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Fluorescent Antibody Responses of Cases and Contacts of Hand, Foot, and Mouth Disease

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Paired sera from clinical cases, familial contacts, and school contacts of hand, foot, and mouth disease were tested by indirect immunofluorescence against a strain of coxsackie A16 virus which had been adapted to human fibroblast tissue-cultured cells. All of the 22 clinical cases developed immunoglobulin (Ig) G antibodies, and 11 responded with IgM and 10 with IgA antibodies. Seventeen of 21 familial contacts of cases showed subclinical infection as determined by development of IgG antibodies. Only seven and nine, respectively, of these had demonstrable IgM and IgA antibodies. Thirteen of 16 school classmates of three clinical cases were shown to have experienced subclinical infection by the development of IgG antibodies. Only five had antibodies in the IgM fraction, and three had antibodies in the IgA fraction. A comparison of IgG titers with those obtained by neutralization tests provides further evidence that the indirect fluorescent antibody technique represents a rapid diagnostic procedure for this disease.

The first demonstration that the etiological agent of hand, foot, and mouth disease was coxsackievirus type A16 appeared in 1958 (12). The few published reports on epidemic occurrences since then have shown that the majority of cases occurred in children under 4 years of age, that the virus was best isolated from stool specimens in suckling mice, and that serum antibodies could be demonstrated by neutralization tests in suckling mice or in certain tissue cultures when viruses could be adapted to the latter (1, 2, 9, 10). Serological findings by these methods indicated that, although most clinical cases showed a rise in titer, many had unchanging high titers at onset, suggesting early development of antibodies during the incubation period (2, 10). No reports on the development and detection of antibodies in various classes of immunoglobulin have appeared. Because of the difficulty in establishing virus strains in mice or tissue cultures and the time consumed in performing neutralization tests, a rapid and simple method of measuring these antibodies is highly desirable.

An unusual opportunity to study specimens from a large epidemic of this disease presented itself in the fall of 1971, when 207 cases occurred in Michigan between July and October (11). The present report describes the adaptation of the indirect fluorescent antibody (FA) technique for the diagnosis of hand, foot, and mouth disease and for the measurement of antibodies in the immunoglobulin (Ig) G, IgA, and IgM fractions of human serum from clinical cases, family contacts, and school contacts, and compares the results with those obtained by the neutralization test.

MATERIALS AND METHODS

FA test. Through the cooperation of personnel in the epidemiology section of the Michigan State Department of Health who performed the field studies with the assistance of an epidemic intelligence officer from the National Center for Disease Control, paired sera were obtained from 22 clinical cases, 21 family contacts of cases, and 16 school contacts. As with previous studies of fluorescent antibodies against various viruses in different classes of globulin (3-5), it was first necessary to adapt the virus to tissue cultures and determine the optimal conditions for the preparation of suitable antigens. This was successfully accomplished, after studying various types of cells, with a prototype strain (New York) of suckling mouse-adapted coxsackie A16 virus by first passing it one time in primary monkey kidney cells from which a pool of virus was made in human fibroblast tissue. These cells were first grown in WI-10 medium (Earle basic salt solution with 10% fetal calf serum and 1% glutamine) and maintained in Earle minimal essential medium with 2% fetal calf serum and 2% glutamine. Suitable antigens were obtained 16 h after infection of cover-slip cultures of the latter tissue in Leighton tubes with 0.1 ml of undiluted virus. The cover slips were fixed in acetone and cut into several

small pieces, which were placed in shallow depressions of plastic plates and covered with the serum dilution to be used. After 1 h at 37 C, the slips were washed three times in phosphate-buffered saline at pH 7.2 and incubated for an additional 1 h with an appropriate dilution of commercial fluorescein-labeled goat antiserum (Hyland, Inc.) directed against human IgG, IgA, or IgM. The purity and specificity of each fluorescein-labeled antiserum was determined by several techniques. Immunoelectrophoresis was performed by testing all conjugates against purified human IgM, IgA, IgG, and normal whole serum. The anti-IgM conjugate gave a single band with IgM and whole serum and did not react with IgA or IgG. The anti-IgA formed a single band with IgA and with whole serum and none with the other globulins. The anti-IgG formed a single band with IgG and whole serum and none with the other globulins. Further evidence of monospecificity was obtained by immunodiffusion analysis. Again only single bands appeared at the interface of the homologous reactants. The slips were washed again three times, counterstained for 10 s with a 1:30 dilution of Eriochrome black, drained, washed five times, and mounted on glass slides under Elvanol at pH 7.2. They were examined under a binocular microscope with a dark-field condenser by use of an Osram HBO-200 light source with a BG-12 exciter and OG-4 and OG-5 barrier filters. Results were recorded from 1+ to 4+, depending on the brilliance of the diffuse stain which occurred in the cytoplasm, and the titers were expressed as the highest dilution of serum with which at least a 2+ reaction was obtained. Uninfected tissue controls were always included, and each preparation of antigen slips was checked against known positive human sera as well as against hyperimmune horse anti-A16 serum (NIH).

Neutralization test. Neutralizing antibody tests in tissue cultures of primary monkey kidney cells had been performed under the direction of M. Becker at the Laboratories of the Michigan State Health Department, and the results were kindly made available by him.

RESULTS

Clinical cases. The 22 cases from whom blood specimens had been obtained were all between the ages of 1 and 19 years, and all had experienced typical symptoms of the disease. Coxsackie A16 virus had been isolated and identified from 12 cases by M. Becker. Table 1 compares the neutralizing antibody titers with FA titers in all three globulin fractions of blood specimens obtained during the acute and convalescent stages of disease. All 18 for whom both acute and convalescent sera were available showed increased neutralizing antibody titers, and 10 of these also showed corresponding increases in FA titers in the IgG fractions. The remaining eight all had high titers in both specimens, a finding consistent with early development as noted by others (2, 10) and suggesting either a longer incubation period than supposed or very rapid development of these antibodies.

TABLE 1. *Comparison of neutralizing antibody titers to coxsackie A16 virus with FA titers in various fractions of sera from clinical cases of hand, foot, and mouth disease*

Days after onset (acute stage/convalescent stage)	Neutralization[a]	FA		
		IgG	IgM	IgA
0/34	8/16	8/64	<8/<8	<8/<8
0/34	8/32	8/128	<8/8	<8/8
0/21	16/64	64/256	<8/<8	16/16
1/24	<8/128	<8/128	<8/16	<8/<8
1/36	<8/32	8/128	<8/<8	<8/<8
2/19	8/32	8/32	<8/<8	<8/<8
2/25	<8/16	64/128	8/<8	16/16
3/24	8/128	8/64	<8/8	8/16
3/26	<8/32	256/256	8/8	16/8
3/10	<8/32	128/512	8/16	<8/32
6/27	<8/32	256/256	8/<8	<8/<8
6/27	16/64	256/64	<8/<8	16/16
7/17	<8/32	128/128	8/8	16/16
7/28	16/128	128/128	<8/<8	<8/<8
9/42	<8/32	64/128	8/<8	8/8
9/43	<8/32	256/128	16/16	32/16
16/50	8/32	64/64	<8/<8	<8/<8
16/50	8/32	512/512	<8/<8	<8/<8
NS/27	NS/8	NS/16	NS/<8	NS/<8
NS/27	NS/32	NS/32	NS/<8	NS/<8
NS/34	NS/32	NS/64	NS/8	NS/<8
NS/36	NS/16	NS/128	NS/<8	NS/<8

[a] Reciprocal of serum dilution. NS, No specimen.

IgM antibodies were demonstrated in 11 cases. Seven of these were positive at the time of the first blood specimen within a few days of onset. The titers never exceeded 1:16 and tended to disappear early.

Antibodies of the IgA class were detected in 10 patients, and in eight of these they were present in both acute and convalescent specimens. These titers were also low, and only two were as high as 1:32. Immunoglobulin responses tended to occur independently, since both IgA and IgM antibodies were demonstrated in only six patients.

Family contacts. Paired blood specimens were received from 21 individuals ranging in age from 4 to 38 years who were living in close contact with at least one clinical case in each family. None had been ill. All family contacts were first bled from 1 to 7 days after onset of the first case in the family, and the second blood

was collected 2 to 3 weeks later. Of the 21 contacts, 15 showed increased neutralizing antibody titers in the second specimens (Table 2). Eight of these same individuals also showed increased FA titers in the IgG class, whereas the remaining seven had stationary titers, four at high levels. Two individuals had unchanging levels in both specimens by both techniques, and the remaining four contacts showed no demonstrable antibody on either test. Antibodies were found in the IgM fraction of serum in only seven of the 21 and in the IgA fraction of nine. Although the numbers are too small for significance, there was a suggestion that the IgA antibodies tended to persist longer than those of the IgM class.

School contacts. Three of the confirmed cases with onsets on 7, 10, and 13 October attended a small school where they exposed classmates in kindergarten through the third grade. Sixteen of these school contacts were bled on 13 October, within 6 days or less of exposure, and again 21 days later, on 3 November. Although none became ill during that period, serological evidence suggests that as many as 13 of these children may have experienced infection; certainly 10 showed significant fourfold or greater increases in titer of IgG antibodies (Table 3). Nine of these same children also had significantly increased neutralizing antibody titers. Again, as was the case with some contacts shown in Table 2, three individuals lacked detectable antibodies by either technique. As with the familial type of contact, few showed the development of IgM or IgA antibodies, since only five and three, respectively, were found to be positive in these classes.

TABLE 2. *Comparison of neutralizing antibody titers to coxsackie A-16 virus with fluorescent antibody titers in various fractions of sera from family contacts of hand, foot, and mouth disease*

Age (years)	Neutralization (1st serum/2nd serum)[a]	FA		
		IgG	IgM	IgA
29	<8/8	32/64	8/16	8/16
33	<8/8	32/32	<8/<8	16/32
7	<8/8	64/256	8/<8	8/8
32	16/64	<8/32	<8/<8	<8/<8
38	<8/<8	<8/<8	<8/<8	<8/<8
9	32/32	128/128	8/<8	8/<8
8	<8/16	32/32	<8/<8	<8/<8
27	<8/<8	<8/<8	<8/<8	<8/<8
25	<8/64	16/32	<8/<8	<8/<8
35	<8/8	16/16	<8/<8	<8/<8
14	<8/16	<8/64	<8/8	<8/32
12	<8/32	<8/512	<8/16	8/32
6	<8/16	8/8	<8/<8	<8/<8
10	8/8	16/16	<8/<8	<8/<8
9	<8/<8	<8/<8	<8/<8	<8/<8
30	<8/<8	<8/<8	<8/<8	<8/<8
23	<8/16	128/256	8/<8	8/<8
6	8/32	256/256	16/<8	8/<8
4	16/64	16/64	<8/<8	<8/<8
35	<8/8	8/8	<8/<8	<8/<8
8	8/32	64/64	<8/<8	<8/<8

[a] Reciprocal of serum dilution.

DISCUSSION

The present study demonstrates that the indirect fluorescent antibody test can be employed in the diagnosis of hand, foot, and mouth disease. Although substitution of this technique for the neutralizing antibody test is not necessarily being recommended, it should be emphasized that the FA technique is a much more rapid and simple method than virus neutralization in either suckling mice or tissue culture. Furthermore, the investigator is able to measure, if desired, IgM and IgA, as well as IgG, antibodies without recourse to cumbersome physicochemical techniques.

In general, there was good correlation of findings with serum specimens when the two different techniques were employed. Some of the acute bleedings from cases and first sera from contacts had titers of <1:8 by neutralization, whereas antibodies, sometimes in high titer, were detected in IgG globulin by the FA

TABLE 3. *Comparison of neutralizing antibody titers to coxsackie A-16 virus with fluorescent antibody titers in various fractions of sera from school contacts of cases of hand, foot, and mouth disease.*

Neutralization (1st serum/2nd serum)[a]	FA		
	IgG	IgM	IgA
<8/32	8/32	<8/<8	<8/<8
8/32	<8/16	<8/<8	<8/<8
32/256	<8/128	<8/8	<8/<8
<8/<8	<8/<8	<8/<8	<8/<8
<8/128	16/256	<8/<8	<8/<8
<8/16	32/64	8/<8	<8/<8
8/32	<8/32	<8/<8	<8/<8
16/128	32/128	8/<8	8/<8
<8/16	<8/32	<8/<8	<8/<8
<8/<8	<8/<8	<8/<8	<8/<8
<8/<8	<8/<8	<8/<8	<8/<8
16/16	128/128	8/<8	16/<8
<8/8	16/32	<8/<8	<8/<8
<8/16	16/64	<8/<8	<8/<8
<8/16	<8/128	<8/8	<8/8
<8/8	16/64	<8/<8	<8/<8

[a] Reciprocal of serum dilution.

method. This is not an entirely unexpected finding in light of the known greater sensitivity of the FA test and of the fact that antibodies determined by various serological techniques show different curves of development in both time and titer. However, the data show clearly that the majority of individuals on whom serological evidence of infection was seen showed increases in titer by both methods. On the other hand, those persons whose paired sera showed FA titers of $<1:8$ in both also were lacking in neutralizing antibodies.

The availability of serum specimens from familial and nonfamilial (school) contacts of cases, as well as from clinically ill persons, made possible a comparison of the serological response of these three categories of individuals. As expected, all of the clinically ill cases showed serological evidence confirming infection. Although most of the familial contacts (71%) experienced subclinical infection concurrently with their index case relative, a surprisingly large number of nonfamilial, school contacts also were found to have significant serological changes. In fact, the extent of infection in contacts was definitely greater than that previously reported by others (1, 9).

A surprising finding was the fact that only half of the clinical cases and approximately one-third of the contacts developed IgM and IgA antibodies. This is in marked contrast to the results of studies with other virus infections (3–5) which employed these same FA techniques. Although the presence of IgM or IgA antibodies undoubtedly reflects recent infection, the diagnostic significance of their absence appears relatively unimportant in this disease.

Previous reports by others have made note of the occasional recovery of viruses other than coxsackie A16 from typical cases of hand, foot, and mouth disease. Thus, coxsackieviruses A4 (10), A5 (8), and A10 (6, 7) as well as herpes simplex virus have been suggested as etiological agents. In the epidemic described herein, several other viruses, including coxsackie A4, echo 9, herpes simplex, poliovirus 3, and adenovirus 2, had been recovered in the laboratory of the State Health Department. However, neither the symptomatology nor the serology associated with these particular individuals indicates that they were implicated in this particular outbreak of the disease. Additional evidence of the specificity of reaction and coxsackie A16 etiology described in the present study can be cited. Several paired sera from coxsackie infections with types A9 and A4 available in this laboratory were tested against the A16 FA antigen and failed to show any diagnostic increases. Conversely, when persons in the present study were negative for coxsackie A16 antibodies in both sera by neutralization tests, they also lacked fluorescent antibodies in the same specimens.

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