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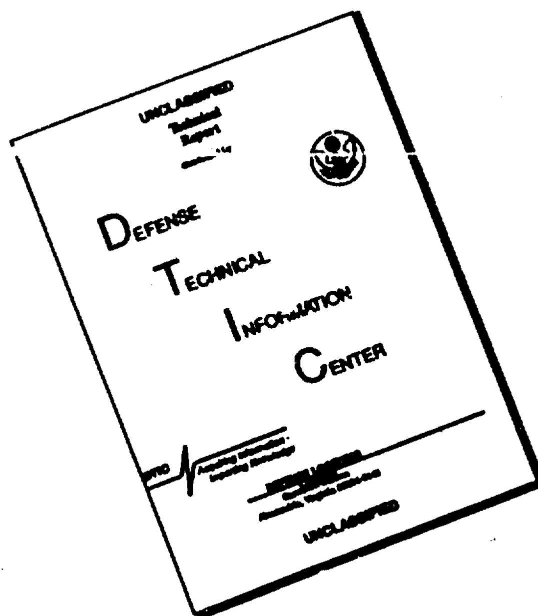

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SPECIFIC ANTIGEN PROPERTIES
OF KERATOCONJUNCTIVITIS EPIDEMICA INCLUSIONS

(Tests Using the Immunofluorescent Method)

Orvosi Hetilap
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No. 105, 1964, pages 439-441

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Isolation of virus, serological tests and independent experimental infections prove that keratoconjunctivitis epidemica (in the following: k.c.e.) is caused by adenovirus type 8 (Jawetz et al. (11), Mitsui and Jaweta (18), Bietti and Bruna (2), Mitsui et al. (17), Quilligan et al. (23)). Other adenoviruses can cause similar diseases (Fowle et al. (5), Frezotti (6), Huebner and Rowe (?), Kahan and Beladi (12), Mitsui et al. (17), Kosecki (14), Nasz et al. (10), but these may be differentiated on the basis of clinical signs. This was demonstrated during the course of the k.c.e. epidemic that occurred in Hungary in 1961-1962 (Beladi et al. (1), Nasz et al. (20)). Nasz et al. (19), and Korchmaros and Imre (15) reported on a triple laboratory infection by type 8 adenovirus; typical k.c.e. formed on both eyes of the infected.

During the acute conjunctivitis phase of k.c.e. characteristic inclusions may be seen in the epithelial cytoplasm of the conjunctiva (Wright (28), zur Nedden (22), Haumence et al. (16), Sie-Boen-Lian (25, 26), Silva (27), Lepri (15), Hofmann (7), Imre et al. (10), and Nocsel (21)). The rela-

tionship between the k.c.e. inclusions and the causative agent of the disease has not been established; it is unclear whether they are identical with agglomerations of virus particles, or whether they are metabolic products resulting from interaction between the virus and the host cell. In an investigation of this question we applied the immunofluorescent method described by Coons and Kaplan (4). In a previous report we described demonstration of k.c.e. inclusion bodies by the immunofluorescent method (Imre, Korchmaros and Geck (9)). Using convalescent serum we established that the k.c.e. inclusion bodies have an antigen character and that the corresponding antibodies may be found in the serum of patients convalescing from k.c.e. The object of this work consists of immunofluorescent examinations made with immunosera, produced in rabbits, against various adenoviruses. Through these examinations we intended to prove the specific antigen nature of k.c.e. inclusion bodies.

Materials and Method

Collecting Scrapings: Scrapings were taken by means of a knife or brush from the lower overlapping fold of the conjunctiva and from the plica semilunaris region. The smears were dried at room temperature, and were fixed for ten minutes with methylalcohol.

Virus Strains Used for Producing the Immunoserum: Immunosera were produced against adenovirus strains 3, 4, 5, 6, 7, 8, and 11. With the exception of type 8 the virus strains used were type-strains, provided by Dr. Krech (Switzerland) and Dr. Dreyzin (Soviet Union). The type 8 strain was isolated in the course of the k.c.e. epidemic in Hungary during 1961 and 1962 (Nasz et al. (20)), and with the use of type specific immunoserum provided by Dr. Huebner (USA) it was established that it belonged to type 8. The virus antigen was produced on Detroit-6 tissue culture, and following repeated freezing and thawing the cell remnants were separated fromthe virus suspension by centrifuging, after which it was used for immunization.

Immunoserum Production: Immunoserum was produced in rabbits by intravenous injection of a total of eight doses at weekly intervals for a period of four weeks. Immunization was begun with 1 milliliter of virus suspension, and the dose was increased by one-half milliliter up to a maximum of three milliliters, which dose was continued. The rabbits were sacrificed by draining the blood eight days after the last innoculation. The immunosera were controlled with complement fixing and hemagglutionation inhibition tests, and

partly with neutralization tests, and then were stored at -10 degrees Centigrade until used.

Immunofluorescent Staining Method: The immunofluorescent staining method described by Coons and Kaplan (4) was utilized. The smears were stained indirectly with a high titer rabbit antiglobulin produced in goats, which was marked partly with Lissamine-Rhodamine B-200 fluorochrome and partly with fluoresceineisothiocyanate, according to the methodsof Chadwick (3) and Riggs (24). The fixed smears first were processed with unmarked immunoserum in a damp chamber for 30 minutes, and following washing and drying they were brought into contact with marked rabbit antiglobulin in a damp chamber for 30 minutes. This was followed by washing and drying again. The stained smears were examined with a Leitz fluorescence microscope, at 250 to 400 magnification. Blue light was used.

Table 1.

	On 1st-10th Days of k.c.e.	On 10th-20th Days of k.c.e.
Conjunctival Scraping Positive	88 (88 %)	7 (31.8 %)
Conjunctival Scraping Negative	12 (12 %)	15 (68.2 %)

Results

Scrapings were taken from the conjunctiva of k.c.e. patients on 122 occasions. In 26 cases two smears were made at the same time, and in two cases eight smears were prepared. Giemsa stain was used in staining 122 smears. The appearance of the inclusions is shown in Table 1.

The k.c.e. inclusion bodies were uniformly stained blue by the Giemsa; they are round or oval in shape, and always are located in the cytoplasm of epithelial cells (Figures 1-4). These inclusion bodies also were found in k.c.e. cases caused by laboratory infection with type 8 adenovirus (13, 19). As a control, 20 other scrapings of conjunctival inflammations were examined, using Giemsa stain, including 8 from acute conjunctivitis cases, 3 chronic conjunctivitis, 1 each from allergic conjunctivitis and blepharoconjunctivitis angularis, 2 trachomatous conjunctiva, 1 pemphigus conjunctiva, 3 keratitis herpetica, and 1 smear was made from a healthy conjunctiva. No inclusion bodies were found in any of these 20 cases, including the cases clinically considered to be trachoma.

GRAPHIC NOT REPRODUCIBLE

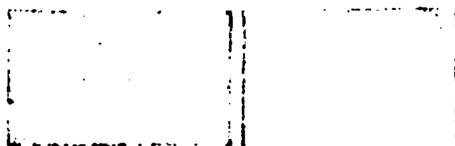
Figures 1-3. K.c.e. inclusion bodies in conjunctival epithelial cells (Giemsa stain).

GRAPHIC NOT REPRODUCIBLE

Figure 4. K.c.e. inclusion in corneal epithelial cell (Giemsa stain).

For the purpose of immunofluorescent staining scrapings were taken on 38 occasions from the conjunctiva of 28 k.c.e. patients. The course of the disease was typical in all 28 cases, and typical inclusion bodies were found in all 28, using the Giemsa stained smears. In the course of immunofluorescent staining 17 smears were made with the type 8 adenovirus immunoserum. Examination under the fluorescence microscope revealed strongly fluorescent formations indicating a specific bond in the cytoplasm of individual epithelial cells in 15 of the 17 smears. Their shape, size, location and frequency of appearance corresponded to those of k.c.e. inclusion bodies. Their shape was round or oval, and the border of the cellular nucleus generally appeared concave in the vicinity of the inclusions (Figures 5 and 6). The contour of the bodies and the cell nuclei was fairly

readily distinguishable, primarily because of their autofluorescence. In several cases cells containing shapes with strong fluorescence indicating the specific bond were marked off on the smear and later stained with Giemsa. In every case typical inclusion bodies were found at the site of the strongly fluorescing formation, exactly in the same cell and at the same point (Figures 7-8 and 9-10). In no instance was strong fluorescence indicating specific bond found in the cell nuclei.



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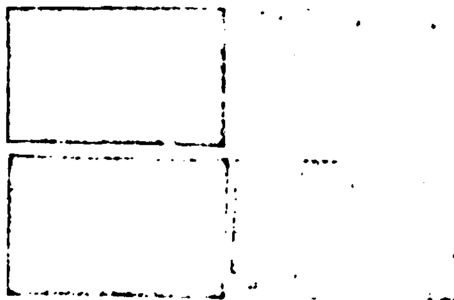
Figures 5-6. K.c.e. inclusions in conjunctival epithelial cells (immunofluorescent staining, using type 8 adenovirus immunoserum).



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Figures 7-8, and 9-10. Identical cells or inclusion bodies stained by the immunofluorescent method (with type 8 adenovirus immunoserum) and with Giemsa stain.

As a control, immunosera produced with other types of adenovirus were used in 21 cases. Immunoserum of adenovirus types 3, 4, 5, 6, 7, and 11 was used in staining three smears, each, and three smears also were made with normal rabbit serum. The Giemsa-treated pairs of the smears in this case also were inclusion-positive. However, immunofluorescent staining was negative in all 21 cases. In no instance was strong fluorescence indicating specific bond found within cells in the control smears. In view of the fact that eight scrapings,

each, were taken from the conjunctiva of two patients, we were able to stain smears from the same patient with immunosera of types 3, 4, 5, 6, 7, 8 and 11 adenovirus, and with Giemsa stain. In both series the stain was positive (typical inclusion bodies could be seen in smears stained with Giemsa) only in the smear treated with the type 8 adenovirus immunoserum. As a control five smears made of other conjunctival inflammations also were stained with type 8 adenovirus immunoserum. No specific staining was found in these control cases.

Conclusions

In the course of the k.c.e. epidemic in Hungary in 1961-1962 characteristic inclusion bodies were found in 88 percent of conjunctival scrapings taken from first to tenth day patients. The k.c.e. inclusion bodies may be well distinguished from trachoma inclusions, and cannot be found in other conjunctival inflammations, either. Because of this examination of conjunctival scrapings may be well used for the purpose of differential diagnosis. Through immunofluorescence tests it was established that specific staining may be observed in the cytoplasm of individual epithelial cells in smears deriving from the conjunctiva of k.c.e. patients, which had been treated with type 8 adenovirus immunoserum. These formations were strongly fluorescent, indicating that an antigen-antibody bond had been formed at the site. Their identity with k.c.e. inclusions is proved by the following: (1) The smears were derived from the conjunctiva of patients with typical k.c.e., and characteristic inclusion bodies were found in their paired slides that had been stained with Giemsa. (2) The shape, size, location, and frequency of occurrence corresponded to those of k.c.e. inclusion bodies. (3) When cells containing fluorescing formations were marked on the slide and the smear was stained with Giemsa, typical inclusion bodies became visible at the exact same points of the same cells, at the site of the fluorescing formation. (4) The immunofluorescent staining of inclusion negative scrapings also was negative.

Other types of adenoviruses also have been cultured from k.c.e. or clinically similar cases, in addition to type 8 adenovirus (types 5, 6, 8, 11, 14, and 18). In conformance with this, in the examination of the antigen specificity of the inclusion bodies it appeared of interest to conduct control tests with types 3, 4, 5, 6, 7, and 11 adenovirus immunosera. All the tests performed with the control sera were negative; the inclusion bodies were not stained in the cases of treatment with heterologous immunosera. The extraordinary sensitivity of the immunofluorescent staining

method is indicated by the fact that only the inclusion bodies of smears treated with type 8 adenovirus immunoserum fluoresced. The above proves the similarity of the antigen properties of the inclusion bodies to the antigen struction of type 8 adenovirus, and indicates that the inclusions in part or entirely contain pathogenic virus particles in addition to the presence of any possible metabolic products. This agrees with the observation that inclusion bodies rarely may be found in scrapings taken after the tenth day of k.c.e. Small acidophylic granules occasionally may be observed surrounding the inclusion bodies. Fluorescence corresponding to these formations was not found in any of the smears, and thus we cannot substantiate the concept of Sie-Boen-Lian (26), to the effect that these granules are elementary pathogenic bodies. According to the foregoing the immunofluorescent procedure enables early and rapid identification of the pathogen at the beginning of k.c.e. epidemics, or in the initial phase of the disease.

Summary: A keratoconjunctivitis epidemic occurred in Hungary in 1961-1962. Characteristic inclusion bodies were found in the epithelial cytoplasm of 83 percent of the scrapings taken during the acute phase of the disease. Through immunofluorescent tests it was established that these inclusion bodies have specific antigen properties which conform to the antigenic nature of type 8 adenovirus, which may be considered as the main pathogenic agent in this epidemic. According to this the inclusions of keratoconjunctivitis epidemic partly or entirely contain the pathogenic virus particles.

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