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Enterovirus type 70: the etiologic agent of pandemic acute haemorrhagic conjunctivitis

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A new enterovirus, now classified as enterovirus type 70, was isolated from the conjunctiva of patients with acute haemorrhagic conjunctivitis during the 1971 epidemics that occurred in Japan, Singapore, and Morocco. These epidemics were parts of a pandemic involving Africa (Algeria, Ghana, Morocco, Nigeria, and Tunisia), Asia (Cambodia, China (Province of Taiwan), Hong Kong, India, Indonesia, Japan, Malaysia, the Philippines, Singapore, and Thailand), and England during 1969–71. A representative strain from each of the three epidemic areas was studied cooperatively. The strains exhibited the physicochemical characteristics of enteroviruses. Cross-neutralization tests showed that these viruses were distinct from all known human enterovirus immunotypes, but that they were antigenically closely related. The human origin of the viruses was demonstrated by the appearance of homologous neutralizing antibodies during convalescence in patients with acute haemorrhagic conjunctivitis.

A pandemic of a distinctive form of conjunctivitis occurred in different parts of Africa, South-East Asia, Japan, India, and England during 1969–71. The symptoms of this eye infection, termed acute haemorrhagic conjunctivitis (AHC) (10), are sudden swelling, congestion, watering, and pain in the eyes. The incubation period appears to be about 24 hours. The most characteristic sign is subconjunctival haemorrhage, varying from discrete petechiae to large blotches of frank haemorrhage covering the whole bulbar conjunctiva. Corneal involvement often occurs in a form of epithelial keratitis, but it is transient, seldom leaves subepithelial opacities, and the prognosis is generally favourable. The symptoms subside rapidly, usually by the third day of the disease, and recovery even in the absence of any treatment is complete within the following week. There are usually no other systemic involvements. However, neurological complications such as acute, essentially lumbar, radiculomyelopathy were reported in some patients during the 1971 epidemic in Bombay (2, 20, 28). The disease seems to be highly contagious in unhygienic and crowded conditions, and in its rapidity of spread resembles influenza. The majority of cases in Japan and elsewhere occurred in adults, with a somewhat higher incidence in males (5, 10).

The first reports on the epidemic prevalence of a new type of conjunctivitis (sometimes called Apollo 11 disease) during 1969 came from Ghana (3, 4) and Nigeria (1, 16). Then in the course of 1970–71 a disease with clinical and epidemiological features similar to the one observed in the two African countries spread to involve practically the whole of South-East Asia. At a WHO Regional Centre seminar held in Manila in December 1971 and from the epidemiological reports on the occurrence of the disease (22) it appeared that the countries affected were Cambodia, China (Province of Taiwan), Hong Kong, India (17), Indonesia, Japan (10), Korea (9), Malaysia, the Philippines, Singapore (11, 12, 23-25), and Thailand. A similar epidemic was also observed during this period in Tunisia (13), Algeria, Morocco (5, 29), and London (7).

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Laboratory investigations to determine the causative agents of AHC were carried out in several parts of the world. Although Chaterjee et al. failed to recover the virus from their epidemic material, they demonstrated that adenoviruses, trachoma agents, and bacteria were not etiologically involved (3, 4). In contrast, virological examination of conjunctival swabs from patients in Japan (10), Singapore (12, 23–25), Morocco (5, 29), Bangkok (Thongcharoen, personal communication to Kono), Hong Kong (Chang, personal communication to Yin-Murphy), and London (6) resulted in the isolation of several cytopathogenic agents with the general characteristics of an enterovirus. However, in none of the above laboratories could the isolates be neutralized with antisera against known enteroviruses and there remained the possibility that a new enterovirus type was the causative agent of the 1971 AHC epidemics.

Three representative isolates originating from the 1971 outbreaks of AHC in Japan, Singapore, and Morocco were submitted to the WHO International Virus Reference Centre, Houston, Texas, for further investigation and comparative antigenic studies. The results of this collaborative study, in which the California Viral and Rickettsial Disease Laboratory also participated, are presented in this paper. They support the findings of the earlier investigators (5, 10, 12, 24, 25, 29) that closely related strains of an unrecognized enterovirus were responsible for the 1971 epidemics of AHC in Japan, Singapore, and Morocco. The new agent is classified as enterovirus type 70.

MATERIALS AND METHODS

Tissue culture

All tests were conducted in tube cultures of serially passaged human embryonic lung fibroblasts (HEL) of Houston origin. Cell cultures were maintained in Eagle's minimum essential medium, supplemented with 2% inactivated fetal bovine serum.

Viruses

The isolates J 670/71 (Japan), EC 146/71 (Singapore), and R 6/71 (Morocco) were received as either human embryonic kidney (HEK) or HeLa cell passages; they are referred to as the J, S, and M strains, respectively. The J and M viruses propagated well in HEL cells. In contrast, the S strain required intermediate passages in primary cynomolgus kidney (Cyno) cells before it could replicate in HEL cells. The virus titres were calculated 7 days after inoculation and averaged $10^{5.5}$ TCD_{50} per ml.

Typing antisera

Typing sera prepared in horses against the 42 enterovirus immunotypes were used in the form of the Lim–Benyesh–Melnick (LBM) typing pools A–H (19). Horse antisera to the group A coxsackievirus prototypes, apart from those already included in the LBM pools (14, 15), were used either as 7 combination typing pools prepared at the 50 neutralizing unit level or as individual sera. The immune sera against representative 1971 AHC isolates were prepared in cynomolgus monkeys and were made available by Kono, Yin-Murphy, and Sohier. Schmidt supplied serum of hamster origin against coxsackievirus A19 (Dohi), as well as monkey serum against enterovirus 68 (Fermon). Monkey serum against DN19 (coxsackievirus A24 variant) used in the Baylor laboratory was prepared by McCrae at the Central Public Health Laboratory, London (18). In Schmidt's laboratory, hamster antisera to the variants of coxsackievirus A24 (Baltazar, Pett, Hu39 and DN19) were employed, as well as immune sera against rhinovirus types 1–89.

Neutralization tests

Typing sera (pooled or single) were inactivated at 56°C for 30 min and then mixed with an equal volume of test virus that had been diluted to contain approximately 100 TCD_{50} of virus per 0.1 ml. The mixtures were incubated for 2 h in a 37°C water bath, and then overnight at 4°C. Virus–serum mixtures were inoculated in 0.2-ml amounts into each of 4 HEL cell culture tubes. Control virus titrations were included in each test. Inoculated cultures were held in roller drums at 37°C for 7 days during which period several readings were made in the course of cytopathologic investigation.

RESULTS

Recovery of the 1971 AHC viruses from patients in Japan, Singapore, and Morocco

According to the reports from the participating laboratories, the virus isolations were made from conjunctival scrapings or conjunctival swabs by inoculation in HEK cells in Japan, or HeLa cells in Singapore and Morocco. Primary monkey cells were found unsuitable for virus isolation, but once isolated in cells of human origin, the strains could be easily adapted to grow in primary monkey cells.

Characterization of the 1971 AHC viruses

The viruses isolated in Japan, Singapore, and Morocco (as was shown in the respective laborato-

ries) shared the general properties of an enterovirus. Electron microscopic examination of the viruses revealed virions with cubic symmetry and a diameter of 25–30 nm. Infectivity of the viruses was not affected by treatment with 20% diethyl ether for 18 h at 4°C, or by pH 3.0 for 4 h at room temperature. The isolates were found to contain a ribonucleic acid genome as demonstrated by (*a*) failure of 5-iodo-2'-deoxyuridine to inhibit virus replication (10) and (*b*) staining the infected cells with acridine orange (Yin-Murphy, personal communication). The J strain treated with 1M $MgCl_2$ was stabilized to thermal inactivation at 50°C for 1 h. All 3 isolates were tested for pathogenicity in newborn mice, with negative results. However, the J strain proved to possess some neurovirulence in cynomolgus monkeys that were inoculated intraspinally as well as intrathalamically (27). In Sohier's laboratory, haematoxylin and eosin staining of HeLa cell cultures infected with the M strain virus disclosed eosinophilic cytoplasmic inclusions. The J strain was banded by gradient centrifugation in cesium chloride at a density of 1.34 g/ml (Yamazaki et al., unpublished data).

Serologic response in patients with AHC

It was shown in Japan that patients with AHC from two different areas (Tokyo and Hokkaido) responded in their convalescence to the J strain with four-fold or greater increases in neutralizing antibodies. In Singapore, the virus neutralization tests were extended and carried out with both the S and J strains: 18 paired sera, collected during the 1971 AHC epidemic in Singapore, were thus tested. The increase in neutralizing antibody in convalescence was the same to both strains in 11 cases. In 7 instances, however, the antibody rise to the S strain was higher by four-fold or more than the antibody rise to the J strain isolate (24, 25).

Attempts to type the 1971 AHC isolates with known enterovirus antisera

The J and S strains were tested against 42 typing sera in pools, but no neutralization of virus infectivity occurred (10, 25). The M isolate was tested in the Lyon laboratory against sera to the entire group of enteroviruses (except enterovirus 68), with negative results. Conversely, the M antiserum did not neutralize any of the presently known enterovirus prototypes.

At the Baylor laboratory the 3 isolates were retested against the LBM typing pools (horse sera) with no demonstrable neutralization. However, a certain degree of inhibition of all 3 viruses was obtained with some of the LBM pools. This was particularly pronounced with the M and S isolates. A partial neutralization (i.e., up to 3 of the 4 inoculated tubes) or a delayed appearance of cytopathic effect occurred in the presence of pools that did not form a correct pattern for identification. In additional tests with the LBM pools diluted to contain 10–20 instead of the standard 50 neutralizing units, or in tests with a higher virus test dose (>320 TCD_{50}) neutralization was not observed. It was concluded, therefore, that the erratic inhibition, in the presence of 50 neutralizing units of antibody and a test virus dose of <100 TCD_{50}, did not represent antigenic relatedness. None of the three 1971 AHC viruses was neutralized by the monkey serum against enterovirus 68.

When tested with 7 preliminary combination pools of coxsackievirus group A antisera, scattered neutralization of all three isolates occurred. In the presence of pools with reduced numbers of neutralizing units (20 instead of 50) no inhibition of M and S isolates was apparent. In contrast, 3 pools (1, 2, and 4) that contained antisera to coxsackie A19 still neutralized the J isolate, thus tentatively identifying it as coxsackievirus A19. The J isolate was subsequently retested against the individual antisera to coxsackievirus A group, and the results of these neutralization tests are presented in Table 1. As expected, the coxsackievirus A19 antiserum (used at 1 : 240 dilution in pools 1, 2, and 4) neutralized the J virus to a titre of 1 : 320, but the titre against homologous virus was $\geq$1 : 4 800. Further tests were carried out with the pooled sera collected from the horses prior to immunization with coxsackievirus A19, and with an antiserum to coxsackievirus A19 from hamsters immunized with the same strain (Dohi) as that used for the inoculation of horses. Preimmune horse serum pool neutralized the J virus to a titre of 1 : 60, whereas the A19 hamster antiserum failed to do so in a 1 : 10 dilution. Mouse neutralization tests performed in Kono's laboratory showed that J antiserum did not neutralize coxsackievirus A19 (Dohi strain) in the lowest dilution tested (1 : 10). Moreover, Kono was able to demonstrate neutralizing antibody to J virus in 8 of 13 preimmunization horse sera[1] tested, in titres ranging from 1 : 40 to 1 : 320. These findings on the whole suggest that occasionally horses may be naturally infected by a virus sharing antigens with the J isolate.

[1] Obtained from the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., USA.

Table 1. Neutralizing antibody titres (50% end-points) of coxsackievirus group A (CA) antisera against J and homologous CA viruses

Antiserum [a]	Antibody titres against: Virus J 670/71	Homologous CA virus [b] (tissue culture or mouse neutralization tests)
CA1–CA12	negative [c]	1 : 1 550–1 : 12 000
CA13	1 : 10	1 : 3 770
CA14	negative	1 : 14 500
CA15	1 : 10	1 : 2 580
CA16	1 : 10	1 : 2 300
CA17	negative	1 : 2 000
CA18	1 : 32	1 : 4 520
CA19, preimmune horse	1 : 60	negative
CA19, immune horse	1 : 320	1 : 4 120
CA19, immune hamster	negative	1 : 4 096 [d]
CA20	1 : 20	1 : 7 150
CA21–CA22	negative	1 : 3 700–1 : 6 400
CA24	negative	1 : 406
DN19 (CA24) immune monkey	1 : 100	1 : 1 000
DN19 (CA24) immune hamster [d]	negative	1 : 256

[a] Unless otherwise stated, the antiserum was prepared in horses.
[b] Data from Melnick & Hampil (14, 15).
[c] Negative in 1 : 8 or 1 : 10 dilution.
[d] Results from Schmidt's laboratory.

The DN19 monkey antiserum used in the Baylor laboratory was found to neutralize the J virus to a titre of 1 : 100, whereas the serum neutralized the homologous virus to a titre of 1 : 1 000. In contrast, neutralization of the DN19 virus was not demonstrable by J antiserum at a dilution of 1 : 10. The cross-neutralization tests were repeated in Schmidt's laboratory with a DN19 immune serum produced in hamsters. Although the homologous neutralizing titre was 1 : 256, the serum did not contain neutralizing antibodies to the J virus at a dilution of 1 : 8. In view of these findings the S and M 1971 AHC isolates were not tested against DN19 immune sera.

Furthermore, in neutralization tests performed in Schmidt's laboratory, none of the antisera to other coxsackievirus A24 variants (Baltazar, Pett, and Hu39) neutralized the J isolate, nor did any of the immune sera against rhinovirus types 1–89. The indication for testing the J virus with the latter sera was the observation by Kono, later confirmed by Schmidt, that the J virus grew better at 33°C than at 37°C.

Cross-neutralization tests with the 1971 AHC viruses

The three 1971 AHC viruses J, M, and S were compared with a 1970 isolate, EH 24/70, recovered during the first outbreak of AHC in Singapore and still unidentified when this paper was being written. As illustrated in Table 2, the 1971 AHC isolates form one antigenically related group, clearly distinct from the 1970 Singapore virus. These results support the

Table 2. Cross-neutralization tests with the Singapore 1970 AHC epidemic virus (EH 24/70), viruses of the 1971 AHC epidemics (J 670/71, EC 146/71, and R 6/71), and coxsackieviruses A19 and A24

Virus	Neutralizing antibody titres (50 % end-points) of immune serum prepared against: Singapore 1970 EH 24/70 monkey	Singapore 1971 EC 146/71 monkey	Japan 1971 J 670/71 monkey	Morocco 1971 R 6/71 monkey	CA19 preimmune horse	CA19 immune horse	CA19 immune hamster	DN19 (CA24) immune monkey	DN19 (CA24) immune hamster
EH 24/70	320 [a]	< 10	< 10	< 100	24	16		< 10	
EC 146/71 [b] (HeLa)	< 5 [b]	640 [b]	320 [b]						
EC 146/71 (HeLa-Cyno-HEL)	< 10	20	640	4 000	< 10	< 10			
J 670/71	< 10	160	1 600	9 600	60	320	< 10	100	< 8 [c]
R 6/71	< 10	10	800	16 000	< 10	40	< 10		
CA19			< 10		< 10	4 800	4 096		
DN19 (CA24)	< 10		< 10					1 000	256 [c]

[a] Reciprocal of 50 % end-point dilutions.
[b] Yin-Murphy's results.
[c] Schmidt's results.

earlier laboratory findings of Yin-Murphy " that the second, 1971, outbreak of AHC in Singapore was caused by a virus closely related to Japanese isolate J 670/71 and not by reappearance of the Singapore 1970 virus " (24). Although a relationship between all three 1971 AHC isolates was shown in cross-neutralization tests, the strongest crossing was obtained between the J and M viruses. Yin-Murphy obtained a neutralizing titre of 1 : 640 for the homologous S strain grown in HeLa cells (Table 2). At the Baylor laboratory, where a HeLa-Cyno-HEL cell-passaged virus was used, the homologous neutralizing antibody titre was reduced to 1 : 20, even against virus filtered through a 50-nm Millipore membrane. In contrast, J and M antisera neutralized the Baylor HeLa-Cyno-HEL cell-propagated S virus readily, and to significant titres (1 : 640 and 1 : 4 000, respectively). Thus, inability of the homologous antiserum to neutralize the S virus propagated in three different host cell systems could not be explained by the phenomenon of virus aggregation (21). Obviously, additional passages through Cyno and HEL cells had made the S virus antigenically broader than the original virus (isolated and grown in HeLa cells) and thus rendered the virus less neutralizable by the antibody raised against the HeLa cell-passaged " parent " virus.

DISCUSSION

Certain adenoviruses have been implicated in outbreaks in which conjunctivitis or kerato-conjunctivitis was the predominating symptom. Although conjunctivitis may sometimes appear as an accompanying symptom of echovirus or coxsackievirus infections (8), a pandemic of a distinctive form of conjunctivitis caused by an enterovirus has never been recorded before. The viruses of the 1971 AHC epidemics occurring in Japan, Singapore, and Morocco share the general physicochemical properties of enteroviruses. The viruses are antigenically closely related and are distinct from any of the currently recognized human enterovirus immunotypes. Serologic responses in AHC patients supplied substantial evidence for the causative role of these viruses in the human disease. However, the exact site of multiplication of the 1971 AHC viruses has not been defined. Since an adenovirus infection was anticipated, only conjunctival materials were usually taken for virus isolation.

During a hospital outbreak of AHC in Lyon, France, in July 1973, Sohier was successful in isolating a virus closely related to the M virus from stool specimens from four patients. In general, however, the isolation of AHC virus from stool does not seem as easy as that of ordinary enteroviruses. This suggests that the human conjunctiva is not the sole habitat of the 1971 AHC virus. Nevertheless, it may be the primary site of virus infection and multiplication, since AHC usually develops within 24 hours after exposure.

The current epidemiologic information is too sparse to ascertain the original focus of the pandemic although it seems that it started in West Africa. Retrospective seroepidemiological studies in the affected countries are indicated in order to establish how widespread the AHC virus distribution was. Such studies should also include the Singapore 1970 AHC isolate, for its etiologic role in the new AHC syndrome need not have been of strictly local significance. Actually, this was evident during the 1971 AHC epidemic in Hong Kong where 2 viruses, one antigenically related to the J isolate and the other to the Singapore 1970 epidemic strains, were circulating in the community (26).

The 1971 AHC agent is now classified as enterovirus type 70 by the WHO enterovirus reference centres. Since the J strain has been studied more intensively than any of the other 1971 AHC isolates, it is recommended as the prototype.

RÉSUMÉ

ENTÉROVIRUS TYPE 70: AGENT ÉTIOLOGIQUE DE LA CONJONCTIVITE HÉMORRAGIQUE AIGUË PANDÉMIQUE

En 1969-71, une pandémie d'une forme particulière de conjonctivite a atteint différentes régions d'Afrique, d'Asie du Sud-Est, du Japon, de l'Inde et d'Angleterre. Le symptôme dominant de l'affection, appelée conjonctivite hémorragique aiguë (CHA), consiste en une hémorragie sous-conjonctivale, allant des pétéchies localisées aux grandes suffusions hémorragiques couvrant la totalité de la conjonctive oculaire. L'atteinte cornéenne prend généralement l'aspect d'une kératite épithéliale, transitoire, guérissant le plus souvent sans laisser d'opacités.

Le pronostic de la CHA est habituellement favorable. Il semble que l'affection soit extrêmement contagieuse en milieu surpeuplé et de faible niveau hygiénique, et se propage aussi rapidement que la grippe. La période d'incubation, courte, est d'environ 24 heures.

Plusieurs isolats d'entérovirus ont été obtenus à partir de la conjonctive de personnes atteintes de CHA pendant les épidémies de 1971 au Japon, à Singapour et au Maroc, et trois souches représentatives de chacune de ces régions ont été étudiées. Toutes présentaient les caractéristiques physico-chimiques des entérovirus. Les épreuves de neutralisation croisée ont montré qu'elles étaient distinctes de tous les entérovirus précédemment identifiés chez l'homme, et étaient antigéniquement étroitement apparentées. Ces trois souches ont été classées comme entérovirus type 70 par les centres OMS de référence pour les entérovirus et il est proposé que la souche japonaise (J 670/71), la mieux étudiée, soit considérée comme souche prototype du nouveau sérotype.

Les examens sérologiques effectués chez des malades ont apporté la preuve du rôle étiologique de ces virus. Cependant, on n'a pas encore clairement déterminé l'endroit où se multiplie l'entérovirus type 70. Ce dernier a été occasionnellement isolé dans les selles de malades; la conjonctive, bien qu'étant peut-être l'endroit où il se multiplie en premier lieu, n'est pas le siège unique de l'infection.

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