THE INFLUENZA VIRUS FLOCCULATION REACTION AS A METHOD OF ANTIGENIC TYPING

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(With 1 Figure in the Text)

The influenza virus serum flocculation previously reported (Belyavin, 1955) opened up a number of lines of investigation. One of obvious importance was extension of the reaction to other viruses belonging to both related and unrelated groups. Indeed, other workers in this laboratory have already achieved the flocculation of poliomyelitis viruses by specific antisera (Smith, Sheffield, Lee & Churcher, 1956) and flocculation of both mumps and Newcastle disease viruses is now reported in this communication. The ease with which the viruses of the mumps-influenza group can be flocculated by homologous rabbit antisera suggested that the technique may be applicable as a method of antigenic analysis. If so, it would have the advantage of being much simpler than the standard haemagglutination inhibition and complement-fixation tests. The exploration of this possibility forms the basis of this paper. A large-scale antigenic survey involving numerous virus strains has not been attempted, greater emphasis being placed on the examination of techniques and their applicability to the end in view. The investigation has also revealed new phenomena peculiar to the direct virus flocculation reaction.

MATERIAL AND METHODS

Virus strains. A representative selection of virus strains from the influenza group, including mumps and NDV, were used. They were:

Mumps. Strain EMA41 (Enders).

NDV. Is believed to be a line originally derived from the Weybridge 'Herts' strain.

Influenza Type A. Classical Strains PR8 and MEL.

Influenza Type A'. Representative type strains FMI, CAM (1946). A.Eng. 1/51. Strains isolated in this laboratory: MW. (1951), ASH (1953), SHEFF (1956).

Influenza Type B. Classical strain LEE; strains ROB and BUT isolated in this laboratory in 1955.

With the exception of the mumps strain, these viruses were maintained by inoculating 11-day-old embryonated hens' eggs allantoically with suitable dilutions of allantoic fluid, stored in dry ice. The infected allantoic fluids were harvested after 40 hr. incubation at 36° C.

The mumps strain was inoculated into the allantoic cavity of 7-day embryonated eggs, and growth was allowed to proceed for 5 days at 36° C. before harvesting.

Haemagglutination titrations. Virus titrations were done by the method of Belyavin, Westwood, Please & Smith (1951). For titration of specific antisera the

pattern test of haemagglutination inhibition was used. This was performed in M.R.C. plastic trays, using 0·25 ml. vol. of serum dilutions and eight agglutinating doses of virus, and adding 0·5 ml. of 0·5% suspension of red cells to each dilution mixture. Fowl red cells were used for all titrations.

Technique of the flocculation test. Serial dilutions of antiserum in physiological saline were pipetted in 0·3 ml. amounts into Dreyer's tubes. 0·3 ml. of appropriate antigen, diluted to give a standard dose of virus, was added and the mixtures placed in a water-bath at 37° C. The water-level was adjusted so as to fall between a third and half-way up the columns of liquid. After $4\frac{1}{2}$–5 hr. incubation flocculation was assessed with the help of a low-power hand-lens and a viewing box. The degree of flocculation in each tube was expressed by means of an arbitrary notation derived from that originally introduced by Dreyer for bacterial agglutination (Belyavin, 1955). The end-point dilution was taken as the highest dilution of serum giving tr + flocculation as defined on the arbitrary scale. Where the end-point fell clearly between two dilutions, the geometric mean of these was taken, without attempting interpolation in fractions of the dilution interval. Although this relatively crude method has been improved upon more recently by the use of a geometrical interpolation, it was considered adequate for the purpose of antigenic analysis.

EXPERIMENTAL

Preparation of flocculating antigens

Although it has been found that the concentration of virus obtained in an infected allantoic fluid is adequate for flocculation, crude fluids are not suitable for direct use as antigens because they often contain inhibitory substances (Belyavin, 1955). Some degree of purification is necessary therefore, and absorption and elution from either human or fowl cells has been found the most suitable technique for all the influenza virus strains. One cycle of absorption and elution will in general yield an antigen suitable for the flocculation test, but, in the present work, two such cycles have been used. In the case of ND virus, a single cycle of adsorption and elution has been followed by further purification by high-speed centrifugation, in order to exclude the products of the haemolysis which this virus sometimes induces during elution. The Enders strain of mumps virus eluted so poorly from fowl cells, that the mumps antigens had to be prepared entirely by high-speed centrifugation.

Comparison of human and fowl cell eluates

Most of the early flocculation work was carried out using antigens eluted from human group O cells, but many of them proved to be of very low potency. The probable reason for this variability was suggested by the work of Smith & Cohen (1956), who found that some influenza virus strains elute poorly from human red cells, but much better from fowl cells, with which there is greater regularity of elution behaviour. Comparative studies were made, therefore, of flocculating antigens prepared from both types of red cell. Infected allantoic fluids were divided into two equal volumes which were processed in parallel with an overall volumetric concentration factor of 10. The eluates were adjusted by dilution to

a haemagglutination titre of between 100 and 150 by the densitometer method, (Belyavin *et al.*, 1951). This is roughly equivalent to a titre of between 300 and 400 by 'pattern' titration using 1·0 % fowl cells, and gives a final dose of virus used in the test of round about 20 agglutinating units (densitometer). Some of the human cell eluates were of too low a titre to allow of adjustment and were therefore used undiluted at doses of about 10 to 15 agglutinating units.

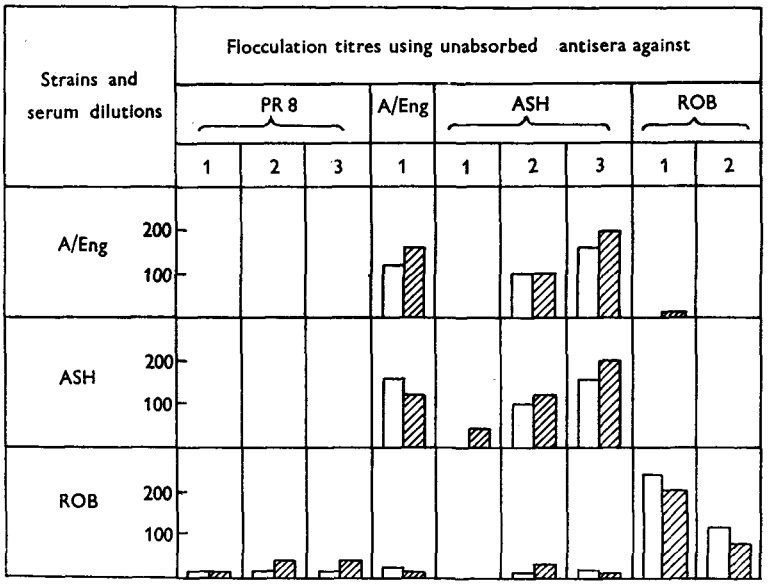

Fig. 1. Human and fowl cell virus eluants compared for readiness and specificity of flocculation against immune rabbit sera. □, Human cell eluates; ▨, fowl cell eluates.

The two types of preparation were then put up in parallel against serial dilutions of homologous and heterologous sera and the resulting titres compared. Such comparisons, using two A′ strains, A Eng/1–51, and ASH, and one type B strain, ROB, are illustrated in Fig. 1. The results showed that fowl cell eluates are in no way inferior to human cell eluates, as flocculating antigens, and they were, therefore, adopted for all subsequent work.

Normal serum flocculation of 'sensitive' antigens

In all flocculation titrations physiological saline controls were included, as in orthodox bacterial agglutination tests. In no single instance was spontaneous flocculation of a virus suspension observed. It was found, however, that occasional preparations of a virus strain gave well-marked flocculation when mixed with normal (non-immune) rabbit serum. The aggregates in these circumstances were large, appeared more quickly than in an antibody flocculation and the reaction could be demonstrated with high dilutions of normal serum. It was originally thought that this was due to storage deterioration of the antigen; however, it has been observed not only with freshly harvested antigens, but also with recently isolated strains. 'Sensitive' preparations of this nature have been found amongst

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antigenically diverse strains of influenza virus, and three of these have been tested against the normal sera of different species and also against 20% egg-white saline (Table 1).

Table 1. *The 'sensitivity' of three influenza virus strains to non-immune serum of different species*

			Serum species								
			Rabbit					Pooled mouse			20%
Expt. no.	Strain	Status	1	2	3	Human	Pooled G.P.	1	2	Horse	egg white saline
1	PR8	Sensitive	640	480	—	320	320	<5	—	—	>320
		Stable	<5	<5	—	<5	<5	<5	—	—	<5
2	PR8	Sensitive	—	—	480	—	—	—	<10	40	>128
		Stable	—	—	<10	—	—	—	<10	<10	<2
	MW	Sensitive	—	—	160	—	—	—	<10	480	<2
	FMI	Sensitive	—	—	<10	—	—	—	<10	20	<2

In this and subsequent tables, titres are expressed as the reciprocals of the highest serum dilutions, giving tr. + flocculation.

The three strains tested were flocculated by non-immune horse, guinea-pig, human and rabbit sera but sensitivity to the different sera varied amongst the strains. The type A strain PR8, for instance, appeared most sensitive to rabbit and the two A prime strains to horse serum. None of these strains was flocculated by mouse serum and only PR8 was sensitive to egg-white. The pattern of flocculation with sensitive preparations of PR8 suggested that the virus was being aggregated by non-specific haemagglutination inhibitor of the α type, and abolition of the reaction by pre-treatment of non-immune rabbit serum with the receptor destroying enzyme of *Vibrio cholerae* supports this view (Table 2). It is not yet known, however, whether the same serum component is responsible for the flocculation of sensitive strains of other antigenic groups.

Table 2. *Effect of treatment with receptor destroying enzyme on the flocculating effect of non-immune rabbit serum*

			Sensitive flocculation titre against serum held at 37° C. for 1 hr. with			
Expt. no.	Serum	Virus	No treatment	Heat 56° 30′	RDE+ 56° 30′	Inactive RDE+ 56° 30′
1	Rabbit	PR8 sensitive	640	500	15	480
2	Rabbit	PR8 normal	<5	<5	<5	<5
		PR8 sensitive	640	500	<5	320

As yet no means has been found of inducing sensitivity at will, and it is not certain that a virus line that has become permanently sensitive can be rendered completely stable again. For the purpose of the present investigation, therefore,

choice of virus strains has been restricted to those which were proved fully stable in normal rabbit serum, and dilute rabbit serum controls were included in every test.

Storage stability of antigen preparations

Antigen preparations were usually made in relatively small quantities and quickly used so that accurate assessments of the stability of flocculation potency on storage have not been made. A few virus suspensions however had fortuitously been stored for long periods at ordinary refrigerator temperature, and these appeared to be fully active against their homologous antisera up to two years after preparation. A limited number of thermal inactivation tests, in demonstrating that the flocculating potency of influenza virus suspensions is at least as stable as the haemagglutinin, would appear to support this observation. The mumps virus suspensions seem to be exceptional in this respect, as loss of flocculability with homologous antisera has been observed after a few days storage at +4° C.

Preparation of antisera

Immunization of rabbits

Rabbits were immunized with two intravenous doses of purified virus. The first sensitizing dose was small, of the order of 100 to 300 agglutinating doses. Three weeks later, a second dose of virus, about ten times greater than the first was administered, and serum bled off on the 5th or 6th, and 10th or 12th days afterwards. The peak of the secondary response, measured as flocculation titre, was usually quickly passed and came between the 5th and 12th day after the second inoculation. Either of the two serum bleeds, therefore, might be found to have the greater flocculating potency. Serum samples taken during the primary response have been found to show little if any flocculating activity. As rabbits vary considerably in their immune response, pairs of rabbits were always immunized with each antigen, and the most powerful flocculating serum selected out of the four available.

Inactivation of sera

After the sera had been separated from the clot, they were inactivated by heating at 56° C. for 1 hr. The long period of inactivation was found necessary to destroy thermolabile flocculation-inhibiting substances that are present in the sera of many animal species (Belyavin, 1956).

Antibodies to host tissue

Rabbits immunized with elution-purified living virus, by the technique described above, do not commonly produce much host tissue component antibody (Smith, Belyavin & Sheffield, 1955) and such sera will behave specifically in the flocculation test without further treatment. It has been found, however, that occasional rabbits, even when immunized by the two-injection technique, will develop appreciable amounts of such antibody. This can be absorbed out with suspensions of minced chorio-allantoic membrane. For the purpose, chorio-allantoic membranes

were harvested from 13-day-old embryonated eggs, and washed thoroughly in two changes of distilled water, and three changes of physiological saline. The pooled membranes were finely minced with scissors, suspended in a small volume of saline (about 5 ml. per membrane) and the suspension pipetted into centrifuge tubes in volumes equal to the volume of diluted serum to be absorbed. After centrifugation at 20,000 *g* for 45 min., the supernatant fluid was discarded, the serum diluted 1/10 added, and the deposit resuspended and placed in the refrigerator at +4° C. overnight. The following morning the minced tissue was re-sedimented at 20,000 *g* for 45 min. The supernatant fluid constituted the absorbed serum. The results of two absorption experiments are given in Table 3.

Table 3. *Absorption of host-tissue antibodies from flocculating antisera*

			Flocculating titres after absorption by									
				Virus			Human RBC		Sheep RBC		Fowl RBC	
Expt. no.	Serum	Tested against	Nil	PR8	LEE	CAM ×1	×1	×2	×1	×2	×1	×2
1	PR8	PR8	240	<10	120	160	—	—	—	—	—	—
		LEE	120	<10	<10	<10	—	—	—	—	—	—
	LEE	PR8	20	<10	<10	<10	—	—	—	—	—	—
		LEE	120	60	<10	60	—	—	—	—	—	—
2	PR8	PR8	120	—	—	60	120	80	80	80	80	80
		LEE	40	—	—	<10	40	40	40	30	30	40
	MEL	PR8	240	—	—	120	240	160	160	160	120	160
		LEE	120	—	—	20	80	80	80	80	80	80

They show that the non-specific antibodies cannot be readily absorbed out by either fowl cells or sheep cells, although they are removed by absorption with CAM or with heterologous virus. They probably represent therefore the host-tissue component antibodies investigated in detail by Smith *et al* (1955). Treatment of flocculating antisera with heterologous virus strains in order to produce mono-specific antisera, as described by Hirst (1952), would automatically render the sera free from such cross-reacting antibodies.

Flocculation titrations

The flocculation of mumps and Newcastle disease viruses

Mumps and Newcastle disease virus suspensions were mixed with dilutions of the respective homologous and heterologous rabbit sera, both unabsorbed and absorbed with chorioallantoic membrane suspensions (Abs. CAM). Control mixtures were made with a serum prepared against influenza virus (LEE). The results are summarized in Table 4. Flocculation titres are low but they clearly demonstrate the specificity of the reaction. Subsequent experiments show that these viruses are antigenically distinct from all the influenza virus strains tested.

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Table 4. *Flocculation of mumps and Newcastle disease (ND) viruses by immune rabbit sera*

	Sera					
	Mumps		NDV		LEE	
Antigen	Unabs.	Abs CAM	Unabs.	Abs. CAM	Unabs.	Abs. CAM
Mumps	30	40	< 10	< 10	< 10	< 10
NDV	15	< 10	80	20	10	10
LEE	< 10	< 10	< 10	< 10	40	30

Cross-flocculation tests with influenza and related viruses

Cross-titrations were put up between eleven virus strains, including mumps and NDV, and nine representative rabbit antisera. All the antisera were absorbed with minced chorioallantoic membrane (Table 5).

Table 5. *Cross-flocculation titres between eleven virus strains of the mumps-influenza group*

	Sera (absorbed with chorioallantoic membrane)								
Strains	Mumps	NDV	MEL	PR8	FMI	A/Eng	ASH	ROB	LEE
Mumps	30	*	*	*	*	*	*	*	*
NDV	*	320	*	*	*	*	*	*	*
Type A									
MEL	*	*	240	10	*	*	*	*	*
PR8	*	*	40	120	*	*	20	*	*
Type A′									
CAM	*	*	*	*	30	60	60	*	*
FMI	*	*	*	*	120	160	120	*	*
A/Eng	*	*	*	*	15	120	120	*	*
ASH	*	*	*	*	*	120	240	*	*
SHEFF	*	*	*	*	*	> 320	320	*	*
Type B									
ROB	*	*	*	*	*	*	*	120	10
LEE	*	*	*	*	*	*	*	40	80

* = < 10.

The major antigenic groups are clearly demarcated. Mumps and Newcastle disease viruses are sharply distinguished from the influenza group, and within the latter, the A, A′ and B subgroups are also well differentiated. Bearing in mind that the strains A/England and ASH represent the 'Liverpool' and 'Scandinavian' A′ subtypes (Isaacs & Andrewes, 1951), the flocculation patterns of these strains and strain FMI, were compared with their haemagglutination-inhibition patterns using the same sera for both types of test. Tables 5 and 6 show that these two strains are distinguishable from FMI both by haemaglutination inhibition and flocculation, but are not clearly differentiated from each other by either test with the rabbit antisera employed.

Table 6. *Cross-haemagglutination-inhibition titres between five influenza virus strains and homologous rabbit antisera*

	Sera (trypsinized)				
Strains	PR8	FMI	A/Eng	ASH	ROB
PR8	7,680	240	*	480	*
FMI	*	>20,480	7,680	2,560	*
A/Eng	*	2,920	15,360	3,840	*
ASH	*	480	3,840	7,680	*
ROB	*	*	*	*	7,680

* = <160.

Qualitative variations in the flocculum

As has been described elsewhere (Belyavin, 1955) influenza virus flocculation develops as a fine granular precipitate closely resembling the classical bacterial O agglutination. The flocculations observed with the strains of mumps and ND viruses used have accorded entirely with this general picture. With the A′ influenza strain SHEFF, and with two other related strains, particulation was of a different nature. In these cases, flocculation developed very rapidly and coarse, loose floccules formed, which were exactly comparable with those of bacterial H agglutination. Similar differences were observed with tobacco mosaic virus by Bawden & Pirie (1945) and were attributed to the presence of filamentous or elongated particles. Preparations of strain SHEFF have been examined by dark field microscopy and many filamentous forms observed, (Chu, Dawson & Elford 1949) but it is not yet certain whether this is the factor determining the altered nature of the flocculum with the strains in question.

DISCUSSION

The demonstration of specific flocculation with mumps and Newcastle disease viruses makes it reasonably certain that the reaction is applicable to all members of the mumps, NDV, influenza group, which are already accepted as being antigenically related on the basis of their haemagglutination behaviour. Nor is the phenomenon limited to viruses of this group or of this intermediate order of size, for it has been observed with one of the largest viruses (vaccinia) by Ledingham (1931) and with one of the smallest (poliomyelitis) by Smith *et al* (1956). It is, therefore, likely that eventually the direct flocculation of many, if not all, known viruses by specific antisera, will be achieved and used for practical purposes such as virus typing and serological diagnosis.

For the serological typing of viruses of the influenza group, the haemagglutination inhibition test is both efficient and convenient, but direct virus flocculation would seem to be equally valid, and is technically much simpler. Antigenic analysis, by its means, gives results broadly similar to those given by haemagglutination inhibition, the main virus types and subtypes being very readily differentiated. Failure to differentiate strains representative of the Liverpool and

Scandinavian A′ subtypes was probably due to the use of rabbit antisera, rather than any relative insensitivity of the flocculation reaction. Haemagglutination inhibition tests with the same reagents gave no additional information, and it has been reported that these subtypes are not readily distinguishable when rabbit sera are employed, but are so with the sera of other species such as the hamster (Sampaio, 1952). Whether this would apply in the flocculation test is not yet known. Perhaps, its chief advantage as a means of virus typing lies in its possible applicability to other non-agglutinating viruses.

Apart from purely practical applications, the flocculation reaction opens up a new field of investigation. Indeed, two new phenomena have been observed in the course of the present study, namely, the occurrence of different types of flocculation with different influenza virus strains and the susceptibility of some strains to non-specific flocculation by the normal sera of some species. Both of these must reflect peculiarities of virus surface structure, and their further investigation should help to clarify the relation between structure and biological behaviour.

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