EPIDEMIC KERATO-CONJUNCTIVITIS—AN ADENOVIRUS INFECTION

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Although many claims have been made for the isolation of a viral agent from various outbreaks of epidemic kerato-conjunctivitis (EKC), no single agent could be regarded as the causal organism until the prototype strain of adenovirus (APC virus) Type 8 was isolated from a typical case in U.S.A. by Jawetz, Kimura, Hanna, Coleman, Thygeson & Nicholas (1955). Serological tests by these authors and Jawetz, Thygeson, Hanna, Nicholas & Kimura (1956) showed that neutralizing antibodies were present in a high proportion of the cases of EKC from various countries, whereas such antibody was absent in other individuals from the same areas. About the same time, Ormsby, Fowle & Cockeram (1955) isolated four strains of adenovirus Type 3 from patients suffering from follicular kerato-conjunctivitis; only one of these patients had neutralizing antibody for the prototype strain of adenovirus Type 8. More recently further conflicting evidence has been produced by Jawetz, Hanna, Kimura & Thygeson (1956) who isolated two Type 6 adenoviruses, and found rising titres of homotypic neutralizing antibody in two cases of follicular conjunctivitis. The association between adenoviruses and other forms of conjunctivitis is well established. Adenovirus Type 3 was identified as the causal organism of pharyngo-conjunctival fever by Bell, Rowe, Engler, Parrott & Huebner (1955), and Type 7 has been isolated by Andrews & McDonald (1957) from cases of punctate subepithelial keratitis.

During a recent outbreak of EKC in the West of Scotland, two groups of patients were subjected to detailed laboratory investigations with a view to assessing the aetiological role of the adenoviruses in this outbreak, and also with a view to assessing the adenovirus type involved. In this paper the results of the laboratory investigations will be described.

The clinical aspects of the outbreak which involved more than 4000 persons between October 1955 and August 1956 have been summarized by Sommerville (1957) and will be described elsewhere in detail by Dr J. Winning.

MATERIALS AND METHODS

The first group of patients was used for virus isolation studies. It consisted of fifty-nine persons who were attending ophthalmological clinics in Glasgow during the early stages of the illness. The cases were selected on clinical grounds as typical early acute cases of EKC.

The second group consisted of fifty-nine male workers in heavy industry, the majority of whom were employed in shipyards. Blood specimens were obtained

from all for antibody determinations and no attempts were made to isolate the virus. The duration of the disease was less than 10 days at the time of bleeding in seven patients who were selected from outpatients attending eye clinics with EKC, and from these second serum specimens were obtained between the second and fourth week of illness. One serum specimen only was obtained from each of thirty-six patients attending works ambulance rooms with EKC, and from a further sixteen similar patients a second sample of serum was obtained 6 months after the first.

Virus isolation methods

Specimens for virus isolations consisted of cotton-tipped swabs which had been rubbed on the affected conjunctival surface, tears and faecal specimens. In every instance the specimen was delivered to the virus laboratory within an hour or two of collection. Each conjunctival swab was extracted in a few ml. of sterile broth containing antibiotics. Specimens of tears were collected into a sterile capillary tube and virus isolation was attempted without dilution. A suspension of faecal material (approximately 10 %) in Hanks' solution with added antibiotics, was clarified by centrifugation at 3000 r.p.m. for 30 min. The supernatant was used for virus isolation.

0·2 ml. of the prepared specimen was inoculated into each of two tube cultures of HeLa cells maintained in Hanks' solution containing 0·5 % lactalbumen hydrolysate and 2 % calf serum. After 7–10 days' incubation at 37° C. in a roller-drum machine the fluid from the two tubes was pooled and 0·2 ml. inoculated into each of two fresh tubes. Examination of each specimen was continued for a total of 21 days in tissue culture, unless specific virus degeneration had occurred before this.

Viruses were identified by neutralization tests in HeLa cells using high-titre antiserum prepared in rabbits against type strains of adenovirus.

In an attempt to exclude the possibility that some patients might be suffering from infection with the virus of herpes simplex, small pools containing an aliquot from each of four or five conjunctival swab extracts were inoculated into suckling and adult mice; 0·1 ml. was injected by the intraperitoneal and 0·025 ml. by the intracerebral route. The mice were observed daily for signs of illness and were killed after 21 days of observation.

Serological methods

Sera were examined by complement fixation (C.F.) tests with the adenovirus group antigen. This was derived from the inactivated (56° C. for 30 min.) supernatant of HeLa cell cultures infected with adenovirus Type 4. A small-volume cold-overnight C.F. technique was used.

Neutralization tests were performed in HeLa cell cultures. Serum, inactivated at 56° C. for 30 min., was diluted 1 in 8 in HeLa cell maintenance medium, and 0·2 ml. of this dilution was mixed with an equal volume of virus suspension. The volume was made up to 2·0 ml. with maintenance medium, and the mixture incubated for 1 hr. at room temperature before adding 1 ml. to each of two cultures of HeLa cells. Each serum was examined for neutralizing antibody against adeno-

virus Types 3, 6, 7 and 8. Where neutralization occurred titration of the serum in serial dilutions was performed. Normally the viruses were diluted between 1 in 2 and 1 in 10; each dilution contained between twenty and fifty tissue culture infectious doses (T.C.D. 50). Considerable difficulty was experienced in obtaining consistent results with Type 8 adenovirus. Batches of HeLa cells appeared to vary in susceptibility and the virus titre was always very low. Tests frequently had to be discarded because of lack of cytopathogenic effect in the Type 8 virus control, and results were recorded only from tests in which the control tubes degenerated satisfactorily. Similar difficulties in handling Type 8 adenovirus have been reported by Jawetz *et al.* (1955). Neutralization tests were read 24 hr. after the virus control tubes showed 4+ (++++) degeneration and the test results were scored 0 to 4+ depending on the degree of cytopathogenic effect present.

C.F. and neutralization titres are expressed in each case as the reciprocal of the final serum dilution.

RESULTS

The results of virus-isolation studies are shown in Table 1. The absence of virus growth from conjunctival swabs is especially noteworthy. The Type 3 adenoviruses were isolated from different individuals, but in neither instance was serum available for antibody determinations to establish the pathogenic relationship of the virus.

Table 1. *Results of virus-isolation studies on fifty-nine patients with epidemic kerato-conjunctivitis*

	HeLa cell culture			Suckling and adult mouse inoculation	
Specimen	Total examined	No. positive	Virus type isolated	Total examined	No. positive
Conjunctival swab	49	0	0	49	0
Tears	2	1	Adenovirus Type 3	N.T.*	0
Stool	8	1	Adenovirus Type 3	N.T.	0

* N.T. = not tested.

To facilitate description, the cases examined serologically have been separated into three subgroups. The first contained seven patients whose illness had been present for less than 10 days at the time of first bleeding, and from whom a second blood specimen was obtained between the second and fourth weeks of illness. The results of serological examinations on these patients are recorded in Table 2. Fourfold or greater increases in titre of antibody to adenovirus were found by C.F. test in five patients; six showed a similar rise in neutralizing antibody to adenovirus Type 8, and none developed neutralizing antibody to Types 3, 6 or 7.

One serum specimen only was obtained from each of the thirty-six cases in subgroup 2, and the results are shown in Table 3. In 3 instances sufficient serum was obtained to allow examination by C.F. test only. The disease had been present for 14 days or less in nine of the remaining thirty-three cases and in these C.F. tests

Table 2. *Adenovirus antibody titres by complement fixation and neutralization tests on acute and convalescent phase sera from seven patients with epidemic kerato-conjunctivitis*

Case no.	Interval between specimens (weeks)	C.F. titre	Neutralization titres against adenovirus Types 3	6	7	8
3	4	<8:16	8:8	<8:<8	<8:<8	<8:16
6	4	<8:32	4:8	<8:<8	<8:<8	<4:256
7	1	16:16	4:4	<8:<8	<8:<8	8:16
8	2	<8:16	16:16	16:16	<8:<8	<8:64
11	2	<8:16	<4:4	8:8	<8:<8	<8:32
12	3	16:64	16:16	<8:<8	<8:<8	<8:32
16	1	8:16	8:8	<8:<8	8:8	<8:16

Table 3. *Results of complement fixation and neutralization tests on single serum specimens from thirty-six patients with epidemic kerato-conjunctivitis*

Duration of disease	Adenovirus complement fixation titre	Adenovirus neutralization titres: Type 8 <8–8	16	32–>32	Type 3* <8–8	16	32–>32	Type 6* <8–8	16	32–>32	Type 7* <8–8	16	32–>32
Less than 14 days	<8–8	6	0	0	2	4	0	6	0	0	6	0	0
	16–>16	3	0	0	2	1	0	3	0	0	3	0	0
More than 14 days and up to 112 days	<8–8	3	0	0	0	0	0	0	0	0	0	0	0
	16–>16	5	6	13	9	11	1	21	0	0	21	0	0

* Insufficient serum prevented examination by neutralization tests against adenoviruses Types 3, 6 and 7 in three instances.

revealed an adenovirus antibody titre of sixteen or greater in only three instances. None contained significant amounts of neutralizing antibody to adenovirus Types 3, 6, 7 or 8. In the absence of second serum specimens C.F. titres of 16 or over and neutralization titres of 32 or over were regarded as significant.

The disease had been present for longer than 2 weeks at the time of bleeding in the remaining twenty-four patients and in these the antibody titre by C.F. tests was 16 or greater in all. Neutralizing antibody to Type 8 adenovirus was present to a titre of 32 or greater in thirteen cases while a similar level to Type 3 was present in only one. No serum contained significant amounts of neutralizing antibody to either Type 6 or 7 adenovirus.

The third subgroup to be examined serologically contained sixteen patients and resembled group 2 in the variable duration of the disease at the time of first bleeding, although it differed in that a second serum specimen was obtained from each case 6 months after the first. In each instance the duration was less than 2 months when the first blood specimen was obtained and the serological results are shown in Table 4. A titre of 16 or greater was obtained by C.F. tests with nine sera and of these six and three neutralized Types 8 and 3 adenoviruses, respectively, to a similar titre. In this group of first specimens a significant C.F.

Table 4. *Results of complement fixation and neutralization tests on paired serum specimens taken at an interval of 6 months from sixteen patients with epidemic kerato-conjunctivitis*

Duration of disease	Adenovirus complement fixation titre	Adenovirus neutralization titres: Type 8			Type 3*			Type 6*			Type 7*		
		<8–8	16	32–>32	<8–8	16	32–>32	<8–8	16	32–>32	<8–8	16	32–>32
1–2 months	<8–8	4	0	3	5	2	0	5	0	0	5	0	0
	16–>16	3	4	2	6	3	0	8	1	0	8	0	1
6–8 months	<8–8	1	2	8	8	3	0	8	0	0	7	1	0
	16–>16	0	2	3	3	2	0	4	0	0	4	0	0

* Insufficient serum prevented examination by neutralization test against adenovirus Types 3, 6 and 7 in some instances.

titre was associated with a neutralization titre of 16 and 32 with each of Types 6 and 7 adenovirus, respectively, in one instance, although the patient with Type 7 antibody also gave a titre of 64 against adenovirus Type 8.

After an interval of 6 months five sera still gave a titre of 16 or over both by C.F. and by neutralization tests against adenovirus Type 8, while only two neutralized Type 3 adenovirus to a titre of 16. No neutralizing antibody to Type 6 or 7 virus was present in the second specimens.

DISCUSSION

These results appear to indicate that the causal agent in the majority of persons who were examined serologically was adenovirus Type 8. The failure to isolate virus in some ways supports these serological findings, because the Type 8 adenovirus is probably the most difficult member of this group to propagate in tissue culture. Jawetz (1957), in a personal communication, has suggested that conjunctival scrapings are preferable to material obtained by cotton-tipped swabs for isolation of this virus, and also that fowl serum should replace calf serum in the HeLa cell maintenance medium. In contrast the Type 3 adenoviruses were isolated without difficulty during the first pass in HeLa cells.

The negative results of animal inoculation and the lack of growth in HeLa cells are taken to exclude the possibility of infection with Herpes simplex.

One of the difficulties associated with the identification of the specific antibody type by neutralization tests is the non-specific rise in antibody to other types of adenovirus which may appear during the course of infection. It is possible that this increase in titre of heterotypic antibody may represent a form of anamnestic reaction indicating previous infections. A series of forty patients with various illnesses other than conjunctivitis has been examined in this laboratory for neutralizing antibody to adenovirus Types 1–8, but no person with antibody to Type 8 has been found. In the same series low levels of antibody to Type 3 were encountered frequently (Sommerville, to be published). The presence of neutralizing antibody to Type 8 adenovirus in a high proportion of cases in the present series therefore assumes increased significance.

Fourfold rising titres obtained by C.F. test (Table 2) are reflected in similar titres

of neutralizing antibody to adenovirus Type 8, but the lack of association between titres obtained by C.F. and by neutralization tests with this virus in single-serum specimens is striking (Table 4). In the early phase of illness three patients out of seven, with insignificant C.F. antibody titres, showed neutralizing antibody titres of 16 or over against Type 8 virus, and the proportion was raised to ten out of eleven in the second serum specimens. Similarly, with Type 3 virus, two out of five and three out of six, with insignificant C.F. titres, showed neutralizing antibody titres of 16 in first and second serum specimens, respectively. If C.F. antibody titre alone is used as the criterion of infection with an adenovirus a considerable number of positive cases will clearly be missed altogether. A similar observation has been reported by Balducci, Zaiman & Tyrrell (1956) who found negative C.F., but positive neutralizing antibody titres in two children from whose adenoids adenoviruses had been isolated.

The presence of neutralizing antibody to Type 3 adenovirus in some cases could be due to previous antigenic experience with this virus, and therefore may represent a non-specific effect of reinfection with a heterologous strain. On the other hand, the isolation of two Type 3 adenoviruses must raise the possibility that in at least some cases the Type 3 virus may have been the infecting agent. It is indeed not unlikely that a few cases of pharyngo-conjunctival fever which would have been diagnosed clinically at a non-epidemic period, may have been included accidentally in the presence of a large epidemic of EKC. A similar explanation may well account for the two cases described in Table 4 with antibody to Type 6 or Type 7 adenovirus, although the short duration of the antibody response and the associated Type 8 antibody in one suggests that in at least one instance the antibody represents a non-specific effect to stimulation by a heterotypic adenovirus.

SUMMARY

Two strains of adenovirus Type 3 were isolated from fifty-nine patients suffering from epidemic kerato-conjunctivitis. Serological examination of a further group of fifty-nine patients revealed that neutralizing antibody to adenovirus Type 8 usually developed when the duration of the disease exceeded 14 days, and that at the same time a smaller number of patients also developed low titre neutralizing antibody to Type 3 adenovirus. From the evidence presented it is suggested that adenovirus Type 8 was the infecting organism in the majority of cases, and that when neutralizing antibody to Types 3, 6 or 7 adenovirus was present this probably represented a non-specific effect of stimulation by a heterotypic strain of adenovirus. Insignificant antibody titres by C.F. tests were frequently associated with high titres of neutralizing antibody in the late convalescent phase of the illness.

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