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EXPERIMENTAL KERATO-CONJUNCTIVITIS OF THE GUINEA-PIG BY ENTEROBACTERIA

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SUMMARY

Three hundred twelve cultures of enterobacteria were inoculated in the guinea-pig eye, namely: *Shigella*, freshly isolated cultures (104); *Shigella*, collection cultures (31); enteropathogenic *Escherichia coli* 0124: K72 (B17) (2), other enteropathologenic ones (32); non-pathogenic *Escherichia coli* (44); *Escherichia coli* of group *Alkalescens-Dispar* (13); *Salmonella* (28); *Arizona* (1); *Citrobacter* (6); *Klebsiella* (12); *Aerobacter* (8); *Serratia* (1); *Hafnia* (1); *Proteus mirabilis* (8); *Proteus vulgaris* (4); *Proteus morganii* (7); *Proteus rettgeri* (1); *Providencia* (9); Culture 185T-64 (1) and Culture 193T-64 (1).

Of the lot, only *Shigella*, *Escherichia coli* 0124: K72 (B17) and the cultures 185T-64 and 193T-64 actually produced a kerato-conjunctivitis. All the remaining cultures caused no lesions on the guinea-pig eye or determined only a mild and fleety kind of conjunctivitis, apparently of non-infectious nature. A sharp correlation was observed between virulence and age of the cultures, in *Shigella*. The cultures sampled from the Department collection were all non-virulent. Much on the contrary, of the 104 recent isolates, only two strains of *Shigella sonnei*, form II, failed to induce kerato-conjunctivitis in the guinea-pig.

The serological response of the infected animals was steady and uniform. The highest levels of agglutinins were attained between the 10th and the 20th day of infection and in many of the subjects they were maintained up to the 40th day.

From the clinical, bacteriological and serological point of view, no appreciable differences were detected among the infections caused by *Shigella*, *Escherichia coli* 0124: K72 (B17) and cultures 185T-64 and 193T-64.

INTRODUCTION

Experimental infections in suitable laboratory animals have been of considerable assistance in clarifying the role of host defense mechanisms and the specific bacterial virulence factors, with a variety of infectious agents. A sensitive experimental model would be of great value as a tool for the better understanding of the relationships between pathogenic enterobacteria and man, a subject which has not been extensively investigated. Very little is known about most of the defense mechanisms and traditionally the pathogenic properties of these bacteria have been explored mainly by determination of the physiological disturbances created by their endotoxins. These observations may be pertinent to the understanding of the problem but similar effects are

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obtained with endotoxins derived either from pathogenic or non-pathogenic organisms. Therefore, the experimental model devised by SERÉNY [7, 8] may be of assistance in elucidating the determinants of the pathogenic effects of *Shigella* and other enterobacteria and contribute as well, for the better recognition of the disease these bacteria usually cause in man.

In view of the potentialities of the method, we decided to undertake the present investigation with the main purposes of determinating the enterobacteria which are pathogenic for the guinea-pig eye and studying with more details the serological response of the infected animals.

MATERIAL AND METHODS

Animals. Albino guinea-pigs without regard for age or sex were used throughout the experiments. Prior to infection the animal eyes were carefully examined and those with any ocular alteration were excluded. After inoculation, the animals were kept in individual cages.

Bacteria cultures. The following freshly isolated enterobacteria cultures were employed:

Shigella dysenteriae,	type 2	2
	" 4	1
	" 5	5
Shigella flexneri,	" 1	6
	" 2	26
	" 3	15
	" 4	10
	" 5	3
	" 6	6
Shigella boydii,	" 2	3
Shigella sonnei	(form I)	25
	(form II)	2
Escherichia coli,	026: K60 (B 6)	2
	086: K61 (B 7)	2
	0111: K58 (B 4)	17
	0119: K69 (B14)	7
	0124: K63 (B 8)	1
	0128: K67 (B12)	1
	non-enteropathogenic	44
	Alkalescens-Dispar	13
Salmonella sp.		9
typhi-murium		10
anatum		3
typhi		6
Arizona		1
Citrobacter		6
Klebsiella		12
Aerobacter		8
Hafnia		1
Serratia		1
Proteus mirabilis		8
vulgaris		4
morganii		7
rettgeri		1
Providencia		9
Culture 185T-64		1
Culture 193T-64		1

In addition to these, the following standard *Shigella* cultures were inoculated in the guinea-pig eye:

Shigella dysenteriae — types 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10.

Shigella flexneri — types 1a, 1b, 2a, 3, 4a, 4b, 5 and 6.

Shigella boydii — types 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14 and 15.

Shigella sonnei — form I.

Most of the freshly isolated cultures were obtained from the feaces of children or adults patients suffering from acute or chronic intestinal disturbances. All of them were identified by the methods recommended by EDWARDS & EWING [1]. Both the freshly isolated and the standard *Shigella* strains were submitted to slide agglutination tests in Trypaflavine (1:500) before inoculation.

Inoculation of the animals. A suspension containing 2.7×10^6 cells per ml (tube number 9 of McFarland's scale) was obtained by harvesting the growth of 16 to 20 hours nutrient agar slants in sterile saline. 0.05 ml of this suspension were instillated in one of the guinea-pig eyes after lifting the upper and lower lids, and both lids then gently massaged to insure distribution of the organisms over the eye-ball. Each of the new cultures was inoculated within a week after isolation and one guinea-pig was used for testing each strain.

Observations on the course and bacteriology of the infection. Following inoculation, the animal eye was inspected 24 hours later and then every 2 or 3 days until healing of the kerato-conjunctivitis took place. If signs of infection were not present after 7 days of the inoculation, the animals were discarded.

Bacteriological examination of the material obtained from the conjunctiva and cornea was performed 24 hours later and then every 4 or 5 days until negativation, in 45 guinea-pigs inoculated with *Shigella, Escherichia coli* 0124: K72 (B17), cultures 185T-64 and 193T-64 and in 26 inoculated with other enterobacteria.

Serological examination. Before infection and 10, 20, 30 and 40 days later, blood samples were taken by heart puncture for titration of serum antibodies by agglutination, hemagglutination, gel and tube precipitation and complement-fixation methods. A total of 31 guinea-pigs inoculated with different serotypes of *Shigella flexneri, Shigella sonnei, Escherichia coli* 0124: K72 (B17) and cultures 185T-64 and 193T-64 was studied. Each of the nine cultures employed were inoculated in groups of 2 to 5 animals. Only the results obtained by the hemagglutination method (FEELEY et al. [2]) will be reported here. A comparative study of all the methods employed in the present research will be published elsewhere.

RESULTS

Effects of enterobacteria culture inoculation on the guinea-pig eye. Typical kerato-conjunctivitis developed when the animals were inoculated with *Shigella, Escherichia coli* 0124: K72 (B17), and cultures 185T-64 and 193T-64. However, the standard *Shigella cultures* as well as 2 recently isolated strains of *Shigella sonnei,* form II, were non-pathogenic for the guinea-pig eye.

Kerato-conjunctivitis was not produced in the animals inoculated with *Salmonella, Arizona, Citrobacter, Klebsiella, Aerobacter, Hafnia, Serratia, Proteus, Providencia, Escherichia coli* 026: K60 (B6), 086: K61 (B7), 0111: K18 (B4), 0119: K69 (B14), 0128: K67 (B12), *Alkalescens-Dispar* and other *E. coli* not belonging to these groups. The correspondent results are shown in Table I.

Several animals inoculated with *Salmonella, Citrobacter, Klebsiella, Proteus* and with some *E. coli* strains developed mild conjunctivitis which lasted for 24 to 72 hours in most instances. However, plating of the ocular secretion from 26 of these animals failed to show the inoculated bacteria in most of them. No manifestations at all were detected in the eyes of the guinea-pigs submitted to infection with non-virulent *Shigella* cultures and bacteriological examinations performed 24 hours later were systematically negative.

Clinical manifestations and bacteriology of kerato-conjunctivitis. The incubation period lasted for 16 to 24 hours but in a few animals the symptoms evolved only after 2 to 3 days. Infection manifested itself as a mucopurulent conjunctivitis followed by keratitis, both very marked. In general, acute manifestations began to subside after 5 to 7 days, recovering occurring in 2 to 3 weeks.

Conjunctivitis disappeared always before keratitis. Often a mild corneal opacity lasted for large periods.

In one of the animals inoculated with *Shigella flexneri,* type 6, kerato-conjunctivitis lasted for 2 months, the animal dying with unaltered ocular lesions, and abundant bacteria present in the ocular secretion. In two others, one inoculated with *Shigella sonnei* and the other with the 185T-64 culture, infection spread to the healthy eye, an identical evolution taking place in both eyes.

Bacteriological examination of the ocular secretion showed almost always the inoculated bacteria in pure cultures and in great amounts at the 2nd, 4th and 5th days following inoculation. From that moment on, a progressive decrease in number occurred in the organisms produced, until their complete disappearance. This always happened before total healing of the keratitis and sometimes, of the conjunctivitis. In 6 of the 45 cases studied, other enterobacteria, chiefly *E. coli,* were isolated in association with the inoculated *Shigella* and always coincidently with an increase in ocular secretion.

The mortality rate of the animals with kerato-conjunctivitis was similar to that of the animals inoculated with non-pathogenic cultures.

Table II includes the incubation periods, duration of clinical manifestations and bacteria elimination in 45 animals inoculated with *S. dysenteriae, S. flexneri, S. boydii, S. sonnei, E. coli* 0124: K72 (B17), cultures 185T-64 and 193T-64, all of which were overlooked for the presence of bacteria until negativation of cultures took place.

TABLE I

Effects on the guinea-pig eye, of the inoculation with 312 enterobacteria cultures

Enterobacteria	No. of strains	Kerato-conjunctivitis	
		+	—
Shigella (freshly isolated)	104	102	2 *
Shigella (collection cultures)	31	0	31
Escherichia coli 0124: K72 (B17)	2	2	0
Escherichia coli (groups 026, 086, 0111, 119 and 0128)	31	0	31
Escherichia coli (non-enteropathogenic)	44	0	44
Escherichia coli (*Alkalescens-Dispar* group)	13	0	13
Salmonella and *Arizona*	29	0	29
Citrobacter	6	0	6
Klebsiella	12	0	12
Aerobacter	8	0	8
Hafnia	1	0	1
Proteus (*mirabilis, vulgaris, morganii* and *rettgeri*)	20	0	20
Providencia	9	0	9
Culture 185T-64	1	1	0
Culture 193T-64	1	1	0

* *Shigella sonnei*, form II.

TABLE II

Duration of incubation period, clinical manifestations and bacterial excretion in 45 guinea-pigs with kerato-conjunctivitis caused by *Shigella* species, *Escherichia coli* 0124: K72 (B17) and cultures 185T-64 and 193T-64

Enterobacteria	No. of strains	Duration in days of		
		Incubation period	Clinical manifestations	Bacterial excretion
Shigella dysenteriae	7	1-3	13-14	10-15
	1	1	10	6-10
Shigella flexneri	3	1	18	12-16
	6	1	15	10-15
	5	1	13-14	10-15
Shigella boydii	2	1-2	12-13	8-12
Shigella sonnei	7	1-2	14-15	8-12
	10	1-2	12-13	6-12
Escherichia coli 0124: K72 (B17) Cultures 185T-64 and 193T-64	4	1-3	12	6-10

Serological response of the infected animals. By the hemagglutination method, a rise in antibody titre was recorded in all the 31 guinea-pigs. The highest titres were reached between the 10th and the 20th day of observation. A decrease was noticed on the 30th day but in approximately 70% of the animals, antibodies were still present on the 40th day. The serological response was always very similar for the animals of each group.

In 10 guinea-pigs, antibodies were found in the serum collected before inoculation. The titres were 1/4 in nine and 1/64 in one. Nevertheless, these preexisting agglutinins apparently had no influence on the extent of antibody titres shown by these animals. Table II presents the results obtained for the first animal of each group.

As a further consideration of the serological reactions, it is interesting to refer that one guinea-pig which did not develop kerato-conjunctivitis showed an antibody response very similar to that of two others which actually acquired the disease (Table III).

TABLE III

Reciprocal of the highest serum dilutions containing hemagglutinins, of 9 guinea-pigs (one of each group) presenting kerato-conjunctivitis induced by different enterobacteria

Enterobacteria	Before inoculation	Observation day			
		10th	20th	30th	40th
Escherichia coli 0124: K72 (B17)	0	16	16	8	8
Culture 185T-64	0	512	32	4	16
Culture 193T-64	4	256	64	32	4
Shigella sonnei	0	4	4	0	0
Shigella flexneri 2	0	128	128	16	16
Shigella flexneri 6	4	1.024	256	64	4
Shigella flexneri 3	0	256	128	32	4
Shigella flexneri 2	0	512	64	64	8
Shigella flexneri 2	0	32	32	16	0

TABLE IV

Reciprocal of the highest serum dilution presenting hemagglutinins, of one guinea-pig affected and one not affected by the inoculation of *Shigella flexneri*, type 6

Guinea-pig	Before inoculation	Observation day			
		10th	20th	30th	40th
With kerato-conjunctivitis	4	1.024	256	64	8
Normal	0	512	512	32	8

DISCUSSION

The results obtained (Table I) make it evident that only *Shigella, E. coli* 0124: K72 (B17) and cultures 185T-64 and 193T-64 were effective in producing kerato-conjunctivitis in the guinea-pig. The other enterobacteria were non-pathogenic or induced only a mild conjunctivitis apparently of non-infectious origin since the subsequent bacteriological surveys failed to show the inoculated bacteria in most of the animals. Thus, these results regarding the pathogenicity of enterobacteria for the guinea-pig eye are in accordance, in most aspects, with those referred by other authors (SERÉNY [6, 7, 8]; SZTURM-RUBINSTEN et al. [11]; SIROKO [9]; MACKEL [5]) except for cultures 185T-64 and 193T-64 which apparently are being recognized as pathogenic agents only now. In a forthcoming paper we shall be presenting results demonstrating that both cultures are antigenically different from the enterobacteria recently studied by STENZEL [10] and SZTURM-RUBINSTEN et al. [12] and which, together with *Shigella* and *E. coli* 0124: K72 (B17) were so far the only enterobacteria known to cause kerato-conjunctivitis in the guinea-pig.

A closed correlation between the pathogenic action of the shigellae and the age of the cultures could be established. We can thus infer from same Table I, that of the 104 recently isolated cultures, only two (less than 2%) identified as *Shigella sonnei*, form II, caused no lesions on the animal eye even after repeated inoculation. On the other hand, none of the shigellae belonging to our collection was effective in bringing about any kerato-conjunctivitis; they were, obviously, of older age but other factors may also have influenced their loss of virulence. Anyhow, a complete different line of behaviour was observed in the two groups of cultures, special stress being put on the high efficiency of Serény's method for the demonstration of virulence of *Shigella*. As a matter of fact, except for the two strains of *S. sonnei*, form II, a form already known to be non-virulent (SERÉNY [7]), all the remaining cultures freshly isolated were pathogenic. This means to say that, excluding the rough mutants, practically 100% of the shigellae do produce a kerato-conjunctivitis in the guinea-pig when inoculated in proper concentrations and within one to seven days after isolation.

Experimenting on a material similar to ours, SERÉNY [7] attained results fairly comparable to those of the present paper. However, SZTURM-RUBINSTEN et al. [11] pointed no correlation between age and virulence of their strains of *Shigella*, although these authors did not employ cultures as recent or as aged as ours, which could, probably, count for the different results yielded. A close relation age-virulence was also referred by SIROKO [9], who detected pathogenicity only in shigellae inoculated within six months of isolation. Nevertheless, more detailed studies must be carried out to clear up the problem, the age of the cultures and their methods of preservation as well as other intervening factors, being carefully surveyed. It must be added, however, that 50% of the standard cultures were stable in Trypaflavine.

In what concerns clinical, bacteriological and serological characteristics, emphasis must be laid on the steadiness and uniformity of the manifestations. The disease settles in usually after 16 to 24 hours of incubation, abundant symptoms being present during the first 5 to 7 days; a gradual recovery takes place then and is completed in 2 to 3 weeks. Serial cultures prepared with strains of ocular secretion, revealed the presence of high amounts of organisms during the first week of infection, progressively smaller populations thereafter and its total sterilization usually before the thorough disappearance of the keratitis and sometimes, of the conjunctivitis. The highest levels of antibodies are attained between the 10th and the 20th day of disease, dropping to lower values on the 30th, the antibodies finally disappearing from the sera of some of the experimental animals around the 40th day. In most instances, however, a low antibody titre is maintained for longer periods of time. Other phenomena such as a chronical condition or transmission to the healthy, non-inoculated eye, occurred relatively seldom in our experiments. The clinical and bacteriological (Table II) as well as serological behaviour (Table III) of the experimental animals was about the same, independently of the inoculum consisting of *Shigella, E. coli* 0124: K72

(B17) or cultures 185T-64 and 193T-64. Probably, the only exception observed was that represented by the kerato-conjunctivitis determined by *S. flexneri*, which lasted for a longer period. A similar phenomenon was noticed by SERÉNY [7] and GEKKER & BELAIA [4] during a comparative study of different species of *Shigella* as causative agents of kerato-conjunctivitis.

It is interesting to observe that one of the guinea-pigs inoculated with *Shigella* showed no evidences of kerato-conjunctivitis but did, nevertheless, develop antibodies just in the same way as those that underwent the infection (Table IV). This statement would suggest that a previous proliferation of the inoculated bacteria is not a necessary condition for the establishment of an immunological response. The stimulus represented by the inoculum would suffice. SERÉNY [7] refers that the instillation of endotoxins in the conjunctival sac of guinea-pigs induces the production of antibodies. In what concerns the immunological response, we wish further to lay special stress on the fact that in our experiments a usually high titre of antibodies was attained, and in a regular way, in opposition to the results reported by other researchers (SERÉNY [7]; GEKKER & BELAIA [4]; MACKEL et al. [5]; FEI-CH'ING et al. [3]). However, this may be in part related to the method (agglutination) used by those authors. As will be demonstrated, hemagglutination was the most sensitive of the methods employed.

Before closing these comments, we wish to point out the usefulness of SERÉNY's experimental model, not only for studying the different aspects of infections by enterobacteria pathogenic for the guinea-pig eye, but as a practical process to be routinely employed in the diagnosis of the intestinal infections, as well. It is an easy and low-cost process. Evidently, this method cannot be recommended as exclusive for the identification of *Shigella* since other enterobacteria can also bring about kerato-conjunctivitis and, on the other hand, the rough mutants of shigellae are non-virulent. Nevertheless, its judicious use can afford a valuable help for the bacteriologist who cannot count on the resources actually necessary for the identification of *Shigella*, and the remaining enterobacteria virulent for the guinea-pig eye.

RESUMO

Cérato-conjuntivite experimental do cobaio por enterobactérias

Foram inoculadas no ôlho de cobaio 312 culturas de enterobactérias, assim distribuídas: *Shigella*, culturas recém-isoladas (104); *Shigella*, culturas de coleção (31); *Escherichia coli* enteropatogênica 0124: K72 (B17) (2), outras enteropatogênicas (32); *Escherichia coli* não enteropatogênica (44); *Escherichia coli*, grupo *Alkalescens-Dispar* (13); *Salmonella* (28); *Arizona* (1); *Citrobacter* (6); *Klebsiella* (12); *Aerobacter* (8); *Serratia* (1); *Hafnia* (1); *Proteus mirabilis* (8); *Proteus vulgaris* (4); *Proteus morganii* (7); *Proteus rettgeri* (1); *Providencia* (9); cultura 185T-64 (1) e cultura 193T-64 (1).

De tôdas, apenas as pertencentes ao gênero *Shigella*, a *Escherichia coli* 0124: K72 (B17) e as culturas 185T-64 e 193T-64 causaram cérato-conjuntivite. As demais enterobactérias não lesaram o ôlho do cobaio ou causaram conjuntivite discreta e fugaz, aparentemente não infecciosa. Encontrou-se nítida correlação entre virulência e idade das culturas, de *Shigella*. As culturas de coleção foram tôdas avirulentas. Ao contrário, das 104 recém-isoladas, apenas duas amostras de *Shigella sonnei*, forma II, deixaram de causar cérato-conjuntivite no cobaio.

A resposta sorológica dos animais infetados foi constante e uniforme. As aglutininas atingiram níveis mais elevados entre o 10.° e o 20.° dia de observação.

Diferenças clínicas, bacteriológicas e sorológicas apreciáveis não foram encontradas entre as infecções causadas por *Shigella*, *Escherichia coli* 0124: K72 (B17) e pelas culturas 185T-64 e 193T-64.

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REFERENCES

1. EDWARDS, P. R. & EWING, W. H. — *Identification of Enterobacteriaceae.* 2nd. ed. Atlanta, Georgia, Burgess, 1962.

2. FEELEY, J. C.; MANCLARK, C. R. & PICKETT, M. X. — The use of formalin-preserved erythrocytes in the enterobacterial hemagglutination test. *Am. J. Clin. Path.* **30**:77-82, 1958.

3. FEI-CH'ING, L.; TEH-LING, Y. & SHU-HUA, Y. — Experimental keratoconjunctivitis shigellosis in guinea pigs. I. Immune phenomenon and antibody formation. *Chinese Med. J.* **83**:454-462, 1964.

4. GEKKER, V. D. & BELAIA, J. A. — Immunity in experimental dysenteric keratoconjuntivitis. *J. Microbiol. Epidemiol. Immunol.* **29**:351-356, 1958.

5. MACKEL, D. C.; LORREINE, F.; LANGLEY, F. & VENICE, L. A. — The use of the guinea-pig conjunctivae as an experimental model for the study of virulence of *Shighella* organisms. *Am. J. Hyg.* **73**:219-223, 1961.

6. SERÉNY, B. — Experimental *Shigella* keratoconjunctivitis. *Acta Microbiol. Hung.* **2**:293-296, 1955.

7. SERÉNY, B. — Experimental keratoconjunctivitis shigellosa. *Acta Microbiol. Hung.* **4**:367-376, 1957.

8. SERÉNY, B. — Biochemical reactions and virulence of *E. coli* 0124: K72 (B17). *Acta Microbiol. Hung.* **10**-11-18, 1963.

9. SIROKO, I. A. — Keratoconjunctivitis in guinea-pigs as a biological test for the identification of dysentery bacilli. *J. Microbiol. Epidemiol. Immunol.* **29**:567-573, 1958.

10. STENZEL, W. — *Sh. scholtensii* und *Sh. manolovii* — zwei zur Untergruppe D zu stellende provisorische *Shigella*-Typen. *Ztschr. Hyg.* **148**:433-444, 1962.

11. SZTURM-RUBINSTEN, S.; PIÉCHAUD, D. & THIBAULT, P. — Inoculation conjonctivale au cobaye de bacilles dysentériques. *Ann. Inst. Pasteur* (Paris) **93**:463-467, 1957.

12. SZTURM-RUBINSTEN, S.; PIÉCHAUD, D. & CHARPAK, M. — *Shigella-Alkalescens-Dispar*; souches intermédiaires. *Ann. Inst. Pasteur* (Paris) **106**:122-126, 1964.

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