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An outbreak of aseptic meningitis associated with Coxsackie B5 and A9 viruses in Northern Japan, 1961. Virological and serological studies

By YORIO HINUMA, YOSHIMICHI MURAI, MINORU FUKUDA,* YOSHIO NUMAZAKI† and NAKAO ISHIDA

Department of Bacteriology, Tohoku University School of Medicine, Sendai, Japan

and TOORU NAKAO

Paediatric Clinic, Aomori Prefectural Central Hospital, Aomori, Japan

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In the summer of 1961, an outbreak of aseptic meningitis took place in Northern Japan. A total of 655 cases, mostly infants or children, was reported by three local paediatricians and by one of the authors (T.N.). It is probable that the total number of cases in the entire district was more than 2000. The highest incidence was observed in Aomori Prefecture, which is in the northern end of Honshu (Japan proper), and a peak incidence occurred between June and August.

From many of the specimens of spinal fluids and faeces, Coxsackie B5 virus and, to a lesser extent, Coxsackie A9 virus were isolated. Serological tests with paired sera also suggested infections with these viruses.

The present paper is concerned with the aetiological studies on 295 cases and with some properties of the viruses isolated. Clinical and epidemiological aspects of the epidemic will be reported in detail in a paper by Nakao *et al.* (1964).

MATERIALS AND METHODS

Patients

Of the 295 cases studied, 234 were examined clinically by one of the authors (T.N.) at the Paediatric Clinic, Aomori Prefectural Central Hospital, Aomori City.

Specimens

Spinal fluids were collected from 270 cases, faeces from 147, and paired sera from 184 cases. The spinal fluids and faeces were obtained upon admission to the hospital. The specimens of blood were taken on admission and more than 7 days later to provide acute and convalescent phase sera. These sera were collected from May to October 1961 and stored at $-25°$ C. until use.

Isolation and identification of viruses

For the isolation of viruses, the stationary cultures of cynomolgus monkey kidney cells and/or HeLa (S3) cells were used. Unweaned mice were not employed

* Died on 23 October 1963.

† Present address: Virology Laboratories, Children's Hospital, Buffalo, U.S.A.

in the present studies. One-tenth millilitre of clarified 10% suspension of faeces or 0·2 ml. of spinal fluid was inoculated into two replicate tubes of cell culture which already contained 1·0 ml. of a maintenance medium. For the identification of viruses, the conventional neutralization test was performed. In the early stages of this study, reference sera against Coxsackie A 9, Coxsackie B 1–5, Echo 1–19 and Polio 1–3, all of which were kindly supplied by the National Foundation of the United States, were used. Later, rabbit antisera prepared in this laboratory against B 5 and A 9 viruses by immunization with the current strains, AM-69-L-61 and AM-85-L-61 respectively, were used for the typing of the viruses.

Antibody titration

Neutralizing antibody in paired sera from the patients was titrated against the current strain (AM-69-L-61) of Coxsackie B 5 virus, Poliovirus type 1 (Mahoney), type 2 (MEF 1) and type 3 (Saukett) in HeLa cell culture. For titration of neutralizing antibody against Coxsackie A 9 virus, the current strain (AM-85-L-61) was used in the monkey kidney cell cultures. The virus dose used was approximately 100 TCID 50.

RESULTS

Virus isolation

As shown in Table 1, Coxsackie B 5 virus was isolated from the spinal fluids of 60 patients out of 270 examined, and Coxsackie A 9 from 1 patient. Cytopathogenic agents were also isolated from the faeces of 82 cases out of 147; Coxsackie B 5 from 51 cases, Coxsackie A 9 from 13, type 3 poliovirus from 3, and unidentified viruses from 15 cases. The large number of isolations of Coxsackie B 5 virus strongly suggested that this was the main causative agent of the epidemic.

That Coxsackie A 9 virus was isolated from the spinal fluid of one case (case no. AM-85) in Aomori and also from the faeces of 13 cases, including case no. AM-85, in the same region, may indicate that Coxsackie A 9 was also partly the cause of the epidemic in this region.

Of the poliovirus excreted by three patients in Aomori (see also Table 8), one (that of no. AM-108) is possibly a wild strain because the patient had not received the live poliovaccine. The virus isolated from two other patients (AM-5 and AM-113), however, might have originated from the vaccine strains since these patients had received the live oral vaccine (Sabin's triple) within 2 weeks before onset of the aseptic meningitis.

Properties of the viruses isolated

Coxsackie B 5 virus

It is evident from Table 2 that this virus was more readily isolated in HeLa cells than in monkey kidney cells. Out of a total of 21 isolations from the spinal fluid, 10 grew only in HeLa cells and 1 grew only in monkey kidney cells. The remaining 10 were isolated in both cells.

The results of assay of viruses in the spinal fluids and faeces seemed to be concordant with the above results, i.e. the virus titres obtained in HeLa cells were generally higher than those in monkey cells (Table 3).

Table 1. *Virus isolation from the patients with aseptic meningitis*

Region	No. of cases	Spinal fluids: No. of specimens tested	Spinal fluids: No. of virus isolated: Coxsackie B5	Spinal fluids: No. of virus isolated: Coxsackie A9	Spinal fluids: No. of virus isolated: Total	Faeces: No. of specimens tested	Faeces: No. of virus isolated: Coxsackie B5	Faeces: No. of virus isolated: Coxsackie A9	Faeces: No. of virus isolated: Polio 3	Faeces: No. of virus isolated: Un-identified	Faeces: No. of virus isolated: Total
Aomori City	234	217	52	1	53	115	43	13	3	15	74
Hirosaki	17	14	5	0	5	17	5	0	0	0	5
Hachinohe	10	6	0	0	0	8	0	0	0	0	0
Mutsu	18	18	0	0	0	0	0	0	0	0	0
Kuzumaki	16	15	3	0	3	7	3	0	0	0	3
Total	295	270	60	1	61	147	51	13	3	15	82

Table 2. *Comparative sensitivity of HeLa cells and monkey kidney cells in isolation of Coxsackie B5 virus from the clinical specimens**

Specimen	Number of virus isolated in following culture: HeLa only	MK only	HeLa and MK	Total
Spinal fluids	10	1	10	21
Faeces	1	0	19	20
Total	11	1	29	41

* Individual specimens were inoculated into HeLa cell and monkey kidney (MK) cell cultures separately.

Table 3. *Assay of amount of Coxsackie B5 virus in clinical specimens by using HeLa cell and monkey kidney cell cultures*

		Amount of virus			
		Tube culture method		Plaque method	
Specimen	Case no.	HeLa cell (TCID 50/ml. or g. (−log))	Monkey kidney cell (TCID 50/ml. or g. (−log))	HeLa cell (p.f.u./ml. or g.)	
Spinal fluids	AM-69	< 1·8	< 1·8	10	(0)*
	AM-74	1·8	< 1·8	65	(0)
	AM-84	1·8	< 1·8	20	(0)
Faeces	AM-95	6·2	3·8	10×10^5	$(1{\cdot}5 \times 10^4)$
	AM-96	5·8	3·5	$8{\cdot}3 \times 10^5$	$(2{\cdot}0 \times 10^4)$
	AM-101	4·9	2·8	$2{\cdot}8 \times 10^5$	$(2{\cdot}0 \times 10^4)$

* The number in parentheses represents the number of large plaques.

To examine plaque characteristics of the virus in these clinical specimens, undiluted spinal fluids and appropriately diluted faecal suspensions, from the same specimens as were used for virus isolation, were inoculated into HeLa cell monolayers. The plaque formation was done principally according to the procedure described by Ketler, Hinuma & Hummeler (1961). Two morphologically different types of plaques were formed from faecal specimens; one was a minute, irregular but well-defined plaque with sharp boundary and another a large, round plaque with diffuse boundary. When plaques were observed on the 8th day after inoculation, the minute type and the large type were 0·5–1·0 mm. and 3–16 mm. in diameter respectively. The minute plaque was predominant in number; the ratio of minute plaques to large in three specimens examined was 65, 40 and 13 as shown in Table 3. From spinal fluids, only minute plaques were formed. Further studies on the plaque variants are now in progress. Strain AM-69-L-61, which was isolated from the spinal fluid of a 2-year-old girl (case no. AM-69) with aseptic meningitis was selected as a representative strain among 111 new strains of Coxsackie B5 virus, and was examined for its characteristics after three to four passages in HeLa cell cultures. This strain gave cytopathic effect on HeLa, FL and monkey kidney cells in monolayer cultures, yielding 10^5–10^7 TCID 50/ml. when titrated in HeLa cell cultures. Antigenicity of AM-69-L-61 virus and two other B5 strains isolated in Japan in 1960 was compared with that of the prototype Faulkner strain, by the cross-neutralization technique. Results are shown in Table 4. AM-69-L-61 strain did not completely cross with the Faulkner strain. On the other hand, the AM strain seemed to be very close to the other Japanese strains, although examination was done only from one side. Further antigenic analysis with both Faulkner and AM-69-L-61 strains is now being carried out. AM-69-L61 virus produced typical spastic paralysis in day-old mice when inoculated intraperitoneally with $10^{5{\cdot}3}$ TCID 50 of virus, and the mice died 4–10 days after inoculation. Marked colour changes in the interscapular fat pad were evident upon autopsy as described by

Dalldorf, Melnick & Curnen (1959). Five other Coxsackie B 5 strains so far examined in unweaned mice showed similar pathogenicity.

Table 4. *Antigenic variation of Coxsackie B 5 viruses*

Strain (year isolated)	Neutralizing-antibody titre*	
	Anti-Faulkner†	Anti-AM-69-L‡
Faulkner§ (1952)	320	80
AM-69-L (1961)	40	160
STS-604-L‖ (1960)	80	160
SD-58-K¶ (1960)	80	320

* Reciprocals of serum dilution which completely inhibited cytopathic effect of 100 TCID 50 of virus on HeLa cell culture.

† Anti-Faulkner rabbit serum obtained from the National Foundation, U.S.A.

‡ Anti-AM-69-L rabbit serum prepared in this laboratory by immunization with HeLa cell-grown virus which was pre-treated with fluorocarbon twice.

§ The strain was kindly supplied by Dr A. L. Barron, University of Buffalo. This had been passaged numerous times on suckling mice and then monkey kidney cells. Upon receipt, the virus was passaged twice on HeLa cells in this laboratory.

‖ The strain was kindly supplied by Dr R. Kono, Kyoto University. This had been isolated and passaged on FL cells. This was passed twice on HeLa cells in this laboratory.

¶ The strain was isolated from the faeces of a patient with aseptic meningitis in Sendai and passaged on FL cells in this laboratory. The virus was used after passage twice on HeLa cells.

Coxsackie A 9 virus

All the Coxsackie A 9 viruses were isolated only in monkey kidney cell cultures. The properties of strain AM-85-L-61 isolated from the spinal fluid of a 5-year-old boy with aseptic meningitis were examined. This virus was cytopathogenic to monkey kidney cells but not to HeLa and FL cells. Virus yield in monolayer culture of monkey kidney cells was usually 10^8–10^9 TCID 50/ml. Plaques formed on monkey kidney cell cultures by the strain, which had been passaged twice in monkey kidney cells, were characteristically large and round with diffuse boundaries, and the growth of the strain was less rapid than the Mahoney strain of poliovirus. Morphologically the plaques were not distinguishable from those produced by the PB strain of a prototype Coxsackie A 9 virus which was obtained by the courtesy of Dr A. L. Barron of the University of Buffalo. The plaques of AM-85-L-61 were also very closely related to those of the Grigg strain of Coxsackie A 9 illustrated in the paper by Melnick (1958). When the antigenic compositions of AM-85-L-61 and PB strains were examined by cross-neutralization test, no significant differences were seen. Day-old mice inoculated intraperitoneally with $10^{7.7}$ TCID 50 of the AM-85-L-61 developed a prostrating paralysis and death followed within 2–5 days. A similar effect was observed after inoculation of 5 other freshly isolated strains of Coxsackie A 9.

Serological studies

Neutralizing-antibody titration against Coxsackie B5 virus was performed with the paired sera obtained from 184 patients with aseptic meningitis (Table 5). A fourfold or greater rise in antibody titre was demonstrated in 76% of the patients from the spinal fluid or faeces of whom B5 virus was recovered. An additional 15% of Coxsackie B5 virus-positive patients demonstrated the presence of antibody in a titre of 4 or greater without a rise in titre between the paired sera However, there were 4 patients without detectable antibody in the paired sera, from whom Coxsackie B5 virus was recovered from the spinal fluid. Of 100 patients with negative isolation of virus, 39% showed a fourfold rise in antibody titre, 29% showed antibodies present in a titre of 4 or greater without a rise in titre, and 32% showed no detectable antibody titres.

Table 5. *Neutralizing antibody against Coxsackie B5 virus in the paired sera of* 184 *patients with aseptic meningitis*

Virus recovery		No. of patients	Antibody absent*		Antibody present			
					No rise†		Rise‡	
			No. of subjects	%	No.	%	No.	%
Positive B5 virus	Spinal fluids	52	4	8	6	12	42	80
	Faeces	32	3	9	7	22	22	69
	Total	84	7	8	13	15	64	76
Negative		100	32	32	29	29	39	39

* Less than 4. † 4 or greater with no rise. ‡ Fourfold or greater rise.

Table 6. *Neutralizing antibody against Coxsackie A9 virus in the paired sera of* 66 *patients with aseptic meningitis*

Virus recovery		No. of patients	Antibody absent		Antibody present			
					No rise		Rise	
			No. of subjects	%	No.	%	No.	%
Positive A9 virus	Spinal fluids	1	0	.	0	.	1	.
	Faeces	11	8*	73	1	9	2	18
Negative		54†	23	42	17	32	14	26

* One (case no. AM-91) of 8 was examined on a single serum obtained at the 9th day of disease.

† In the 54 patients, no viruses were isolated and rise of Coxsackie B5 antibody was not proved.

Neutralizing-antibody titration against Coxsackie A9 virus was performed with paired sera from 66 patients. The results are shown in Table 6. One patient (case no. AM-85) from whom Coxsackie A9 virus was isolated from both spinal fluid and faeces showed significant antibody rise. Of the other 11 patients who excreted

Coxsackie A 9 virus in the faeces, only 2 showed a significant rise of antibody titre, 1 showed antibody with no rise of titre and 8 showed no detectable antibody in paired sera.

Table 7. *Antibodies against Coxsackie A 9 and B 5 viruses in the paired sera of patients excreting Coxsackie A 9 virus*

		Antibody titre*						
		Coxsackie A 9				Coxsackie B 5		
		NT†		CFT‡		NT		
Case no.	Age (year-month)	A§	C‖	A	C	A	C	Serodiagnosis
AM-85	5-7	0¶	64	0	4	16	16	Coxsackie A 9
AM-95-1	5-2	16	256	0	0	64	64	Coxsackie A 9
AM-81	3-3	64	256	0	0	16	64	Coxsackie A 9 or B 5
AM-79	9-3	64	64	0	4	64	64	Coxsackie A 9
AM-82-1	0-10	0	0	0	0	ND**	0	Unknown
AM-83-1	2-0	0	0	0	0	16	64	Coxsackie B 5
AM-83-2	2-2	0	0	0	0	64	64	Unknown
AM-84-1	0-9	0	0	0	0	16	256	Coxsackie B 5
AM-86-1	0-8	0	0	0	0	16	64	Coxsackie B 5
AM-97	5-7	0	0	ND	0	256	256	Unknown
AM-104	2-7	0	0	ND	ND	64	64	Unknown
AM-91	5-11	ND	0	ND	ND	ND	> 4	Unknown
AM-99	1-0	0	0	ND	ND	ND	> 4	Unknown

* Reciprocals of serum dilution.
† Neutralization test.
‡ Complement-fixation test.
§ Acute serum.
‖ Convalescent serum.
¶ Less than 4.
** Not done.

Complement-fixation tests were performed on paired sera of the Coxsackie A 9 virus-positive patients, using the antigen prepared from the monkey kidney tissue culture fluid infected with AM-85-L-61 virus. Negative results were obtained which were comparable with the results obtained in neutralizing-antibody determination as shown in Table 7. These results suggested that a large proportion of persons with alimentary tract infection with Coxsackie A 9 virus did not show a good antibody response. They also suggested that the aseptic meningitis of these patients might not be caused by Coxsackie A 9 virus but by other agents, even though Coxsackie A 9 virus was recovered from the faeces. Therefore, neutralizing antibody against Coxsackie B 5 virus in these paired sera was determined, because Coxsackie B 5 virus was considered to be the main causative agent for the epidemic. Results are shown in Table 7. Four patients showed a fourfold or greater rise in Coxsackie B 5 antibody titre, although one of them also demonstrated a significant rise of antibody against Coxsackie A 9. On the other hand, 54 patients in whom no viruses were recovered and a rise of Coxsackie B 5 antibody was not detected were selected and the paired sera were tested for Coxsackie A 9 antibody. Among them, 14 showed a fourfold rise in antibody against Coxsackie A 9 (Table 6). Thus the serodiagnostic results suggested that a portion of the population of patients with aseptic meningitis were caused by Coxsackie A 9 virus.

Neutralizing-antibody titration against polioviruses types 1, 2 and 3 was performed with the paired sera obtained from 12 selected patients. Among them, 3 were positive in isolation of type 3 poliovirus but the remainder had no evidence of infection with either Coxsackie B5 or Coxsackie A9. As shown by Table 8, a significant antibody rise was shown in 2 patients for type 2 poliovirus, and in one for type 3 poliovirus. In one case among the rest, simultaneous antibody rise was shown against types 2 and 3 poliovirus. The remaining 6 cases, not cited in Table 8, did not show a rise of antibody against any type of poliovirus. From these results, polioviruses type 2 and type 3 were also assumed to be the causative virus for a small portion of the aseptic meningitis, at the time of this epidemic.

Table 8. *Neutralizing antibody against polioviruses in the paired sera of patients with aseptic meningitis*

Case no.	Live polio vaccination*	Virus recovered from faeces		Neutralizing-antibody titre against poliovirus Type 1	Type 2	Type 3	Serodiagnosis
AM-5	+	Polio 3	A	256	256	64	Unknown
			C	256	256	64	
AM-108	–	Polio 3	A	Serum not available			Unknown
			C	0	0	64	
AM-109	–	—	A	0	0	0	Polio type 2
			C	0	16	0	
AM-113	+	Polio 3	A	0	0	0	Polio type 3†
			C	0	0	16	
AM-344	–	—	A	0	4	0	Polio type 2
			C	0	64	0	
AM-366	–	—	A	0	64	4	Polio type 2 or 3
			C	0	1024	16	

* Both AM-5 and AM-113 received Sabin's oral triple live vaccine within 2 weeks before onset of the aseptic meningitis.

† Probably vaccination effect.

DISCUSSION

On the basis of the results of virus isolation, especially from spinal fluids of the patients, and of serological tests, causative agents of the aseptic meningitis occurring in Northern Japan in the summer of 1961 were determined to be Coxsackie B5 virus, and Coxsackie A9 virus to a lesser extent. The incidence of the illness attributable to polioviruses seemed to be very low. Yamada and his colleagues (personal communication) reported that more than 300 Coxsackie B5 virus strains were isolated from faeces of healthy children and patients with either acute febrile illness or aseptic meningitis in Hokkaido during the summer of the same year. This evidence suggests that there was an extensive seeding of Coxsackie B5 virus in the population of both northern Honshu and Hokkaido. On the other hand, there were no reports suggesting the spreading of Coxsackie B5 in other areas of Japan, except a few isolations of the virus from healthy children. It should be mentioned that an outbreak of aseptic meningitis on a comparable

scale to that in Northern Japan in 1961 had occurred in Western Japan during the summer of 1960 and the causative agent was determined to be Coxsackie B5 virus, as reported by Kono *et al.* (1960). In that year, we found 9 patients with aseptic meningitis associated with Coxsackie B5 virus in Aomori during August to October, but the spreading pattern of the infection was not likely to be epidemic. However, it was apparent that Coxsackie B5 virus had begun to spread in this region at that time. The sero-immunity against Coxsackie B5 virus in children in Aomori had been very poor before the 1961 epidemic as shown in Fig. 1. From these facts, it was considered reasonable to assume that Coxsackie B5 virus began to invade children in Aomori in the late summer to early autumn of 1960, and in the following summer the virus spread out explosively in this region. In addition, attempts to isolate Coxsackie B5 virus were not successful from clinical specimens of aseptic meningitis obtained in the years before and after 1960 and 1961 in Northern Japan.

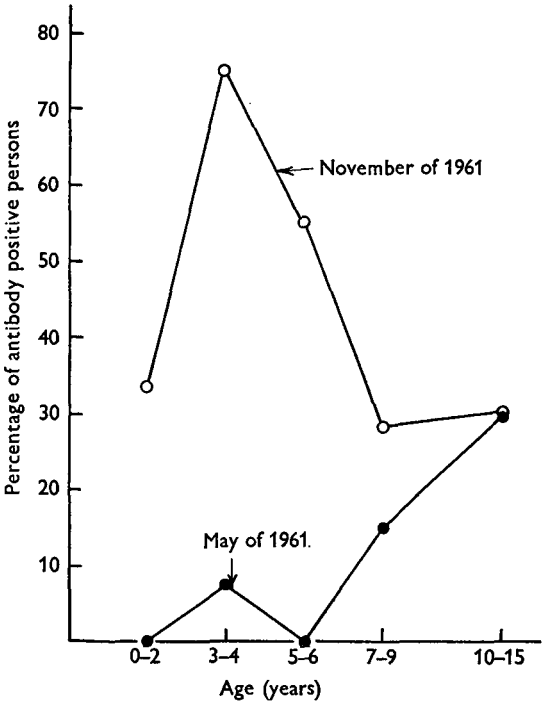

Fig. 1. Age-specific antibody patterns to Coxsackie B5 virus of the children in Aomori, before and after epidemic in summer of 1961. The children bled on the two occasions were not the same. Positive antibody was determined as a titre of 4 or more against 100 TCID50 of the AM-69-L-61 virus.

Coxsackie B5 has caused fairly large epidemics of aseptic meningitis in the U.S.A. in 1956 and 1957 (Syverton *et al.* 1957; Rubin *et al.* 1958; Curnen *et al.* 1958; Gordon, Lennette & Sandrock, 1959); in Canada in 1958 (Walker, McNaughton & McLean, 1959; Cooper, Lesiak, Belbin & Labzoffsky, 1961) and in Japan in 1960 (Kono *et al.* 1960). The accumulated evidence thus appears to show that

Coxsackie B5 virus is one of the important enteroviruses which can induce extensive epidemic aseptic meningitis.

The fact that 17 patients were diagnosed as Coxsackie A9 aseptic meningitis by a serological test should not be ignored in discussing the causative agents in the epidemic described here. Although aseptic meningitis attributable to Coxsackie A9 has been described in many reports, there has been no large epidemic like that due to Coxsackie B5. However, it was interesting that Lerner, Klein, Levin & Finland (1960) reported 15 cases of Coxsackie A9 infection in Boston during July to October 1962 and Lerner, Klein & Finland (1960) also reported a small outbreak which occurred in a laboratory. It seems worth noting that the A9 virus has sometimes produced severe or fatal illness, as in the latter outbreak. The report of Hughes, Webb, Chang & Hart (1963) concerns an outbreak caused by A9 virus in Hong Kong during June to August, 1961. They isolated the virus from spinal fluids of 9 patients. The Coxsackie A9 virus described here was the first isolation in Japan and the strain AM-85-L-61 was one of relatively few strains isolated from spinal fluids so far reported (Davis & Melnick, 1958; Sabin, Krumbiegel & Wigand, 1958; Godtfredsen, 1959; Lerner, Klein & Finland, 1960; Cooper *et al.* 1961; Hughes *et al.* 1963). In addition, we have isolated Coxsackie A9 virus from the spinal fluids of 2 cases with aseptic meningitis in Aomori, 1962. The details of this study will be reported elsewhere.

During the course of the present studies, we have encountered several interesting virological and serological facts. One of them was that Coxsackie B5 virus antigen was detected by immunofluorescence in the urinary cells excreted from the patients with aseptic meningitis, as reported by Hinuma, Miyamoto, Murai & Ishida (1962). Demonstration of Coxsackie B5 plaque variants isolated from the alimentary tract in HeLa cells was also interesting, in a comparison with plaque variants of echovirus 6 in faeces observed by Suto, Karzon & Bussel (1962). As for the effect of subsequent passage upon the plaque variants, the answer should await further studies.

In general, viral infection produces the homologous antibody in the course of the illness. However, the Coxsackie A9 virus infection observed in this study was unusual. Eight of 11 patients who excreted Coxsackie A9 virus did not show detectable neutralizing antibody against 100 TCID50 of the virus. The complement-fixing antibody was also lacking. Johnsson, Böttiger & Löfdahl (1958) reported that presence of neutralizing antibody was not demonstrated, by the conventional tube test, in the convalescent serum of a patient from whose faeces echovirus 4 was recovered. In their case, however, the complement-fixing antibody was detectable. Since both neutralizing and complement-fixing antibodies were not detectable in the serum of the Coxsackie A9 positive patient, the Coxsackie A9 infections appear to be different in their pathogenesis from the echovirus 4 infections. Why a large proportion of persons having alimentary tract infection with Coxsackie A9 did not produce detectable antibodies is unsolved as yet.

SUMMARY

Virological and serological studies on an outbreak of aseptic meningitis in association with Coxsackie B 5 virus, and Coxsackie A 9 virus to a lesser extent, are reported. Coxsackie B 5 virus was isolated from spinal fluids of 60 patients and Coxsackie A 9 virus from one spinal fluid. Fifty Coxsackie B 5 and 13 Coxsackie A 9 viruses were recovered from patients' faeces. The evidence that the epidemic was caused by these viruses was confirmed by determination of neutralizing antibody in paired sera.

The plaque variants of Coxsackie B 5 virus isolated from the faecal specimens and the poor antibody response in Coxsackie A 9 infection were reported as points of virological and serological interest.

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