Application of Immunodiffusion Methods for Typing Members of the Phlebotomus Group of Arboviruses[1] (34979)

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Until 1959 two immunologically distinct etiological agents of phlebotomus fever were recognized in man. These agents, known as Sicilian (SFS) and Naples (SFN) sandfly fever viruses, were thought to be different, but perhaps related types which produced a similar clinical entity. It was not until 1954 that these viruses were adapted to suckling mice by Sabin and Sweet (1–4) allowing the investigators to study these agents outside the human host. They showed that, although in nature these viruses cause similar disease syndromes in man, no immunological relationship could be detected, and they were entirely separate entities. During the last decade, increasing numbers of new agents have been isolated from widespread geographical areas from man, rodents, and Phlebotomus flies. Several of the newer agents, such as Chagres (CHG), Punta Toro (PT), and Candiru (CDU), have been isolated from human clinical cases. Others—Salehabad (SAL), Karimabad (KAR), and I-47 have been isolated from Phlebotomus flies. Still others, including Bujaru (BUJ), Anhanga (ANH), Icoaraci (ICO), and Itaporanga (ITP), have been isolated from various wild rodents or sentinel mice (5–8). The "lumping" of these agents into a single group (Phlebotomus - PHL) is based, in part, on the similarity of their clinical disease syndromes, isolation from Phlebotomus flies, and/or a degree of immunologic relationship to one or more of the old members of the group.

Three conventional serological methods—the hemagglutination-inhibition (HAI), mouse-neutralization (MN), and complement-fixation (CF) tests—have been used for diagnosis, typing, and identification. The HAI test appears to be the broadest in cross-reactivity and has been the prime tool in grouping them. However, failure to prepare a useful hemagglutinin (HA) for all members of this group of viruses is not uncommon. The CF test has been reported to be much more specific (5).

The present investigation was undertaken to evaluate the use of immunodiffusion (ID) and immunoelectrophoresis (IE) for typing and/or identifying this group of viruses, as well as to determine whether antigenic relationships among members of the group can be demonstrated by these techniques.

Materials and Methods. Viruses. Table I lists the viruses of the phlebotomus fever group used in this study, mouse-passage level, host from which they were originally isolated, country of origin, year of isolation, name of investigator, and reference.

Antigens. The following antigen preparations were used: (1) Sucrose–acetone-extracted (SA) antigens were prepared from normal (NMB) and infected suckling mouse brains (ISMB) by the method of Clarke and Casals (9) except that after the final centrifugation, the supernatant fluids were used without lyophilization. (2) Crude supernate antigens were prepared as 40% suspensions of NMB and ISMB after centrifugation at 13,200*g* for 30 min. The diluent was Tris-buffered saline (0.05 *M* Tris, 0.15 *M* NaCl) at pH 8.0.

Antisera and immune ascitic fluids. (1) Hyperimmune mouse ascitic fluids (IAF) against each virus listed in Table I were prepared by multiple injections into adult white Swiss albino mice of ISMB suspensions emulsified in complete Freund adjuvant. Sarcoma 180/TG cells were injected ip to

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TABLE I. Viruses Used in Immunodiffusion Studies.

Virus	Mouse passage	Isolated from	Isolated by	Year	Reference
Sicilian (SFS)[a]	37	Man	Sabin	1943	1–4
Naples (SFN)	35	Man	Sabin	1944	1–4
Chagres (CHG)	20	Man	Shelokov	1960	6
Icoaraci (ICO)	4	Rodent	Belem Labs	1960	7
Candiru (CDU)	13	Man	Belem Labs	1962	5
Anhanga (ANH)	5	Rodent	Belem Labs	1962	5
Bujaru (BUJ)	16	Rodent	Belem Labs	1962	5
Itaporanga (ITP)	5	Swiss mouse	Trupp and Shope	1962	8
Punta Toro (PT)	14	Man	Sather	1966	5
Karimabad (KAR)	20	Phlebotomus pool	Barnett	1959	5
Salehabad (SAL)	19	Phlebotomus pool	Barnett	1959	5
Iran-47 (I-47)	23	Phlebotomus pool	Barnett	1959	5

[a] Abbreviations recommended by the American Committee on Arthropod-Borne Viruses (1969).

enhance the ascitic fluid production prior to the last virus injection. When the mice became distended, paracentesis was performed. The collected IAF was allowed to clot, centrifuged at 13,200*g* for 1 hr, and the supernatant fluid was stored at ± 70°. (2) Hyperimmune mouse sera were either supplied by the Research Reference Reagents Branch (NIH-NIAID) or prepared by multiple ip injections of ISMB suspensions into adult white Swiss albino mice. (3) Human sera obtained from volunteers experimentally infected in 1953–1955 (by one of us in collaboration with Dr. A. B. Sabin), with unadapted and mouse-adapted SFS and SFN viruses were also used.

Immunodiffusion. A micromodification of the Ouchterlony double diffusion in two dimensions was used. Glass slides 3 × 1.5 in. were precoated with 0.2% Noble agar (Difco Laboratories, Detroit) in distilled water and allowed to air dry. Each slide then was coated with 4.5 ml of 1% Noble agar dissolved in a 0.1 *M* borate buffer at pH 8.6. as soon as the agar coat had hardened, the slides were transferred to a humidified chamber, stored at 4°, and used within 1 week.

A stainless-steel cutter was used to cut the wells into the agar-coated slides. A pattern of a central well (7 mm diam), and 6 peripheral wells (5 mm diam) with a distance of 5 mm between the central and peripheral wells was used. The antiserum or IAF was placed in the central well; the peripheral wells were filled with the homologous, heterologous, and NMB antigens. Multiple slides were used for each antiserum. Slides then were placed in humidified chambers and incubated at 30°. Incubation of the slides at room temperature and at 4° was also tried. Permanent records of the reactions were made by photography of the unstained slides.

Cross-absorption tests. When an antiserum or IAF cross reacted with more than one antigen, ID cross-absorption tests were carried out using the method described by Ibrahim (10).

Immunoelectrophoresis. The micromethod of Scheidegger (11) was followed except for minor modifications described by Ibrahim and Hammon (12). As in ID each antiserum or IAF was tested against all test antigens, including the NMB control.

Complement fixation. Nine IAF's (Table IV) were tested by CF against all test antigens. The human anti-Naples and Sicilian sera used in ID tests were also tested by CF. The micro CF test, described by Casey (13), except that four times the optimal dilution of antigen was used.

Results. Normal mouse serum and ascitic fluid as well as the preinfection human sera did not react with any of the test antigens in ID, IE, or CF tests. Fig. 1A shows the precipitin reaction obtained when mouse anti-Sicilian ascitic fluid was reacted with its homologous and five of the heterologous antigens. It reacted monospecifically with its ho-

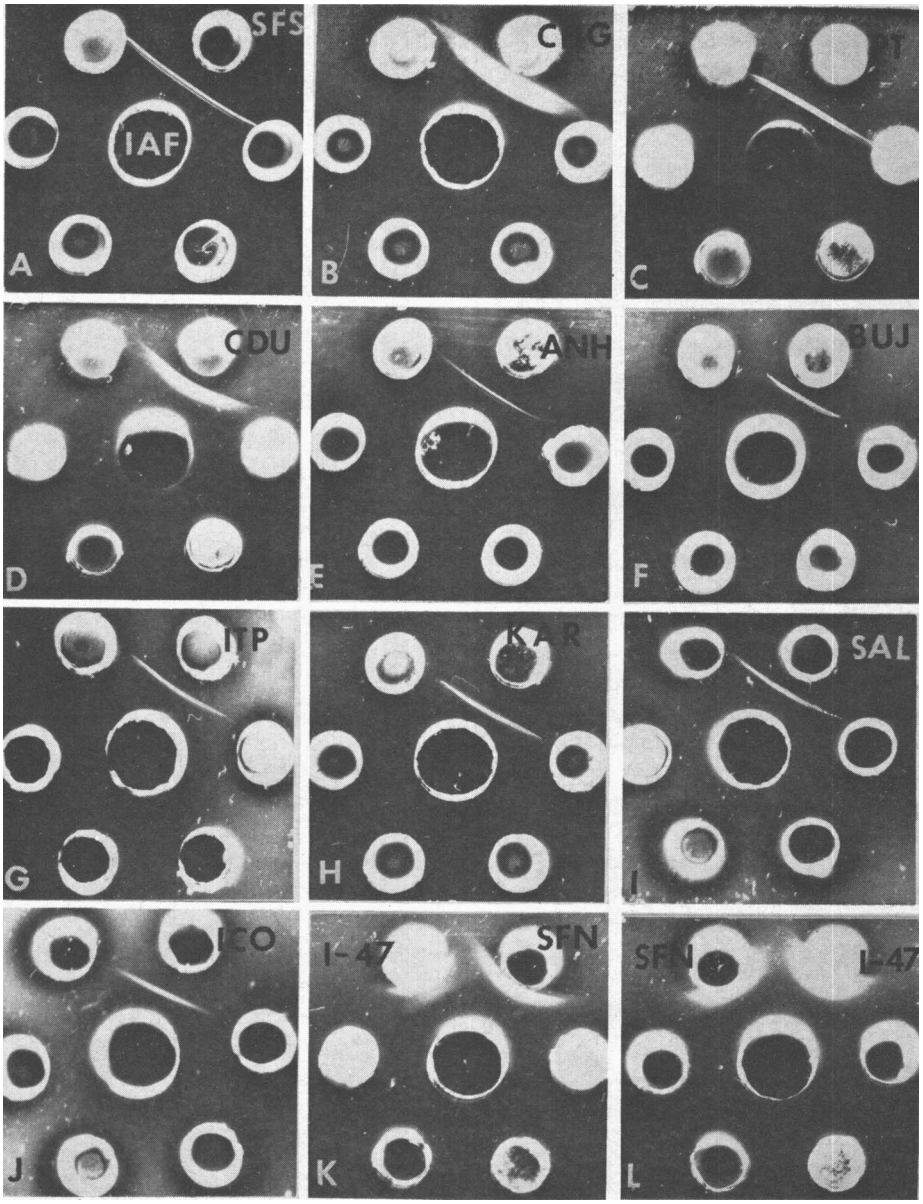

FIG. 1. Immunodiffusion patterns. Central wells contain mouse immune ascitic fluids (IAF). A:SFS; B:CHG; C:PT; D:CDU; E:ANH; F:BUJ; G:ITP; H:KAR; I:SAL; J:ICO; K:SFN; L:I-47. The peripheral wells contain the homologous (1 o'clock position) and the heterologous test antigens.

mologous antigen. Although not shown in the figure, the same IAF did not react with any of the remaining test antigens, including NMB. The reaction appeared within 18 hr and reached maximum intensity in 48 hr. Similarly, each mouse IAF of CHG, PT, CDU, ANH, BUJ, ITP, KAR, SAL, and ICO reacted only with its homologous antigen (Fig. 1B–J). Naples and I-47 were the only IAF's which showed cross reactions; each reacted with both Naples and I-47, but not with any other antigen (Fig. 1K, L).

Examples of IE patterns are shown in Fig. 2A and B. Each IAF of SFS and CHG react-

ed with its homologous antigen (upper wells), but not with that of NMB (lower wells). Although not illustrated, all immune mouse sera and ascitic fluids reacted monospecifically except those of NSF and I-47, which cross reacted with their antigens but not with any other antigen.

It is apparent, from Fig. 1, that the precipitin lines obtained with each antigen–antibody system varied in number and curvature. Each homologous system of SFS, CHG, PT, CDU, ANH, SFN, and I-47 showed at least two lines, whereas only one line appeared with BUJ, ITP, ICO, KAR, and SAL. In IE, three precipitin arcs could be observed with each system of SFS, and CHG (Fig. 2); two with CDU, PT and SFN; and only one with ANH, BUJ, KAR, SAL, ITP, and ICO (not shown in the figure). No appreciable difference was noted in the intensity or number of lines when the slides were incubated at 4, 25, or 30°. The precipitin reactions were essentially identical using the SA or the Tris-buffered saline-extracted antigens. The same was true whether mouse hyperimmune sera or ascitic fluids were used.

The results of the ID cross-absorption tests carried out using IAF's of SFN, and I-47 and their antigens, are summarized in Table II. Each IAF, when adequately absorbed, reacted only with its homologous antigen, indicating lack of complete identity.

TABLE II. Immunodiffusion Reactions of Naples and Iran-47 Immune Ascitic Fluids after Absorption with the Heterologous Antigens.

IAF	Procedure	Immunodiffusion reactions	
		Naples antigen	I-47 antigen
Naples	Unabsorbed	+	+
	Absorbed with I-47	+	0
I-47	Unabsorbed	+	+
	Absorbed with Naples	0	+

Table III shows the homologous MN indices and CF titers of selected sera obtained from human volunteers after experimental infection with unadapted or mouse-adapted SFS and SFN viruses. In ID tests, each serum reacted with its homologous antigen exclusively. Only one human anti-SFS serum of eight, and one anti-SFN of seven, gave no ID reaction. It is interesting to note that none of the human anti-SFN sera reacted with I-47 antigen.

Figure 3 shows that a polyvalent IAF, consisting of equal volumes of CDU, SFN, SFS, PT, and I-47 IAF's (central well), reacted with all corresponding antigens (peripheral wells).

The CF results shown in Table IV indicate that each IAF used reacted only with its homologous antigen. As in ID and IE, SFN and I-47 cross reacted in a reciprocal manner.

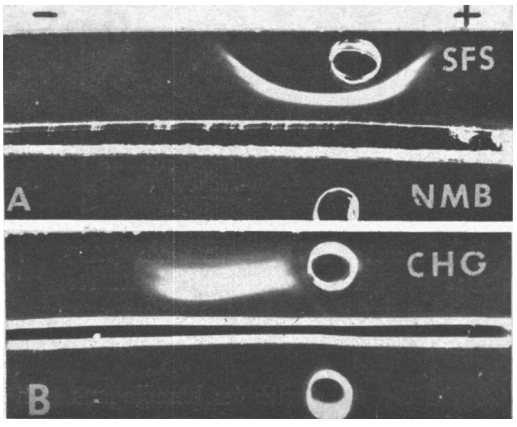

FIG. 2. Representative immunoelectrophoretic patterns. Troughs contain mouse ascitic fluids: A:SFS; B:CHG. Upper wells contain the homologous antigens and lower cells contain normal mouse-brain antigen.

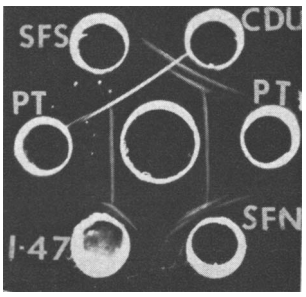

FIG. 3. Immunodiffusion patterns. Central well contains polyvalent mouse IAF of CDU, SFS, PT, I-47, and SFN. Peripheral wells contain their corresponding antigens.

TABLE III. Homologous Neutralization Indices, Complement-Fixation Titers, and Immunodiffusion Reactions of Sera Collected from Human Volunteers Inoculated with Unadapted or Mouse-Adapted Sicilian and Naples Sandfly Fever Viruses.

Virus	Inoculum[a]	Vol.	Post inoc.	Homolog. NI[a]	Homolog. CF titers	Immunodiffusion Homolog.	Immunodiffusion Hetero-log.[b]
Sicilian	Unadapted Sicilian virus (acute phase serum)	Lu	3 weeks	200	<8	0	0
		Gr	3 weeks	1000	8	+	0
		Mo	2 months	200	<8	+	0
		Au	5 months	630	<8	+	0
	MP10 Sicilian sandfly fever $10^{6.4}$ SMLD50	La	3 weeks	2000	—[c]	+	0
		Ro	3 weeks	3000	—	+	0
	MP20 Sicilian sandfly fever virus $10^{8.0}$ SMLD50	Fr	3 weeks	160	—	+	0
		Me	3 weeks	2000	—	+	0
Naples	Unadapted Naples virus (acute phase serum)	Ha	8 weeks	10	<8	+	0
		Hu	1 month	100	16	+	0
		Ca	1 week	160	<8	+	0
		Cl	2 weeks	20	8	+	0
	MP30 Naples $10^{5.9}$ SMLD50	Fl	1 month	32	16	+	0
	MP30 Naples followed 28 days later with unadapted Naples	Fa	18 days post challenge with unadapted virus	250	<8	0	0
		Mi		320	8	+	0

[a] We are indebted to Dr. B. H. Sweet and Dr. Albert B. Sabin for these data.

[b] Six to eleven heterologous antigens.

[c] Not tested.

Discussion. The results of both ID and IE indicate clearly that each mouse hyperimmune ascitic fluid or serum against 12 phlebotomus fever viruses reacted monospecifically with its homologous antigen preparation with the exception of Naples and I-47. Each of Naples and I-47 IAF reacted with both Naples and I-47 antigens, but not with any other heterologous antigen. These results strongly suggest that this test procedure is exceedingly useful in typing the viruses of this group. The precipitin lines appeared within 18 hr and were very distinct. The method appears to be extremely valuable since the test is rapid, easy to perform, reliable, and highly reproducible. Furthermore, the polyvalent IAF, prepared by mixing equal volumes of each of five IAF's, reacted with all the corresponding antigens. This indicates its usefulness in identification of a new isolate, provided that it is not a new type.

Naples and I-47 IAF's cross reacted with their antigens. It was difficult to determine whether they were antigenically identical until the cross-absorption tests were carried out.

TABLE IV. Complement-Fixation Titers of Mouse IAF's of Phlebotomus Fever Viruses with Homologous and Heterologous Antigens.

Anti-gens[a]	IAF: SFN	I-47	SFS	CHG	CDU	PT	ITP	ICO	KAR
Homolog.	2048	2048	512	2048	512	64	64	1024	256
Heterolog.	512 with I-47 only	2048 with SFN only	<8	<8	<8	<8	<8	<8	<8

[a] Antigens of all viruses listed in Table I.

The results indicated that although they are closely related, yet they are not identical. Whether this relationship would be shown if cross-protection tests were carried out in man remains to be answered. Further evidence that Naples and I-47 are not identical is that those human anti-Naples sera tested, reacted only with Naples antigen, but not with I-47. Although not presented, these sera also failed to react to a significant degree ($<1:8$) with I-47 antigen in CF tests.

The CF results are in agreement with those of ID and IE except that by using ID cross-absorption test, antigenic differences between SFN and I-47 could be detected. However, the superiority of the ID tests is evident since it is simple, less time consuming, and more informative as regards the number of antigenic components and their physical properties. The simple ID cross-absorption technique, in particular, led to valuable information which could not be obtained except by using much more time consuming and costly methods.

The results obtained, so far, with human anti-Sicilian and Naples sera, indicate strongly the potential use of ID as a diagnostic tool. These results, either in typing, diagnosis, or identification, are quite encouraging to extend these studies to other groups and subgroups of arboviruses.

Immunoelectrophoresis, in general, would afford the advantage of better separation of the antigenic components of a viral antigen preparation if there are differences in their electrophoretic mobilities. However, immunoelectrophoretic studies of phlebotomus fever viruses using mouse IAF did not afford a significant advantage over the simpler double-diffusion method. It appears that the antigenic components detected by mouse IAF have essentially or approximately the same isoelectric points and hence carry about the same electric charge, but with different diffusion rates. Examples are shown in Fig. 2A and B, where more than one arc is seen. Each antigen migrated in the electric field to approximately the same distance from the antigen well suggesting that each component has about the same electric charge. However, the separation was in the vertical direction, one after the other, indicating differences in diffusion rates.

As indicated by the number of precipitin lines appearing with each antigen–antibody system, the number of the antigenic components varied from 1 to 3. According to Korngold and van Leeuwen (14), the degree of curvature of the precipitin lines suggests variation in molecular weights. On this assumption, it appears that the antigens of CHG, I-47, SFN, and CDU seem to have higher molecular weights than those of their corresponding antibodies. Antigens of SFS, PT, ANH, BUJ, ITP, SAL, KAR, and ICO appear to have approximately the same molecular weight as their respective antibodies. Whether this finding can be applied to eventual subgrouping remains to be determined.

Summary. Studies were undertaken to evaluate the use of immunodiffusion methods (ID) for typing and/or identification of the phlebotomus fever group of viruses as well as for diagnosing sandfly fever. Hyperimmune mouse ascitic fluids (IAF) to twelve viruses were tested against the homologous and heterologous antigens. Each reacted monospecifically with its homologous antigen preparation. Naples and I-47 IAF's were the only exceptions; each cross reacted with both Naples and I-47 antigens, but not with any other heterologous antigen. By cross-absorption tests, these two agents were shown not to be identical. Further evidence of lack of identity was that human anti-Naples sera reacted only with Naples, but not with I-47 antigen. These results strongly suggest that ID is exceedingly useful for typing these viruses. Human anti-Naples and Sicilian sera obtained after experimental infection with unadapted or mouse-adapted viruses also reacted monospecifically with the homologous antigen. This indicates the potential use of this technique in the diagnosis of sandfly fever.

1. Sabin, A. B., Arch. Gesamte Virusforsh. **4**, 367 (1951).

2. Sabin, A. B., *in* "Viral and Rickettsial Infections of Man" (T. M. Rivers and F. L. Horsfall, eds.) 3rd Ed., P-374, Lippincott, Philadelphia (1959).

3. Sabin, A. B., and Sweet, B. H., Unpublished

observations and Reports to the Armed Forces Epidemiological Board (1952–1955).

4. Sabin, A. B., Amer. J. Trop. Med. Hyg. **1**, 198 (1955).

5. Taylor, R. M. (Comp.), "Catalogue of Arthropod-Borne Viruses of the World," 1st Ed., U.S. Public Health Service, Publication No. 1760 (1967).

6. Peralta, P. H., Shelokov, A., and Brody, J. A., Amer. J. Trop. Med. Hyg. **14**, 146 (1965).

7. Causey, O. R., and Shope, R. E., Proc. Soc. Exp. Biol. Med. **118**, 420 (1965).

8. Trapp, E. E., Raes de Andrade, A. H., and Shope, R. E., Proc. Soc. Exp. Biol. Med. **118**, 421 (1965).

9. Clarke, D. H., and Casals, J., Amer. J. Trop. Med. Hyg. **7**, 561 (1958).

10. Ibrahim, A. N., Proc. Soc. Exp. Biol. Med. **130**, 784 (1969).

11. Scheidegger, J. J., Int. Arch. Allergy Appl. Immunol. **7**, 103 (1955).

12. Ibrahim, A. N., and Hammon, W. M., II, J. Immunol. **100**, 93 (1968).

13. Casey, H. L., U.S. Public Health Service Publication No. 1228, Public Health Monograph No. 74 (1965).

14. Korngold, L., and van Leeuwen, G., J. Immunol. **78**, 172 (1957).

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