8	128	<8	<8	<8	<8	<8
1052/79	1	<1	<1	<1	32	<1	<1	<1	<1
1052/79	3	<32	<32	<32	256	<32	<32	<32	<32
1430/80	2	<8	<8	<8	<8	64	<8	<8	<8
1141/78	2	<32	<32	<32	<32	256	<32	<32	<32
1591/79	1	<2	<2	<2	<2	<2	128	<2	<2
1418/81	1	<2	<2	<2	<2	<2	<2	64	<2
1076/81	1	<8	<8	<8	<8	<8	<8	512	<8
1592/79	1	<1	<1	<1	<1	<1	<1	<1	32
1353/80	2	<1	<1	<1	<1	<1	<1	<1	128

*: Equivocal results

TABLE VI

Removal of nonspecific reactions by treatment with fluorocarbon or kaolin

Strains	Number of passages in mice	Identification	Treatment with			
			Fluorocarbon		Kaolin	
			Titers		Titers	
			Specific*	Nonspecific**	Specific	Nonspecific
2321/78	1	Cox. A 6	16	<1	16	<1
1052/79	1	Cox. A 5	32	<1	16	<1
1051/79	2	Cox. A 5	128	2	128	1
1051/79	3	Cox. A 5	1024	2	512	2
1091/80	2	Cox. A 4	128	4	16	1
1082/80	2	Cox. A 4	128	4	32	1
1077/81	1	Cox. A 10	256	4	256	1
1076/81	1	Cox. A 10	512	4	256	1
1570/78	1	Cox. A 10	128	8	128	<1
1052/79	3	Cox. A 5	256	16	128	2

*: Specific hemagglutination titers with antibody in the IAHA test.

**: Nonspecific hemagglutination titers without antibody.

CF antigen titers determined with immune ascites to								Identification by	
Cox. A2	A3	A4	A5	A6	A8	A10	A16	IAHA	CF
8	<1	<1	<1	<1	<1	<1	<1	Cox. A 2	Cox. A 2
32	<1	<1	<1	<1	<1	<1	<1	A 2	A 2
<1	4	<1	<1	<1	<1	<1	<1	A 3	A 3
<1	<1	8	<1	<1	<1	<1	<1	A 4	A 4
<1	<1	16	<1	<1	<1	<1	<1	A 4	A 4
<1	<1	<1	2	<1	<1	<1	<1	A 5	—*
<1	<1	<1	16	<1	<1	<1	<1	A 5	A 5
<1	<1	<1	<1	<1	<1	<1	<1	A 6	—
<1	<1	<1	<1	8	<1	<1	<1	A 6	A 6
<1	<1	<1	<1	<1	4	<1	<1	A 8	A 8
<1	<1	<1	<1	<1	<1	8	<1	A 10	A 10
<1	<1	<1	<1	<1	<1	16	<1	A 10	A 10
<1	<1	<1	<1	<1	<1	<1	2	A 16	—
<1	<1	<1	<1	<1	<1	<1	16	A 16	A 16

titers were somewhat decreased. However, since IAHA antigen titers after kaolin treatment was still high and the specific hemagglutination pattern appeared more distinct after kaolin than fluorocarbon treatment, we now prefer the former to the latter treatment.

DISCUSSION

Several investigators have used IAHA for the detection of virus antigens in serum (Mayumi et al., 1971), tissue culture fluids (Suzuki et al., 1979; Inouye et al., 1980; Suntharee et al., 1981) and stools (Matsuno et al., 1978; Nishio et al., 1981) because of its higher sensitivity than CF.

In this study, we also found that IAHA was 4- to 64-fold more sensitive than CF to detect Cox. A antigens in torso homogenates of infected suckling mice. Even if virus antigen could not be detected by CF because of low antigen titers, serotypes of isolates were easily identified by IAHA.

The cross-reaction observed in this study between Cox. A 8 and Cox. A 3 is in accordance with the results by CF and NT reported by other investigators (Kamitsuka et al., 1965; Munch, 1962; Suzuki et al., 1978). However, IAHA reaction was more type-specific than CF reaction (Table IV); this is in accordance with the report of Inouye et al. (1981). Yolken and Torsh (1981) observed cross-reactions between low-number and high-number Cox. A viruses

by enzyme-linked immunosorbent assay. We are now intending to study on the cross-reactions by IAHA.

One problem of the IAHA technique lies in the fact that nonspecific hemagglutination was observed in 10% mouse torso suspension treated with fluorocarbon. This nonspecific activity was more readily removed by kaolin treatment. Although antigen titers were somewhat decreased by the kaolin treatment, this treatment can safely be used in identification by IAHA since IAHA is sensitive enough for the antigen detection.

The most important point in the IAHA test is the selection of human erythrocytes. When animal sera are used in the IAHA test, human erythrocytes of any blood type can be used. In this study, we always used erythrocytes from a healthy type B donor.

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REFERENCES

- BEHABEHANI, A. M. (1967): Growth and cationic stabilization of group A coxsackievirus. *Proc. Soc. Exptl. Med. Biol.*, **125**, 983-988.
- INOUE, S., MATSUNO, S., HASEGAWA, A., MIYAMURA, K., KONO, R. AND ROSEN, L. (1980): Serotyping of dengue viruses by an immune adherence hemagglutination test. *Amer. J. Trop. Hyg.*, **29**, 1389-1393.
- INOUE, S., MATSUNO, S. AND KONO, R. (1981): Difference in antibody reactivity between complement fixation and immune adherence hemagglutination tests with virus antigen. *J. Clin. Microbiol.*, **14**, 241-246.
- ITO, M. AND TAGAYA, I. (1966): Immune adherence hemagglutination test as a new sensitive method for titration of animal virus antigens and antibodies. *Japan. J. Med. Sci. Biol.*, **19**, 109-126.
- KAMITSUKA, P., LOU, T. Y. AND WENNER, H. A. (1965): Preparation and standardization of coxsackievirus reference antisera. 1. For twenty-four group A viruses. *Amer. J. Epidemiol.*, **81**, 283-306.
- MATSUNO, M. AND NAGAYOSHI, S. (1978): Quantitative estimation of infantile gastroenteritis virus antigens in stools by immune adherence hemagglutination test. *J. Clin. Microbiol.*, **7**, 310-311.
- MAYUMI, M., OKOCHI, K. AND NISHIOKA, K. (1971): Detection of Australia antigen by means of immune adherence haemagglutination test. *Vox. Sanguinis*, **20**, 178-181.
- MUNCH, B. S. (1962): On the specificity of complement fixation tests for typing of coxsackievirus strains. *Acta Pathol. Microbiol. Scand.*, **56**, 89-104.
- NISHIO, O., SAKAE, K., KUNO, A., ISHIHARA, Y. AND INOUE, H. (1978): A continuing surveillance of enteroviruses in Aichi Prefecture. *Clin. Virol.*, **6**, 243-246 (in Japanese).
- NISHIO, O., ISHIHARA, Y., SUMI, J., MIYAKE, T., SAKAE, K., FUJIURA, A. AND INOUE, H. (1981): Problem points and feasibility of rotavirus detection by IAHA test. *Clin. Virol.*, **9**, 64-68 (in Japanese).
- SUZUKI, T., SAITO, N., MOCHIDA, T., KOBAYASHI, I., TAKAMIYA, A. AND KANAYAMA, F. (1978): Cases of isolation of coxsackie A 3 virus. *Japan. J. Publ. Hlth.*, **25**, 288-290 (in Japanese).
- SUZUKI, T., YOSHIDA, Y., ODA, K. AND TAKAMIYA, A. (1979): Application of immune adherence hemagglutination test for typing of enteroviruses. *Virus*, **20**, 153-154 (in Japanese).

- SUNTHAREE, R., CHARNCHUDHI, C., SOMPOP, A., KANAI, C., IGARASHI, A. AND INOUE, S. (1981): Isolation and identification of dengue viruses by combined use of C6/36 cells and the immune adherence hemagglutination test. *Japan. J. Med. Sci. Biol.*, *34*, 375-379.
- YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWHORNE, L. AND TREIBER, G. (1970): Rapid bacteriophage sedimentation in the presence of polyethyleneglycol and its application to large scale virus purification. *Virology*, *40*, 734-744.
- YOLKEN, R. AND TORSCH, V. (1981): Enzyme-linked immunosorbent assay for detection of coxsackieviruses A. *Infect. Immun.*, *31*, 742-750.