THE USE OF FILTRATES OF *VIBRIO CHOLERAE* IN THE CLASSIFICATION OF INFLUENZA VIRUS STRAINS.

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SINCE the original isolation of influenza A virus in 1933 there has been considerable evidence to show that this virus is not a single biological entity, but that antigenic differences exist between various strains—particularly between those isolated from different outbreaks. These differences have been demonstrated by neutralization tests *in vivo*, by complement-fixation tests *in vitro*, and by the agglutination-inhibition test of Hirst (1942). In the latter reaction, as shown by Hirst (1942), human and animal sera may yield high titres of inhibition due to non-specific inhibitory substances in addition to those derived from true antiviral antibodies. As a result no firm basis exists for classification of strains by simple titration of immune sera for their power of haemagglutination inhibition *in vitro*, and the results of such titrations may not be confirmed by *in vivo* neutralization tests. Moreover, though amounts of non-specific potentially inhibitory substances in normal sera are themselves variable and unpredictable, the titres found depend to a large extent upon the particular virus used for titration. Francis (1947) considers that the capacity of B strains for combining with non-specific serum inhibitors diminishes on repeated egg passage, but while this may not be a general rule with A strains (Stuart-Harris and Miller, 1947 ; van der Veen and Mulder, 1950) it seems clear that with any one virus strain this property can be markedly influenced by passage.

It is essential, therefore, in order to increase the precision and the reliability of haemagglutination-inhibition as a means of classifying influenza strains, to find some means of reducing non-specific effects in immune sera. It has been reported by van der Veen and Mulder (1950) that though these can be reduced by treatment of sera by various physical or chemical agents (heat, periodate, removal of calcium ions) these methods are not entirely satisfactory. These authors have found that better results could be obtained by treating serum with crude filtrate of *Vibrio cholerae*, as was first suggested by Mulder and van der Veen (1948). We have been able to confirm this.

METHODS.

Preparation of cholera filtrate.

The medium used for the preparation of cholera filtrate was a semi-solid tryptic digest of heart, without added phosphates, at pH 6·8, as recommended by Mulder (personal communication). This was poured into petri dishes to form a very thin soft layer, and inoculated with approx. 0·2 ml. per plate of an 8-hour broth culture of *V. cholerae* 4Z. After about 16 hours' incubation at 37° C. the soft agar cultures were filtered through filter paper and cotton wool in a Buchner

funnel, and the filtrate clarified, Seitz-filtered, and neutralized. Though the optimal pH for the receptor destroying enzyme (R.D.E.) of this organism has been found by Burnet (1948) to be around 6·0, this was found to be too low for complete elimination of ferret serum inhibitors for influenza A strains, and better results were obtained with a neutral filtrate.

Filtrates were titrated by finding the minimal amount which, with a given amount of normal ferret serum, would eliminate inhibition of virus haemagglutination. Filtrates can be stored at 4° C. for at least six months without loss of activity.

Treatment and Titration of Sera.

Each immune serum to be investigated for antibody level was titrated in duplicate, one portion with added cholera filtrate, and one without, the conditions for both titrations being otherwise identical. For each serum there was thus obtained titres of untreated serum and titres after treatment with filtrate when non-specific inhibition had been eliminated.

The procedure adopted was to add to 0·1 ml. serum (for each virus) either 0·5 ml. calcium saline (0·85 per cent NaCl, 0·1 per cent $CaCl_2$) or 0·5 ml. cholera filtrate appropriately diluted with calcium saline. With each batch of immune sera one normal serum showing a good titre of non-specific inhibition with the particular virus to be used was treated similarly, as an indicator of the satisfactory behaviour of the filtrate. All serum dilutions were incubated at 37° C. overnight, and then heated at 58–59° C. for 50 minutes to inactivate the filtrate. This amount of heating may diminish slightly the level of non-specific inhibition, but does not by any means eliminate it.

The amount of heating required can be decreased if calcium is not incorporated in the diluent (or is subsequently removed by addition of citrate) as the thermal stability of R.D.E. is enhanced by the presence of added calcium (Burnet and Stone, 1947). That there should be no residual active R.D.E. in the titrated sera is important, to obviate destruction of the red cell receptors with resultant loss of virus agglutination which can occur at room temperature during the performance of the titration. Calcium saline was used as diluent in the first experiments because of the reported effect of added calcium upon destruction by R.D.E. of mucopolysaccharide inhibitors (Edney, 1949). In later experiments with crude filtrate it was found that normal saline gave satisfactory results, and addition of calcium was unnecessary for elimination of non-specific inhibitors. The calcium content of the diluent did, however, affect the demonstrable non-specific inhibition of a serum. Thus, as illustrated in Table I, rather higher titres were found in normal sera diluted initially with calcium saline, even though the rest of the titrations were carried out with normal saline.

Virus strains.—Influenza A strains were represented by N-W.S. (a neurotropic variant of W.S. (Stuart-Harris, 1939)); PR8—the classical influenza A strain isolated in 1934; Kunz—isolated during a local outbreak in this country in 1947 (Stuart-Harris and Miller, 1947); NED/1/49—a strain from Holland isolated in 1949 and found by Chu, Andrewes and Gledhill (1950), to belong to the so-called influenza A prime group; and a number of other strains isolated locally during the outbreak of 1949, whose origin is described elsewhere (Stuart-Harris, Laird, Tyrrell, Kelsall, Franks and Pownall, 1949).

TABLE I.—*Effect of Calcium upon the Inhibitory Titres of Normal Ferret Sera for Virus Haemagglutination.*

Virus.	Normal ferret serum.	Serum diluent.	Incubation 37° C.	Heating.	Agglutination at serum dilutions of						
					1/24	1/48	1/96	1/192	1/384	1/768	1/1536
KUNZ	1	N.S.	18 hrs.	59° C., 50 min.	0	0	0	0	+++	+++	
		Ca.S.	,,	,, ,,	0	0	0	0	0	++	+++
		N.S.	None	None	0	0	0	0	++	+++	
		Ca.S.	,,	,,	0	0	0	0	0	+++	
KUNZ	2	N.S.	18 hrs.	59° C., 50 min.	0	0	0	+	+++	+++	+++
		Ca.S.	,,	,, ,,	0	0	0	0	0	++	+++
		N.S.	None	None	0	0	0	0	++	+++	+++
		Ca.S.	,,	,,	0	0	0	0	0	++	+++
NED	3	N.S.	18 hrs.	59° C., 50 min.	0	0	+++				
		Ca.S.	,,	,, ,,	0	0	0	0	+++		

N.S. = Normal saline. Ca.S. = Calcium saline.

(After the initial serum solution (0·2 ml. serum + 1·0 ml. diluent) the diluting fluid was in all cases N.S.)

All viruses were egg-adapted, used either in the form of pooled allantoic fluids or of concentrates prepared from these by elution from erythrocytes.

Immune sera.—Convalescent sera were obtained from ferrets by cardiac puncture 10 to 14 days after intranasal inoculation. In some cases a serum sample obtained from the same ferrets before inoculation was available for comparison (pre-infection sample).

Method of titration.—Titrations were carried out by a method similar to that described by Salk (1944), using fowl cells and (with a few exceptions) 8 haemagglutinating doses of virus.

RESULTS.

Results of cross titrations, both with and without the use of cholera filtrate, are presented in Table II. Inhibition titres demonstrated diagrammatically in Fig. 1 represent the results with cholera-treated sera only.

It is evident that crude filtrate can, under the conditions of these experiments, eliminate all non-specific inhibition of red cell agglutination. The findings also show that, when non-specific effects were eliminated, the influenza strains which were examined fell into three distinct antigenic groups with little apparent cross-reaction : W.S., PR8, and the group whose representatives here appeared in 1947 and 1949, and which is probably the same as that termed by American authors the influenza A prime group.

Of the latter group it would appear that some strains, notably Shef./10/49 and Shef./8/49, resemble the " Q strains " of Mulder, van der Veen, Brans and Enserink (1949) in that, though not poor antibody producers, they will only detect low levels of antibodies in homologous and heterologous sera. A glance at the titres obtained with the same virus strain with different sera, all of which exhibit some residual inhibition shows, however, the impossibility of drawing conclusions in regard to the degree of antigenic overlap from the quantitative level of inhibition obtained in this modified inhibition test. For example, the 1947 KUNZ virus gave high titres of inhibition with many of the sera from 1949 strains, yet the KUNZ antiserum was relatively feeble in its action on the 1949 strains compared with its titre for homologous virus.

Because so many variable factors are involved in the inhibition test it seems improbable that the method will reveal fine differences between related strains until greater precision and standardization can be achieved.

Treatment of Sera in Parallel with Crude Filtrate and with R.D.E.

A preparation of " purified " R.D.E. was obtained by the courtesy of Professor F. M. Burnet, and compared with crude cholera filtrate for its capacity to remove non-specific inhibition for influenza A strains from normal ferret sera. Results obtained with two sera, titrated with a virus sensitive to non-specific inhibition, are shown in Table III. It is quite evident that while the filtrate destroyed in each case all non-specific inhibition of haemagglutination the enzyme preparation reduced it only slightly. That the higher titre in the latter cases was not due to destruction of red cell virus receptors by residual R.D.E. is shown by the fact that no titre was obtained in the absence of serum.

Ferret antisera	Virus strains							
	N-W.S.	PR8	Kunz	Catt./1/49	Shef./22/49	Shef./10/49	Shef./8/49	Ned/1/49
N-W.S. 1933								
PR8 1934								
Kunz 1947								
Ned/1/49								
Shef./8/49								..
Shef./10/49								
Shef./5/49					..			
Shef./4/49						..	..	
Shef./22/49			..	..			..	

FIG. 1.—Antibody titres of ferret sera, treated with cholera filtrate, for homologous and heterologous strains of virus. The shaded areas represent logarithms of titre increases as multiples, where the reciprocal of the lowest dilution (1/24) is taken as unity. Actual titres are given in Table II. Titres with NED virus were sometimes difficult to interpret owing to rapid elution. .. = No test. Titres with PR8 virus and NWS sera were variable but some inhibition was obtained with three of four sera tested.

Investigation of Influenza B Strains.

As it became clear that the recently isolated influenza A strains examined here showed little antigenic relationship in haemagglutination inhibition titrations to the early strains of 16 years ago it was decided to investigate a limited number of influenza B strains in order to determine whether a similar deviation from the parent type had occurred amongst this group.

The methods of preparation and titration of ferret convalescent sera were the same as for influenza A strains. Viruses representative of the B group were LEE 1940 and two strains isolated locally from a school outbreak of influenza during the early months of 1950—TOD and COW. Acute and convalescent

TABLE II.—*Titres of Ferret Antisera, with and without Treatment with* V. cholerae *Filtrate, for Homologous and Heterologous Viruses.*

Treatment with cholera filtrate	N-W.S.		PR8		KUNZ		CATT/1/49		SHEF./22/49		SHEF./10/49		SHEF./8/49		NED/1/49	
	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+
LEE B strain	768	<24	*<24*	*<24*	*192*	*<24*	384	<24								
N-W.S. 1933																
Pre-inf.	48	<24	*<24*	*<24*	*320*	*<24*										
Post-inf.	3072	1536	*160*	*160*	*768*	*<24*	192	<24	192	<24	96	<24	<24	24	48	<24
PR8 1934	1536	<24	*1536*	*1536*	*768*	*<24*	384	<24	384	<24	192	<24	24	24	192	<24
KUNZ 1947	768	<24	*<24*	*<24*	*1536*	*768*	192	96	192	48	192	48	<96	96	192	96[a]
NED/1/49	1536	<24	*<24*	*<24*	*768*	*384*	384	192	384	192	192	96	96	96	768	384[a]
SHEF./9/49	1536	<24	*<24*	*<24*	*1536*	*384*	384	96	384	192						
SHEF./7/49	768	<24	*<24*	*<24*	*1536*	*768*	768	384							384	96
SHEF./8/49	768	<24	*<24*	*<24*	*768*	*192*	768	192	768	192	192	96	96	48		
SHEF./10/49	768	<24	*<24*	*<24*	*1536*	*768*	768	384	384	384	384	192	96	96	48	48
SHEF./18/49	192	<24	*<24*	*<24*	*1536*	*384*	768	192								
SHEF./1/49	192	<24	*<24*	*<24*	*768*	*192*	192	48							96	24
SHEF./5/49	192	<24	*<24*	*<24*	*1536*	*192*	384	192			384	96	96	48	48	48
SHEF./4/49	96	<24	*<24*	*<24*	*1536*	*384*	384	192	384	96					192	48
SHEF./6/49																
Pre-inf.	192	<24	*<24*	*<24*	*384*	*<24*									**48**	**<24**
Post-inf.	192	<24	*<24*	*<24*	*1536*	*768*	768	768							**96**	**48**
SHEF./22/49																
Pre-inf.	48	<24	*<24*	*<24*					<24	<24						
Post-inf.	48	<24	*<24*	*<24*					96	96	384	192			768	768[a]
Normal ferrets	48	<24			*768*	*24*	384	<24	<24	<24	192	<24	24	<24	192	<24

(a) = 4 haemagglutinating doses of virus. Pre-infection serum was obtained immediately before inoculation. Post-infection serum 10–14 days after inoculation. Figures in italics are titres obtained with virus in the form of an eluate; in all other cases the virus preparation consisted of pooled egg-allantoic fluids. (Results with NED virus were sometimes difficult to interpret owing to the great readiness with which this virus elutes from red cells at room temperature.)

TABLE III.—*Inhibitor Titres of Normal Ferret Sera Treated with "Purified" R.D.E. and with Cholera Filtrate.*

Serum.	Components—incubated and heated.	Haemagglutination at dilutions							
		1/24	1/48	1/96	1/192	1/384	1/768	1/1536	1/3072
Normal ferret (24)	0·2 ml. serum + 1·0 ml. Ca. saline	0	0	0	0	0	0	±	+++
	0·2 „ + 1·0 ml. filtrate[1]	+++	+++						
	0·2 „ + 1·0 ml. R.D.E.[2]	0	0	0	0	0	++	+++	+++
Normal ferret (15)	0·2 ml. serum + 1·0 ml. Ca. saline	0	0	0	0	0	0	+++	+++
	0·2 „ + 1·0 ml. filtrate[1]	+++	+++						
	0·2 „ + 1·0 ml. R.D.E.[2]	0	0	0	0	0	+++	+++	
None	0·2 „ + 1·0 ml. R.D.E.[2]	+++	+++						

Virus = KUNZ ; 4 H.D.

(1) An equivalent amount of calcium added.

(2) In solution in calcium acetate buffer, containing 200 units R.D.E./ml.

serum samples from the two patients from whom the latter viruses were recovered had already been found, by complement fixation test, to show a significant rise in antibodies to influenza B virus. However, in haemagglutination inhibition tests using LEE virus, while COW sera showed a parallel rise in inhibitory titre from 20 to 160, TOD sera did not inhibit haemagglutination at all.

For titrations of ferret sera all viruses were used in the form of pooled infected egg allantoic fluids.

Results of cross titrations are presented in Table IV. It is apparent that as two of the viruses concerned are affected by non-specific serum inhibitors it is only from results with cholera filtrate treated sera that their relationships are clarified. Thus it can be seen from Table V that the recently isolated strains of influenza B differ immunologically from the original parent LEE, but resemble each other closely.

TABLE IV.—*Titres of Ferret Antisera, with and without Treatment with* V. cholerae *Filtrate, for Homologous and Heterologous B Viruses.*

		Virus strains					
		LEE		TOD		COW	
	Treatment with cholera filtrate	−	+	−	+	−	+
Ferret Antisera.	LEE	768	768	96	<24	192	<24
	TOD Pre-inf.	—	—	192	<24	—	—
	Post-inf.	24	24	768	768	768	768
	COW Pre-inf.	—	—	—	—	384	<24
	Post-inf.	24	24	768	384	768	384
	Normal ferrets	<24	<24	96	<24	192	<24

TABLE V.—*Titres of Antisera Treated with* V. cholerae *Filtrate for B Viruses.*

		Virus strains.		
		LEE	TOD	COW
Ferret Antisera.	LEE	768	0	0
	TOD	24	768	768
	COW	24	384	384

DISCUSSION.

Since the discovery by Francis (1947) that a component of normal serum can inhibit haemagglutination by heated LEE virus, much attention has been focused upon the properties of the " Francis inhibitor " (found to be a muco-polysaccharide) and its related red cell virus receptors, both of which are a substrate for influenza virus and for R.D.E. The Francis inhibitor can therefore be eliminated by treatment of serum with this enzyme. Less is known about a second component of normal sera—the " non-specific inhibitor "—which prevents haemagglutination by living influenza A and B strains, and which possesses quite different properties from the Francis inhibitor. Some of the differences have been investigated by McCrea (1948). The non-specific inhibitor, associated with the γ-globulin fraction of serum, is not noticeably changed by R.D.E. or by periodate, but is more heat labile.

" Purified " R.D.E. alone, therefore, is not adequate as a tool for eliminating

non-specific inhibition of haemagglutination by influenza strains. There is present in cholera filtrate a second unidentified substance which is essential, and the conditions required are not precisely those prescribed by other workers for the optimum activity of R.D.E. Nor can a true estimate of the potency of a filtrate be made by titrating the red cell receptor-destroying capacity, as described by Burnet and Stone (1947) for R.D.E.

The question of antibody destruction by enzymes of the filtrate remains uncertain. Though Wilson Smith and Westwood (1948) have found crude cholera filtrates to have too high a content of proteinase to be practicable for use with immune serum, this may well vary with different strains of *V. cholerae*, and with the conditions of cultivation. In fact, during the present investigation, two indications were found suggesting that the antibody loss is not sufficient to be detected by this method of titration: Firstly, the PR8 and LEE strains were not capable of detecting non-specific inhibitors in any serum tested, and in titrations of homologous ferret sera with these viruses no reduction in titre was ever found as the result of filtrate treatment; and secondly, immune hamster sera, which were not demonstrated to contain non-specific inhibitory substances, showed no reduction in homologous titre after contact with filtrate, although normal ferret sera titrated in parallel showed no residual inhibition of haemagglutination after treatment with cholera filtrate.

SUMMARY.

1. By treating immune ferret sera with crude filtrates of *V. cholerae* 4Z under specified conditions all demonstrable non-specific inhibitors of virus haemagglutination were removed. This required the presence in filtrates of an unidentified substance other than receptor-destroying enzyme. It would not appear that there was much, if any, concomitant loss of antibody.

2. The findings of van der Veen and Mulder (1950) were confirmed in that, when non-specific titres were eliminated, influenza A and B strains could be broadly classified by the haemagglutination test using convalescent ferret sera.

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