Differentiation of Group A Arboviruses Chikungunya, Mayaro, and Semliki Forest by the Fluorescent Antibody Technique[1] (35089)

SONJA M. BUCKLEY AND DELPHINE H. CLARKE
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Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510; and The Rockefeller Foundation, New York, New York 10020

The fluorescent antibody (FA) technique (1) for antigen localization was first applied to arboviruses by Noyes (2), who studied West Nile virus (Egypt 101 strain) and found that the cellular localization of the antigen was strictly cytoplasmic. Subsequently, specific cytoplasmic localization was described for many other viruses, representing a number of different groups of arboviruses (3–11).

In 1961, Wiebenga (12) used the FA method to study cross-reactions between arboviruses and was able to distinguish between Japanese encephalitis and dengue type 1. Specific differences among dengue virus types 1–4 by immunofluorescence were reported in 1964 by Bhamarapravati *et al.* (13). In the same year, Kunz (14) was able to make quantitative differentiation of viruses of the tick-borne encephalitis complex by combined application of the FA method and microphotometry.

This communication reports the differentiation of three closely related group A arboviruses—Chikungunya (CHIK), Mayaro (MAY), and Semliki Forest (SF)—by the indirect FA method (15), using a conjugate subjected to diethylaminoethyl (DEAE)-cellulose chromatography and gradient elution to eliminate both underconjugated and overconjugated antibody molecules (16).

Materials and Methods. Viruses. Prototype strains of CHIK, MAY, and SF viruses were propagated serially in HeLa cells (17). Viral transfers from infected to new cultures were carried out as soon as a cytopathic effect (CPE) was detected, *i.e.*, 24–48 hr after inoculation. Infected *in vitro* stocks (CHIK, 3rd passage; MAY, 14th passage; SF, 17th passage) were prepared from combined cell and fluid phases, lyophilized in 1-ml amounts, and stored at −65°. The viruses were rehydrated freshly for each test, and before dilution were allowed to stand at room temperature for 20 min.

Cell cultures. Conditions pertaining to propagation and maintenance of HeLa cells, as well as the methods of preparing stationary tube cultures, have been described (18, 19). For tinctorial and FA studies, HeLa cells (100,000 cells/ml) were grown on cover glasses (11 × 22 mm, No. 1 thinness) placed in Leighton-type tubes. Monolayers were present within 48–72 hr after incubation at 37°. Preparations inoculated with large virus doses, *i.e.*, 0.1 ml of undiluted stock material per Leighton-type tube, were harvested as soon as CPE began to appear, which was about 18–24 hr after inoculation. Uninoculated control cultures were harvested at the same time.

Diluent. The diluent consisted of 0.75% bovine albumin (Fraction V) in phosphate-buffered saline, pH 7.2 (20).

Titration of viruses. Increasing 10-fold dilutions of virus were inoculated into replicate stationary tube cultures which were exam-

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ined daily for 4 days for development of CPE. Titers were calculated by the method of Reed and Muench (21) and represent the virus doses required to induce CPE in 50% of the inoculated cultures (TCD_{50}). TCD_{50} titers/1.0 ml of virus stock were 6.4 dex (22) for CHIK virus, 7.4 dex for MAY, and 6.5 dex for SF.

Antisera. Antisera were prepared in mice given repeated intraperitoneal inoculations of a suspension of infected newborn mouse brain (23) and bled out 1 week after the last inoculation. Sera were pooled by virus and stored at −20°; sera of uninoculated mice were collected at the same time, pooled, and stored. In hemagglutination-inhibition (HI) tests, the antisera reacted with their homologous antigens (8 units) as follows: CHIK, 1:>1280; MAY, 1:2560; and SF, 1:1280.

Mouse antisera for Hughes virus (Dry Tortugas) and Junin virus (strain F) were kindly supplied by Dr. Jordi Casals.

Antimouse gamma globulin rabbit serum. Rabbits were given 3 intramuscular injections of a mixture consisting of 5 parts mouse gamma globulin (prepared by ammonium sulfate precipitation) and 7 parts Freund's adjuvant (24). Three weeks after the last inoculation. they were test bled and checked by a short-term precipitin test; those giving satisfactory results were then bled out, and the sera were pooled and stored at 4°.

Preparation of purified antimouse rabbit gamma globulin and fluorescent antibody reagent.[2] The techniques described by Goldstein *et al.* (16) were followed, with two modifications, namely: (a) proteins were concentrated, not by pressure dialysis, but with an LKB 6300 A ultrafilter (LKB Instruments, Inc., 4840 Rugby Ave., Washington, D.C.); and (b) fractions I plus II, as well as III plus IV, were combined. These steps resulted in two conjugates, of which conjugate I was characterized by a fluorescein:protein (F/P) ratio of 3.4×10^{-3} with a protein concentration of 10.0 mg/ml, and conjugate II by a F/P ratio of 5.6×10^{-3} with a protein concentration of 7.5 mg/ml. Both preparations were stored at 4° after sterilization by filtration through a Millipore filter (220 mμ).

Method of fixation. For parallel tinctorial studies, coverglasses were fixed in Bouin's fluid and stained by Giemsa's method as described in detail elsewhere (25). For visualization of antigens by the FA method, preparations were first drained on tissue paper and immersed for 15 min in acetone prechilled to −65°; they were then removed and stored in prechilled acetone in the Revco freezer (−65°) for from 18 hr to 1 week before being tested.

Fluorescent antibody staining. Fixed cultures were removed from the acetone not more than 15 min prior to staining. Each coverglass was placed in an individual Petri dish, where traces of the fixative were allowed to evaporate at 23°. The coverglasses were then immersed briefly in buffered saline, pH 7.2, drained of excess, and placed in new Petri dishes having lids lined with moistened filter paper. Two drops (0.25-ml syringe fitted with a 27-gauge needle) of diluent or of nonfluorescent homologous, heterologous, or normal mouse serum diluted as indicated with buffered saline were then placed on infected and uninoculated cells, and spread evenly by applicator over the entire surface of each coverglass. After the cells had reacted for 20 min at 23°, the coverglass was washed with 3 changes of buffered saline, each used for 4 min; it was then drained of excess saline, placed in a new Petri dish, and overlaid for 20 min at 23° with 2 drops of conjugate, diluted as indicated. After again being washed with 3 changes of buffered saline, the coverglass was mounted in a mixture of buffered saline (9 parts) and glycerine (1 part) on a 1 × 3-in. slide, cells facing down. Examination under the fluorescence microscope was carried out immediately.

Fluorescence microscopy. The large Zeiss equipment was used, with exciter filter UG_2 and a combination of barrier filters BG_{23} and GG_4. Maximal ultraviolet excitation was thus utilized. Photomicrographs were taken with

[2] The authors thank Dr. Merrill W. Chase of The Rockefeller University, New York, for his guidance in the preparation of the fluorescent reagents.

TABLE I. Titration of Conjugates I and II in the Presence of a Constant Dilution of Nonfluorescent Anti-SF Mouse Serum.

Prep. no.	Cell culture inoculum	Dilution of anti-SF serum	Conjugate		Staining[a]	
			No. and dilution	Protein (mg/ml)	Specific	Nonspecific
1	SF virus	1:5	I, 1:5	2	2+ to 3+	0
2	Uninoculated	1:5	I, 1:5	2	0	Trace
3	SF virus	1:5	I, 1:10	1	3+	0
4	Uninoculated	1:5	I, 1:10	1	0	Trace
5	SF virus	1:5	I, 1:20	0.5	2+ to 3+	0
6	Uninoculated	1:5	I, 1:20	0.5	0	Trace
7	SF virus	1:5	I, 1:40	0.25	2+	0
8	Uninoculated	1:5	I, 1:40	0.25	0	Trace
9	SF virus	1:5	I, 1:80	0.125	+	0
10	Uninoculated	1:5	I, 1:80	0.125	0	Trace
11	SF virus	1:5	II, 1:3.75	2	4+	0
12	Uninoculated	1:5	II, 1:3.75	2	0	+
13	SF virus	1:5	II, 1:7.5	1	4+	0
14	Uninoculated	1:5	II, 1:7.5	1	0	+
15	SF virus	1:5	II, 1:15	0.5	3+	0
16	Uninoculated	1:5	II, 1:15	0.5	0	+
17	SF virus	1:5	II, 1:30	0.25	2+	0
18	Uninoculated	1:5	II, 1:30	0.25	0	Trace
19	SF virus	1:5	II, 1:60	0.125	+	0
20	Uninoculated	1:5	II, 1:60	0.125	0	Trace

[a] On a scale of 4+.

the Zeiss camera and Ektachrome ASA 125 type 3 film.

Results. At the time of harvest, *i.e.*, 18–24 hr after inoculation of 5.4 to 6.4 dex TCD_{50} of virus, about 30% of the cells in unstained cultures inoculated with CHIK, MAY, and SF viruses were rounded and granulated, and 30% of the cells in Giemsa-stained preparations showed pyknosis. In uninoculated control cultures at this time, about 1% of the cells were rounded and granulated as a result of necrobiosis.

Evaluation of conjugates I and II. A SF virus test system was used to evaluate conjugate I (F/P ratio of 3.4×10^{-3}) and conjugate II (F/P ratio of 5.6×10^{-3}). Anti-SF mouse serum in a 1:5 dilution was allowed to react with infected and uninoculated cultures; 2-fold serial dilutions of conjugates I and II, starting at 1:5 and 1:3.75, respectively, were overlaid subsequently. The results are shown in Table I. SF antigen was localized specifically in the cytoplasm of about 30% of the cells. The character of the fluorescence was diffuse. As regards brightness of specific staining and degree of nonspecific staining, conjugate I proved to be satisfactory at dilutions 1:5 through 1:40, with optimal reaction obtained at dilution 1:10 (protein concentration of 1 mg/ml). Conjugate II, although giving more brilliant specific staining than conjugate I at protein concentrations of 1 and 2 mg/ml, stained nonspecifically even at a protein concentration of 0.5 mg/ml; a satisfactory separation of specific from nonspecific staining was obtained only at a protein concentration of 0.25 mg/ml (dilution 1:30). Further experiments were done with conjugate I at dilution 1:10.

Reactions obtained with heterologous mouse antisera and diluent. Table II shows the results of an experiment in which cells infected with SF virus were reacted with antisera for Hughes and Junin viruses, as well

TABLE II. Indirect FA Reactions Observed with Homologous and Heterologous Mouse Sera and with Diluent.

Prep. no.	Cell culture inoculum	Mouse serum and dilution	Conjugate and dilution	Staining[a]	
				Specific	Nonspecific
1	SF virus	Anti-Hughes, 1:20	I, 1:10	0	Trace
2		Anti-Hughes, 1:20	I, 1:10	0	Trace
3		Anti-Junin, 1:20	I, 1:10	0	Trace
4		Anti-Junin, 1:20	I, 1:10	0	Trace
5		Anti-SF, 1:20	I, 1:10	3+	0
6		Anti-SF, 1:20	I, 1:10	2+	0
7		Normal, 1:20	I, 1:10	0	Trace
8		Normal, 1:20	I, 1:10	0	Trace
9		Diluent	I, 1:10	0	0
10		Diluent	I, 1:10	0	0

[a] On a scale of 3+.

as with diluent, before being stained with conjugate; anti-SF and normal mouse sera were included for comparison purposes. Whereas bright yellow-green specific fluorescence was obtained with the homologous system, minimal dull-green staining was observed with the heterologous and normal mouse sera. Infected cells overlaid with diluent followed by conjugate were dull blue-gray in color.

FA titers obtained in homologous and cross-FA testing. The next point investigated was the localization and degree of fluorescence to be observed with CHIK, MAY, and SF viruses in the presence of 4-fold dilutions of mouse antisera beginning at 1:8. Dilutions of pooled normal mouse serum as well as uninoculated cultures were included for control purposes. Reactions were graded from 0 (no specific fluorescence) to 3+; the highest dilution of serum giving a specific fluorescence of 2+ in about 30% of the cells was taken as the FA titer.

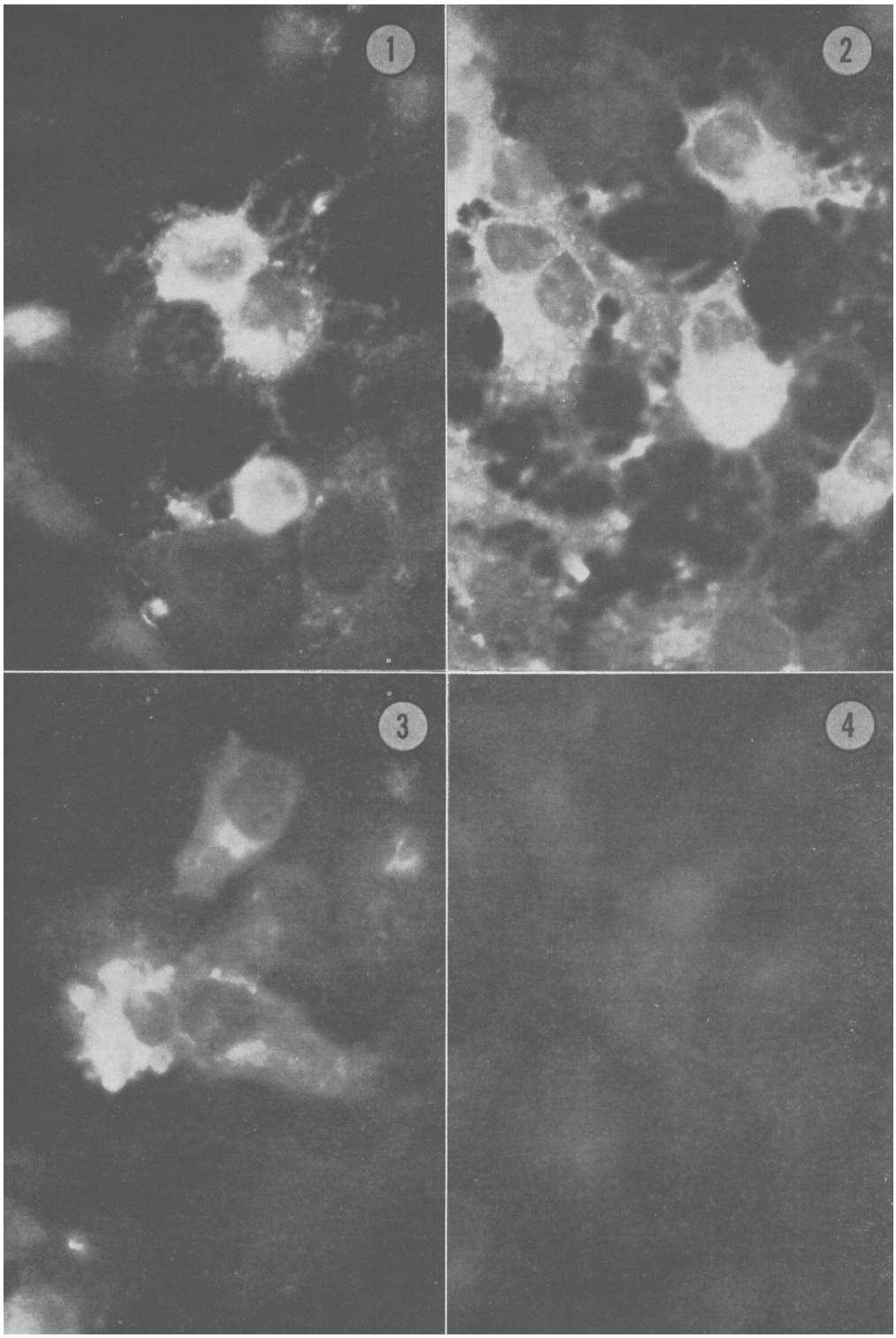
As shown in Table III, the homologous FA titers obtained were: CHIK, 1:32; MAY, 1:32; and SF, 1:128. The antigens were localized specifically in the cytoplasm (Figs. 1–3); inoculated cultures overlaid with normal mouse serum prior to reaction with the conjugate showed absence of specific staining, as illustrated for CHIK virus in Fig. 4. Uninoculated control preparations overlaid with homologous mouse sera before staining with conjugate also showed absence of specific staining.

Table IV summarizes the results obtained when cultures inoculated with CHIK, MAY, and SF viruses were tested in duplicate cross-FA tests against 4-fold dilutions of mouse antisera. Although cross-reactions occurred at the lower serum dilutions, the FA titers of homologous systems were both reproducible (see Table III) and uniformly higher than the FA titers obtained in the heterologous systems. Thus, the three closely related group A viruses could be readily distinguished by the indirect FA method.

TABLE III. FA Titers in Homologous FA Tests with CHIK, MAY, and SF Viruses.

Cell culture inoculum	Mouse serum	FA titer[a]
CHIK virus	Anti-CHIK	1:32
	Normal	<1:8
Uninoculated	Anti-CHIK	<1:8
MAY virus	Anti-MAY	1:32
	Normal	<1:8
Uninoculated	Anti-MAY	<1:8
SF virus	Anti-SF	1:128
	Normal	<1:8
Uninoculated	Anti-SF	<1:8

[a] Highest dilution of serum giving a specific fluorescence of 2+ on a scale of 3+ in about 30% of cells.



FIGS. 1–4. Infected HeLa cells harvested 18 hr after inoculation; stained by the indirect FA method; ×400. (1) CHIK virus reacted with anti-CHIK mouse serum; bright, diffuse fluorescence in cytoplasm. (2) MAY virus reacted with anti-MAY mouse serum; bright, diffuse fluorescence in cytoplasm. (3) SF virus reacted with anti-SF mouse serum; bright, diffuse fluorescence in cytoplasm. (4) CHIK virus reacted with normal mouse serum; absence of fluorescence.

TABLE IV. FA Titers in Cross-FA Tests with CHIK, MAY, and SF Viruses.

Cell culture inoculum	Mouse serum	FA titer[a]
CHIK virus	Anti-CHIK	*1:32*
	Anti-MAY	1:8
	Anti-SF	1:8
MAY virus	Anti-CHIK	1:8
	Anti-MAY	*1:32*
	Anti-SF	1:8
SF virus	Anti-CHIK	1:32
	Anti-MAY	1:32
	Anti-SF	*1:128*

[a] See Table III.

Discussion. Classification of arboviruses on a serological basis is usually carried out by neutralization, HI, and complement-fixation tests. Although immunofluorescence is mainly a tracing method with a fluorescent label, the fact that an antibody is part of the reaction implies the possibility of FA serology. For arboviruses, the immunofluorescence technique has been complementary to conventional serological methods in the distinction of some group B viruses (12, 13); Kunz (14) showed that the method compares favorably in sensitivity with techniques involving HI with virus-absorbed sera and cross-precipitin reactions in agar gel. This is not surprising, since the FA method effects a combination of immunology and microscopy.

In the present experiments, both precision of microscopy and specificity of immunology have been emphasized. As to the first, it has been demonstrated that CHIK, MAY, and SF virus antigens are specifically localized in the cytoplasm of infected HeLa cells; the nature of the specific fluorescence was diffuse, and nuclear specific staining was absent. These results are in agreement with previous reports of intracytoplasmic localization of arboviruses (1, 3–11).

Second, it has been demonstrated that these three closely related group A arboviruses are readily distinguishable by the indirect FA method. In repeat staining experiments, the serum for each virus gave the same FA titer with its homologous antigen but reacted in uniformly lower titers with cells infected with heterologous viruses prior to staining with conjugate.

Nonspecific staining was reduced to nearly undetectable levels by use of a conjugate subjected to DEAE-cellulose chromatography and gradient elution as described by Goldstein *et al.* (16). In addition to fractionation, dilution of the conjugate proved to be an excellent means of reducing nonspecific protein interactions (26); thus, a dilution of 1:10 (1 mg of protein/ml) resulted in bright specific staining and negligible nonspecific staining.

Summary. A conjugate subjected to diethylaminoethyl-cellulose chromatography and gradient elution to eliminate both underconjugated and overconjugated antibody molecules has been used to localize specifically Chikungunya, Mayaro, and Semliki Forest viruses in HeLa cells. Immunofluorescence was present in the cytoplasm only and was diffuse in nature; nonspecific staining was negligible. The three viruses, closely related members of arbovirus group A, were readily distinguishable by the indirect fluorescent antibody (FA) technique. The FA titers of homologous systems were reproducible and uniformly higher than the FA titers obtained in heterologous systems.

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