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Biological and Serological Properties of Frater Virus — a Cytopathogenic Agent Associated with Aseptic Meningitis

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With 2 Figures

(Received February 13, 1961)

During the late summer and autumn of 1959 an epidemic of aseptic meningitis took place in Scotland due to a cytopathogenic agent which could not be identified as any known virus. Sixty nine cases were found which showed definite evidence of infection with the virus and a typical clinical picture of aseptic meningitis (1, 2). Increases in antibodies to the virus during the course of the illness in the sera of these patients, together with a much higher rate of isolation of the virus from aseptic meningitis patients than from control patients, strongly suggested that the virus did cause the disease. The virus is meantime being referred to as Frater virus after the type strain. In a short preliminary account of the epidemic and of the virus (1) brief reference was made to the biological and serological properties of Frater virus which indicated that it probably belonged to the ECHO group. These properties are now reported in full, and in the light of the findings the classification of Frater virus is discussed.

Materials and Methods

Virus strains. Seventy five strains of Frater virus were examined; all were isolated in human thyroid, human amnion, or monkey kidney tissue cultures from specimens of faeces examined in this laboratory. Strains 1 to 6, and 1 and 2 in particular, were studied most intensively. Strain 1 was isolated from an infant with a disease clinically typical of paralytic poliomyelitis, who showed no virological evidence of infection with poliovirus. The purity of this strain was ensured by making a series of 3 terminal dilutions. Sixty two strains including strains 2, 3, 4 and 6 were from patients with aseptic meningitis, but as 2 of them were obtained from the same patient they really repre-

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sent only 61 different strains. The remaining 12, including strain 5, were from patients treated in hospital for diseases other than aseptic meningitis. The strains of polioviruses and Coxsackie and ECHO viruses were the same as those used in previous work (3) except that the Brunenders strain of poliovirus type 1 was used instead of the Mahoney strain.

Sera. An antiserum to each of strains 1, 2, 3, 4, 5 and 6 of Frater virus was prepared by injecting a guinea pig intra-muscularly at 4-day intervals with 1 ml. of virus seed in a course of 7 injections. Antisera to polioviruses types 1, 2, and 3 were hyperimmune monkey sera obtained from the Virus Reference Laboratory, Colindale, England. Antisera to Coxsackie viruses A 9, B 1, 2, 3, 4, and 5 and ECHO viruses 1 to 20 except 10 and 16 were prepared in this laboratory by immunising rabbits.

Pathogenicity tests. a) *Animals.* 0.03 ml. of 10th tissue-culture-pass seed of Frater virus strain 1 was inoculated intracerebrally into each of 6 adult mice which were observed for 4 weeks. Tissue culture fluid of between the 6th and 10th pass of each of 12 strains of Frater virus was injected intraperitoneally into a family of suckling mice in a dosage of 0.03 ml. per mouse. With 3 strains the mice were given 0.01 ml. of virus seed intra-cerebrally in addition to the 0.03 ml. intra-peritoneally. Aliquots of 23 faecal extracts from which strains of Frater virus were isolated were each injected into a family of suckling mice intra-peritoneally in a dose of 0.03 ml. per mouse. All suckling mice were observed for at least 2 weeks.

b) Chick embryos. 0.1 ml. of 10th pass seed of Frater virus strain 1 was inoculated on to the chorio-allantoic membranes of 3 14-day hen's eggs, intra-amniotically to 3 12-day eggs, intra-allantoically to 3 12-day eggs, and into the yolk sac of 3 7-day eggs. The eggs inoculated by the yolk sac route were observed until just short of hatching and the others were killed and examined at 3 and 4 days.

Haemagglutination. 7 strains of Frater virus including strains 1 and 2 were grown in tubes of the same batch of human amnion tissue cultures. A control preparation was made by harvesting uninoculated tubes of this batch containing the same maintenance medium in the same way as the virus-infected cultures. The 7 viruses and the control fluid were tested for haemagglutination by mixing 0.3 ml. volumes of tissue culture fluids and red cell suspensions in plastic plates. Each was tested against a 1% saline suspension of human group 0 red cells and against a 0.5% suspension of fowl red cells of a type known to be strongly agglutinable by vaccinia virus. All these tests were done in replicate at 4° C, 23° C, and 37° C.

Preparation of tissue cultures. All the types of tissue used were grown in 4 × ½ inch test tubes in stationary racks. Human thyroid, human amnion, rhesus monkey kidney and guinea pig kidney cells were obtained by trypsinization and used to prepare first generation cultures. Propagation medium was Hanks' solution + 10% human serum for thyroid, Hanks' + 20% human serum for amnion, Hanks' + 10% human and 10% calf serum for monkey kidney, and Hanks' + 10% calf serum for guinea pig kidney. Details of the methods of growing thyroid (3) and amnion (4) are given elsewhere. All 4 tissues were maintained in Earle's solution + 5% calf serum. HeLa cell tube cultures were propagated and maintained in Hanks' solution + 4% calf serum from stock cultures grown in 340 ml. flat bottles in Hanks' solution + 20% human serum. All these media contained also 0.5% lactalbumin hydrolysate and antibiotics.

Titrations. Tissue culture adapted strains of Frater virus were titrated in 10-fold dilutions and 3 tubes of each type of tissue were used for each dilution. Stool extracts containing Frater virus were titrated in half log. steps and 2 tubes of each tissue used per dilution. In the growth curve experiment titrations were performed with 10-fold dilutions and 5 tubes of human amnion per dilution, and in the adsorption experiment with half log. steps and 5 tubes per dilution. The titrations were read after 7 days' incubation and the media in the tubes were not changed during this time. TCD_{50} end points were calculated by *Kärber*'s method.

Neutralization tests. Equal volumes of virus suspension, containing 100 TCD_{50} per 0.1 ml., and diluted serum were mixed, and after 1 hour at room temperature 0.2 ml. volumes of the mixture were transferred to each of 2 or more tubes of tissue culture which were incubated at 37° C. Control tubes were inoculated with 100 TCD_{50} doses of virus unmixed with serum and these usually showed complete degeneration of the monolayer after about 3 days. Tubes containing serum were read 24 hours after the controls showed complete degeneration, and if cytopathogenic changes were absent the serum was considered to have neutralized the virus. The antibody titre of a serum was taken as the reciprocal of the greatest dilution neutralizing the virus. Dilutions are expressed as final dilutions of serum in serum-virus mixtures.

Complement fixation tests. These were performed in plastic plates. Reagents were diluted in sodium veronal buffer, and a constant volume of 0.1 ml. each of serum, virus, complement, and sensitised red cells was employed in each test. The dose of complement in this volume was 4 units, 1 unit being the amount which gave haemolysis of 50% of the red cells.

Growth experiments. 100 TCD_{50} of Frater virus strain 1 was added to 10 ml. maintenance medium in each of two 6 oz. bottles, one containing a monolayer of human amnion cells and the other a monolayer of monkey kidney cells. The cultures were incubated at 37° C and 0.3 ml. samples of tissue culture fluid were removed at 4 hours and 8 hours, and then daily until 1 day after complete degeneration of the tissue. The samples were stored at −40° C until the end of the experiment when they were all titrated in tubes of human amnion tissue culture.

Adsorption experiments. 10^4 TCD_{50} of Frater virus strain 1 in 10 ml. maintenance medium was added to each of two 6 oz. bottles, one containing a monolayer of human amnion and the other a monolayer of monkey kidney tissue culture, and to a sterile empty bottle which served as a control. The bottles were incubated at 37° C and 0.35 ml. samples of fluid removed from the tissue culture bottles at 30 minutes, 1 hour, and then hourly to 8 hours. The control bottle was sampled at 1, 2, 6 and 8 hours. Samples were stored at −40° C until titrated in human amnion cultures.

Results

Animal pathogenicity

The adult mice inoculated intra-cerebrally with strain 1 Frater virus all remained healthy. None of the 12 strains of Frater virus injected in the form of tissue-culture fluid or the 23 strains of wild virus in stool extracts caused paralysis of suckling mice. The doses of virus in tissue-culture fluids injected into each mouse varied with different virus strains

from 10^3 to $10^{4.5}$ TCD_{50}. Histological sections were made of carcasses of mice injected with 6 of the tissue-culture fluids. No lesions were seen in brain, fat, pancreas, or muscle as would be found in Coxsackie virus infections. The 6 guinea pigs injected intramuscularly with strains 1 to 6 of Frater virus for the preparation of sera, all remained healthy during the 5 weeks between their first injection and slaughter.

Pathogenicity for chick embryos

Lesions were not observed on the chorio-allantoic membranes of eggs examined 3 days and 4 days after inoculation. After 3 and 4 days' incubation of the eggs, the amniotic and allantoic fluids from eggs inoculated by the amniotic route and from those inoculated by the allantoic route failed to agglutinate human and fowl red cells. The chick embryos survived in all the eggs whether inoculated by one of these 3 routes or by the yolk-sac.

Haemagglutination

Haemagglutination was not observed with any of the 7 strains tested with human and fowl cells at 4° C, 23° C, and 37° C.

Cytopathogenicity

As has been reported previously (1) Frater virus was isolated from 75 stools which were tested in parallel in human thyroid, human amnion, and monkey kidney tissue cultures. Sixty five isolations were made in thyroid, 58 in amnion, and 26 in kidney. All 75 strains were successfully passed in amnion tissue cultures, this seed being used for neutralization tests. Tissue culture adapted seed of 1 of the 10 strains not originally isolated in thyroid was later tested in thyroid tissue, and seed of each of 4 of the 49 strains not originally isolated in kidney was later passed in kidney. In all cases typical cytopathic changes were seen in these passes. Thus every strain of Frater virus produced typical cytopathic changes in all of these 3 types of tissue culture in which it was tested.

Tissue-culture-adapted seed of 15 strains was tested in HeLa cell cultures. Cytopathic changes were not observed. Seed of 11 strains was tested in tissue cultures of guinea pig kidney. As a control the Lang strain of ECHO type 10, now classified as a Reovirus (5), which is known (6) to be cytopathogenic for guinea pig kidney cultures, was tested in parallel with the Frater viruses. Cytopathic changes were seen with Lang strain virus but not with any of the Frater virus strains.

The cytopathic changes produced by Frater viruses in human thyroid and amnion, and in monkey kidney tissue cultures were identical with those produced in these tissues by known strains of polioviruses, Coxsackie

viruses, and ECHO viruses. Cytopathic changes developed much more slowly in monkey kidney cultures after infection with Frater virus than in cultures of human thyroid and amnion.

Relative infectivity for different tissue cultures

The very different number of isolations obtained when stool extracts were tested in human thyroid, human amnion, and monkey kidney cultures seemed to indicate considerable differences in the sensitivity of these tissue cultures to infection by Frater virus. To establish these differences, 12 stool extracts known to contain the virus were titrated in all 3 types of tissue. The titres are given in Table 1. Tissue culture fluid seed of each of 5 strains was titrated in the same way and the results are given in Table 2. With minor exceptions strains of both wild and tissue culture adapted virus gave the highest titre in human thyroid, the next highest in human amnion, and the lowest in monkey kidney tissue cultures.

Table 1. Titres (as $\log_{10}$ TCD_{50}) in thyroid, amnion, and kidney of 12 wild strains of Frater virus

Thyroid	3.0	3.0	5.0	4.2	2.5	1.2
Amnion	1.7	1.5	5.0	4.5	0.7	0
Kidney	1.7	0.2	3.5	4.2	0	0
Thyroid	0.5	0.2	0.7	0	0	0
Amnion	0	0	0.7	1.2	0	0.5
Kidney	0	0	0	0	0.2	0

Table 2. Titres (as $\log_{10}$ TCD_{50}) in thyroid, amnion, and kidney of 5 tissue culture adapted strains of Frater virus

Thyroid	5.8	6.5	6.5	6.5	5.5
Amnion	4.2	5.8	5.2	5.2	4.5
Kidney	1.8	3.8	2.8	3.5	2.5

Growth characteristics in amnion and kidney tissue cultures

A number of experiments were done in an attempt to explain the differences in titre obtained when the same seed was titrated in human amnion and monkey kidney cultures. Strain 1 Frater virus was used for this work. A lower titre in monkey kidney cultures might be found if the virus grew in the monkey kidney cells without producing a cytopathic effect when the virus inoculum per tube was small, although it produced normal cytopathic changes when the inoculum was large. This phenomenon has been described in the case of polioviruses titrated in human thyroid cultures (7). To test this possibility Frater virus seed with a titre of $10^{-5.5}$ in human amnion and in human thyroid was titrated in monkey kidney tissue using 6 tubes per log dilution. All 6 tubes of the 10^{-3}

dilution, 1 tube of the 10^{-4} dilution, and none of the 10^{-5} or 10^{-6} tubes showed cytopathic effects. The tissue culture fluids from the 10^{-5} tubes, the 10^{-6} tubes and the 5 of the 10^{-4} tubes not showing cytopathic changes were titrated in thyroid cultures, and no virus was detected in any of them.

It seemed probable, therefore, that the differences in titre were due to a smaller proportion of the virus particles in a suspension of Frater virus being able successfully to infect kidney cells than amnion cells. This might be due to poorer adsorption of virus to kidney cells, and experiments were done to determine the speed of adsorption of virus to monolayers of kidney and of amnion cells. The results are given in Figure 1, which shows that the rate of adsorption to amnion is distinctly more rapid than to kidney. At 7 hours the original titre of 10^2 had fallen to $10^{0.6}$ in the medium of the amnion culture but only to $10^{1.5}$ in the kidney culture. The experiment was stopped at 8 hours because then virus began to be freed from infected amnion cells into the medium.

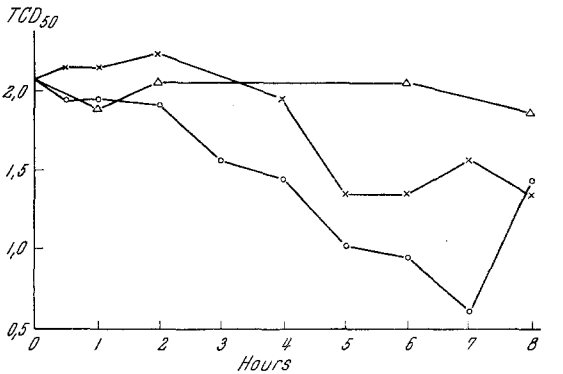

Fig. 1. Adsorption of Frater virus from supernatant medium to monolayers of human amnion and monkey kidney cells. Titres expressed as $\log_{10}$ TCD_{50} per 0.1 ml.
△——△ control ○——○ amnion ×——× kidney.

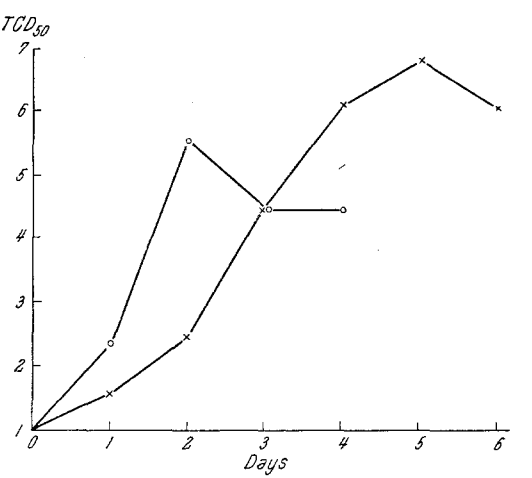

Fig. 2. Release of Frater virus into the medium in tissue cultures of human amnion and monkey kidney. Titres expressed as $\log_{10}$ TCD_{50} per ml.
○——○ amnion ×——× kidney.

The slower release of Frater virus into the medium in kidney than in amnion tissue cultures is illustrated in Figure 2, which shows a maximum yield in amnion at 2 days and in kidney not until 5 days. The higher yield in kidney than in amnion in this particular experiment was peculiar to

these particular batches of tissues. In many experiments with strain 1 virus similar yields of virus were obtained in both kidney and amnion tissue cultures.

Complement fixation tests

Tissue culture fluid seed of strain 1 Frater virus was used as antigen in complement fixation tests with herpes simplex and adenovirus antisera. All these tests were negative. Complement fixation tests were also carried out with this antigen and human and guinea pig sera known to contain neutralizing antibodies to Frater virus. Complement fixing antibodies to Frater virus were found in these sera. The levels were lower than those of neutralizing antibodies in sera of both species, and relatively the guinea pig sera contained more complement fixing antibodies than the human sera.

Neutralization tests of Frater isolates with known enteroviral antisera

Before it became apparent that they were strains of a new type of enterovirus, 19 isolates, later shown to be Frater virus, were tested against antisera to the 3 strains of poliovirus, Coxsackie viruses A 9 and B 1 to 5, and ECHO viruses types 1 to 9, 11 to 15, and 17 to 20. All the tests were negative.

Dr. *A. D. Macrae* of the Central Public Health Laboratory, Colindale, most kindly agreed to examine strain 1 Frater virus. His antisera to Coxsackie viruses A 9 and B 1 to 5, and ECHO viruses types 1 to 27 failed to neutralize the virus.

Neutralization tests of prototype enteroviruses with Frater antisera

Seed of the 3 types of polioviruses, Coxsackie viruses types A 9 and B 1 to 6, and ECHO viruses types 1 to 9 and 11 to 27 were tested against antisera to Frater virus strains 1 and 2. None of these viruses were neutralized by either serum.

Neutralization tests of Frater virus by gamma globulin

Frater virus strain 1 was tested against 8 samples of gamma globulin prepared from the blood of Scottish blood donors in the years between 1951 and 1959. Six of the samples neutralized the virus to a titre of 1:32 or more, and the other 2 to a titre of 1:16.

Cross neutralization of 6 strains of Frater virus

Homologous and heterologous neutralization tests were carried out with strains 1, 2, 3, 4, 5, and 6 of Frater virus and their corresponding antisera. The titre of neutralizing antibodies for the homologous strains of virus was 1024 in each of the sera except strain 2 serum which had a

titre of 256. For the purpose of comparing both homologous and heterologous titres of different sera, the titre of any one serum with homologous virus was considered as being 1 unit. The titres of the 5 heterologous viruses tested with this serum were calculated in terms of units. For example with serum 4 the titre with virus 4 was 1024 and with virus 2 was 64. The titre of virus 2 with serum 4 would then be 16 units. The results of these heterologous neutralization tests expressed in terms of units are given in Table 3. Strains 3, 4, 5 and 6 show almost complete cross neutralization

Table 3. Serological relationships of 6 strains of Frater virus

Frater virus strain number	Antiserum* to Frater virus strain number					
	1	2	3	4	5	6
1	1	2	4	4	4	1
2	1	1	16	16	8	8
3	1	2	1	4	1	1
4	1	2	2	1	1	2
5	1	2	4	1	1	2
6	1	2	2	2	1	1

* Expressed as number of units of antiserum, 1 unit being titre of antiserum with homologous virus.

and appear to be antigenically closely similar. Strain 2 shows high-titred neutralization in one direction only with strains 3, 4, 5 and 6 and therefore resembles the prime strains of ECHO viruses described by the Committee on the Enteroviruses (8), *Melnick* (9) and *Karzon* et al. (10). Strain 1 not only shows cross-neutralization with the strain 3, 4, 5, 6 group but also with strain 2. In these tests 8th pass seed of strain 2 was used. Since *Karzon* et al. (10) found that serial passage in tissue culture altered strains of ECHO 6 from the "specific" to the "broad" antigenic phase, strain 2 Frater virus was similarly passaged and the neutralization tests repeated with 30th pass seed. The results were the same as with the 8th pass seed used in the original tests.

Discussion

The occurrence of Frater virus in specimens of human faeces, its cytopathogenicity for cultures of human and monkey tissues, and the type of cytopathic change produced in these tissue cultures, suggest that it should be grouped with the enteroviruses. Its ether resistance, temperature stability, and size, which will be reported fully later, provide further evidence that it belongs to this group. Moreover in its seasonal epidemicity

and in the nature of the disease associated with it (1, 2), Frater virus is similar to known enteroviruses. Within the enterovirus family it appears to belong to the ECHO group as defined by the Committee on the ECHO viruses (11). The characteristics of the ECHO viruses are 1) cytopathogenicity for monkey and human cells in culture with the exception that HeLa cells are not readily susceptible 2) failure to be neutralized by poliovirus antisera 3) failure to be neutralized by Coxsackie virus antisera and lack of pathogenicity of suckling mice 4) lack of relationship to other viruses recoverable from the alimentary tract by inoculation of tissue cultures, such as adenoviruses, herpes simplex virus and influenza virus 5) neutralization by human gamma globulin and individual human sera. Frater virus satisfies all these criteria.

Serological investigations in this laboratory and in that of Dr. *A. D. Macrae* failed to identify it as one of the ECHO types 1 to 27. Because of the poor growth in tissue culture of ECHO type 28 and the difficulty of preparing an antiserum to it, serological tests were not done with this type. ECHO 28 is now (12) the designation of the virus described by *Mogabgab* and *Pelon* as 2060 (13). The ready growth of Frater virus and the type of cytopathic effect it produces in monkey kidney tissue in no way resemble the behaviour of ECHO type 28. It is possible that more delicate serological methods, such as those by which the U-virus was shown by *Philipson* and *Rosen* (14) to be related to ECHO type 11, may show a relationship between Frater virus and one of the known ECHO types. On the other hand it may prove to be an entirely new type of ECHO virus. The existance of some antigenic variation among Frater viruses is of particular interest as this phenomenon has been described in various ECHO viruses (8) and in particular in ECHO type 6 (10).

From the diagnostic viewpoint the greater success of human thyroid and human amnion than of monkey kidney cultures for the isolation of Frater virus is important. Human amnion is more susceptible than monkey kidney to infection by several other ECHO viruses (4) and it is probably unwise to rely on monkey kidney tissue cultures entirely as a single tissue culture system for the investigation of enteroviral infections.

Summary

Frater virus, a cytopathogenic agent isolated from cases of aseptic meningitis in Scotland in 1959, was found to resemble the ECHO viruses in its properties. It was not pathogenic for laboratory animals or for hens' eggs, but produced cytopathic changes in cultures of human and monkey tissues. Human thyroid was more susceptible to infection than human amnion, and amnion more than monkey kidney, while HeLa cell cultures failed to show any cytopathic effects. Serologically no relationship was detected between Frater virus and polioviruses, Coxsackie viruses,

ECHO virus types 1 to 27, herpes simplex virus and adenoviruses. Among different strains of Frater virus some antigenic variation was detected. Frater virus is thought to be a new type of ECHO virus.

Acknowledgements

I am most grateful to Dr. *A. D. Macrae* for gifts of prototype virus strains and for testing Frater virus with his sera, to Dr. *R. A. Cumming*, Regional Blood Transfusion Service, Edinburgh, for gamma globulin samples, to Dr. *E. J. Bell* and Dr. *M. C. Timbury* for their cooperation in various aspects of the work, and to Miss *M. M. Buchanan*, Miss *M. F. Macfarlane*, and Mr. *H. G. Carson*, F. I. M. L. T. for technical assistance.

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