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Studies on Serological Homogeneity of Asian Influenza Strains. (28485)

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Several groups of investigators(1,2,3) have reported evidence of what was interpreted to represent serological heterogeneity of different strains of Asian influenza virus isolated during 1957. Interpretation of these findings has, however, been complicated by the demonstration by Fukumi(4), and others, that Asian strains of influenza virus display marked variations in antibody avidity, perhaps analogous to the PQ variations described by van der Veen and Mulder(5). Fukumi, in a study of 300 strains of Asian virus isolated in Japan during 1957, demonstrated that the strains differed markedly in avidity for antibody and for inhibitors. In an effort to determine whether some of the extreme variations were related to antigenic difference, cross absorption experiments were carried out. These experiments did not support the hypothesis of antigenic diversity as underlying the difference in antibody combining power manifested by these strains.

Preliminary analyses of strains isolated at the Medical General Laboratory (406) in 1960 by hemagglutination inhibition technique suggested possible differences between 1957 and 1960 Asian influenza strains. Since complement fixation techniques employing purified "viral" antigen(6) are thought to offer a means for circumventing the avidity problems associated with the hemagglutination inhibition technique, it appeared of interest to evaluate these differences by these methods. The present report summarizes comparison by hemagglutination inhibition and specific viral complement fixation techniques of 6 Asian influenza strains with 6 such strains isolated during 1960.

Materials and methods. Virus strains. The prototype 1957 strain employed for immunization was A_2/Formosa/313/57, in third allantoic passage. This strain had been isolated in Formosa by Dr. S. P. Wang. The prototype 1960 strain employed for immunization was A_2/Japan/775/60. This strain was isolated at the Medical General Laboratory (406) in April 1960 from a patient at the Tachikawa Air Force Base Hospital near Tokyo. Third allantoic passage material was employed for immunization. In addition to the above strain, the following 1957 strains were examined: A_2/Japan/Adachi-2/1957 and A_2/Japan/Kumamoto/Y-5/1957 strains described by Fukumi(4), which were kindly supplied by Colonel T. Sonoguchi, Japan Self Defense Forces Hospital; A_2/Formosa/T-22/57, which was employed on the initial assumption that it represented an independent isolate but was subsequently found to represent a derivative of the same isolate as A_2/Formosa/313/57: A_2/Japan/115/57, a strain isolated at Medical General Laboratory (406).

The 1960 strains examined included, in addition to the prototype described above: A_2/Japan/538/60, isolated from a patient in Kyushu during March, 1960 at Medical General Laboratory (406); A_2/Japan/691/60, a strain isolated from a patient at Johnson Air

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Force Base near Tokyo at Medical General Laboratory (406) during February, 1960; A_2/Korea/805/60, a strain isolated from a patient at the 121st Evacuation Hospital near Seoul, Korea at Medical General Laboratory (406) during March, 1960; A_2/California/Cradock/60 and A_2/Formosa/T-60/60 representing American and Formosan 1960 prototypes, which were kindly made available by Dr. Fred Rasmussen, U. S. Naval Med. Research Unit No. 2, Taiwan.

Preparation of immune sera. Rabbit immune sera were prepared by inoculation with 10 cc of undiluted allantoic fluid intraperitoneally and 5 cc intravenously. Rabbits were boosted with 10 cc of allantoic fluid inoculated intraperitoneally on the seventh day and 5 cc inoculated on the fourteenth day. Animals were exsanguinated on the twenty-first day.

Guinea pig anti "V" sera were prepared by the methods described by Lief and Henle (6). Animals were inoculated with irradiated viral antigen 3 times at 7-day intervals and a fourth time 14 days after the third inoculation. They were exsanguinated 7 days after the final inoculation.

Hemagglutination inhibition tests. Hemagglutination inhibition tests were carried out by standard methods employing 4 units of antigen and goose erythrocytes. All sera were kaolin adsorbed(7) prior to use. This treatment completely removed hemagglutination inhibiting antibodies from preimmunization sera.

Complement fixation technique. Standard block tests were carried out with the micro complement fixation technique described by Fulton and Dumbell(8). With this technique it was found that commercial lyophylized complement contained normal hemolysin for sheep erythrocytes. This was removed by 3 successive adsorptions of complement with equal volumes of washed sheep erythrocytes for 30 minutes at 2 – 4°C. Two full units of complement were employed in the tests. Preliminary incubation was for 16 hours in the refrigerator. The hemolytic reaction was carried out in a humidified 37°C incubator for 60 minutes. Antigens for the complement fixation technique were prepared by 10:1 concentration of allantoic fluid by centrifugation at 34,850 $\times$ g at 4°C. The sedimented virus was resuspended in 0.1% bovine serum albumin veronal buffered saline. Antigens were stored at 4°C after addition of one part in 10,000 of merthiolate.

Red cell adsorption-elution was not employed for preparation of "V" antigens for serological testing, since the low elutability of many Asian strains made it difficult to obtain adequate antigen concentrations for complement fixation analyses. Use of simple ultracentrifugal concentration was justified by the absence of demonstrable anti-soluble antigen titer in the guinea pig sera employed when these were tested against high titered soluble antigen from which "viral" antigens had been removed by red cell adsorption.

Results. Table I summarizes the comparison of 1957 and 1960 strains by hemagglutination inhibition with rabbit antisera. It was noted that while the strains could not be distinguished by the use of the antiserum against the 1957 prototype, when antiserum against the 1960 strain was employed there appeared to be a difference between some of the 1957 strains and the 1960 strains. In particular, the Kumamoto, 313, T-22, and 305 strains appeared to be only weakly reactive with anti-1960 prototype antisera. It was, however, obvious that this did not necessarily imply true serological difference but could be interpreted as a manifestation of avidity differences, since the studies of Fukumi(4) had indicated that Adachi and Kumamoto strains could not be distinguished by cross absorption and hemagglutination inhibition techniques.

The results of block complement fixation tests using the guinea pig antiserum against the "viral" antigen of the 1957 prototype are presented in Fig. 1. In agreement with the hemagglutination inhibition results, these data did not distinguish between any of the 12 strains. In contrast, when antiserum against 1960 prototype strains was employed (Fig. 2), the T-22 and 313 strains, as well as the Kumamoto strain, appeared markedly less able to combine with the anti-1960 prototype antisera than did the remainder of the viruses tested.

These results are summarized quantitatively in Table II on the basis of antigen and antibody titers estimated as the highest dilution reached at 2 or more dilutions of the other reagent. If a significant difference in *antigen* titer is taken as a 4-fold or greater reduction in titer as compared to the titer obtained with antiserum prepared against the prototype strain of the same year, it may be concluded that 4 strains from the 1957 group reacted in a heterologous manner to the 1960 antisera. Similarly, if it is assumed that a significant difference of *antibody* titer exists when there is a 4-fold or greater reduction in titer as compared to that obtained with homologous prototype antigen, then it can be concluded that three 1957 strains were significantly dissimilar from the 1960 prototype employed. It is of interest that the 305 strain, which appeared to be relatively "non-avid" in hemagglutination inhibition tests, revealed a reaction of identity by complement fixation.

Discussion. These studies have indicated

TABLE I. Comparison of 1957 and 1960 Asian Influenza Strains by Hemagglutination Inhibition.

Virus			Reciprocal of HI titer	
Year	Country	Strain	Rabbit anti A_2/Formosa/313/57*	Rabbit anti A_2/Japan/775/60*
1957	Japan	305	320	40
"	"	Adachi-2	1280	160
"	"	Kumamoto Y-5	640	20
"	"	1155	1280	160
"	Formosa	T-22	640	20
"	"	313	640	20
1960	Japan	775	640	320
"	"	691	640	160
"	"	538	640	160
"	Formosa	T-60	640	160
"	Korea	805	640	80
"	Calif.	Cradock	5120	320
Controls		A/PR8	<20	<20
		B/Lee	<20	<20

* Sera were kaolin adsorbed prior to testing.

TABLE II. Numerical Summary of Cross of Test Comparison Between 1957 and 1960 Asian Influenza Strains.

Virus			Reciprocal of CF titer*			
			Guinea pig anti A_2/Formosa/313/57		Guinea pig anti A_2/Japan/775/60	
Year	Country	Strain	Antigen titer	Antibody titer	Antigen titer	Antibody titer
1957	Japan	305	4, 8	512, 512	4, 4	64, 128§
1957	"	Adachi-2	8, 16	512, 512	2, 4†	64, 128
1957	"	Kumamoto Y-5	64, 128	512, 512	16, 16†	32, 32‡§
1957	"	1155	2, 4	256, 512	2, 1	64, 64
1957	Formosa	T-22	32, 64	512, 512	2, 2†	16, 16‡§
1957	"	313	32, 32	512, 512	4, 2†	8, 8‡§
1960	Japan	775	4, 4	128, 128‡	8, 8	128, 128
1960	"	691	—	—	8, 8	512, 512
1960	"	538	2, 4	128, 256	4, 4	128, 128
1960	Formosa	T-60	32, 64	256, 256	32, 32	64, 64
1960	Korea	805	4, 8	512, 1024	8, 4	256, 256
1960	Calif	Cradock	32	2048	32, 32	64, 64
Controls		A/PR8	<1	<8	<1	<8
		B/Lee	<1	<8	<1	<8

* Titers taken as highest reached at 2 or more dilutions of other reagent.
† 4-fold or greater reduction in titer as compared to titer with homologous (1957) antiserum.
‡ 4-fold or greater reduction in titer as compared to titer with homologous antigen.
§ Relative non-avid strains by hemagglutination inhibition. See Table I.

that with an antiserum prepared against a 1960 prototype Asian influenza virus, differences can be detected between some 1957 Asian strains, and 1960 strains by both hemagglutination and complement fixation techniques. The question must be considered as to whether these differences reflect true antigenic diversity or whether they indicate only differences in "avidity." The data presented do not unequivocally answer this question. However, the finding that the 305 strain, which is relatively non-avid in respect to heterologous serum by the hemagglutination inhibition technique, shows a reaction of identity by the complement fixation technique would suggest that avidity differences seen in the hemagglutination inhibition test are not necessarily paralleled in the complement fixation technique. A conclusive answer to the question whether the complement fixation technique with "viral" influenza antigens is independent of avidity variations could best be obtained by carrying out complement fixation studies with stable homogenous, avid and

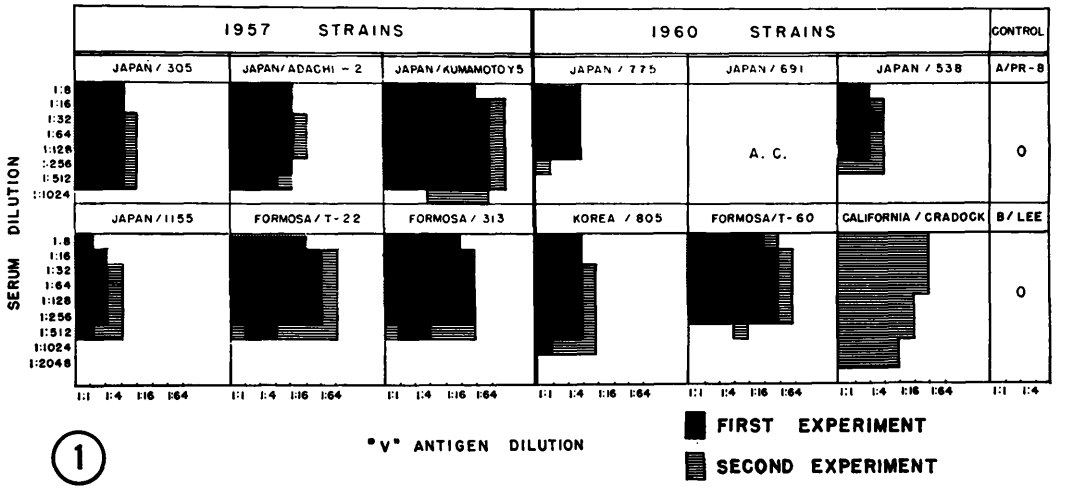

FIG. 1. Analysis of 1957 and 1960 Asian influenza strains by complement fixation with guinea pig anti A_2/Formosa/313/1957 ("V" antigen) antiserum.

FIG. 2. Analysis of 1957 and 1960 Asian influenza strains by complement fixation with guinea pig anti A_2/Japan/775/60 ("V" antigen) antiserum.

non-avid substrains derived from the same strain by the procedures described by Choppin and Tamm(9).

It would appear likely that the variations between the strains observed in these experiments, even if they reflect true antigenic dissimilarity, are not of epidemiological significance, since these data suggest that the newer strains react well with antisera to the original 1957 agent.

Summary. Comparison of 1957 and 1960 Asian influenza strains indicated that differences could be detected by both hemagglutination inhibition and complement fixation between some 1957 strains and the 1960 prototype. These differences were detected with antisera against a 1960 prototype virus, but not with antisera against the 1957 prototype employed.

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Procedure for Bioassaying Mosquito Repellents in Laboratory Animals.* (28486)

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Until recently, insect repellents have mainly been evaluated after surface application on the human arm and visual determination of insect bites by either the experimental subject or the observing investigator(1). This type of evaluation not only suffers from uncertainty of visual determinations but does not take into account the amount of blood ingested by the mosquitoes. Moreover, testing of new compounds with poorly known acute and chronic toxicity, particularly for internal administration, excludes use of the human arm as a testing bait. In our laboratory, the following procedure was found useful in determining the number of mosquitoes biting the mouse used as a bait and the amount of blood taken in by the mosquitoes.

Procedure. The eggs of yellow fever mosquitoes (*Aedes aegypti*) are hatched in deoxygenated water, and the larvae are raised in an incubator (80°F, 80% relative humidity). Pupae are separated according to sex. The adult mosquitoes are housed in the incubator for 4 to 6 days, before testing. Three days before testing, the female mosquitoes are temporarily immobilized by cold exposure (–4°C) and are randomly distributed, 50 in each cage, and fed with 10% sucrose. The following day the sucrose solution is removed. Water is left in the cages until about 4 hours before testing. The prospective repellent is administered to a mouse by an appropriate route at a predetermined time prior to testing. The mouse is injected intraperitoneally 10 minutes before testing with 0.005 ml per gram of body weight of an anesthetic mixture. Each milliliter of the anesthetic mixture contains 80 mg of allobarbital, 320 mg of urethane, and 320 mg of monoethyl urea. Five minutes before testing the indicator solu-

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