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STUDIES ON TRACHOMA*

1. Isolation and Identification of Strains of Elementary Bodies from Saudi Arabia and Egypt

by

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INTRODUCTION

Trachoma and related diseases of the eye afflict millions of people and cause serious impairment of vision in many parts of the world(1). Most of the countries of the Middle East have been severely involved. In 1954 a study was begun in Saudi Arabia to establish, if possible, the etiologic agents of the common diseases of the external eye, including trachoma, the cause of which has been much disputed in the past. The investigations consisted of clinical, epidemiological and laboratory observations, and recently have been extended to Egypt and other countries of the Mediterranean Region. Attempts to recover etiologic agents from conjunctival scrapings of persons with clinical trachoma have revealed a range of potential pathogens(2), including bacteria, adenoviruses(3,4,5) and elementary bodies(6). It is the purpose of this series of papers to record our observations and experiments with the viruses of the latter category; the first paper presents the data pertaining to the properties of several strains of elementary bodies isolated from Saudi Arabia and Egypt.

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Resumé of Previous Work on Elementary Bodies

Many attempts have been made to culture trachoma viruses[7]. Viruses were successfully transferred by inoculation of the conjunctivae of the higher anthropoid apes, but lower animals were resistant to infection. In 1944 Macchiavella reported the growth of a virus in the yolk sac of embryonated eggs[8]; his strain was lost. In recent years there have been intensive trials by several investigators to cultivate trachoma viruses in cell culture, but without success.

In 1957, T'ang, Chang, Huang, and Wang reported the isolation of strains of elementary bodies from conjunctival cells obtained from patients with clinical trachoma[9]. They inoculated specimens of infected conjunctival cells into chick embryos using a modification of the technique of Cox[10] and Macchiavello[8]. The findings of T'ang and his colleagues were confirmed and extended by Collier and Sowa who isolated strains from trachoma patients in Gambia, West Africa[11]. Collier, Duke-Elder, and Jones inoculated the Gambian strain into the eye of a blind human volunteer who developed typical microscopic and clinical findings of classical trachoma[12].

Giroud, Renoux, and Netaf described the isolation of elementary bodies by intranasal inoculation of mice, and they reported success in establishing a strain on the chorio-allantoic membrane of chick embryos[13]. They reported that their strains, after several passages in mice or eggs, induced clinical trachoma in two human subjects. Murray, Snyder and Bell isolated a strain of elementary bodies from Saudi Arabia in the yolk sac membrane of chick embryos; they showed that the strain was toxic for mice and induced inclusion bodies in a chimpanzee[6].

METHODS

The clinical and epidemiological studies were conducted principally among residents of Al Hasa and Qatif, two large oases of eastern Saudi Arabia. In a few instances material was obtained from patients attending eye clinics, either at the Dhahran Health Centre, or in villages. The Egyptian strains were obtained from patients of the outpatient clinic of the Giza Memorial Ophthalmological Institute in Cairo through the courtesy of our good friend and colleague, Dr. Ibrahim Ahmed, and I wish to take this opportunity to thank him on behalf of the staff of the Harvard School of Public Health.

Clinical and Epidemological Features

A case of "pure" trachoma is a rarity in Saudi Arabia or in Egypt. Under ordinary village conditions an infant becomes infected with one or more pathogenic agents usually during the first few months of life. The resulting conjunctivitides produce chronic mucopurulent discharges and papillary hypertrophy, tending to mask the symptoms and signs of "pure" trachoma. Under these conditions it is difficult to establish accurately the time of onset of trachoma on the basis of clinical findings. Insofar as possible, however, diagnoses were based on the criteria as stated by the Expert Committee of the World Health Organization[14,15] and as presented by Thygeson[16]. Attempts at isolation of elementary bodies were made using specimens from forty-four children whose conjunctival scrapings met the morphologic criteria in the next paragraph, and whose clinical findings were those of trachoma II or III, with a single exception as noted in the attached Table. The age of the children ranged from four months to seven years. Thirty-nine were residents of eastern Saudi Arabia and five lived in Egypt. Twenty-four individuals were among the large groups which were encountered in survey type visits, whereas twenty were children brought to a clinic because of their parents' concern over their eyes.

Microscopic Features of Conjunctival Scrapings.

The sole criterion for considering a slide as positive was the findings of one or more intracellular aggregates of elementary bodies. In most cases the scrapings contained both elementary and initial bodies. Inclusions consisting entirely of initial bodies were not considered as positive since the latter are frequently similar in appearance to the intracytoplasmic aggregates of bacteria sometimes found in bacterial conjunctivitides. Occasionally the elementary bodies appeared singly in the cytoplasm, but the slides were not considered as positive unless aggregates consisted of more than ten elementary bodies, closely packed in an inclusion.

Toxicity Tests

After establishing strains by repeated passages in eggs, those which were very rich in elementary bodies were tested for toxicity, as follows: yolk sacs from embryos surviving on the sixth to the eighth day after inoculation were selected. The membranes were mixed thoroughly, either mechanically or by shaking with glass beads, using sucrose PG as the diluent. The suspensions were diluted to 30% yolk sac in sucrose PG and

centrifuged at 1,500 rpm for fifteen minutes to eliminate gross particles. Young albino Swiss mice were lightly anaesthetized with ether and inoculated intravenously with 30%, 20% and 10% suspensions of yolk sac in sucrose PG on the basis of 0.25 ml of yolk sac suspension per 10 grams of body weight. The mice recovered promptly from anaesthesia and were normally active for at least fifty-five minutes after inoculation. In an occasional instance a mouse was eliminated from consideration because of abnormal signs in the hour immediately after inoculation or because of failure to recover from the anaesthesia. The test was arbitrarily terminated at the end of twenty-four hours. Deaths attributed to toxin regularly occurred in the interval from two to eight hours after inoculation, and haemorrhagic exudate in the upper small intestine was the characteristic abnormality at autopsy(6). Differences between some of these strains with respect to active immunization of mice against toxic death have been noted elsewhere(17).

Complement Fixation Tests

Antigens were prepared from pools of those strains which after several passages in eggs were toxic for mice and which contained in Giemsa stained smears at least 500 elementary bodies per oil immersion field. The antigens were prepared by boiling, using Terzin's modification(18) of Bedson's method(19). Three or four antigen units were used in the CF test. Controls were prepared from normal yolk sac suspensions. Standard positive reference psittacosis sera were supplied through the courtesy of the Rocky Mountain Laboratory of the U.S. Public Health Service and Dr. Fritz Jocklang of the Institute of Tropical Hygiene, Amsterdam, Holland.

Miscellaneous Observations

(a) Pleuropneumonia-like organisms. The presence of these micro-organisms was suspected in every strain until negative cultures(20) with appropriate controls had been obtained.

(b) Other viruses of the psittacosis-LGV group. Each suspected strain was inoculated into weanling mice by the intracerebral route; the test mice were observed for three weeks for signs suggestive of psittacosis or other viruses of the group which are pathogenic for mice.

(c) Pathogenicity for human epithelial cells. Eight strains were inoculated into roller tubes containing either conjunctival cells(21) maintained in MS and horse serum(22), or HEP cells(23). The inocula

were suspensions of infected yolk sacs at 10 to 30% in sucrose PG. The tubes were observed for at least three weeks and transfers to new tubes were made for three passages in some instances. On a few occasions highly concentrated suspensions of elementary bodies were also used as inocula. Several of the tissue cultures were subsequently inoculated into eggs as an assay for viable elementary bodies.

RESULTS

Forty-five attempts were made to establish strains by inoculation of chick embryos with conjunctival scrapings, aliquots of which showed typical inclusions. All of the patients had clinical trachoma except one (see SA-5 in the attached Table). This nine month old patient on repeated examinations had numerous inclusions in her scrapings, but her clinical findings were noted only as "mild conjunctivitis".

Elementary bodies were isolated from nineteen of the forty-five trials; nine of these strains have been subjected to the various procedures reported above. The elementary bodies of each of the strains had the morphologic properties which are regarded as characteristic of viruses of the psittacosis-lymphogranuloma group. Their appearance in Giemsa stained smears was identical to the description given by Collier and Sowa[11]. The iodine stain was not applied to any of the yolk sac material. The elementary bodies stained bright reddish pink by Macchiavello's stain. The attached Table presents the data pertaining to the nine strains which were thoroughly studied.

The data in the table indicate various points of interest. Strains were isolated from several different localities in eastern Saudi Arabia, during the years 1956, 1957, and 1958. Storage of the conjunctival scrapings in sucrose PG for periods up to twenty and a half months at -60°C did not destroy the viability of the elementary bodies. Isolation attempts with specimens stored in sucrose PG were more successful than with those stored in the other media, six of the nine strains in the attached Table were initiated from scrapings placed in sucrose PG. (If the incompletely studied isolates are also included, the positives from sucrose PG were fifteen out of sixteen attempts, whereas there were only four positives out of twenty-nine attempts with other media).

Strains SA-2 and SA-4 were obtained from the same subject, a three-year old child whose conjunctivae were scraped on two occasions, four weeks apart.

Isolation of strains was successful not only with children in the clinics, (brought because of the parents' concern over their eyes), but also with individuals observed in surveys. (The ratios of positives to total attempts, combining both the nine completely studied and the ten other isolates, were: 13/25 for surveys, 6/20 for clinics).

Miscellaneous Tests

Each of the strains was shown to be free of pleuropneumonia-like organisms. None was pathogenic for mice by intracerebral inoculation of 10 to 30% yolk sac suspensions. Although a few intracellular aggregates of elementary bodies, very similar to classical inclusions, were occasionally observed in tissue cultures within a few days after exposure to 10 to 20% yolk sac suspensions of the various strains, in no instance was any of these strains established by serial passage in conjunctival or HEP cells maintained in MS and horse serum. None of the eggs died after receiving tissue culture passage material.

Pathogenicity

It was reported previously that strain SA-1 induced conjunctival inflammation in a chimpanzee[2,6]. Figure 1* is a photograph of the inclusions observed by Giemsa stain in the conjunctival scraping of the chimpanzee on the second day of clinical disease.

During the period when our laboratory staff was working exclusively with egg passages of strains SA-1, 2, 5, and Egypt-2 a severe case of trachoma occurred in the technician who was inoculating eggs and processing the yolk sac membranes. No overt break in technic was recalled, and the infection was presumed to have been caused by airborne droplets of infected yolk sac membranes, probably strain SA-1, a large pool of which was subjected to several procedures by the technician just three days prior to the onset of her infection. The details of the case will be reported elsewhere[24]. Figure 2* shows the appearance of Giemsa stained conjunctival cells scraped from the left eye of the technician on the fourth day of clinical disease. Strains of elementary bodies were isolated from conjunctival scrapings obtained on the second and fourth days after onset of signs and symptoms. The infection responded to treatment with oral sulfonamides and topical tetracycline.

*as shown to the meeting

DISCUSSION

The data reported above constitute a confirmation of the reports by T'ang et al.[9] and Collier et al.[11,12] of the successful isolation of elementary bodies from trachoma cases using streptomycin and the yolk sac route of inoculation of chick embryos. In our tests successful isolation was obtained in fewer than half the attempts even though the specimens were all inclusion positive. This low rate may be attributable to adverse environmental conditions during storage of the specimens, since one preserving fluid was clearly superior to the three others which were included in the tests. This point is being investigated with particular reference to the influence of freezing and storage on viability.

The morphologic identity of the strains and their content of common group antigen might lead one to assume identity in other properties as well, but it is the conviction of the authors that the rather impressive differences among the strains in respect to regularity of attainment of high titers in eggs probably indicates basic differences rather than chance variations. This view is supported by the experiments on active immunization of mice against toxic challenge[17].

The failure to obtain unequivocal propagation of the trachoma elementary bodies in cultures of human conjunctival cells is a very challenging observation, the explanation of which, if elucidated, may throw light on the complex relationships of the elementary body parasite to the tissue cells, the nutrients, and other factors which are still to be identified.

The data now available justify the view that the elementary bodies which have been propagated in yolk sac of the chick embryo by T'ang et al.[9], Collier et al.[11,12] and as reported above, can induce clinical trachoma in man and that the organisms can again be recovered in laboratory passage. It is not possible, however, to justify an assertion that clinical trachoma is a disease entity with a single etiologic agent. There are several observations which suggest important differences from one region to another, in respect to (a) success in isolation of strains of elementary bodies, and (b) abundance of inclusions in conjunctival scrapings in some regions as opposed to extreme rarity in other regions, despite identity of clinical findings and standard techniques of investigation. It is also possible that agents other than viruses of the elementary body group can induce conjunctival reactions which mimic early clinical stages of classical elementary body induced trachoma[4,5]. Our current studies did not

include simultaneous search for adenoviruses and elementary bodies from the same specimen because the quantity of infectious material was so small that we hesitated to divide the specimens. It may be noted, however, that several adenoviruses (types 8 and 15, and Strains 931 and BAR-2) have been isolated from conjunctival scrapings of children who had classical inclusions at the time the specimens were taken for virus isolation attempts[25].

SUMMARY

This paper reports the isolation of strains of elementary bodies from trachoma cases in Saudi Arabia and Egypt by inoculation of the yolk sacs of chick embryos. Nineteen strains were obtained in forty-five attempts using conjunctival scrapings from children who were shown to have typical inclusions at the time of the scraping. Nine of the strains were subjected to a series of tests which showed that they were indistinguishable morphologically from the elementary bodies of the psittacosis-lymphogranuloma group of viruses, that they were not sensitive to streptomycin, that they were free of other viruses and of pleuropneumonia-like organisms, that they were toxic for white mice by the intravenous route, and that they contained the heat stable antigen which is common to the psittacosis-lymphogranuloma group of viruses. Although each strain was capable of multiplication in yolk sac membranes of chick embryos, irregularities of growth were frequently observed, being more characteristic of certain strains, than others.

Viability of the trachoma elementary bodies in conjunctival scrapings persisted at -60°C for many months if the specimens were stored in a medium containing sucrose, glutamate, and phosphate. One of the strains induced conjunctivitis in a chimpanzee whose conjunctival cells showed typical inclusions at the height of the conjunctival reaction. A laboratory worker contracted severe trachoma probably as a consequence of air-borne droplets arising from manipulations of concentrated suspensions of the elementary bodies.

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REFERENCES

(1) Thygeson, Phillips, 1959. Trachoma and Inclusion Conjunctivitis. Chapter 34, Viral and Rickettsial Infections of Man, 3rd Edition. Edited by Rivers and Horsfall. J.B. Lippincott Co., Philadelphia.

(2) Snyder, J.C., Page, R.C., Murray, E.S., Daggy, R.H., Bell, S.D., Jr., Nichols, R.L., Haddad, N.A., Hanna, A.T., McComb, D., 1959. Observations on the etiology of trachoma. Am. J. Ophth. In press.

(3) Murray, E.S., Chang, R.S., Bell, S.D., Jr., Tarizzo, M.L., and Snyder, J.C., 1957. Agents recovered from acute conjunctivitis cases in Saudi Arabia. Am. J. Ophth. 43:32.

(4) Bell, S.D., Jr., McComb, D.E., Murray, E.S., Chang, R.S., and Snyder, J.C., 1959. Adenoviruses isolated from Saudi Arabia 1. Epidemiologic features. Am. J. Trop. Med. and Hyg. 8:492-500.

(5) Feng, Marie, Chang, R.S., Smith, T.R., Snyder, J.C., 1959. Adenoviruses isolated from Saudi Arabia 2. Pathogenicity of certain strains for man. Am. J. Trop. Med. and Hyg. 8:501-504.

(6) Murray, E.S., Snyder, J.C. and Bell, S.D., Jr., 1958. A note on the toxicity for white mice and gerbilles of a strain of elementary bodies isolated from a patient with trachoma in eastern Saudi Arabia. Proceedings of the Sixth International Congress on Tropical Medicine, Lisbon, Portugal. September 12, 1958. In press.

(7) Thygeson, Phillips, 1939. Cultivation of trachomatous conjunctival epithelium in vitro. Arch. Ophth. 21:229.

(8) Macchiavello, A., 1944. El virus del trachoma y su cultivo en el saco vitelino del huevo de gallina. Rev. Ecuatoriana de Hig. y Med. Trop. Guayaquil. 1:1-33.

(9) Tang, F., Chang, H., Huang, Y., and Wang, K., 1957. Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. Chinese Medical Journal, 75: 429.

(10) Cox, H.R., 1941. Cultivation of rickettsiae of the Rocky Mountain spotted fever, typhus, and Q fever groups in the embryonic tissues of developing chicks, Science 94:399.

(11) Collier, L.H., Sowe, J., 1958. Isolation of trachoma virus in embryonate eggs. The Lancet, 1:993.

(12) Collier, L.H., Duke-Elder, S., Jones, B.R., 1958. Experimental trachoma produced by cultured virus. Brit. J. Ophthal., 42:705

(13) Giroud, P., Renoux, G., et Nataf, R., 1958. I. Cultures de produits trachomateux sur souris, merions, gerbilles et oeufs embryonnes, Extrait des Archives de l'Institut Pasteur de Tunis, 35:327.

(14) World Health Organization. Expert Committee on Trachoma. First Report, Technical Report Series. 1952, 59:4.

(15) World Health Organization. Expert Committee on Trachoma. Second Report, Series 106, May 1956.

(16) Thygeson, Phillips, 1958. Trachoma Manual and Atlas. Public Health Service Publication No. 541. U.S. Government Printing Office, Washington 25, D.C.

(17) Bell, S.D., Jr., Snyder, J.C., and Murray, E.S., 1959. Immunization of mice against toxic doses of homologous elementary bodies of trachoma. Science 130:626-627.

(18) Terzin, A.L., 1958. Psittacosis-ornithosis-mammallian pneumonitis (POMP) viruses in man mammals and birds, J. Hyg., Epi., Micro. and Immunol., 11:129.

(19) Bedson, S.P., Barwell, C.F., King, E.J. and Bishop, L.W.J., 1949. The laboratory diagnosis of lymphogranuloma venereum. J. Clinic. Path. 2:241.

(20) Dienes, L., Weinberger, H.J., 1951. The L forms of bacteria. Bacteriological Reviews, 15:245-288.

(21) Chang, R.S., 1954. Continuous subcultivation of epithelial-like cells from normal human tissues. Proc. Soc. Exp. Biol. Med., 87:440.

(22) Scherer, W.F., 1953. The utilization of a pure strain of mammalian cells (Earle) for the cultivation of viruses in vitro. I. Multiplication of pseudorabies and herpes simplex viruses. Am. J. Path., 29:113.

(23) Moore, A.E., Sabachewsky, L. and Toolan, H.W., 1955. Culture characteristics of four permanent lines of human cancer cells. Cancer Res., 15:598-602.

(24) Murray, E.S., Bell, S.D., Jr., and Allen, H.A. Trachoma in a laboratory worker. To be published.

(25) Bell, S.D., Jr., and Murray, E.S. Unpublished observations.

T A B L E

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DATA PERTAINING TO CERTAIN STRAINS OF ELEMENTARY BODIES ISOLATED FROM SAUDI ARABIA AND EGYPT

Strain Designation	SA-1	SA-2	SA-4	SA-3	SA-5	SA-6	SA-8	SA-13	EGYPT-2
Patient: Home	Al Umran	Um es Sahich	Um es Sahich	Hazim Ujam	Al Awamieh	Mubarrez	Safwa	Kilabia	Giza
Age	18 mo.	3 yr.	3 yr.	5.5 yr.	9 mo.	5 yr.	5.5 mo.	7 yr.	4 yr.
Sex	F	M	M	F	F	M	F	M	F
Clinic	Survey	Survey	Survey	Survey	Survey	Survey	Survey	Clinic	Clinic
Clinical data					Mild conjunctivitis				
Right eye	Tr.I+P	Tr.II+F++P	Tr.II+F++P	Tr.II++F+P		Tr.III	Tr.II	Tr.II	Tr.II+F
Left eye	Tr.I+P	Tr.II+F++P	Tr.II+F++P	Tr.II++F+P		Tr.III	Tr.II	Tr.II	Tr.II+F
Inclusions in conjunctival scrapings (see text)									
Right eye	2	2	1	1	48/600	5/100	15/400	3/1000	9/300
Left eye	1	3	1	1	12/600	10/450	14/300	12/1600	3/150
Date scraping obtained	26.7.57	23.10.56	20.11.56	20.11.56	3.7.58	2.8.58	9.8.58	19.3.58	31.8.58
Preserving fluid (see text)	II	II	I	I	I	I	I	II	I
Duration of storage at -60°C before egg inoculation	10 mo.	20.5 mo.	19.5 mo.	19.5 mo.	7 days	None	2 days	7 mo.	None
Egg Inoculations: Day of death, D, or sacrifice - S	See Footnotes								
Number elementary bodies in smears									
1st passage	3,S,A	8,D,B	9,S,C	9,D,C	7,S,C	9,D,A	8,S,B	8,S,C	6,D,A
2nd passage	8,D,B	7,D,B	5,D,C	6,D,B	5,D,B	7,S,B	8,D,B	7,S,C	6,D,B
3rd passage	5,D,C	5,D,B	6,D,C	11,D,B	5,D,B	7,S,A	6,D,C	4,D,A	8,D,B

T A B L E (continued)

Strain Designation	SA-1	SA-2	SA-4	SA-3	SA-5	SA-6	SA-8	SA-13	EGYPT-2
Number of egg passages to date 9/22/59	27	35	6	16	25	9	8	10	16
LD_{50} for eggs[b]	10^6	10^8	10^6	10^6	10^6	q	q	q	q
Toxicity for mice (per cent yolk sac)	25%	20%	25%	r	30%	30%	30%	30%	20%
CF titer of boiled antigen vs. standard positive psittacosis serum	1/160	1/80	1/160	1/40	1/320	1/10	1/320	1/10	1/80

FOOTNOTES

O - Elementary bodies not observed in 30 minutes' search of smear
A - 1 to 10 elementary bodies per oil immersion field (8X oculars, 100X objectives)
B - 10 to 100 elementary bodies per oil immersion field (8X oculars, 100X objectives)
C - More than 100 elementary bodies per oil immersion field (8X oculars, 100X objectives)
p - Maximum number of egg LD_{50} units per gram of yolk sac
q - Titration not attempted. Dilutions of 10^{-2} were lethal in routine passages
r - Toxic only when highly concentrated (see text)