

A survey of coxsackie A16 virus antibodies in human sera

By G. E. D. URQUHART*

Regional Virus Laboratory, Ruchill Hospital, Glasgow G20 9NB

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SUMMARY

A comparison of neutralizing and immunofluorescent (IF) IgG antibody tests in 18 sera from 10 cases of hand, foot and mouth disease (HFMD) showed a variety of responses but all sera taken more than one week after infection had both neutralizing and IF IgG antibodies.

A survey of IF IgG antibody in 80 paediatric and 80 adult non-HFMD case sera gave antibody detection rates of 47.5% and 11.3% respectively. This difference could be attributed to a decline in IF IgG antibody with time after infection. Three point nine per cent of 26 sera from possible adult carditis cases had IF antibody suggesting that coxsackie A16 virus was not a common cause of adult carditis. Forty-eight point three per cent of 29 sera from cases of spontaneous abortion had detectable IF antibody, a rate similar to the paediatric sera and significantly greater than that in adult male (7.9%) and other adult female (13%) sera tested. This interesting observation requires further study.

INTRODUCTION

Coxsackie A16 virus is the cause of epidemic hand, foot and mouth disease (HFMD), a highly characteristic exanthematous infection occurring mainly in children. Epidemics of disease occur about every three years, the last in 1980 in the United Kingdom (World Health Organisation, 1981). The disease is usually benign and transmission of infection occurs at a high rate to close susceptible contacts. Thirty-seven per cent of nursery contacts may develop the disease (World Health Organisation, 1981) while 81% of school contacts and between 71 to 81% of family contacts may show evidence of infection, usually asymptomatic (Brown & O'Leary, 1974; Goh *et al.* 1982). Serious manifestations of infection can occur and both fatal myocarditis (Goldberg & McAdams, 1963) and spontaneous abortions (Ogilvie & Tearne, 1980) have been associated with coxsackie A16 virus infection. One case of chronic infection, where the virus was isolated over a two and a half year period, has been reported (Evans & Waddington, 1967).

Isolation of the virus from faeces during the acute phase of HFMD illness is the diagnostic method of choice and can give positive results in up to 93% of cases (Urquhart, 1980), but Brown & O'Leary (1974) showed that immunofluorescent antibody (IF) tests are useful for diagnosis in the absence of specimens for virus isolation.

* Present address - Virus Unit, Microbiology Department, Ninewells Hospital and Medical School, Dundee DD1 9SY.

This study describes a survey of IF antibody to coxsackie A16 virus in 215 paediatric and adult sera from a wide variety of non-HFMD cases, including carditis and spontaneous abortions, to investigate the possible role of the virus in diseases other than clinical HFMD. Eighteen sera from 10 cases of clinical HFMD due to coxsackie A16 virus were also investigated for comparison.

MATERIALS AND METHODS

Virus isolation and identification procedures

Coxsackie A16 viruses were isolated from the faeces of the 10 cases of HFMD in secondary rhesus monkey kidney or by inoculation of newborn mice and identified by neutralization with specific antisera in the isolation system, using methods described by Grist *et al.* (1979).

Newborn mouse neutralising antibody test

0.3 ml of a second pooled mouse passage of a field strain of coxsackie A16 virus (12603-80), containing 100 MLD₅₀, was incubated with an equal volume of a 1 in 10 dilution of inactivated serum for 1 h at room temperature. 0.03 ml of the mixture was inoculated subcutaneously into each mouse of a litter of at least seven newborn mice which were observed for pathogenic effect for 14 days. If 50% or more mice survived the test was repeated with a two-fold high serum dilution until an end-point was obtained. Virus control preparations killed all mice in 6-9 days.

Immunofluorescent IgG antibody test

Secondary rhesus monkey kidney cell culture tubes, inoculated with 0.2 ml of a 1 in 2 dilution of a virus stock containing 10^{7.6} MLD₅₀ per ml, and incubated at 37 °C for 48 h were found optimal for use as infected cell substrate. Sera were tested initially at a 1 in 10 dilution for antibody using anti-human IgG conjugate (Nordic Immunological Laboratories Ltd), and titrated as necessary to the last dilution giving good (2+) fluorescence. Specific IgM antibody tests were also carried out, using an appropriate conjugate, on all IgG antibody positive sera.

Test sera

Eighteen sera from 10 cases of HFMD from which coxsackie A16 virus had been isolated were tested for neutralising and IF IgG antibodies. Eighty 'random' paediatric and 80 adult sera, taken after the first week of onset of illness, from a wide variety of clinical syndromes, not typical of HFMD, were tested for specific IF IgG antibody. Thirty-seven of the paediatric sera were from cases of respiratory tract infection, 12 were from diseases of the central nervous system and 31 were from miscellaneous cases, including pyrexia, lymphadenopathy, gastroenteritis etc. Of the 80 adult sera, 30 were from cases of respiratory tract infection, 14 from cases of disease of the central nervous system and 36 from miscellaneous diseases. Half of these 160 sera had been submitted for routine viral tests in the second half of 1979, the remainder during and after the coxsackie A16 epidemic of 1980. Approximately half of the sera were from males.

In addition 26 sera from cases of possible adult clinical carditis in 1981, and 29 sera from cases of spontaneous abortion collected between 1977 and 1982 were also

Table 1. *Coxsackie A16 neutralizing and immunofluorescent antibodies in 18 sera from 10 cases of hand, foot and mouth disease*

Case	Age/Sex	Time of serum After onset (days)	Antibody titre		
			Neut	IF IgG	IF IgM
1	3M	1	<10	<10	<5
2	30M	5	80	80	10
		16	≥640	80	5
3	22M	3	40	<10	<5
		17	20	40	<5
4	11F	-17	<10	20	<5
		-7	<10	20	<5
5	32F	1	NT	80	<5
		10	NT	80	<5
6	3M	1	80	80	NT
		28	80	80	NT
7	4F	21	320	160	NT
		28	320	160	NT
8	45M	-150	<10	<10	NT
		+120	640	40	NT
9	20M	5	40	<10	NT
		10	20	10	NT
10	16F	2	20	NT	NT

NT = Not tested.

examined for IF IgG antibody. In none of these 55 sera was clinical HFMD noted. The mean ages and age ranges of the different groups are shown in Table 2.

RESULTS

Specific antibody responses in HFMD cases

From five of the 10 cases of proven coxsackie A16 infection paired sera were available for estimation of neutralising antibody and might have been expected to have antibody rises (Table 1, cases 2, 3, 6, 8 & 9). In only two cases were rises detected. Likewise, of six pairs of sera tested for IF IgG antibody rises, only two had rises, and in only one case was there a simultaneous rise by both methods. Thus only three of six cases had antibody rises when tested by both neutralizing and IF techniques.

Five of six sera taken the first week of onset of disease had 'high' (≥ 40) levels of neutralising antibody and three of six had high levels of IF IgG antibody. Also, Flewett & Foster (1960), and Brown & O'Leary (1974) also found high antibody levels in some early sera and suggested that, in these cases, a possible previous infection had occurred by a serologically related virus.

In four antibody positive sera, neutralising titres were significantly higher (four-fold or greater) than corresponding IF IgG titres, and in six sera neutralising and IF IgG titres were similar. In two sera from one case, IF IgG antibody was present but neutralising antibody was undetectable. Both these sera were

Table 2. *Coxsackie A16 immunofluorescent IgG antibody in paediatric and adult sera*

Clinical category of sera	Age (years)		Number tested	Number Positive (%)	Titres	
	Mean	Range			GMT	Range
Random paediatric	7.5	1-13	80	38 (47.5)	24	10-160
Random adult	39.1	17-77	80	9 (11.3)	11	10-20
Adult carditis	40.7	15-78	26	1 (3.9)	10	10
Abortion	28.8	19-37	29	14 (48.3)	19	10-160

GMT = geometric mean titre.

Table 3. *Comparison of Coxsackie A16 immunofluorescent IgG and neutralizing antibody in 20 random adult sera*

IF IgG titre	Number tested	Neutralising titre							Number positive
		<10	10	20	40	80	160	640	
<10	11	5	3	1	1	0	1	0	6
10	8	0	0	1	0	5	1	1	8
20	1	0	1	0	0	0	0	0	1
Total	20	5	4	2	1	5	2	1	15

taken before the onset of disease suggesting, in this case, a possible serological cross reaction with another, presumed enterovirus. Ogilvie & Tearne (1980) reported similar findings in three cases of coxsackie A16 infection.

Two of the seven post-onset sera were positive for specific IgM antibody (one of four cases) at low dilution and this low rate of specific IgM antibody response has been reported by Brown & O'Leary (1974).

However, after the first week of illness all seven sera tested for neutralising antibody were positive (titre range 20 - \geq 640) and all eight tested for IF IgG antibody were also positive (titre range 10-160).

Antibodies in 'random' paediatric and adult sera

Thirty-eight of 80 (47.5%) paediatric sera and nine of 80 (11.3%) adult sera had detectable IF IgG antibody with geometric mean titres of 24 and 11 respectively (Table 2). This difference is highly significant ($P < 0.001$) and may be attributed either to different rates of past infection with the virus between children and adults or to loss of IF IgG antibody with time after infection. To test the latter hypothesis a comparison of neutralising and IF IgG antibodies was made into 20 of the random adult sera (Table 3). All nine sera with IF IgG antibody had neutralising antibody but six of the 11 sera without IF IgG antibody had detectable neutralising antibody, suggesting that IF IgG antibodies decline with time after infection. Table 4 shows the incidence of IF IgG antibody in seven age groups. There is no difference in the percentage with antibody up to the age of 20 years but thereafter there is a significant decline. As the majority of clinical infections occur between the age of one and 15 years, the significant difference in the percentage with

Table 4. *Coxsackie A16 immunofluorescent IgG antibody in random sera according to age*

Age group (years)	Number tested	Number positive (%)
1-5	28	16 (57.1)
6-10	28	11 (39.3)
11-15	24	11 (45.8)
16-20	10	3 (30)
21-30	16	2 (12.5)
31-40	21	3 (14.5)
41+	33	1 (3.0)

Table 5. *Coxsackie A16 immunofluorescent IgG antibody according to sex*

Clinical category of sera	Male		Female	
	Number positive/ Number tested (%)	Mean age (years)	Number positive/ Number tested (%)	Mean age (years)
Random paediatric	21/43 (48.8)	6.6	17/37 (46)	8.5
Random adult	3/38 (7.9)	40.6	6/42 (14.3)	37.4
Adult carditis	0/14 (0)	42.9	1/12 (8.3)	38.1
Abortion	—	—	14/29 (48.3)	28.8
Abortion age matched* Adult random and carditis	3/22 (13.6)	28.4	6/33 (18.2)	27.6

* Adult sera from adult random and carditis groups selected to cover age range 19-37 years.

antibody between the 11-15 year age group and the 21-30 year age group suggests that IF IgG antibody is detectable for at least five years. The presence of IF IgG antibody probably indicates that infection has occurred within recent months or years, but the actual frequency of past infection with coxsackie A16 virus will be greater than that indicated by the 11.3% of adults with IF IgG antibody (Table 3). None of the 47 paediatric or adult sera with IF IgG antibody (Table 2) had detectable specific IgM antibody suggesting that coxsackie A16 does not commonly cause the clinical diseases sampled.

There was no significant difference between the percentage of IF IgG positive sera before and after the epidemic of 1980 or between males and females (Table 5) in these random groups of sera.

Antibodies in adult carditis and spontaneous abortion sera

One of 26 (3.9%) sera from cases of possible adult carditis (Table 2) had IF IgG antibody at a titre of 10, but no specific IgM antibody was detected. There was no significant difference in the frequency of IF IgG antibody between the carditis and random adult groups indicating no association between coxsackie A16 infection and possible carditis.

Nineteen of 29 (48.3%) of sera from cases of spontaneous abortion had IF IgG antibody with a geometric mean titre of 19. This frequency of antibodies was

similar to that in random paediatric sera and significantly greater than that in the adult random and carditis groups ($P < 0.001$). Specific IgM antibody was not detected in any IF IgG antibody positive sera from the abortion cases.

Further analysis according to sex is shown in Table 5. There was no significant difference in the percentage of sera with IF IgG antibody between males and females within the random paediatric, random adult or carditis groups. There was a significant difference between random adult females plus female carditis cases with antibody (7 of 54) compared to sera from abortion cases (14 of 29, $P < 0.001$). Because the mean age of adult female carditis and adult female random cases was about 10 years greater than the abortion cases, further analysis was carried out comparing those carditis and adult random sera whose ages were similar to abortion cases (19–37 years). Although there was no difference between males and females within these groups there was a significant difference between age matched females (6 of 33) compared to abortion cases (14 of 29, $P: 0.02-0.01$). However, although the difference in the proportion with antibody is still significant, the trend is towards a reduction in significance ($P < 0.001$ to $P: 0.002-0.1$), which might disappear if cases and controls were closely matched for age, parity and number and age of children in the family.

DISCUSSION

Previous study has shown (Brown & O'Leary, 1974) that the immunofluorescent antibody test is useful for the diagnosis of coxsackie A16 infections, but only 10 of their 18 cases with neutralizing antibody rises had corresponding IF IgG rises, the remainder having high static levels of antibody. Alsop *et al.* (1960), using a complement fixation test, also found high levels of antibody in some early sera. Brown & O'Leary (1974) reported the presence of specific IgM antibody in only 39% of cases with antibody rises. The findings presented here confirm this wide range of responses in proven coxsackie A16 infections. Only three of six cases had antibody rises, six of seven cases had high levels of antibody in early sera, and one of four cases had detectable specific IgM antibody. It can be concluded that antibody rises, detected by neutralisation or IF IgG, or the presence of specific IgM antibody can confirm a specific infection but, serology cannot be relied on alone to detect all infections.

Early high titres of complement fixing or IF IgG antibody might indicate a previous infection with the same or a related virus (Alsop *et al.* 1960; Brown & O'Leary, 1974), and in one case this was suggested by the presence of IF IgG antibody and absence of neutralising antibody in two sera taken before onset of disease. Ogilvie & Tearne (1980) reported three similar cases. Cross-reactivity of coxsackie A16 immunofluorescence antibody with another virus is a more likely explanation of these findings because the results show that immunofluorescent IgG antibody to coxsackie A16 virus declines with time after infection while specific neutralising antibody persists in the absence of immunofluorescent IgG antibody. Thus the finding of IF IgG antibody without neutralising antibody is more likely to result from cross-reactivity. The nature and extent of this possible cross-reactivity is unknown.

Nevertheless, the finding here that all sera tested after the first week of HFMD

did contain antibody both by neutralisation and immunofluorescence (titre range 10 to ≥ 640) indicated that the latter technique would be useful for the survey of specific antibody in sera from different groups of cases.

The percentage of random paediatric sera containing IF IgG antibody was 47.5% compared to 11.3% in a similar group of adult sera. This significant difference can be explained by the decline of IF IgG antibody with time compared to neutralising antibody. IF IgG antibody persists for possibly five years so that detection of IF antibody gives a falsely low incidence of total past infection with the virus.

The incidence of IF IgG antibody in 26 sera from possible adult carditis cases (3.9%) was not significantly different from the random adult cases (11.3%) suggesting that coxsackie A16 virus is not significantly associated with adult carditis. Fourteen of 29 (48.3%) sera from cases of spontaneous abortion had detectable IF IgG antibody which was similar to the rate found in random paediatric sera (47.5%) and the geometric mean titres were 19 and 24 respectively. Sera from abortion cases also had a significantly higher percentage of antibody than other adult sera tested. There was no difference in the incidence of antibody between males and females within the paediatric and adult random cases or the adult carditis cases. There was a significant difference in antibody incidence between all adult female carditis plus random cases (13%), and abortion cases (48.3%), and also between age-matched female carditis plus random cases (18.2%). This difference between the antibody content of abortion case sera and other adult female sera is an interesting observation, the significance of which requires further investigation using control cases matched specifically for age, parity and number of children in the family. Although the three abortion cases reported by Ogilvie & Tearne (1980) had clinical HFMD, infection with coxsackie A16 virus can be asymptomatic and minor clinical manifestations may not be reported. Indeed Adler *et al.* (1970) noted that, in young children, 89% of coxsackie A16 infections were symptomatic but only 18% were symptomatic in an older age group.

Coxsackie A16 virus can persist in individuals for one month (Higgins *et al.* 1965) and chronic infection over a two and a half year period has been described (Evans & Waddington, 1967). In the light of these findings and possible cross-reactivity between IF IgG coxsackie A16 antibody and another presumed enterovirus, further studies are indicated to fully evaluate these observations.

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REFERENCES

- ADLER, J. L., MOSTOW, S. R., MELLIN, H., JANNEY, J. H. & JOSEPH, J. M. (1970). Epidemiologic investigation of hand, foot and mouth disease. *American Journal of Diseases of Children* **120**, 309-313.
- ALSO, J., FLEWETT, T. H. & FOSTER, J. R. (1960). Hand, foot and mouth disease in Birmingham in 1959. *British Medical Journal* **ii**, 1708-1711.
- BROWN, G. C. & O'LEARY, T. P. (1974). Fluorescent antibody responses of cases and contacts of hand, foot and mouth disease. *Infection and Immunity* **9**, 1098-1101.
- EVANS, A. D. & WADDINGTON, E. (1967). Hand, foot and mouth disease in South Wales, 1964. *The British Journal of Dermatology*, **79**, 309-317.
- GOH, K. T., DORAISINGHAM, S., TAN, J. L., LIM, G. N. & CHEW, S. E. (1982). An outbreak of

- hand, foot and mouth disease in Singapore. *Bulletin of the World Health Organisation*, **60**, 965–969.
- GOLDBERG, M. F. & MCADAMS, A. J. (1963). Myocarditis possibly due to coxsackie group A, type 16 virus. *The Journal of Paediatrics*, **62**, 762–765.
- GRIST, N. R., BELL, E. J., FOLLETT, E. A. C. & URQUHART, G. E. D. (1979). *Diagnostic Methods in Clinical Virology*, 3rd ed, Oxford, Blackwell Scientific Publications.
- HIGGINS, P. G., ELLIS, E. M., BOSTON, D. G. & CALNAN, W. L. (1965). Hand, foot and mouth disease, 1963–64. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **24**, 38–45.
- OGILVIE, M. M. & TEARNE, C. F. (1980). Spontaneous abortion after hand, foot and mouth disease caused by coxsackie virus A16. *British Medical Journal* **281**, 1527–1528.
- URQUHART, G. E. D. (1980). A study of hand, foot and mouth disease. *Communicable Diseases Scotland Weekly Report*, **80/25**, vii–ix.
- WORLD HEALTH ORGANISATION (1981). Coxsackievirus A16. *Weekly Epidemiological Record* **56**, 39–40.