SERO-IMMUNITY TO ARTHROPOD-BORNE VIRUSES IN LEBANON

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ABSTRACT

A total of 110 blood samples from Lebanese residents were tested for the presence of antibodies to certain group A and group B arboviruses including Sindbis, West Nile, Dengue 1, Dengue 2 and Yellow Fever by hemagglutination-inhibition and neutralization tests.

Group B arbovirus(es) apparently are present in Lebanon as evidenced by the elevated titers to these viruses observed in hemagglutination-inhibition tests. However, due to extensive antigenic overlapping among these viruses, the exact etiologic agent(s) responsible for these reactions could not be determined by serology. Results with neutralization tests were inconclusive due to the limited number of determinations performed. Future work should attempt to isolate this agent(s) from infected arthropod vectors.

Goose red cells were found to be superior to 1-day chick erythrocytes for use in hemagglutination and hemagglutination-inhibition tests.

Tanned erythrocytes from various species of animals gave identical results in hemagglutination tests as compared with untanned erythrocytes.

The kaolin and acetone methods of extracting the nonspecific inhibitors from serum gave identical results in the hemagglutination-inhibition tests.

Antigenic overlap among various group B arboviruses, indluding West Nile, Dengue 1, Dengue 2 and Yellow Fever was demonstrated by the use of hemagglutination-inhibition tests. Nevertheless, in all instances the homologous serum titer was found to be higher than the heterologous titer.

Mosquitoes caught from various districts in Lebanon were identified as Aedes mariae, Culex hortensis, C. molestus, and C. laticinctus.

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INTRODUCTION

The arthropod-borne animal viruses, often abbreviated as arbor- or arboviruses, are described as those viruses which infect arthropods by their ingestion of infected vertebrate blood, followed by multiplication of the virus in the vector and its transmission to further susceptible hosts by biting, after a period of incubation in the vector known as the extrinsic period (WHO Study Group, 1961; Porterfield, 1962). Viruses carried mechanically by arthropods are excluded from this group of viruses (Casals and Reeves, 1959).

The arboviruses have been divided into groups depending on their serological reactions and the cross reaction patterns they display. The concept of antigenic groups, as introduced by Casals and Brown (1954), has allowed the placement of arboviruses into A, B, C, and Bunyamwera groups based on hemagglutination-inhibition (HI), complement fixation (CF) and neutralization tests (NT) (Casals, 1957). Other arboviruses, not well defined, have been placed in a separate class and designated as "ungrouped" arboviruses.

Arthropod-borne viruses are considered to be widely distributed throughout the world and are important as disease causing agents mainly in the tropical countries (WHO Study Group, 1961). Of the 150 arboviruses available 50 are known

to be pathogenic to man. Though frequently encountered in many parts of the world, exact information on their geographical distribution is limited. Several reports of certain epidemics and studies in endemic areas have shown arboviruses to be present in the Eastern Mediterranean basin.

West Nile virus was first isolated in Uganda from a woman with febrile illness (Smithburn *et al.*, 1940). Smithburn (1952) reported on the presence of West Nile in Sudan and other East African countries. Melnick *et al.* (1951), isolated West Nile virus from Egypt. Taylor *et al.* (1956) isolated the virus from febrile children, *Culex univittatus*, *C. antennatus*, and from the hooded crow. They also showed that 40% of the human samples collected from Sudan and 60% from Egypt neutralized the West Nile virus. Similar serological studies on humans were made by Smithburn *et al.* (1954), and also by Work *et al.* (1955) on the wild birds of Egypt. Results of these studies indicated that West Nile virus is endemic in Egypt. Studies carried out in Israel indicated the presence of this virus in this country (Goldblum *et al.*, 1954; Bernkopf *et al.*, 1953). Epidemics of Dengue fever are said to have occurred in Lebanon in 1861, 1889, 1913, 1918, 1920 and 1921. An unusually severe outbreak involving over 100,000 people was experienced in Beirut during 1945-46 (Graham, 1902; Réunion du Groupement des Medecins de Culture Francaise du 27 Novembre 1945, Consacreé à la Dengue,

1945-46; Berberian, 1945; Hitti and Khairallah, 1945). Theiler *et al.* (1960) reported about a Dengue epidemic in Greece which had occurred in 1927-28. Isolation in Egypt of Sindbis virus was carried out by Taylor *et al.* (1955), and Goldblum (1963) reported the presence of a virus similar to Sindbis in Israel.

Serological studies are undertaken to give an understanding as to the presence or absence of a certain virus or viruses in a population, taking into consideration the age group, sex, locality, and the relation of these to the vectors present. Such studies present the means by which a population is known to be immune or not to certain infections. They also add to the existing geographical and microbiological knowledge. When an investigation for the presence of arboviruses is made, an antibody survey of the residents (human and other vertebrates) is necessary. This is followed by a study of the ecological background made in relation to former results, and by attempts to isolate and identify the virus (WHO Study Group, 1961).

The studies reported herein were designed to investigate in Lebanon the presence of antibodies in a limited number of humans selected at random. The sera were tested against group A (Sindbis) and some group B (Dengue 1, Dengue 2, Yellow Fever, and West Nile) viruses utilizing the HI and the NT

tests. A preliminary survey was made, using the HI test only. Nevertheless, due to extensive antigenic overlapping encountered in these tests among group B viruses, NT tests were adopted later as they were considered to be more specific than HI or CF tests.

GENERAL PROPERTIES OF ARBOVIRUSES

Physical and Chemical Characteristics:- The arthropod-borne viruses have certain common characteristics. Generally their size falls within the range of 15-40 millimicra. For example, Yellow Fever virus was shown to have a size of 36 ± 5 mu as determined by electron microscopic studies (Bergold and Wiebel, 1962). Sindbis virus measures 40-80 mu (Taylor et al., 1955), Dengue, 17-25 mu (Schlesinger and Frankell, 1952) and West Nile 21-31 mu (Smithburn et al., 1940).

Arboviruses are readily inactivated at room temperature but at -20°C they are more stable. Normal serum and bovine plasma albumin have a protective action on these viruses and consequently are used in their preservation (Casals and Reeves, 1959). Little loss of initial titer of the virus takes place upon lyophilization. An alkaline pH level of the virus suspension is necessary as arboviruses are unstable at a lower pH. They are inactivated by ultraviolet light and by a temperature of 60°C for 10-30 minutes. Inactivation of the virus by chemicals, such as formaldehyde, ether, and a 1:1000 dilution of sodium desoxycholate is considered as one of the criteria for the identification of these viruses (Theiler, 1957).

Relatively little is known of the detailed chemical

structure of arboviruses. They have a ribonucleic acid core (Clarke, 1960; McAllister, 1962).

Antigenic Property:- All arboviruses act as good antigens and they provoke the formation of antibodies in the susceptible host. However, the presence of a number of common antigens gives serologic cross reaction patterns within the group. Clarke (1960) demonstrated the complex nature of the multiple antigens through serum absorption studies.

Classification:- Table 1 shows the geographic distribution of arboviruses divided into antigenic groups as suggested by Brown and Casals (1954), Casals (1957), and the WHO Study Group (1961).

TABLE 1

Arthropod-borne viruses listed by antigenic groups

Group A

Name or laboratory designation	Where isolated
Aura (BeAr 10315)	Belém (Brazil)
Chikungunya	Tanganyika, Uganda, South Africa, Thailand

TABLE 1. Group A (continued)

Name or laboratory designation	Where isolated
Eastern equine encephalitis	USA, Panama, Brazil, Trinidad, British Guiana
O'nyong-nyong	Uganda
Mayaro	Trinidad, Belém, Colombia
Middelburg	South Africa
Semliki	Uganda, West Africa (Kumba), Mozambique
Sindbis	Egypt, India, South Africa, Malaya (strain AMM 2215)
Una (BeAr 13136)	Belém
Uruma	Bolivia
Venezuelan equine encephalitis	Venezuela, Colombia, Belém, Trinidad, Ecuador
Western equine encephalitis	USA, Argentina, Mexico, Canada, British Guiana
AMM 2021 } identical?	Malaya
Sagiyama } identical?	Japan
AMM 2354	Malaya

Group B

Bat salivary gland (Rio Bravo)	USA
Bussuquara	Belém (Brazil), Colombia
Dengue, type 1	Hawaii, New Guinea, Japan, India, Malaya
Dengue, type 2	New Guinea, India, Trinidad, Thailand
Dengue, type 3	Philippine Islands
Dengue, type 4	Philippine Islands
Ilhéus	Brazil (Ilhéus, Belém), Trinidad, Honduras
Japanese B	Japan, eastern Asian mainland, from USSR to Malaya, India, Guam

TABLE 1. Group B (continued)

Name of laboratory designation	Where isolated
Modoc	USA (California)
Murray Valley encephalitis	Australia, New Guinea
Ntaya	Uganda
Spondweni	South Africa
St Louis	USA, Trinidad, Panama
Turkey meningo-encephalitis	Israel
Uganda S	Uganda
Wesselsbron	South Africa
West Nile	Uganda, South Africa, Egypt, Israel, India
Yellow fever	Africa, Central and South America, Trinidad
Zika	Uganda, Nigeria
AMM 1775	Malaya
SA H 336 (similar to Uganda S)	South Africa
Diphasic meningo-encephalitis	USSR
Central European tick- or milk-borne	Central Europe, Sweden to Balkans
Kyasanur forest disease	India
Langat (TP 21)	Malaya
Louping ill	Great Britain
Omsk haemorrhagic fever	USSR
Powassan	Canada, USA (?)
Russian spring-summer encephalitis	USSR, Central Europe

Group C

Apeu (An 848)	Belém (Brazil)
Caraparu (An 3994)	"
Marituba (An 15)	"
Murutucu (An 974)	"

TABLE 1. Group C (continued)

Name or laboratory designation	Where isolated
Oriboca (An 17)	Belém (Brazil)
Itaqui (An 12797)	"
Bunyamwera Group	
Bunyamwera	Uganda, South Africa
Cache Valley	USA, Trinidad, Belém (Brazil)
Chittoor	India, Malaya
Germiston	South Africa
Guaroa	Colombia, Belém
Ilesha	West Africa
Kairi	Trinidad, Belém
Wyeomyia	Colombia
Other Groups	
California encephalitis	USA (California)
Trivittatus	USA
Melao	Trinidad
BeAr 8033	Belém (Brazil)
Guama	Belém
Catu	Belém
Bimiti	Trinidad
Bwamba	Uganda
Pongola	South Africa
Simbu	South Africa
Oropouche	Trinidad
Sathuperi	India

TABLE 1. Other Groups (continued)

Name or laboratory designation	Where isolated
Turlock	USA (California)
Umbre	India
Anopheles A	Colombia
Anopheles B	Colombia
Tr 10076	Trinidad
AMM 2549	Malaya
AMM 2325	Malaya
Tr 7994	Trinidad
Tr 8762	Trinidad
Tr 9223	Trinidad
Quaranfil	Egypt
Chenuda	Egypt
EgAr 1306	Egypt

Ungrouped

African horse sickness	Africa and the Eastern Mediterranean area
Blue tongue	Africa, North America, Spain, Portugal, Israel
Colorado tick fever	USA
Crimean haemorrhagic fever	USSR
Nairobi sheep disease	Africa
Sandfly fever, Neapolitan strain	Italy
Sandfly fever, Sicilian strain	Italy, Egypt
Hart Park	USA (California)
Manzanilla (Tr 3587)	Trinidad

TABLE 1. Ungrouped (continued)

Name or laboratory designation	Where isolated
Rift Valley fever	Africa
Tacaiuma (BeAn 73)	Belém (Brazil)
Witwatersrand (Sa Ar 1062)	Africa
Argentinian haemorrhagic fever (Junin virus)	Argentina

WHO Study Group, 1961

Host - Vector Relationships:- Maintenance of arboviruses in nature depends on the presence of an adequate number of susceptible vertebrate hosts, arthropod vectors, and upon the association of these with their environment (Smith, 1960).

Certain criteria are necessary for a vector to be recongnized as a transmitting agent. The virus must be recovered from an arthropod that is infected by feeding on a viremic host. It should then transmit the virus to further susceptible hosts. Infections produced by arboviruses may be mosquito - and/or tick-borne in nature. The vectors show no sign of infection following virus multiplication in their tissues. Arboviruses have a wide host range of vertebrates,

mammals and birds.

Several factors may influence transmission of arbovirus infections including, the amount of virus in the host blood necessary to establish an infection in the arthropod (Kissling, 1958), threshold of infection in the vector, the extrinsic incubation period, presence of a non-immune host, and its availability to the vector. Man is only an occasional host to some of the viruses of the arbo group and the survival of these viruses appears to depend on a cycle of transmission between insect and birds or mammalian hosts. Epidemiological and ecological studies point out to a primary avian-mosquito-avian cycle with an occasional infection in man. For example, the pattern of transmission and maintenance in nature of St. Louis Encephalitis virus involves a primary wild bird-mosquito-(Culex pipiens) -bird cycle and a secondary bird-mosquito-(Culex tarsalis) -man cycle. Isolation of West Nile virus from Mansonia mettallica, a bird biting mosquito, in Uganda, indicates that birds may act both as natural hosts and reservoirs with man as a secondary host (Woodall et al., 1962). Further evidence that man is susceptible to infection with arboviruses has been provided by the isolation of these viruses from human blood. For example, West Nile virus isolation was successfully undertaken from patients with viremia (Smithburn et al., 1940). Some viruses have been adapted to a life cycle which does not require a vertebrate,

non-human host, such as Dengue infections where the secondary vector transmits the virus directly from man to man. Contrary to the tick-borne virus infection transovarian passage of the virus in mosquito-borne infection does not occur (Davies and Yosphe-Purer, 1954).

The clinical manifestations of arbovirus infection in humans range from severe encephalitis to mild febrile disease. The Dengue viruses cause malaise, fever, headache, rash of the maculopapular type, with no involvement of the central nervous system (Sabin, 1950; Anderson et al., 1956). West Nile viruses cause a non-fatal febrile illness (Hamilton and Taylor, 1954; Taylor et al., 1956), with viremia (Southam and Moore, 1951); however, occurrence of encephalitis and the presence of neurological signs in humans have been reported (Spigland et al., 1958; Pruzanski and Altman, 1962). Nothing is known of the symptomatology of natural infections in humans with the Sindbis virus (Taylor et al., 1955; Pfefferkorn and Hunter, 1963a).

Experimental Studies on Susceptible Hosts:- Arboviruses infect a variety of laboratory animals. They propagate well in suckling mice, embryonated eggs and cell cultures. The successful propagation of West Nile virus in suckling mice was reported by Smithburn et al. (1940), Southam et al. (1961), and Taylor et al. (1956). The injection of arbo-

viruses by the intracranial, intranasal, intraperitoneal, and subcutaneous routes results in encephalitis and death of white suckling mice. Rhesus monkeys, rabbits, guinea pigs show no signs of overt illness when infected with Dengue and West Nile viruses even though they develop antibodies in their serum. Adult mice show encephalitic involvement only on intracranial injection of the virus (Havens, 1954; Smithburn et al., 1940). Successful propagation of Dengue virus in mice has been reported by Sabin (1952), Schlesinger and Frankell (1952) and that of Sindbis virus by Taylor et al. (1955). Hedgehogs are considered as possible reservoirs of Yellow Fever virus (Dick, 1952).

Tahori et al. (1956), studied the infection rate, incubation period and thershold titer of infection with West Nile virus in the Culex molestus mosquitoes. The extrinsic incubation period varied from 7 to 21 days. A threshold titer for transmission of $10^{-1.5}$ was reported. Transmission did not occur when the titer of the virus suspension was less than $10^{-2.8}$. Evidence for multiplication of virus in this vector was established by showing a rise in titer within 3 days after infection. Results of Work et al. (1955), Southman and Moore (1951) demonstrated that certain mosquitoes were susceptible to Ar-248 strain of West Nile virus in the range of viremia found in human and various animal infections. Hurlbut (1956) showed that Culex univittatus, Culex antennatus,

Culex pipiens and the tick Ornithodorous savignypi allowed the multiplication of West Nile virus and transmitted it after experimental infection. West Nile virus was experimentally transmitted to suckling mice by Aedes aegypti (Goldwasser and Davies, 1953) and by Aedes albopictus (Philip and Smadel, 1943). Studies on West Nile virus distribution in the tissue of infected infant mice showed its appearance first in the blood next in the brain, and last in the liver (Tahori et al., 1956).

Various cell lines have been used for arbovirus studies. Cytopathogenic effect is noticed on multiplication of arboviruses in HeLa cell lines (Buckley, 1961, 1964), and chick fibroblast monolayers (Pfefferkorn and Hunter, 1963b). Trypsinized rhesus monkey kidney cells for Dengue (Hotta and Evans, 1955), hamster kidney cell cultures for Japanese B (Kundin and Diercks, 1960), and kidney cell lines from chicks, guinea pigs and hamsters were used for the 17D strain of Yellow Fever (Hotta et al., 1962) with satisfactory results. Human amnion cell cultures, have shown cytopathogenic effect following growth of many group A and B viruses (Salminen, 1962c). The use of human skin cells for Dengue 1 propagation has been reported by Wiebenga (1961). Furthermore, KB cell monolayers with a methyl cellulose overlay, have been successfully used in the study of group B arboviruses (Schulze and Schlesinger, 1963). The successful propagation of mouse adapted Hawaii strain of dengue

(Sahlesinger, 1950) and West Nile virus (Taylor et al., 1956) in embryonated eggs have also been reported.

Isolation and Identification:- Arbovirus isolations have been carried out from naturally infected birds, mosquitoes and human beings. Smithburn (1952) isolated West Nile virus from a human volunteer who showed no overt symptoms of infection. Isolation of West Nile virus from infected children, from mosquitoes, Culex Univittatus, Culex antennatus, pigeons and the hooded crow has been reported. (Melnick et al., 1951; Taylor and Hurlbut, 1953; Taylor et al., 1956). Sindbis virus isolations were carried out from Culex pipiens, C. univittatus (Theobald) and the hooded crow (Taylor et al., 1955). Active transmission of Yellow Fever infections by Haemagogous spegazzinii, Aedes africanus, Aedes aegypti has been reported (Havens, 1954). Both Sindbis virus and West Nile have been isolated in South Africa from Culex univittatus (Theobald). Sindbis virus was further isolated from Culex africanus (Theobald) suggesting that host specificity of viruses is variable among the vector genus (Worth et al., 1961). Arthropod vectors are identified and classified before virus isolation procedures are started. More detailed outline of the procedures used in the identification and isolation of arboviruses is presented in Table 2.

Overwintering:- The endemic nature of arbovirus infections in

TABLE 2

Isolation and identification of arthropod-borne viruses[1]

SPECIMENS	BLOOD	TISSUE	ARTHROPOD POOL
	(1) heparinized whole blood, plasma or serum (2) macerated clot in serum or diluent[2]	emulsion 10% W/V[3]	emulsion 10-30% W/V[3] in diluent[2] with antibiotics
	bacteriological study		
HOSTS	SUCKLING MICE	EMBRYONATED EGGS[4]	CELL CULTURES[5]
	route/dose[3] IC 0.01 ml IP 0.3 ml	YS 0.1 ml (8-10d) CAM 0.1 ml (13d) EMB 0.03 ml (8-10d)	tube 0.1 ml
	Observation period 14-21 days	3-7 days	7-14 days
	illness or death; bacteriological study		
PASSAGE	brain; pooled viscera; blood	YS, CAM, EMB	cells, fluid

TRANSMISSIBLE AGENTS:

Primary objectives: (1) haemagglutinating agent; (2) CF antigen; (3) seed virus stocks; (4) antisera in laboratory animals — in order to — (1) relate agent to disease in donor patient if possible; (2) provide preliminary serological identification (CF, HI, neutralization tests)

Secondary objectives: (1) chemical characterization; (2) host range; (3) gross filtration (Seitz, sintered glass, Berkfeld filters); (4) immunization-challenge tests if required

[1] WHO Study Group, 1961.

[2] 50% normal animal serum, free from "antibody" in saline.

[3] Weight/volume.

[4] IC = intracerebral; IP = intraperitoneal.

[5] YS = yolk sac; CAM = chorioallantoic membrane; EMB = embryo.

[6] Primary explants from small laboratory animals and fowl embryos: continuous cell lines (?).

temperate and tropical areas suggests the presence of a mechanism by which these viruses may survive adverse environmental conditions. Isolation of West Nile virus during the winter season (Taylor et al., 1956), and the development of antibodies in non-immune children point out to the possibility that the virus may survive during a period when conditions are unsuitable for its transmission by arthropod vectors. Various suggestions to explain the mechanism of overwintering in arboviruses have been presented. The virus is thought to be present in a long lived vector as in ticks which also pass the virus to their offspring transovarially. Chronic latent infection in vertebrate hosts, as in bats and reptiles, is another possibility. Other investigators believe that overwintering adult mosquitoes carry the virus, or that there may be seasonal introduction of the virus by migratory birds. The presence of alternate arthropod vectors and hosts may also explain the situation. A combination of two or more of these mechaniams is probable under natural conditions (McAllister, 1962; Reeves, 1961; Reeves, 1962; Schaeffer et al., 1958).

SEROLOGICAL STUDIES OF ARBOVIRUSES

Serological studies are carried out to assess the sero-immunity of a population group to a specific agent. Serological tests commonly utilized in the study of arboviruses are the HI, NT and CF tests.

Hemagglutination-Inhibition Tests:- Hemagglutination (HA) with influenza virus was first described by Hirst (1941), and by MaClelland and Hare (1941). Independently, they reported that the influenza viruses possess the capacity to agglutinate chick red blood cells. Further work on HA with neurotropic agents showed that the EMC group of Col-MM, and encephalomyelitis viruses agglutinate sheep erythrocytes, (Olitsky and Yager, 1949; Gard and Heller, 1951). Specific HA by the arboviruses was demonstrated by Sabin (1951), Sabin and Buescher (1950), and Macdonald (1952). Each arbovirus has its own specific requirements for the performance of satisfactory HA tests such as the pH of the buffer, the method of antigen extraction, and other factors. The inhibition of hemagglutination by a specific immune serum has made the HI test a useful tool in the classification of arboviruses, diagnosis of infections caused by these viruses, and has further facilitated survey work.

Cheng (1961) carried out a detailed study on Semliki

Forest virus. He showed the hemagglutinating particle to be the same as the infective virus particle which also carries the majority but not all of the complement-fixing activity. The titer of hemagglutinins is affected by chloroform, formalin, acid pH, and by freezing and thawing, but lyophilization has no effect on it (Chanock and Sabin, 1953 I). The hemagglutinins can be prepared from infected mouse brain, liver or serum, and from infected rhesus monkey serum. However, infected suckling mouse brain is the source of choice for hemagglutinin preparation (Clarke and Casals, 1958).

Several methods for the extraction of the hemagglutinins have been described. The preparation of hemagglutinins by centrifugation of alkaline brain suspensions at 10,000 r.p.m. for 1 hour, (Clarke and Casals, 1958), was a modification of a procedure previously utilized by Sabin (1951), Chanock and Sabin (1953 II). Extraction with fluorocarbon (Porterfield and Rowe, 1960), acetone - ether, sucrose - acetone (Clarke and Casals, 1958), are other procedures by which satisfactory antigens can also be obtained. The satisfactory preparation of hemagglutinins in tissue culture was described by Likar et al. (1962). Buckley and Srihongse (1963) prepared arbovirus hemagglutinins in HeLa cells. Salminen (1962c) reported the hemagglutinin production of various arboviruses in continuous human amnion cells in an inhibitor free maintenance medium.

Arboviruses have individual pH and temperature requirments (Sabin, 1951; Smith, 1957). Protamine treatment of the antigen reduces this dependence on pH. (Clarke and Casals, 1958; Salminen, 1962c).

The most satisfactory HA results were obtained by the use of newly hatched chick erythrocytes (Sabin, 1951). However, Porterfield (1957) found that goose red cells gave similar results with higher hemagglutinin titers and with less dependence on variations in the pH. The pattern of sedimented erythrocytes was described by Sabin and Buescher (1950) as being like a shield formed at the bottom of the tube in cells affected by the hemagglutinin and as clumps in non-affected cells. The red blood cells are prepared in an electrolyte suspension for the stability of the sedimentation pattern, and 0.2% bovine albumin is added to improve this sedimentation. Human O and rhesus monkey cells have given negative results in arbovirus HA, while red cells from old chicks and sheep cells demonstrated low titers and irregular results (Sabin and Buescher, 1950; Sabin, 1951; Chanock and Sabin, 1953 I). Various arboviruses require different erythrocytes for HA. For example, the Russian Spring-Summer Encephalitis virus agglutinates sheep red cells (Sabin, 1951); the Murray Valley Encephalitis virus agglutinates pigeon red cells as well as chick red cells (Macdonald, 1952). Salminen (1962a) observed that hen red cells are agglutinated by

arboviruses, only after treatment of arbovirus antigen with protamine sulfate, while cock red cells gave HA readily. He tested the behavior in HA tests of erythrocytes obtained from cocks injected with sex hormones. The results of his experiments indicated that progesterone and testosterone had no effect on the agglutination titers while oestradiol benzoate and diethylstilboestrol administration resulted in loss of HA.

Human, rhesus monkey, mouse and guinea pig normal serum samples were found to contain a non-specific inhibitor which acted as a soluble receptor on the red blood cells (Sabin and Buescher, 1950; Clarke and Casals, 1958). Non-specific inhibitors were also found in normal mouse brain (Chanock and Sabin, 1953 III). These inhibitors were considered to be lipid or lipoprotein in nature (Sabin, 1951). Further studies showed that these inhibitors were cholesterol, phospholipids in combination with free fatty acids (Salminen, 1962b; Porterfield and Rowe, 1960). These inhibitors could be removed from the serum by benzine, chloroform or by Seitz filtration. Treatment of the serum with acetone precipitates the protein, thus removing the inhibitor with no ill effect on the antibody. Removal could also be accomplished by treatment with kaolin or bentonite (Clarke and Casals, 1955; Casals and Brown, 1954; Sabin, 1951; Chanock and Sabin, 1954a).

Sera may also have agglutinins specific for goose cells, and these can be removed by adsorption with goose erythrocytes prior to use (Casals and Brown, 1954).

Neutralization Tests:- Neutralization is defined as loss of infectivity of a virus particle following its reaction with a specific antibody (Hirst, 1959). It is studied by inoculation of serum and virus mixtures into different living host systems. In these studies, laboratory animals as mice, hamsters, and rabbits as well as monkey kidney and HeLa cell lines can be used (Hammon, 1956; Bugher, 1956). The virulence of the virus used is titrated in the host system and its 50% end point calculated. Reed and Muench (1938) designed a statistical method by which this 50% end point could be calculated. This is defined as the dilution of virus which would affect one half of the infected hosts designated in terms of LD50, lethal dose; ID50, infective dose; PD50, paralytic dose for test animals, and TCD50, for cytopathogenic effect in tissue culture. Neutralization tests can be run by two different methods. In the first method the amount of virus is kept constant while varying the amount of serum, and in the second method amount of virus is variable while keeping the test serum at a constant dilution. The test is standardized and adequate serum, virus, animal and other controls are routinely employed in order to obtain comparable results. The results are reported in terms of the neutrali-

zation index (NI) which is the antilogarithm of the difference between two LD50s, in acute and convalescent sera, or in test and control sera. Virus NT may also be reported by recording the ratio of the number of inoculated animals surviving to the total of injected animals and the results are interpreted as protective, inconclusive, and negative (Miles et al., 1964; Taylor et al., 1956). To show different gradations of protection, the average survival time is used as a criterion in the test animals. In tissue culture the degree of cytopathogenic effect recorded as one plus or more is employed.

Results obtained by NT tests, as applied to the study of arboviruses, are more specific than the CF or HI tests (Lennette, 1959). Neutralizing antibodies persist for several years after initial infection. Rosenzwieg et al. (1963), reported on the persistence of Yellow Fever neutralizing antibodies 16-19 years after vaccination with Yellow Fever 17D strain.

In arbovirus NT tests, mice are the animals of choice although other animals could also be used. With mice, the principal variables are the age and strain of mice employed, route of inoculation, time of inactivation of serum, dose of virus used and incubation conditions of the virus-serum mixture (Porterfield, 1962).

Neutralization of arboviruses is demonstrated in tissue

culture by the absence of cytopathogenic changes after treatment of the virus with the immune serum. A more sensitive method is the plaque-reduction technique where reduction in the number of plaque forming units of virus after mixture with the antiserum is observed. Adequate controls are kept to avoid non-specific results (McAllister, 1960). Porterfield (1960) described a plaque-inhibition test for Yellow Fever. In these tests sera reducing plaque counts by 50% were considered as positive (Miles et al., 1964).

Several factors might affect the satisfactory performance of NT tests. For example, virus suspensions could be neutralized by bacterial contamination, thermal inactivation, or by sodium desoxycholate. Likewise unsatisfactory storage of serum samples or their inactivation by heat may cause loss of the labile accessory factor necessary for NT (Sabin, 1950). To overcome this, fresh human serum (Wisseman et al., 1962) and fresh guinea pig serum (Miles et al., 1964; Schlesinger and Frankell, 1952) have been used to provide this factor. Intraperitoneal NT tests in suckling mice have been utilized to minimize the effect of the loss of the serum labile factor (WHO Study Group, 1961).

Complement Fixation and Precipitation Tests:- The standard CF test is applied to the study of arboviruses (Hammon, 1956). In arbovirus study it is mainly used in screening, and when

positive it indicates a recent infection. Complement fixing antigens are prepared from infected suckling mouse brain by the use of different extraction methods. As to the persistence of complement-fixing antibodies, it was shown that in Dengue infection of humans and rhesus monkey, the complement-fixing antibody appeared 2-6 weeks after inoculation, and it stayed in high titer for several months after which it gradually disappeared (Sabin and Young, 1948). The use of combined antigens in CF tests to detect the presence or absence of antibodies in the specimens tested was recently advocated. Sera giving positive results against the combined antigen were found positive with one or more antigens when retested against separate antigens (Carey, 1963).

Gel diffusion technics resulted in the formation of various lines of precipitation which demonstrated cross reaction between different virus antigens and related antibodies (Clarke, 1962).

MATERIALS AND METHODS

Virus Strains:- The virus strains used were, Dengue 1 (Hawaii), and Dengue 2 (New Guinea B) supplied by the Rockefeller Foundation Virus Laboratory, West Nile (Ar-248, M-875), and Sindbis (Ar-339, M-162) supplied by Dr. Taylor, and the 17D strain of Yellow Fever supplied by the South African Institute for Medical Research. Stock virus was prepared by intracerebral inoculation of 0.03 ml. of a 10^{-1} virus suspension into 2-3 days old mice. On paralysis or death, which occurred within 3-4 days with West Nile, 4-5 with Dengue 2, 8-9 with Dengue 1, 6 days with Yellow Fever and 5 days with Sindbis, the brains of infected mice were harvested aseptically, weighed and homogenized in a mortar using alundum. A 20% suspension was made, using 2% bovine albumin in phosphate buffer, pH 8. Suspensions were tested for sterility and either stored at -70° or lyophilized. Each virus suspension was titrated in suckling mice, by inoculation of 0.03 ml. of 10-fold dilutions, 10^{-1}-10^{-9}, prepared in borate saline, pH 9. Titers were $10^{-7.8}$ for West Nile virus, $10^{-6.1}$ for both Dengue 1 and Dengue 2, 10^{-6} for Sindbis and $10^{-5.8}$ for Yellow fever virus. The mice used in the experiment were white suckling Swiss mice bred in this laboratory and were obtained originally from the United States Naval Research Unit 3, Cairo in 1951.

Antigen Preparation:- Antigens for HI were prepared by the sucrose-acetone extraction method described by Clarke and Casals (1958). Extractions were carried out using suckling mouse brains infected with Dengue 1, Dengue 2, Yellow Fever, West Nile and Sindbis viruses. Normal mouse brains were likewise extracted to serve as controls in the HA and HI tests. Antigen suspensions were then lyophilized and kept at -20°C.

Preparation of Sera:- Blood samples, obtained at random from Lebanese residents representing different age-groups, were collected from various areas in Lebanon. Blood samples were kept for one hour at 4°C, after which the clot was broken, and the tubes stored overnight at 4°C. The next day they were centrifuged, the serum collected, and stored at -20°C.

Most human serum samples were tested against the 5 arboviruses described earlier. However, some samples were tested against only 2 or 3 viruses due to shortage of the amount of available serum. A total of 110 samples were tested against West Nile virus, 79 against Sindbis, 70 against Dengue 1, 77 against Dengue 2 and 82 against Yellow Fever.

Immune mouse serum against each virus was prepared using adult mice (Clarke and Casals, 1958). Five intraperi-

toneal virus injections, 0.1 ml., were given to each of three-week old mice at 5-day intervals. The first two injections were given in a dilution of 10^{-2} and the remaining 3 in a dilution of 10^{-1}. The mice were bled 10 days after the last injection. An incision was made through the chest wall exposing the pericardial cavity and blood was drawn from the heart using a 22-gauge needle. The blood was pooled, and the serum separated from the clot as described above. Serum from normal mice was collected likewise to serve as control.

Before use for HI tests non-specific inhibitors were removed from the serum by kaolin extraction, using the method described by Clarke and Casals (1958). For comparative purposes, 10 human sera were extracted both by kaolin and acetone extraction methods as described by Clarke and Casals (1958). Naturally occurring agglutinins to goose red blood cells were removed using 0.1 ml. packed goose erythrocytes with 5 ml. of serum in adsorption tests.

Preparation of Erythrocytes:- Red blood cells from 1-day old chicks and from adult geese were used. Eggs were incubated at 37°C till hatched. The chicks were bled before they were 24 hours old. Goose blood was obtained aseptically by bleeding the bird from the blood vessels in the wing. The blood was collected in an acid-citrate-dextrose anticoagulant. After washing, in dextros-gelatin veronal buffer, a 10%

suspension was prepared in the same diluent and the cells stored at 4°C (Clarke and Casals, 1958). Red blood cells from goose, chick, human, guinea pig and sheep were processed in a like manner. Tanning of the erythrocytes was carried out according to the method described by Garabedian et al. (1957) with some modification. To 9.5 cc of a 1/20,000 tannic acid, 0.5 ml. of packed red blood cells were added. These were then incubated in 37°C water bath for 10 minutes, with occasional shaking. They were centrifuged, washed twice with phosphate buffer and a 0.25% suspension prepared in phosphate buffer. The pH of phosphate buffer used for washing cells and for preparation of the cell suspension varied according to the range indicated for each virus HA tests.

Chemical Reagents:- (Clarke and Casals, 1958)

Borate Saline pH 9 (0.05 M borate, 0.12 M NaCl)

1.5 M NaCl	80 ml.
0.5 M H_3BO_3	100 ml.
1.0 M NaOH	24 ml.

Mixed and made up to 1000 ml. with distilled water.

Acid-Citrate-Dextrose (ACD)

Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	11.26 gm.
Citric acid ($H_3C_6H_5O_7.H_2O$)	4.00 gm.
Dextrose	11.00 gm.

Make up to 500 ml. with a sufficient quantity of water. Sterilized by autoclaving 10 minutes at 10 lb. pressure.

Dextrose-Gelatin-Veronal (DGV)

*Veronal	0.58 gm.
Gelatin	0.60 gm.
**Sodium Veronal	0.38 gm.
$CaCl_2$ (anhydrous)	0.02 mg.
$Mg\ SO_4.7H_2O$	0.12 mg.
NaCl	8.5 mg.
Dextrose	10.00 mg.

The gelatin and veronal were first heated in 250 ml. of water after which other reagents were added and a sufficient quantity of water was added to bring up the volume to 1000 ml. This preparation was then sterilized for 10 minutes at 10 lb. pressure.

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*Veronal was prepared by chilling a saturated commercial veronal solution made in hot ethyl alcohol. One gm. of veronal dissolves in 14 ml. of hot ethyl alcohol. The precipitate was collected on a Buchner funnel.

**Sodium veronal was prepared from a saturated solution of barbital in ethyl alcohol at room temperature to which an alcoholic solution of NaOH 2M was added to precipitate the sodium salt. Addition of alcoholic solution of NaOH was continued till the suspension was alkaline to a universal indicator. Crystals were collected on a Buchner funnel.

Antigen Serum Diluent

The diluent used was borate saline with 0.2% bovine albumin which was prepared from a stock 4% suspension of bovine albumin.

Adjusting Diluents for Red Blood Cell

These are 0.15 M NaCl-0.2 M phosphate.

Basic Buffer - 0.15 M NaCl-0.2 M Na_2HPO_4

NaCl ..... 8.77 gm.

Na_2HPO_4 ..... 28.4 gm.

Make up to 1000 ml. with distilled water.

Acidic buffer - 0.15 M NaCl-0.2 M $NaH_2PO_4.H_2O$

NaCl ..... 8.77 gm.

$NaH_2PO_4.H_2O$ ..... 27.6 gm.

Make up to 1000 ml. with distilled water.

The working solution is prepared according to Table 3.

Hemagglutination:- The method followed was that of Clarke and Casals (1958). The test was run in tubes. Titrations of all the antigens were carried out at different pH ranges (Table 3). The pH at which the highest HA titer was obtained was used in the test. The titers obtained were: 1/320 at pH 6.2 for Yellow Fever; 1/640 at pH 6.6 for West Nile; 1/320 at pH 5.8 for Sindbis; 1/640 at pH 6.0 for Dengue 1,

and 1/320 at pH 6.2 for Dengue 2. Proper controls were kept with each set of determination.

TABLE 3

Adjusting diluents used for erythrocyte suspensions*

Final pH**	% 0.15 M NaCl-0.2 M Na_2HPO_4	% 0.15 M NaCl-0.2 M $NaH_2PO_4.H_2O$
5.75	3.0	97.0
6.0	12.5	87.5
6.2	22.0	78.0
6.4	32.0	68.0
6.6	45.0	55.0
6.8	55.0	45.0
7.0	64.0	36.0
7.2	72.0	28.0
7.4	79.0	21.0

*Clarke and Casals, 1958.

**The final pH was obtained by mixing equal volumes of the adjusting diluent with borate saline, pH 9.

Haemagglutination-Inhibition:- (Clarke and Casals, 1958). Serum dilutions were prepared in borate saline using 0.2% bovine albumin as diluent. Antigen, 0.25 ml. containing 8 units, was then added to each tube and the mixture held overnight at 4°C. Red cell suspension, 0.5 ml., at a pH indicated by titration, was added to each tube in the test. Controls including normal mouse serum, immune mouse serum, normal mouse brain, infected mouse brain, and the diluent used in the test, were kept. The HI titer was recorded, as the highest dilution of serum which showed inhibition of 8 units of antigen.

Neutralization Tests:- These tests were carried out according to the methods described by Wisseman et al. (1962) and Miles et al. (1964) with some modification. Virus suspensions of Dengue 1, Dengue 2, Yellow Fever and West Nile were prepared to contain 200 mouse LD50s. Calculations for LD50 were made according to the method described by Reed and Muench (1938) with some modifications (Smadel, 1952). Virus diluent was borate saline pH 9. Bovine albumin 2% was used for Dengue 1 and Dengue 2, and 1% for Yellow Fever and West Nile. Equal amounts of virus suspension and inactivated serum together with 0.1 ml. of fresh guinea pig serum were mixed. The mixture was kept at 37°C for 2 hours for neutralization to take place, after which it was chilled in an ice box at 4°C until used. Seven suckling mice were inoculated intra-

cerebrally with each serum-virus mixture, using 0.03 ml. of inoculum for each mouse. Normal and immune mouse serum controls were included in each determination. The mice were observed daily for signs of paralysis or death for a period of 13 days.

Mosquito Classification:- Mosquitoes were collected from different localities in Lebanon. They were stored at -20°C deep freeze in closed containers. Identification was carried out according to a key described by Parr (1943), with some modifications.

RESULTS

The results of HI tests presented in Table 4 clearly indicate that arbovirus infection is present in Lebanon. Of 78 samples tested for Sindbis virus, 16 were positive in titers of 1/10-1/20. Of the 110 samples tested 79 were positive for West Nile virus in dilutions of 1/10 and over. Similar studies indicated that of 70 samples tested 38 were positive for Dengue 1 and of 77 samples 60 were positive for Dengue 2. Likewise of a total of 82 samples tested, 72 were positive for Yellow Fever virus in HI tests.

Table 5 shows the distribution in various age-groups of sera showing positive HI against certain arboviruses. The incidence of positive reactors with arboviruses were high in all age-groups. A gradual increase in the percentage of the positive reactors was noted in older age-groups. In the age-group of 0-10 years, 47% were positive against West Nile and 11% against Dengue 1. In the age-group of 40 years and over 90.7% and 91.7% were positive against West Nile and Dengue 1 viruses respectively. A less marked rise in the percentage of positive reactors was noted with Dengue 2 and Yellow Fever viruses.

Thirty six human blood specimens reacting positively or negatively in HI tests to one or more of Group B arboviruses, were analyzed for the presence of neutralizing antibodies.

The results of these determinations are presented in Table 6. Of all the samples that were positive in HI tests relatively few had neutralizing antibodies. For example, only 1 serum sample neutralized West Nile virus; 3 Yellow Fever; 3 Dengue 2 and 14 Dengue 1. Results of NT tests were interpreted using a protective ratio. For example, 7 or 6 mice surviving out of 7 inoculated mice was considered as positive, 5 or 4 surviving as inconclusive, and 1, 2, or 3 as negative or showing no protection.

Table 7 shows the results of HI titrations on 8 human sera using goose and chick red blood cells. It can be seen from these results that the use of goose erythrocytes in HI tests resulted in 2-fold increase in titer.

Only slight differences in titers were observed when tanned and untanned erythrocytes from various species of animals were compared in the HA tests. Untanned goose red cells gave a titer of 1/640 with West Nile virus and a titer of 1/320 with Sindbis virus, while tanned erythrocytes gave a titer of 1/1280 with West Nile and 1/320 with Sindbis. Chick, human, guinea pig red cells gave a titer of $<1/10$ with both Sindbis and West Nile viruses regardless of whether the cells were tanned or not. Sheep cells showed a titer of 1/40 with West Nile and 1/10 with Sindbis viruses with untanned cells, and titers of 1/80 and 1/20 respectively with

tanned erythrocytes (Table 8).

Six human samples were tested by the HI test, using kaolin or acetone for extracting the non-specific inhibitors. The results with these two methods were usually comparable. Nevertheless, 1- or 2-fold differences in titer were observed occasionally.

Preliminary experiments to determine the antigenic relationships among group B arboviruses were carried out by the HI tests (Table 10). It can be noted that Yellow Fever gave a titer of 1/160 with the homologous virus, and a titer of $<1/10$ with Dengue 1, Dengue 2 and West Nile viruses. The West Nile immune serum showed a titer of 1/320 with the homologous virus and titers of 1/80 both with Yellow Fever and Dengue 2, and 1/40 with Dengue 1. Dengue 2 immune serum reacted with the homologous virus and West Nile viruses in a titer of 1/40. Likewise, it gave a 1/20 titer with Dengue 1 and a titer $<1/10$ with Yellow Fever virus. Dengue 1 immune serum gave a titer of 1/80 with the homologous virus and 1/10 with both West Nile and Dengue 2 viruses. Its titer was $<1/10$ with Yellow Fever virus.

Of 5131 mosquitoes studied (Table 11) 988 were identified as Aedes mariae (male), 1478 Aedes mariae (female), 16 Culex hortensis, 960 C. molestus, and 77 C. laticinctus.

Species identification of 542 Culex mosquitoes was not carried out. A total of 1070 mosquitoes were not identified.

TABLE 4

Hemagglutination - inhibition titers of human sera with certain group A and group B arboviruses, Lebanon 1963

Type of Arbovirus Antigen	No. Tested	Positive in Titers of: 1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Total No. Positive	% Positive
Group A													
Sindbis	79	12	4	0	0	0	0	0	0	0	0	16	20.25
Group B													
West Nile	110	5	3	11	11	14	10	10	5	4	6	79	71.81
Dengue 1	70	0	9	8	8	9	3	0	1	0	0	38	54.28
Dengue 2	77	7	15	10	6	10	10	1	1	0	0	60	77.92
Yellow Fever	82	6	9	8	15	9	10	11	2	1	1	72	87.8

TABLE 5

Hemagglutination - inhibition tests by age-groups on human sera with some group A and group B arboviruses, Lebanon 1963

Type of Arbovirus Antigen	Age-groups: 0-10 yrs			11-20 yrs			21-30 yrs			31-40 yrs			41 yrs and over			Total No. Tested
	No. Tested	No. Positive*	% Positive	No. Tested	No. Positive	% Positive	No. Tested	No. Positive	% Positive	No. Tested	No. Positive	% Positive	No. Tested	No. Positive	% Positive	
Group A																
Sindbis	15	3	20	12	1	8.3	16	2	12.5	18	3	16.7	18	7	38.9	79
Group B																
West Nile	17	8	47.1	21	11	52.4	28	25	89.2	22	15	68.2	22	20	90.7	110
Dengue 1	9	1	11.1	15	4	26.7	23	16	69.6	11	6	54.5	12	11	91.7	70
Dengue 2	10	7	70.0	16	9	56.2	24	21	87.5	14	10	71.4	13	13	100	77
Yellow Fever	13	11	84.6	18	12	66.7	23	22	95.6	13	12	92.3	15	15	100	82

*Positive in titers of 1/10 and over.

TABLE 6

Comparison of hemagglutination - inhibition and neutralization antibody titers in human serum samples obtained from Lebanese residents

Age	West Nile		Dengue 1		Dengue 2		Yellow Fever	
	HI	NT	HI	NT	HI	NT	HI	NT
3	< 10	-	< 10	-	20	-	20	-
9	< 10	-	< 10	-	<10	-	<10	-
12	< 10	-	< 10	-	<10	-	<10	-
12	<10	-	< 10	-	<10	-	20	4/7
12	640	-	80	-	160	-	640	-
13	<10	-	<10	-	<10	-	20	-
17	320	-	160	4/7	320	5/7	320	-
17	160	-	20	7/7	20	-	80	7/7
18	< 10	-	< 10	-	<10	-	<10	-
19	<10	-	< 10	-	<10	-	<10	-
19	5120	-	320	7/7	640	-	640	-
19	160	-	< 10	7/7	20	-	80	7/7
21	<10	-	<10	-	<10	-	<10	-

TABLE 6 (continued)

Age	West Nile		Dengue 1		Dengue 2		Yellow Fever	
	HI	NT	HI	NT	HI	NT	HI	NT
23	<10	-	40	7/7	<10	-	<10	-
25	80	-	<10	-	20	-	80	7/7
26	<10	-	<10	-	20	-	40	-
26	80	-	<10	7/7	<10	-	40	-
26	<10	-	40	7/7	<10	-	<10	-
26	40	-	80	7/7	10	4/7	10	-
27	320	-	160	-	320	-	640	-
28	1280	-	320	7/7	320	-	640	-
28	320	-	160	-	320	-	640	-
28	160	-	80	7/7	40	7/7	80	-
28	80	7/7	<10	-	20	-	20	-
28	160	-	160	7/7	160	-	160	-
28	5120	-	1280	-	1280	-	2560	-
29	80	-	20	-	40	-	160	-

TABLE 6 (continued)

Age	West Nile		Dengue 1		Dengue 2		Yellow Fever	
	HI	NT	HI	NT	HI	NT	HI	NT
29	640	-	160	-	160	-	1280	-
31	640	-	160	-	160	-	160	-
32	160	-	160	7/7	320	-	320	-
37	320	-	80	-	320	-	160	-
40	<10	-	160	7/7	10	-	<10	-
42	160	-	160	7/7	40	7/7	160	-
47	160	-	80	-	80	-	320	-
60	40	-	80	7/7	40	7/7	40	-
65	5120	-	320	-	320	-	640	-

1/ No protection.
2/ Reciprocal of serum dilution.
3/ Mice surviving after inoculation.
4/ Mice inoculated.

TABLE 7

Comparative studies on hemagglutination - inhibition titers in human serum against West Nile, using goose and 1-day old chick red blood cells

Laboratory designation	Chick red cells	Goose red cells
AM - 62 - 793	320*	640
AM - 62 - 798	640	640
AM - 62 - 800	2560	5120
AM - 62 - 787	160	640
AM - 63 - 210	320	1280
AM - 63 - 311	160	320
AM - 63 - 309	< 10	40
AM - 63 - 269	160	320

*Reciprocal of serum dilution.

TABLE 8

Agglutinability of erythrocytes from various species of animals with arboviruses before and after treatment with tannic acid

Nature of Red Cells		West Nile (pH 6.6)	Normal MB (pH 6.6)	Sindbis (pH 5.8)	Normal MB (pH 5.8)
Goose	Untanned	640*	<10	320	<10
	Tanned	1280	<10	320	<10
Chick	Untanned	<10	<10	<10	<10
	Tanned	<10	<10	<10	<10
Human O	Untanned	<10	<10	<10	<10
	Tanned	<10	<10	<10	<10
G. Pig	Untanned	<10	<10	<10	<10
	Tanned	<10	<10	<10	<10
Sheep	Untanned	40	<10	10	<10
	Tanned	80	<10	20	<10

*Reciprocal of serum dilution.

TABLE 9

Comparative studies on hemagglutination - inhibition tests using kaolin and acetone extraction methods

Laboratory designation	Yellow Fever Virus		Sindbis Virus	
	Kaolin Extraction	Acetone Extraction	Kaolin Extraction	Acetone Extraction
AM - 64 - 238	10[1]	10	<10	20
AM - 64 - 148	40	40	<10	20
AM - 64 - 249	<10	10	<10	20
AM - 64 - 264	80	40	40	160
AM - 64 - 210	40	40	40	160
AM - 64 - 209	160	80	40	80
Immune Mouse Serum	320	160	640	2560
Normal Mouse Serum	<10	<10	<10	<10

[1] Reciprocal of serum dilution.

TABLE 10

Cross reactivity among various group B arboviruses in immune mouse serum samples

Antigen	Immune Sera				
	Dengue 1	Dengue 2	West Nile	Yellow Fever	Normal Mouse Sera
Yellow Fever	<10	<10	80*	160	<10
Dengue 1	80	20	40	<10	<10
Dengue 2	10	40	80	<10	<10
West Nile	10	40	320	<10	<10
Normal Mouse Brain	<10	<10	<10	<10	<10

*Reciprocal of serum dilution.

TABLE 11

Classification of mosquitoes collected from different localities in Lebanon, 1962-63

Locality	Aedes mariae	Culex	C. hortensis	C. molestus	C. laticinctus	Not Classified	Total No.
Beirut City	M 14 F 23	28		184	46	29	324
Beirut Sea Shore	M 329 F 488	16		29		177	1039
Shoueyfat	M 28 F 51	66		85	13	50	293
Abey		30		257		18	305
Nahr	M 18 F 16					11	54
Bahmiun		95		104		80	279
Monteverde		44		2		1	47
Damour	M 132 F 192					92	416
Rumeyleh	M 59 F 96					46	201
Aitat		60		104		51	215
Ain-Anoub	M 68 F 106	19		10		86	289
Ouzai	M 185 F 258					105	548

TABLE 11 (continued)

Locality	Aedes mariae	Culex	C. hortensis	C. molestus	C. laticinctus	Not Classified	Total No.
Khalde	M 155 F 248					65	468
Baysour		15		7		80	102
Souk el Garb		46		28	2	64	140
Aley		36		35	4	25	100
Barouk		87	16?	115	12	90	320
Total No.	M 988 F 1478	542	16	960	77	1070	5131

✱Species not determined.

M = Male
F = Female

DISCUSSION

Group B arboviruses have been extensively studied. Infection with a group B virus elicits formation of antibodies specific to the virus inoculated and to other related viruses within the group (Casals and Brown, 1954; Pond *et al.*, 1955; Smithburn, 1954). When humans or animals are infected with a group B virus, and later by another virus of the same group, antibodies develop to the second virus, to related viruses, and the titer of the original virus also rises. This pattern is extensively seen in human sera tested by HI (Price *et al.*, 1961; Southam and Greene, 1958). Antibodies to arboviruses persist for a long time. For example, neutralizing antibodies have been reported 15 years after infection while those showing HI were observed thirty years after a Dengue epidemic in Greece. The CF antibodies may be detected during a period of two or three years (Miles *et al.*, 1964; Theiler *et al.*, 1960).

Smithburn (1954) noted that West Nile was related antigenically to Japanese B, Louping ill, and to some extent to Yellow Fever viruses. By CF tests, Casals (1944) and Melnick *et al.* (1951) showed definite relationship between Japanese B, St. Louis, and West Nile viruses. Monkeys immunized against both Ilheus and St. Louis viruses, developed antibodies, in high titers, to both Japanese B and West

Nile viruses. Neutralization tests show a close relationship between Japanese B, St. Louis and Murray Valley Encephalitis viruses to West Nile virus (Goldblum et al., 1957; Smithburn, 1942; Taylor et al., 1956). Sabin (1949) reported that this relation was not proportional to the number of homologous units used against each specific immune serum. The presence of a specific antigen was shown by Clarke (1960) in three different strains of West Nile by the use of serum absorption tests. Japanese B, St. Louis and West Nile viruses were also shown to have a specific antigen, each independent from the cross reactive groups. Wisseman et al. (1962), Wisseman and Sweet (1962), and Sweet et al. (1962), reported the presence in humans, following vaccination with 17D Yellow Fever virus, of heterologous antibodies to West Nile, Dengue 1, Dengue 2, whenever there was previous experience with Japanese B prior to vaccination. No such antibodies were detected when there was no previous experience with a group B infection. The viremia levels were the same in both test groups.

These interactions indicate a common genetic origin for these viruses (Sabin, 1950), and they show a slow evolutionary trend possibly brought about by environmental and epidemiological conditions (Clarke, 1960). Consequently it would be interesting to observe the degree of immunity which this relationship and serologic interaction would confer to to the host. Price et al. (1961, 1963) demonstrated protec-

tion against Russian Spring-Summer Encephalitis, Japanese B, St. Louis, Dengue 1, Dengue 2, Dengue 3, Dengue 4, West Nile, and Murray Valley Encephalitis viruses in monkeys following vaccination. A triple vaccine program starting with 17D Yellow Fever virus followed by West Nile and then by the Russian Spring-Summer Encephalitis virus was instituted. Imam and Hammon (1957a, 1957b) observed that combined vaccination with West Nile virus followed by Japanese B vaccine, protected monkeys against Japanese B. The possibility of using attenuated West Nile virus with subsequent inoculation of a booster dose of a killed viral vaccine against which protection is desired was studied. Administration of Yellow Fever vaccine before or with Dengue infection to human volunteers, prolonged the incubation period of Dengue and decreased the severity of the disease (Sabin, 1950).

Serological studies on arboviruses were carried out in Lebanon in 1955 by Garabedian and Matossian by the use of the CF tests. St. Louis, Eastern Equine Encephalitis, and Western Equine Encephalitis viruses were tested against human serum samples collected from Lebanese residents in various age-groups. A small percentage showed antibodies to some of these viruses. However, later work (1960) by the same workers failed to detect antibodies to these viruses. The reason for this discrepancy was not understood.

Theiler and Casals (1961) studied 60 serum samples from Lebanese residents. These samples showed no antibody response to group A arboviruses. Fifteen per cent in the age-group 0-29 and 43% in the age-group 30-69 were positive to group B arboviruses especially with West Nile and Dengue 1. Theiler and Casals reported that "positive results with group B in general, did not give a clear cut diagnostic pattern."

From the results reported in Tables 4, 5, and 6, it is apparent that a large percentage of the Lebanese population carry antibodies to group B arboviruses, and only a small percentage have antibodies to group A arboviruses. The presence of low titer antibodies to Sindbis virus in various age-groups cannot be explained. This could be due to a past infection with this or a related virus.

Immunological overlap must be considered in the interpretation of serological data. For example Table 4 shows antibodies in many of sera tested to West Nile, Dengue 1, Dengue 2 and Yellow Fever viruses in high titers. Table 5 shows antibodies in the young age-groups, with increasing incidence in the older age-groups which could be attributed to a subsequent reinfection with the same or a related virus. The pattern obtained in this study is difficult to interpret because the HI tests provide no clue to the identity of the virus. Rosenzweig et al. (1963) reported on HI titers as

high and even higher in heterologous reactions than with the homologous one. Sarkar et al. (1962) reported that 89% of the positive sera from India had group antibodies against a majority of group B viruses tested. Sweet and Sabin (1954) suggested that Dengue 1 virus infection stimulates a heterologous antibody response as well as a homologous one.

Neutralization tests carried out on a number of samples indicated a positive reaction mainly with Dengue 1 virus and few with Dengue 2. This could be explained on the basis of the occurrence of the Dengue epidemic of 1945, as the samples tested were collected from individuals within the age-group of 17 to 65 years. Only one sample neutralized the West Nile virus. The fact that 3 samples from Lebanese residents neutralized the Yellow Fever virus can be explained by the frequent travels of the Lebanese people abroad indicating that vaccination could have been given to these individuals with the 17D strain of Yellow Fever virus. One serum sample from an individual of Sudanese origin showed an HI titer of 1/160 to Yellow Fever virus and neutralized only this virus. Another sample gave protection in neutralization tests to West Nile, Dengue 1, and Dengue 2 but not to Yellow Fever. It gave an HI titer of 1/640 with West Nile, 1/160 with Dengue 2 and 1/320 with both Dengue 1 and Yellow Fever antigen. This was thought to be due to laboratory infection as it belonged to an individual working with arboviruses in this laboratory. Hamilton and Taylor (1954)

reported on one case of West Nile infection acquired in the laboratory.

In conclusion, it can be stated that agents responsible for these varied manifestations observed in HI and NT tests possibly did not belong to hitherto recognized arboviruses. It is also possible that some virus or viruses closely related to anyone of group B arboviruses is present in the country and is responsible for these serological vagaries. It is further possible that a multiple infection with a number of group B arboviruses in the same individual could have brought forward this immunological puzzle.

Attempts to isolate virus from mosquitoes caught from various sections of the country were unsuccessful. It should be pointed out that isolation of the etiologic agent(s) is the only means to confirm the results of serologic reactions. Thus, further attempts to isolate the virus(es) responsible for the results reported herein should be carried out. Nevertheless the present investigations have established beyond any doubt the fact that viruses antigenically related to group B arboviruses are present in Lebanon.

Porterfield (1957) reported that the use of goose erythrocytes in hemagglutination tests with arboviruses was superior to that of 1 day chick red cells. In view of relative

unavailability of 1-day old chick red blood cells, goose erythrocytes were used in these experiments. However, before this shift was made an HI titration for comparison purposes was necessary. Experiments indicated that there was a 1- to 2-fold increase in titer when goose red cells were used in place of chick cells (Table 7). Red blood cells from various species of animals have been tested as to their use in arbovirus studies in HA tests. Negative results were obtained when rhesus monkey, human O red cells were used and irregular and low titers when sheep cells were utilized (Sabin and Buescher, 1950; Sabin, 1951). The best results were obtained with goose erythrocytes (Porterfield, 1957) or with red cells of 1-day old chicks (Clarke and Casals, 1958). Tannic acid is known to cause a change on the red blood cell surface potential (Springer, 1963). In our experiments the use of tanned guinea pig, goose, human O, chick and sheep red cells did not improve the HA titer as compared to untanned erythrocytes (Table 8).

The kaolin and the acetone extraction methods for removal of nonspecific inhibitors, gave identical results (Table 9).

Antigenic relationship between group B arboviruses have been studied extensively. Hemagglutination-inhibition tests with immune mouse, guinea pig, and monkey serum demonstrated cross reactions between Dengue 1, Dengue 2, West Nile,

Yellow Fever, Ilheus, St. Louis, Japanese B, and Russian Spring-Summer Encephalitis viruses, (Clarke and Casals, 1954; Chanock and Sabin, 1954b; Woodall et al., 1962). Similar serological cross reactions were observed in the present studies. It was further apparent from our tests that Yellow Fever infection does not give as wide a heterologous antibody response as the Dengue group of viruses (Table 10).

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