son, E. L., *Fed. Proc.*, 1953, v12, 430.

2. DeBusk, B. G., and Williams, R. J., *Arch. Biochem.*, 1955, v55, 587.

3. Stokstad, E. L. R., Seaman, G. R., Davis, R. J., and Hutner, S. H., *Vitamin Assay Methods*, in press.

4. Bullock, M. W., Brockman, J. A., Jr., Patterson, E. L., Pierce, J. V., von Saltza, M. H., Sanders, F., and Stokstad, E. L. R., *J. Am. Chem. Soc.*, 1954, v76, 1828.

5. Olson, R. E., and Dinning, J. S., *Proc. N. Y. Acad. Sci.*, 1954, v57, 889.

6. Combs, G. F., personal communication, 1955.

7. Norris, L. E., personal communication, 1955.

8. Supplee, *et al.*, *Arch. Biochem. Biophy.*, 1956, v61, 140.

9. Snell, E. E., and Quarles, E., *J. Nutrition*, 1941, v22, 483.

10. Walton, E., Wagner, A. F., Bachelor, F. W., Peterson, L. H., Holly, F. W., and Folkers, K., *J. Am. Chem. Soc.*, 1955, v77, 5144.

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## Antibodies to APC Virus Type 8 in Epidemic Keratoconjunctivitis.* (22394)

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Epidemic keratoconjunctivitis (EKC) is an infectious eye disease with well-defined clinical characteristics. After an incubation period of 7 to 10 days there develops an intense follicular conjunctivitis, sometimes with pseudomembrane formation, accompanied by enlarged tender preauricular lymph nodes. In most cases 5 to 10 days later corneal lesions develop. These are characteristically small, round, subepithelial corneal opacities without superficial ulceration or impaired sensitivity of the cornea. These opacities may persist up to 2 years and may seriously interfere with vision. The disease has occurred in large epidemics in Europe, the Far East, Hawaii, and the Pacific Coast of the United States. Smaller outbreaks are seen from time to time all over the world.

It is generally believed that EKC is of viral etiology and many claims have been made for the isolation of a specific causative agent. These were reviewed by Cockburn(1) who concluded that "there is at present no virus available that can be regarded with confidence as the etiologic agent of EKC." In the course of studies on various types of keratoconjunctivitis we isolated a virus from EKC (2) which has been designated the prototype of APC virus type 8(3). Initial serological observations showed a high incidence of neutralizing antibodies to that virus in patients with EKC and a virtual absence of such antibodies in other individuals(4). Consequently, a comprehensive serological survey was undertaken to explore this association. The present paper summarizes the results obtained to date with sera from several widely separated areas of the world.

*Materials and methods. Viruses.* The virus isolated in tissue culture from a patient with typical EKC ("TRIM") was employed in the 4th to 14th passage in the form of supernatant fluid from infected HeLa cell cultures. This virus (APC type 8) was propagated routinely by inoculating twice-washed HeLa cell cultures obtained from Tuskegee Institute, Ala., with 0.1 ml of undiluted stock virus, adding 0.9 ml of maintenance medium† (10% chick serum in mixture 199 containing Penicillin 500 *u*/ml, Streptomycin 500 $\mu$g/ml, and Rimocidin 10 $\mu$g/ml) and incubating the tubes at 36°C for 2-4 days, until cytopathogenic effects had progressed to 3+ or 4+. The fluid from individual tubes (containing de-

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† All tissue culture media were obtained from Microbiological Associates, Bethesda, Md.

generated and detached cells) was pooled and kept at −20°C until used in neutralization tests. The infective titer of pools never exceeded $10^{-2}$, was not diminished by prolonged storage, and was not increased by disrupting the cells with repeated freezing and thawing (2). The "E.K." strain of St. Louis encephalitis virus was kindly supplied in the form of 8th passage mouse brain by Dr. W. McD. Hammon. It was passed intracerebrally in 21 day Swiss mice and a virus pool consisting of a 20% brain suspension in skim milk was kept at −40°C until used in mouse neutralization tests. *Sera.* Whenever possible paired or serial serum specimens were secured from patients seen during acute EKC. However, in many instances only single sera were available which had been obtained from 2 weeks to 8 years after onset of EKC. To estimate the frequency of antibodies to APC virus type 8 in the general population and in other eye diseases, sera were obtained from a variety of individuals from the same geographic areas and age distribution represented by the EKC patients. We are greatly indebted to Dr. H. L. Ormsby for serum specimens from Canada (Windsor 1951 and Toronto 1953-55), to Dr. M. D. Pearlman for sera from Chicago (1954), to Dr. I. Leopold for sera from Philadelphia collected in 1955 after an outbreak in 1953, and to Doctors H. Konig and R. Witmer for sera from Switzerland (1955). Sera from EKC patients and normals from the Como area of Italy (1955) were kindly supplied by Prof. G. Bietti, and from the Kumamoto area (Kyushu) of Japan (1955) by Prof. Y. Mitsui. Most other sera were secured from patients seen by ophthalmologists in California for either EKC or unrelated eye diseases, and from persons without any eye disease. All sera were stored at −20°C, and were inactivated by heating at 56°C for 30 minutes before use in neutralization tests. *Neutralization tests.* These were performed as previously described(2,4). Briefly, the technic consisted in mixing equal volumes of inactivated serum dilution (in mixture 199) and undiluted virus pool, and incubating the mixture for 30 minutes at room temperature. The mixture (0.2 ml) was then inoculated into twice-washed HeLa cell cultures and 0.8 ml maintenance medium was added. The tubes were incubated at 36°C in a stationary position without change of medium and inspected daily for cytopathogenic effects. Readings were taken for at least 4 days after degeneration of the control tubes (virus plus normal rabbit serum) was complete. Tubes were read at random and cytopathogenic changes were graded in the conventional fashion (0 to $4^+$). Agreement between tubes inoculated with the same mixture was good. The results were taken to indicate neutralization only when there was a difference of $3^+$ in readings of experimental and control tubes for at least 2 consecutive days. The results are expressed as the final dilution of serum in the serum-virus mixture which prevented cytopathogenic effects. Some difficulty was encountered because of the very low titers of the available virus pools (the greatest amount of virus per tube being 50 TC50), and because of some variability in the susceptibilities of different lots of HeLa cells. Certain animal sera delayed cytopathogenic effects, even though they contained no antibody, and could therefore not be employed. It was necessary to include in every neutralization test positive control sera of known antibody content, negative control sera lacking antibody, and virus titrations. The results were accepted for inclusion in this report only when all these controls were satisfactory. The titers of virtually all positive human sera were determined more than once. Repeated determinations of antibody in a given serum specimen generally agreed within one 2-fold dilution of serum. Paired serum specimens were always titrated simultaneously. Mouse neutralization tests with the "E.K." strain of St. Louis encephalitis were performed in the conventional manner, using inactivated undiluted serum and tenfold virus dilutions.

*Results. Frequency of APC 8 neutralizing antibodies.* The present series includes 70 cases of EKC and 140 controls. Of the EKC patients 60 had the typical disease, whereas in 10 others, some feature of the complete picture was lacking, most often keratitis or corneal opacities. The serological results are summarized in Table I. It is seen that the vast majority of EKC patients had neutral-

TABLE I. Incidence of Neutralizing Antibodies to APC 8 Virus in EKC Patients and Controls.

| Sera from | No. sera | Neutralizing antibodies in 1:10 dilution: Present No. | Present % | Absent No. | Absent % |
|---|---|---|---|---|---|
| EKC | | | | | |
| U.S.-Canada | 30 | 29 | 96.7 | 1 | 3.3 |
| Switzerland | 15 | 13 | 86.7 | 2 | 13.3 |
| Italy | 13 | 13 | 100.0 | 0 | .0 |
| Japan | 12 | 11 | 91.7 | 1 | 8.3 |
| EKC–Total | 70 | 66 | 94.3 | 4 | 5.7 |
| Controls | | | | | |
| U.S.-Canada | 104 | 5 | 4.8 | 99 | 95.2 |
| Italy | 24 | 1 | 4.2 | 23 | 95.8 |
| Japan | 12 | 4 | 33.3 | 8 | 66.7 |
| Controls–Total | 140 | 10 | 7.1 | 130 | 92.9 |

izing antibodies in a serum dilution of 1:10 or greater. This was the case in 96.7% of cases of typical EKC and 80% of atypical disease. Such antibodies occurred infrequently in controls including normal individuals, patients with urinary tract infections, skin herpes, or a variety of eye diseases such as herpetic keratitis, uveitis, and several types of conjunctivitis. Because of the greater frequency of EKC in Europe and Japan, it was important to examine sera from normal individuals residing in the same area as the EKC patients. Whereas antibodies to APC 8 in the absence of clinical EKC were rare in North America and Italy, they occurred in 4 of 12 individuals chosen at random in the Kumamoto area of Japan. In that region clinical EKC occurs at a high endemic level at the present time.

Sera from EKC and controls were well matched in age distribution, covering a span of from 10 to 75 years. EKC may occur at all ages but typical cases are uncommon in children(5). The Italian EKC patients were from 18 to 70 years old, and among 24 controls ranging from 11 to 75 years, only one person (34 years old) had an antibody titer of 1:10, and 2 others aged 20 and 47 had a titer of 1:5. The Japanese EKC patients ranged from 11 to 64 years and the controls from 14 to 66 years. The single Japanese EKC patient without APC 8 antibodies was 14 years old, whereas the 4 controls with APC 8 antibodies were 14, 23, 49, and 51 years old. The North American EKC patients as well as controls are weighted toward middle age. The low incidence of APC 8 antibodies in the general population in the Western United States and Canada (Table I) was paralleled by a survey of the clinical population in Washington, D.C.(6). There APC 8 antibodies in a serum dilution of 1:8 were found in 1 of 44 sera (2.2%) from the ages 6 to 15, and in 3 of 46 sera (6.5%) from the ages 31 to 77. The comparable age distribution of EKC cases and controls indicates that the observed differences in the incidence of APC 8 antibodies are not due to errors in sampling.

*Titer of APC 8 neutralizing antibodies.* The distribution of neutralizing antibody titers is shown in Table II. Among 60 patients with typical EKC 62% had a titer of 1:40 or greater and 87% had a titer of 1:20 or greater. Similarly, 8 of 10 cases of atypical EKC had a titer of 1:20 or greater. Of 140 controls only 3 (2.2%) had a titer of 1:20 or greater. Some of the EKC patients included in Table II contracted the disease months or even years before the serum was obtained. This circumstance may have influenced the distribution of antibody titers, because antibody levels decline relatively soon after infection. In Table III antibody titers are arranged according to the interval between onset of EKC and the date of securing the serum specimen. In spite of the small number of patients in some groups the trend is evident. While more than 70% of patients bled within 6 months of onset had antibody titers of 1:40 or greater, this occurred in only

TABLE II. Titer of Neutralizing Antibodies to APC 8 Virus in EKC Patients and Controls.

| Serum from | No. sera | Neutralizing antibodies present in a serum dilution of 1 to: <10 | 10 | 20 | 40 | 80 | >80 |
|---|---|---|---|---|---|---|---|
| EKC | | | | | | | |
| U.S.-Canada | 30 | 1 | 6 | 9 | 5 | 7 | 2 |
| Switzerland | 15 | 2 | 0 | 2 | 7 | 3 | 1 |
| Italy | 13 | 0 | 0 | 5 | 4 | 4 | 0 |
| Japan | 12 | 1 | 0 | 2 | 3 | 5 | 1 |
| EKC–Total | 70 | 4 | 6 | 18 | 19 | 19 | 4 |
| Controls | | | | | | | |
| U.S.-Canada | 104 | 99 | 3 | 2 | 0 | 0 | 0 |
| Italy | 24 | 23 | 1 | 0 | 0 | 0 | 0 |
| Japan | 12 | 8 | 3 | 0 | 1 | 0 | 0 |
| Controls–Total | 140 | 130 | 7 | 2 | 1 | 0 | 0 |

20% of those bled 1-2 years after onset, and in none of those bled more than 2 years after onset. Thus it is apparent that the search for APC 8 antibodies could not be applied reasonably to patients who suffered EKC infection prior to 1951 unless their sera had been preserved from the time of active disease.

*Rise in titer of neutralizing antibodies to APC 8 during acute EKC.* Paired sera were secured from 18 individuals whose initial serum specimen had been obtained before the 14th day after onset of EKC. Seventeen of them reached neutralizing antibody titers of 1:10 or greater at some time during their illness. The remaining one (a Japanese boy aged 14) never developed either keratitis or measurable APC 8 antibodies. Fifteen patients had a 4-fold or greater rise in APC 8 antibodies during their illness (examples in Table IV). The speed and magnitude of titer rise and fall varied greatly. One woman had a peak titer of 1:40 in her initial serum obtained on the 13th day after onset of EKC, showed no further rise, and her titer fell to 1:20 by the 60th day. The majority of patients from whom serial serum specimens were available reached peak titer (1:40 to 1:160) in 3 to 6 weeks after onset of EKC, and maintained it for some weeks. The highest APC 8 antibody titer was 1:640 in a Japanese with severe EKC. We observed one patient who reached a maximum titer of 1:80 on the 44th day after onset, maintained it through the 80th day, but gradually declined to 1:20 by the 380th day. That sequence may well occur in many individuals after EKC infection, and may account for the low titer of APC 8 antibodies in patients bled more than 6 months after the onset of EKC.

TABLE III. Presence of Neutralizing Antibodies to APC 8 Virus at Intervals after Onset of Acute EKC.

| Time after onset of EKC | No. patients | Neutralizing antibodies present in a serum dilution of 1 to <10 | 10 | 20 | 40 or greater |
|---|---|---|---|---|---|
| <6 mo | 56 | 3 | 1 | 12 | 40 |
| 1-2 yr | 19 | 1 | 3 | 4 | 2 |
| 2-4 " | 4 | 0 | 2 | 2 | 0 |
| 8 " | 5* | 2 | 2 | 1 | 0 |

* These 5 patients are not included on the present series of 70 patients with EKC.

TABLE IV. Homotypic and Heterotypic Antibody Titers in Patients with EKC Infections.

| Location | Patient | Days after onset | Titer of antibodies to APC 8 | APC 3 |
|---|---|---|---|---|
| Japan | Hira | 3 | 10* | 40 |
| | | 24 | 320 | 40 |
| | | 31 | 640 | ND |
| " | Naga | 3 | 10 | 80 |
| | | 24 | 40 | ND |
| | | 31 | 80 | 80 |
| " | Kota | 13 | 10 | 5 |
| | | 20 | 40 | ND |
| | | 37 | 80 | 10 |
| Chicago | Quar | 4 | <10 | <10 |
| | | 33 | 80 | <20 |
| " | Lars | 3 | <10 | 10 |
| | | 19 | 40 | 20 |
| " | Hans | 2 | <10 | 40 |
| | | 24 | 80 | 40 |
| Calif. | Cott | 6 | <10 | 20 |
| | | 80 | 80 | 40 |

* Reciprocal of highest final serum dilution neutralizing approximately 50 $TC_{50}$ of virus.
ND = Not done.

*Antibodies to other APC types in EKC.* In view of the antigenic relationships which exist within the group of APC viruses, heterotypic antibody rises must be considered. APC type 3 is a common cause of external eye infection. Therefore, it seemed desirable to investigate possible serological cross reactions between APC types 3 and 8. In Table IV are listed the APC 8 antibody titer changes in a few representative patients with EKC. The rise in APC 8 antibody titer was not accompanied by a heterotypic rise in APC 3 antibody titer. We tested 10 serum pairs from individuals with infections due to APC types 2, 3, and 6. There was a significant homotypic antibody titer rise, but no rise to APC 8. Thus it appears that the rise in neutralizing antibody titer to APC 8 is not due to infection by other known APC types, but most likely is caused by APC 8 infection.

*Antibodies to the preserved "E.K." virus or St. Louis encephalitis virus.* A virus prominently associated with EKC in the literature was isolated by Sanders(7). At present its nature and whereabouts are uncertain and the original agent may have been lost. The strains labeled "E.K." virus and preserved in

several laboratories, and perhaps derived from Sanders' isolation, are immunologically closely related to St. Louis encephalitis(8). We tested 15 sera from patients with typical EKC against this virus by mouse neutralization test. All these sera contained antibodies against APC 8 virus. None of them neutralized the "E.K." virus to any significant extent. Thus there is no serological evidence that the virus labeled "E.K." is associated with the disease EKC as it has occurred since 1951(1).

*Discussion.* The serological data presented in this paper reveal a striking and unequivocal association between epidemic keratoconjunctivitis and neutralizing antibody to APC virus type 8. This antibody can be presumed to have arisen as a result of infection with APC virus type 8, or an antigenically closely related agent. The rise in antibody titer which occurs during the acute phase of EKC and the gradual loss of titer thereafter are in agreement with this concept. The low incidence of APC 8 antibodies in the general population in America and Italy contrasts with the more frequent occurrence of such antibodies in individuals without EKC in Japan. In Japan EKC occurs at a high endemic level and subclinical infections are to be expected, whereas in America and Italy, the disease is only seen in occasional circumscribed epidemics. All these features support the concept that infection with APC virus type 8 (or an agent closely related antigenically) is regularly associated with clinical EKC. This statement applies only to patients infected with EKC between 1951 and 1955. Because of the rapid fall in APC 8 antibody titer within a year of onset of EKC, one cannot expect to find with any regularity significant antibody titers to APC 8 in individuals who contracted EKC prior to 1951. Thus it is not possible to state whether APC 8 infection was associated with EKC prior to that time, unless sera collected within one year of onset of EKC could be tested. Unfortunately, we have been unable as yet to secure any such specimens.

The outbreaks of EKC in America prior to 1950 have been generally attributed to the agent described by Sanders(7). The presently available strains of "E.K." virus—possibly derived from Sanders' agent—are not neutralized by sera from patients who contracted EKC since 1951(1,8). Thus it is evident that typical EKC occurring between 1951 and 1955 in Italy, Switzerland, America, and Japan were not associated with infection by that agent.

The present serological study strongly suggests that APC 8 virus (or an antigenically related agent) is involved in the production of the clinical disease EKC. In order to establish its etiologic role it would be necessary to isolate the virus regularly from the disease and to reproduce the typical illness by experimental inoculations of human volunteers. Such studies are currently under way.

*Summary.* The sera from patients with epidemic keratoconjunctivitis (EKC) in Japan, Italy, Switzerland, and North America regularly contain neutralizing antibodies to APC virus type 8, whereas such antibodies are absent from the general population in these areas. Paired sera from patients with EKC show a significant rise in neutralizing antibodies to APC virus type 8. These antibodies persist for only a limited period after the onset of the disease. The constant association of clinical EKC and antibodies to APC virus type 8 suggests that this agent (or antigenically related virus) may play a role in the etiology of this disease.

1. Cockburn, T. A., *Am. J. Ophthal.*, 1954, v38, 476.

2. Jawetz, E., Kimura, S. J., Hanna, L., Coleman, V. R., Thygeson, P., and Nicholas, A., *ibid.*, 1955, v40, 200.

3. Huebner, R. J., Bell, J. A., Rowe, W. P., Ward, T. G., Suskind, R. G., Hartley, J. W., and Pfaffenbarger, R. S., *J. Am. Med. Assn.*, 1955, v159, 986.

4. Jawetz, E., Kimura, S., Nicholas, A., Thygeson, P., and Hanna, L., *Science*, 1955, v122, 1190.

5. Mitsui, Y., Tanaka, C., and Yamashita, K., *Am. J. Ophthal.*, 1955, v39, 540.

6. Rowe, W. P., Huebner, R. J., personal communication.

7. Sanders, M., and Alexander, R. C., *J. Exp. Med.*, 1943, v77, 71.

8. Cheever, F. S., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 125.

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