Infectivity for Man of Cell Culture-Adapted Tric Agents.* (32497)

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The infectious agents causing trachoma-inclusion conjunctivitis in man (TRIC agents) can be grown regularly in the yolk sac of embryonated eggs. This has been the most successful method for isolating TRIC agents from infected eyes. Such "wild" strains are generally incapable of unlimited replication in cultures of primary cells of any type, or of cell lines. In cell culture, "wild" strains can undergo one or two cycles of replication, particularly if inoculated with the aid of centrifugal force(1). However, the yield of infective agent is insufficient to sustain passage in cell culture.

"Wild" strains subjected to prolonged egg passage in the laboratory yield, at random, variants which are capable of unlimited proliferation in cell culture. These variants also grow to higher titer in embryonated eggs and have been called "fast" strains. Such TRIC variants can adsorb to and penetrate cells in culture spontaneously, requiring no application of centrifugal force, and regularly yield a large quantity of infective particles. The question has been asked repeatedly whether such cell culture-adapted mutants are still pathogenic for man, or whether the acquisition of the potential for unlimited replication in cell culture is regularly associated with the loss of the potential for significant replication in human tissues(2). This might be an important consideration in the selection of potential live vaccines.

Limited experiments in the past have yielded irregular results. Strains capable of growth in cell culture have failed to induce eye disease in monkeys in the hands of some investigators(3,4,5), but have induced such monkey disease in the hands of others(6). Three children infected with very large inocula of 3 different cell culture-adapted TRIC strains by Bernkopf developed fairly typical, albeit mild, eye disease(7). Five adult volunteers infected with 3 cell culture-adapted TRIC strains by Mitusi(8) developed very mild self-limited disease. It was concluded that "The tissue culture adapted variants did not lose completely their pathogenicity for human eyes but were apparently attenuated" (8).

Since none of these experiments had employed quantitative methods for the measurement of inocula and had not differentiated infection from disease, we decided to reinvestigate this question. We used 2 cell culture-adapted strains, defined the inoculum in terms of numbers of particles and inclusion-forming units, and followed the process of infection by immunofluorescence applied to conjunctival scrapings, as well as by frequent clinical observations. The results presented here clearly show that the two strains were capable of limited replication in man. However, in only 1 of 20 volunteers was the replication of infectious particles sufficient to result in production of active disease.

Materials and methods. Infective agents. Three TRIC strains were employed.

(a) LB-1 (official designation TRIC//GB/MRC-1/G), originally isolated from the cervix of the mother of an infant with inclusion conjunctivitis was obtained from Professor L. H. Collier in its 30th yolk sac passage. This isolate had spontaneously acquired the ability to proliferate readily in cell culture, after continuous yolk sac passage. A suspension of the 36th yolk sac passage material was used as an inoculum for L 929 cells grown and maintained in an antibiotic-free lactalbumin hydrolysate yeast extract medium containing 10% fetal calf serum (LHY). Cells and fluid from the 9th passage in cell culture were shaken in a bead bottle, the resulting mixture diluted 50% in skim milk, and stored at −60°C. By direct microscopic count(9), this LB-1

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pool contained 1.6×10^8 particles per ml, and 6.4×10^5 inclusion-forming units (IFU) per ml when titrated on HeLa cell monolayers (10).

(b) BOUR-J, a variant of BOUR (TRIC/ /USA-Cal-1/OT) was adapted to cell culture by Mitusi *et al*(11). The original BOUR had been isolated in our laboratory in 1959 from a patient in California with trachoma. Material from the 7th yolk sac passage in our laboratory following Mitsui's cell culture adaptation was used for the inoculation of L 929 cells in LHY medium. The harvest from the BOUR-J 9th cell culture passage contained 9.8×10^7 particles per ml, and 8.9×10^5 IFU per ml when titrated on HeLa cell sheets. Strains LB-1 and BOUR-J which replicate in cell culture and do not require centrifugation of the inoculum for infection will be called "cell culture-adapted" strains.

(c) ICCal-8 (TRIC/ /USA-Cal/Cal 15/ ON) was isolated in our laboratory in 1961 from an infant with inclusion conjunctivitis. It does not proliferate readily in cell culture, but infection can be maintained if, at each transfer, the inoculum is centrifuged onto the cell monolayer(1,12,13). This isolate in its 10th yolk sac passage was centrifuged onto HeLa 229 cells grown in LHY medium. The harvest from the 7th cell culture passage was used for volunteer inoculation without dilution with skim milk or freezing. At this time the IC-Cal-8 had not become adapted to cell culture, and infection could not be maintained without the aid of centrifugation. This pool contained approximately 10^8 particles per ml, and 10^6 IFU per ml when centrifuged onto HeLa cell monolayers.

Volunteer inoculation. The selection of volunteers and the method of their inoculation has been described in detail(14). The LB-1 and BOUR-J pools were taken in the frozen state to the volunteers, thawed immediately before use and diluted in cold skim milk. A portion of the thawed pool was returned to the laboratory for particle counts. The volume of infective inoculum retained in the volunteer's eye was estimated to be 0.02 ml. Thus the effective inoculum of the LB-1 pool (undiluted) contained 3.2×10^6 particles and 1.3×10^4 IFU, whereas the effective inoculum of the BOUR-J pool (undiluted) contained 2×10^6 particles and 1.8×10^4 IFU. The pool of ICCal 8 was used without freezing, or dilution, in one volunteer. His inoculum contained approximately 8 EID50, 2×10^6 particles and 2×10^4 IFU.

Criteria of infection in volunteers. Volunteers were followed by subjective symptoms, objective signs, and laboratory examinations. Each volunteer recorded daily symptoms referable to his eyes or his general health. Detailed ophthalmological examinations were performed and recorded twice weekly for 1 month after inoculation and every 7-10 days thereafter for 5 months. Conjunctival scrapings were collected at intervals from the inoculated eyes and examined by immunofluorescence. The presence of TRIC agent antigen in the cytoplasm of epithelial cells was determined by direct immunofluorescence (FA), employing fluorescein isothiocyanate-labeled serum from a rabbit hyperimmunized with crude yolk sac infected with the TE 55 (T'ang) strain of TRIC agent. The details of the method and the evidence for the specificity of the immunofluorescent stain have been described previously(14,15,16,17). Reisolation in yolk sac of the TRIC agent from volunteers was attempted only a few times. Blood specimens for serologic tests (group complement-fixing antibodies) were collected immediately prior to inoculation and three weeks later from all volunteers. Additional sera were obtained later from a few persons. Conjunctival scrapings were examined regularly for typical Giemsa-staining TRIC intracytoplasmic inclusions in only one volunteer, inoculated with strain ICCal 8.

Results. Twenty volunteers were inoculated with strains LB-1 or BOUR-J grown in cell culture. Only a single volunteer developed objective signs of infection (IX-12). The clinical and laboratory findings in that individual are summarized in Table I. It is noteworthy that FA+ inclusions were demonstrated during the 2nd and 3rd week after inoculation whereas unequivocal symptoms and signs of disease did not appear until the 5th week. The infecting TRIC agent was

TABLE I. Clinical and Laboratory Findings in Volunteer IX-12 Infected with Cell Culture-Adapted Strain LB-1.

	Weeks after inoculation								
	1	2	3	4	5	7	8	13	18
Symptoms	±	±	—	—	+	+	±	±	—
Clinical disease	—	—	—	—	+	+	+*	±	—
Group CF antibody	<1/5		<1/5				1/20	1/40	
TRIC agent re-isolated			—				+	—	
FA+ inclusions	—	+	+	—	+	+	—	—	—

* Systemic tetracycline treatment administered during 8th and 9th week.

re-isolated by the inoculation of conjunctival scrapings into embryonated eggs in the 8th week (day 56) just prior to the inception of tetracycline treatment. The follicular conjunctivitis, discharge, epithelial keratitis and lymphadenopathy in this volunteer was entirely comparable in severity to the disease observed in many other volunteers previously. It differed principally in the extraordinarily long incubation period of 35 days.

While none of the other 19 volunteers inoculated with two cell culture-adapted strains developed objective signs of disease, many did manifest subjective symptoms and laboratory evidence of infection. Between the 3rd day after inoculation and the end of the third week, more than half of the volunteers complained of excessive tearing, foreign body sensation, itching and other forms of intermittent discomfort. The greatest frequency and the highest intensity of these subjective manifestations occurred around the 7th day after inoculation.

The results of immunofluorescence examinations are summarized in Table II. The highest incidence of FA+ inclusions occurred during the 2nd week after inoculation, shortly after the peak incidence of subjective complaints. At that time all volunteers receiving the larger LB-1 inoculum were FA+. Three of the 4 volunteers receiving a 10-fold smaller inoculum of LB-1 were

TABLE II. Incidence of Positive Immunofluorescence Among Volunteers Infected with Cell Culture-Adapted Strains LB-1 and BOUR-J.

	Inoculum		FA+ inclusions present in conjunctival scrapings — Weeks after inoculation						
Volunteer	Strain	Dil.	1	2	3	4	5	7	13
IX-1*	LB-1	10^{0}	+†	+	—	—	—	—	
IX-8			ND	+++	—	+	—	—	
IX-12‡			—	+	+	—	—	+	—
IX-3*			—	+	—	—	—	—	—
IX-14			—	+++	—	—	—	—	
IX-7	LB-1	10^{-1}	—	+	+	+++	—	—	
IX-11			—	—	+	—	—	—	
IX-16			—	—	—	+	+	—	
IX-19			—	—	—	—	—	—	
IX-2*	BOUR-J	10^{0}	ND	—	—	—	—	—	
IX-5*			—	—	—	—	—	—	
IX-10			+	—	+	—	—	—	
IX-15			—	+	—	+	—	—	
IX-20			+	+	—	+++	—	—	
IX-18			—	—	+	+	—	—	
IX-4*	BOUR-J	10^{-2}	—	++	—	+	—	ND	
IX-6*			—	+	—	—	—	—	
IX-9			—	++	—	—	—	—	—
IX-13			—	+++	—	—	—	—	
IX-17			+	—	+	—	—	—	

* Infected during earlier experimental inoculations.
† + = 1-4 inclusions; ++ = 5-10 inclusions; +++ = >10 inclusions.
‡ Volunteer IX-12 is the only one who developed clinical disease.
ND = not done.

FA+ during the 3rd or 4th week after inoculation, as though a longer period had been required for replication to reach a level detectable by immunofluorescence. Nine of the 11 volunteers inoculated with BOUR-J were FA+ some time during the 4 weeks following inoculation, but the interval to the FA+ finding appeared unrelated to the size of the inoculum. Among the 14 volunteers who experienced this as the first TRIC infection, 13 were FA+, 9 of them more than once. By contrast, among 6 volunteers for whom this was a second or third infection, 4 were FA+, only 2 of them more than once.

None of the inoculated volunteers developed a significant rise in group complement fixing antibodies in the first 3 weeks after inoculation. Later serum specimens were examined only in volunteer IX-12, who developed manifest clinical disease. In him the antibody titer showed a significant rise by the 8th week after inoculation, 3 weeks after the inception of disease signs.

The inoculation of strain ICCal 8 into one volunteer gave very different results. In spite of having undergone repeated infections with TRIC strains in the past, this volunteer developed unequivocal signs of follicular conjunctivitis and keratitis on day 14. The symptoms and the clinical eye disease were of moderate severity and will be described in detail elsewhere. Signs of active infection continued for 6 weeks and then subsided without the need for drug therapy. During the 2-week incubation period, conjunctival scrapings were FA+ in 6 of 7 examinations, 2 of which were also positive by Giemsa stain. During the 6 weeks of active disease, conjunctival scrapings were Fa+ 16 times in 22 examinations, 8 of which were also positive by Giemsa stain.

Discussion. Our earlier studies on experimental human infection with recent isolates of TRIC agents have indicated that the inoculum size determined the length of the incubation period but not the severity of the resulting disease(14). Apparently the infecting TRIC agent replicates in conjunctival cells until it reaches the level required for the production of disease. The smaller the inoculum, the longer the time required to reach this level, *i.e.,* the longer the incubation period. However, if replication proceeds to the level of disease production, the subsequent course of the disease is independent of the original inoculum size, and is largely determined by characteristics of the infecting TRIC strain and of host tissues.

The ability of a TRIC agent to replicate to the level of disease production must depend not only on inoculum size but also on the TRIC strain's potential for replication in the host and on the activity of host mechanisms which can restrain such replication. In the course of prolonged passage in the laboratory many microorganisms lose "virulence." This has been observed with TRIC strains upon prolonged egg passage(5). Conversely, fresh TRIC isolates are never capable of profuse replication in cell culture whereas some variants arising in the course of laboratory passage have acquired this characteristic. Does this mean that cell culture-adapted TRIC strains are incapable of infecting man?

The results of this study indicate that the strains LB-1 and BOUR-J which are capable of unlimited replication in cell culture have indeed lost most of the potential for producing disease in man. Their replication in human conjunctiva is greatly limited, so that the level of replication necessary for disease production is reached only very rarely—once in 20 volunteers. However, some degree of replication occurred in virtually all volunteers, as shown by FA+ inclusions present during the second to fourth weeks after inoculation. The strains LB-1 and BOUR-J were "attenuated" in that they had become much more susceptible to the host influences which limit replication, than fresh isolates. One can only speculate on the nature of these host factors. It is known, however, that strains LB-1 and BOUR-J are susceptible to the action of interferon and can induce the formation of interferon(13).

Clinical infection with TRIC agents can induce a partial resistance to reinfection(18). Some of the volunteers in the present study had undergone an experimental TRIC infection prior to their inoculation with cell culture-adapted strains. The frequency of subclini-

cal LB-1 or BOUR-J infection may have been different in volunteers inoculated for the first or for the second time. Nine of 13 volunteers infected for the first time were repeatedly FA+, whereas only 2 of 6 reinfected volunteers were in this category. The role of "immune" factors resulting from prior experience with TRIC agents in the restraint of TRIC replication is difficult to evaluate in this study. Moreover, we do not know at this time whether volunteers who have carried a subclinical TRIC infection for a few weeks might be demonstrably more resistant to challenge with a fresh isolate.

There is an apparent discrepancy between the results of others with similar cell culture-adapted strains and those recorded here. Neither Berkopf(7) nor Mitsui(8) quantitated their inoculum for volunteer's eyes. It is probable that they inoculated somewhat larger numbers of infective particles than we did, since both used undiluted cell culture material applied to the eye with cotton swabs. We employed the same quantitative method of inoculation which had earlier been used to measure the size of the human infective dose. In this comparison, the cell culture-adapted strains LB-1 and BOUR-J were at least 100-1000 times less infective for the human conjunctiva than the unmodified TRIC strain ICCal 8(14). The potential applicability of such "attenuated," cell culture-adapted TRIC strains to problems of trachoma control will be considered in the future.

Summary. Fresh isolates of trachoma-inclusion conjunctivitis (TRIC) agents infect the human eye, but undergo only very limited, if any, replication in cell culture. In the course of laboratory passage, variants arise which proliferate freely in cell culture. The ability of such cell culture-adapted variants to infect the human eye was investigated. Of 20 volunteers infected with 2 such variants, only one developed clinical eye disease. In 16 others, limited replication of the agent was proven by immunofluorescent staining of conjunctival scrapings, with the largest amount of TRIC antigen present during the second week after infection. The cell culture-adapted strains LB-1 and BOUR-J appeared to be 100-1000 times less infective for the human conjunctiva than fresh TRIC isolates. The limitations of such variant strains as potential live vaccines are considered.

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