titration with EDTA, whereas in the present experiment flame photometry by the method of MacIntyre was used(8). In addition Nielsen injected large "unphysiologic" doses of Vasopressin (0.6 to 1.4 units) in his experiments demonstrating an increased calcium excretion(3).

Heaton and Hodgkinson(6) found no relationship between urinary flow and calcium excretion during water load in normal subjects. It is of interest that they measured calcium by flame photometry, a method comparable to the present study. In patients who had therapeutic hypophysectomy for metastatic carcinoma, Gardner *et al*(13) found that water diuresis and Vasopressin-induced antidiuresis did not correlate with calcium excretion. These authors(13) also used a flame photometric methd for analysis. However, the malignant disease and hypophysectomy makes their experiments considerably more difficult to interpret.

The slight decrease in calcium and sodium excretion observed in the present experiments may represent diurnal variation or very slight changes in glomerular filtration rate (Table I, Fig. 2).

Summary. In 9 healthy men doses of Arginine-8-Vasopressin and Lysine-8-Vasopressin from 5.5 to 20 mM which produced decreases in free water clearance (C_{H_2O}) of 4 to 112% had no significant effect on calcium or sodium excretion.

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TRIC Agents Isolated in the United States. X. Immunofluorescence in the Diagnosis of TRIC Agent Infection in Man.* (30282)

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Among the millions of persons afflicted with trachoma the best hope for effective treatment is early diagnosis. For the experienced ophthalmologist the typical case of trachoma (TR) or of inclusion conjunctivitis (IC) is relatively easy to diagnose. There remain many cases in which clinical diagnostic signs are inadequate, and in which laboratory diagnostic tests are needed. To date, the laboratory has been unable to offer adequate help. The most commonly used method has been the demonstration of the typical Giemsa-staining inclusions within epithelial cells of the conjunctiva, first described by Halberstaedter and von Prowazek(1). After Rice(2) demonstrated an iodine-stain-

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ing matrix in these inclusions, some workers (3) considered this technic more satisfactory than the Giemsa stain. Following isolation of the trachoma agent(4) it was reported (5,6,7) that isolation offered a more sensitive method of detecting TRIC infection. Most recently fluorescent antibody (FA) technics were applied by Vozza and Balducci(8) and by Nichols *et al*(9). The latter group surveyed a large number of individuals seen in a Saudi Arabian hospital clinic, and demonstrated that under their conditions FA technics were superior to either Giemsa-stained preparations or egg inoculation.

The major portion of the findings to be reported here was obtained as part of a large study being conducted among experimentally infected volunteers, inmates of Vacaville Medical Facility, California State Department of Corrections(10). This situation provided a unique opportunity to follow closely groups of individuals for whom time and dose of inoculation, onset of disease, and chemotherapy, if any, were known. A second source of materials was patients examined at University of California, San Francisco Medical Center because of manifest eye disease, some of which were highly suggestive of TRIC infection on clinical grounds.

The purpose of this report is to compare immunofluorescence with other methods (TRIC isolation in embryonated eggs, Giemsa stain, and iodine stain) in the laboratory diagnosis of experimental or spontaneous TRIC infections.

Materials and methods. Specimens were collected from the 2 groups of individuals described above. The larger number were volunteers in the inclusion conjunctivitis study, who had been inoculated with varying dilutions of IC-Cal 3† or IC-Cal 8† strains of inclusion conjunctivitis agent isolated in this laboratory from newborn infants(10). Most of the volunteers developed clinical disease, and all inoculated persons were followed very closely. At the beginning of the study each patient was studied exhaustively, with frequent microscopic examination of conjunctival scrapings using iodine and Giemsa staining and immunofluorescence (FA) and attempts to reisolate the infecting agent. Later, as the FA technic proved to be reliable, it was used as the principal laboratory test for infection.

The methods of collecting and handling materials have been described(10). Following ophthalmologic examination conjunctival scrapings were collected from the lower lid by means of a sterile platinum spatula several minutes after instillation of an ophthalmic surface anesthetic. When multiple specimens were collected, an attempt was made to scrape 3 different but comparable areas of the conjunctiva with specimens for isolation, FA, and Giemsa studies collected in that order.

For FA examination the scraping was smeared onto a standard area within the etched circle of a glass slide and air-dried. The slides were then placed into chilled acetone, transported from the volunteer institution to the laboratory (about a 2-hour interval), rinsed in phosphate buffered saline (PBS) at pH 7.1, air-dried and stored at −20°C until they were examined by direct immunofluorescence.

Specimens from individuals examined at University of California, San Francisco Medical Center were handled similarly, except that fixation in cold acetone was limited to 20-30 minutes.

The method for preparing labeled antisera and for staining smears has been described (10,11). All slides from the series of experimental infections were examined using a serum from a single rabbit hyperimmunized with a crude yolk sac suspension of the BOUR† strain of TRIC agent. This serum showed a high FA titer with fluorocarbon-purified preparations of both the homologous antigen and IC-Cal 3, indicating considerable cross-reactivity. In addition, the group specificity of the staining reaction and the optimum serum dilution for the test were determined in titrations in which T'ang strain of TRIC agent in its 35th transfer in a contin-

† According to recommendations of Trachoma Group, International Congress of Microbiology, Montreal, 1962, nomenclature would be as follows:
IC-Cal 3 = TRIC/ /USA-Cal/Cal-9/ON
IC-Cal 8 = TRIC/ /USA-Cal/Cal-15/ON
BOUR = TRIC/ /USA-Cal/Cal-1/OT

uous cell line (HEp 2) was the antigen. The serum was labeled with isothiocyanate by the method of Spendlove *et al*(12). Slides for routine diagnosis were examined with either the same anti-BOUR serum or one prepared against IC-Cal 3 and tested in the same way. Both antisera were equally satisfactory.

Slides were thawed immediately before staining and allowed to air-dry. The smears were covered with a 1:10 dilution of the labeled antiserum in PBS at pH 7.1 and incubated in a moist chamber at 37°C for 30 minutes. They were then rinsed for 10 minutes in PBS at pH 7.1, drained and mounted in 90% glycerol in PBS. The entire area of the smear was scanned with a Zeiss fluorescent microscope equipped with 40 × oil immersion objective, 8 × oculars, an OG 1 barrier filter and a BG 12 exciter filter. "Positive" immunofluorescent findings met the following criteria: (a) A brightly fluorescing, sharply circumscribed body, typical of TRIC inclusions in epithelial cells; and (b) intracellular location within an epithelial cell, clearly located outside the nucleus, and not attached to or part of a polymorphonuclear leucocyte. Fluorescent areas or masses not meeting all the above criteria were considered "doubtful."

The examiner was not aware of the source of the specimen.

Results. Specifically fluorescing inclusions within epithelial cells in conjunctival scrapings were readily identified in the majority of instances, with the 40 × oil immersion objective. Low-power (10 ×) scanning was not particularly helpful in view of the bright fluorescence of polymorphonuclear cells which were frequently present in large numbers, particularly during the acute stage of infection. In addition, epithelial cells often occurred in large clumps, surrounded by fibrin or mucus, and in these circumstances it was impossible to determine cellular detail. Free fluorescein occurred in crystalline form and could be easily differentiated from specific inclusions. Uptake of the fluorescein used for ophthalmologic examination seemed minimal, but scrapings were routinely collected before administration of the fluorescein. Autofluorescence was apparent in some degenerating cells. Since the cells most easily removed by the scraping were those already damaged by disease, nuclear or cytoplasmic detail was sometimes difficult to determine.

TABLE I. FA-Positive Inclusions *vs* Onset of Clinical Disease in Experimental TRIC Infections.

		(%)
FA+ with disease	33/64*	51
FA+ before "	21/64	33
Disease before FA+	9/64	14
" without FA+	1/64	2

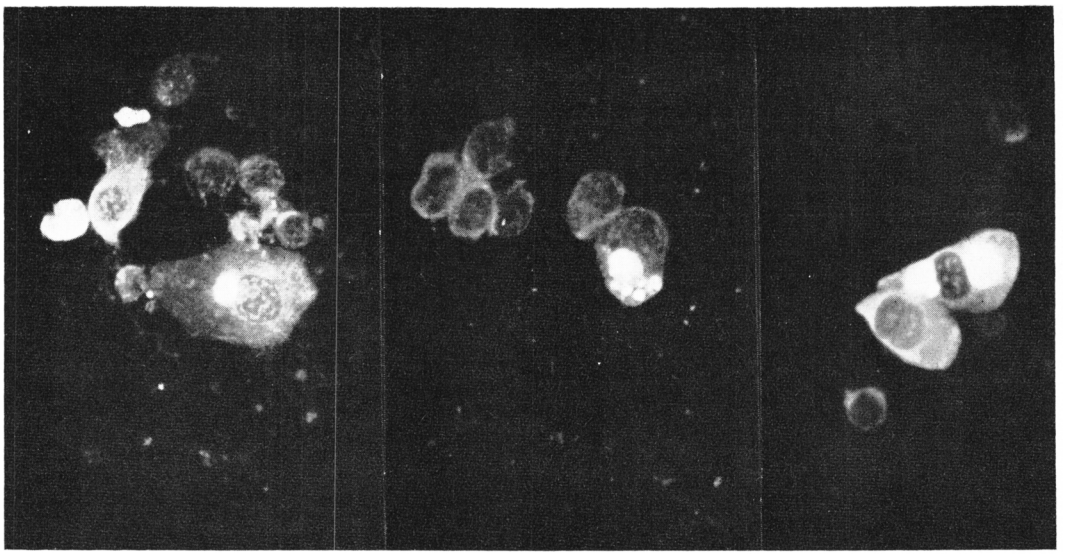
* Positive cases/total cases.

It was common to see 5-10 inclusions per smear during the acute stage of disease, and it was rare to see 20 or more. As the infection became less acute, the number of inclusions decreased, and frequently only 1 or 2 were noted. The most common finding was of one small inclusion within the cytoplasm of a cell, but multiple foci were encountered frequently. When the inclusion was large, or when there were many within one cell, the nucleus was usually distorted (Fig. 1). The frequent presence of clumps of epithelial cells made meaningful cell counts impossible. Consequently, the proportion of inclusion-containing cells could not be determined.

In more than half of the volunteers who developed clinical disease, the appearance of FA+ inclusions paralleled onset of disease, as shown in Table I; in one-third, FA+ inclusions were seen before appearance of clinical disease. Rarely, clinical disease preceded microscopic evidence of infection, and in only one case of clinical disease was it impossible to demonstrate inclusions at any time.

Volunteers not developing clinical disease included those receiving less than one infective dose and those who had developed apparent strain-specific immunity to the agent due to previous infection. In this group two-thirds were consistently FA inclusion negative. In seven, 1 or 2 inclusions were seen on one occasion only, apparently representing a limited replication of the TRIC agent. "Doubtful" inclusions were seen in an additional 3 volunteers without disease.

During clinical disease, and in those instances in which isolated inclusions were seen in the absence of disease, there was close



a b c

FIG. 1. Conjunctical scrapings from volunteers, examined by immunofluorescence. (a) Epithelial cell containing FA+ inclusions surrounded by lymphocytes; (b) Epithelial cell containing multiple FA+ inclusions within the cytoplasm, and several lymphocytes; (c) One epithelial cell containing two FA+ inclusions which have distorted the nucleus, a second, uninfected epithelial cell, and lymphocytes.

correlation between presence of inclusions and increased numbers of polymorphonuclear leucocytes. This correlation was particularly apparent during the early stages of disease.

In one group of experimental infections, specimens were available for isolation studies and microscopy. Iodine and Giemsa stains were used, as well as immunofluorescence. Table II shows the laboratory procedure, alone or in combination, which *first* yielded positive evidence of infection. In 11 of the 13 volunteers of that group, immunofluorescence alone, or in combination with another method, yielded the first proof of infection; whereas, egg isolation provided such proof in 8 instances and Giemsa stain in only 3 instances.

TABLE II. Earliest Laboratory Proof of TRIC Infection in 13 Experimentally Infected Volunteers.

No. patients	TRIC isol	Inclusions FA	Inclusions Giemsa	Inclusions Iodine
6	+	+	—	—
3	—	+	+	—
2	+	—	—	—
2	—	+	—	—
Total 13	8/13	11/13	3/13	0/13

TABLE III. Comparison of Sensitivity of Immunofluorescence and Giemsa Stain in Identification of Inclusions During Experimental Infection with TRIC Agent.

Infected patient	Giemsa stain		FA	
I- 1	6/15*	40%	9/ 9	100%
I- 2	2/ 9	22	2/ 9	22
I- 3	5/ 9	55	4/ 7	57
I- 4	3/14	21	4/10	40
I- 5	9/13	69	5/ 8	63
I- 6	3/12	25	6/ 9	67
I- 7	5/10	50	4/ 6	67
I- 8	2/10	20	5/ 8	63
I- 9	2/ 7	29	1/ 6	17
I-10	3/11	27	7/ 8	88
II- 2	3/ 9	33	1/ 4	25
II- 5	2/ 9	22	3/ 3	100
III- 5	2/ 3	67	3/ 4	75
III- 8	4/ 5	80	4/ 6	67
Total	51/136	38%	58/97	60%

* No. of inclusion-positive slides/total No. of slides examined.

In 14 other volunteers who became infected, scrapings for both Giemsa and FA were collected throughout the course of the disease. Table III compares the relative sensitivity of Giemsa and FA preparations. These data represent only typical inclusions, and include all specimens collected after inoculation until spontaneous healing or treatment.

TABLE IV. Comparison of FA, Giemsa and Egg Isolation Methods for Demonstration of TRIC Agent in Patients with Clinically Diagnosed Spontaneous Eye Disease.

Clinical diagnosis	No. of patients	TRIC isol	Inclusions FA	Giemsa
TRIC	20	7	14	6
Possible TRIC	7	0	2	0
Non-TRIC	9	0	0	0

After inclusions had become demonstrable by FA in a given diseased volunteer, typical inclusions were found quite regularly throughout the infection. In contrast, Giemsa-staining inclusions were observed much less regularly.

Specimens from patients with spontaneous eye disease (seen at University of California, San Francisco Medical Center) were usually obtained on only one occasion, but the patients were examined in close proximity to the laboratory, and the handling of specimens was optimal. Table IV shows the results of TRIC isolation attempts and examination for Giemsa-positive or FA-positive inclusions in 3 categories of patients, designated by the clinicians as typical TRIC, possible TRIC (follicular conjunctivitis or keratoconjunctivitis of unknown etiology), and non-TRIC (herpes simplex, adenovirus, allergic conjunctivitis, corneal ulcer due to periarteritis nodosa, etc.). Among the group of 20 patients with typical TRIC infection (inclusion conjunctivitis and both active and healed trachoma) TRIC agent was isolated in approximately one-third of the cases. Giemsa-staining inclusions were observed in about the same number, but immunofluorescence revealed typical inclusions in two-thirds of this group. In the non-TRIC group none of the laboratory procedures yielded positive findings. Among 7 clinical cases of possible TRIC, typical FA+ inclusions were observed in 2, neither of which yielded a TRIC isolate or Giemsa-positive inclusions.

When results of the 3 technics are considered with reference to individual patients rather than to groups with similar diagnoses, it is apparent that positive immunofluorescence in 14 patients was confirmed by isolation of TRIC agent in 5 and by Giemsa stain in another 3 (Table V). In only 3 patients did the other laboratory methods provide evidence of TRIC infection in the absence of FA+ inclusions.

Discussion. Immunofluorescence appears to be a much better laboratory aid in the diagnosis of TRIC infection than has been available in the past. Our use of immunofluorescence in controlled experimental infections permits a more detailed evaluation of the technic than has been possible in other surveys. Vozza and Balducci(8) considered the technic of doubtful value, because they encountered weak reactions and FA+ inclusions which could not be confirmed by Giemsa stain. Their study suffered from the use of an antiserum against human conjunctival scrapings; their reagent contained antibodies to human cellular antigens and only low titers of TRIC antibodies. They also used convalescent sera from trachoma patients. These human sera also may have contained very little TRIC antibody(10).

Nichols *et al*(9) considered the technic more successful in their survey of 114 clinic patients in a Saudi Arabia hospital. Rigid controls at each step strongly indicated the specificity of the immunofluorescence noted with a labeled high-titer human anti-LGV serum, but they also noted the presence of "excess inclusions" which could not be identified by Giemsa. The demonstration of similar discrepancies in numbers between Giemsa and FA+ inclusions seen in our group of volunteers known to be infected with TRIC agent and to have active disease, is additional support for the belief that these do represent specific immunofluorescent staining.

At the beginning of our study, when FA+

TABLE V. Incidence of Positive Results with FA, Giemsa and Egg Isolation in 20 Patients with Clinically Diagnosed Spontaneous TRIC Infection.

No. patients	TRIC isol	Inclusions FA	Giemsa
2	+	+	+
3	+	+	—
3	—	+	+
2	+	—	—
6	—	+	—
1	—	—	+
3	—	—	—
Total 20	7/20	14/20	6/20

inclusions were at times found in the absence of Giemsa-staining inclusions in parallel slides, the FA preparations (initially fixed in chilled absolute methyl alcohol) were rinsed in distilled water, re-fixed in methyl alcohol and Giemsa-stained. In all instances these slides were Giemsa-negative. Thus, certain FA+ cells apparently failed to contain typical Giemsa-staining inclusions.

It has been shown previously(11,13) that immunization of rabbits with TRIC-infected crude yolk sac results in the production of high levels of antibody. Even with purified antigens there may be considerable cross-reaction among antisera to various TRIC strains. The IC-Cal 3 strain of inclusion conjunctivitis cross-reacts to a very high degree with the BOUR strain of trachoma, and to a lesser degree with all strains of TRIC agent tested. Therefore, it is probable that the two labeled antisera employed in the present study provided sensitive reagents for detection of TRIC antigens. It has been shown earlier(11) that LGV has the broadest spectrum of reactivity among PLT agents tested. Consequently, a high-titer anti-LGV serum such as that used by Nichols *et al*(9) should be as satisfactory a diagnostic reagent as those described here.

It is noteworthy that in 33 of the 64 experimental infections, onset of disease and appearance of FA+ inclusions occurred simultaneously; and that in 21, immunofluorescence permitted prediction of future disease. The failure to find inclusions in the single individual who developed clinical disease without microscopic evidence might be explained by the limited number of specimens obtained (two). Egg inoculation and examination of Giemsa-stained preparations were not done on that individual, but the etiology of his disease was established by a rise in complement-fixing antibody(10).

Among the infected volunteers in whom complete laboratory studies were performed, all procedures employed were capable of confirming the presence of the agent. However, relative incidence of positive findings varied.

The findings in specimens from the smaller group of patients with spontaneous eye disease are more difficult to evaluate. The high incidence of FA+ inclusions in clinically diagnosed TRIC infection and their absence in non-TRIC disease suggests a sensitivity and specificity comparable to that observed in the volunteer study.

The observations on both volunteers and patients with spontaneous eye disease indicate that immunofluorescence offers a valuable aid in diagnosis of TRIC infection. In addition to its higher sensitivity, it has several other advantages over previously used methods. Egg isolation studies are time-consuming and expensive, and large-scale attempts are impractical. Specimens for FA microscopy can be easily collected in the laboratory or in the field, stored, and examined at a convenient time. Aside from a fluorescent microscope, little equipment is necessary, and the examination time is relatively brief.

Summary. Conjunctival scrapings from volunteers experimentally infected with TRIC agents and from patients with spontaneous eye disease were examined by immunofluorescence. Brightly fluorescing inclusions were demonstrated with hyperimmune TRIC antisera. The findings were compared with those obtained by Giemsa stain, iodine stain or isolation of the infectious agent in embryonated eggs. Immunofluorescence appears to offer an improved method for laboratory diagnosis of TRIC infections.

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Discriminative Enumeration of Mixed Anisometric Cell Populations By an Electronic Counter.* (30283)

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During the course of quantitative studies designed to assess the immunological effect *in vitro* of lymphocytes on an established line of "target" tumor cells bearing homologous transplantation antigens, some method was required that would enable the enumeration of the larger target cells present in mixtures with the smaller lymphoid cells. The instrument of choice for this purpose seemed to be an electronic particle counter because of its convenience and simplicity of operation as well as the degree of precision possible(1,2). While the electronic counter has often been used for counting and sizing cells or other particulate matter from relatively homogeneous populations in suspension, including tissue culture cells(3,4), its use in the *discriminative* counting of one cell type when present in mixtures with another has been limited; moreover, the details of calibration of this instrument for this type of counting problem have not been published. This note, then, describes: a) the results obtained when an electronic counter was used to enumerate selectively, on the basis of cell volume, fibroblasts of larger volume from smaller sized lymphoid cells present together in suspension; and b) the procedures used to calibrate the counter for such discriminative counting. Particles having a known mean volume were also employed so as to provide a standard reference for establishing the absolute volumes of the cells counted.

Materials and methods. Enumeration. The Model B Coulter Counter‡ having a 100 μ aperture was employed. The aperture current (APC) and amplification (AMP) settings used depended on the particle being counted; selection of the appropriate settings was made according to the principles suggested by Brecher *et al*(2). Counts were performed on cells or particles suspended in a versene buffered counting fluid.§

Cells and particles employed. a) Lymphoid cells were obtained *via* standard techniques (5) from the axillary and brachial nodes of adult C57BL/6 female mice and from mature female DA rats. These cells were washed 3 times in Hanks' buffered salt solution and a sample taken for enumeration with a hemocytometer and the aid of a phase-contrast microscope.

b) Tissue cultured cells were of two types

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‡ Coulter Electronics Co., Hialeah, Fla. The authors are indebted to Dr. Leonard Hayflick for the use of the counter.

§ The counting fluid was made up as 1 l of a 10X stock solution containing NaCl 79.0 g; KCl 3.7 g; $Na_2EDTA \cdot 2H_2O$ 8.9 g; Na_4 EDTA 9.9 g; 4 ml 0.5% phenol red. One part of the stock was diluted with 9 parts distilled water and calf serum added to a final concentration of 0.2%.