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STUDIES OF THE COMPLEMENT-FIXATION REACTION IN VIRUS SYSTEMS

V: IN FOOT AND MOUTH DISEASE USING DIRECT AND INDIRECT METHODS

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Complement-fixation tests of the "*direct*" type have become essential aids in the early diagnosis of foot-and-mouth disease in cattle, and in typing the virus. These methods are less useful at later stages of the disease when infected tissue can no longer be obtained, since only a small proportion of cattle develop in their sera complement-fixing activity for foot-and-mouth disease viral antigens. The preliminary studies presented here, however, suggest that the antibodies in such convalescent sera can be detected by complement-fixation tests of the "*indirect*" form. If more extensive studies confirm the results of these initial investigations, the *indirect* test may prove to be an epidemiological tool of some importance.

The previous literature on the use of the *direct* complement-fixation technique in the diagnosis of foot-and-mouth disease (FMD) has been recently reviewed by one of us, (1). This test was applied in 1929 by Cuica (2) in identifying virus of guinea-pig origin by the reaction with type-specific guinea-pig antisera, and more recently by Traub and his co-workers (3–5) in the study of variant bovine strains recovered from outbreaks in Germany. A complement-fixation technique which depends on the use of hyperimmune guinea-pig antisera has been developed in Pirbright, and has been of value in identifying and typing FMD virus strains from outbreaks not only in England, but in many other parts of the world (6–9). Workers in several other countries have also reported favourably on the use of *direct* complement-fixation methods in the diagnosis of foot-and-mouth disease (10–15).

Such tests, however, are possible only during the limited interval before the infected epithelium exfoliates from the tongue. Subsequently, although the presence of antibodies may be demonstrated in the sera of cattle by neutralization or protection tests in animals, only a very small proportion show definite complement-fixing activity with FMD virus antigen. Through the use of unheated serum (16) or higher salt concentration (17), certain workers claim to have increased the proportion of convalescent bovine specimens fixing complement with these viral antigens, but these modifications have not proved so successful in other laboratories.

The *indirect* complement-fixation test was initially developed to meet such a situation, that is to detect antibody in non-complement-fixing antisera (18–20). It has since been applied not only in titrating antibodies in antipullorum chicken, turkey, and duck sera (19), but also in detecting antibodies for the virus of Newcastle disease (21), ornithosis-psittacosis (22, 23), and variola (24) in chicken antisera. It may also be used to demonstrate anticarbohydrate antibody in antipneumococcal equine and human sera.

The first section of this paper presents the results of a quantitative study by two *direct* complement-fixation methods of the behaviour of pooled sera of guinea pigs and cattle immunized with different strains of A type FMD virus, including that responsible for the recent outbreak in western Canada, designated as Ca-I. The second section describes the behavior in *indirect* tests of sera from cattle that had been naturally or experimentally infected with FMD virus of the A, O, or C types.

The earlier experiments were made in Canada by C. E. R., the later ones at the Research Institute (Animal Virus Diseases) Pirbright, Surrey, England.

METHODS

Preparation of Antisera (Pirbright Series)

In guinea pigs. Adult guinea pigs were infected in the plantar pads of the hind feet by the multi-

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ple tracking method. Three to four months after infection, all surviving animals were inoculated intramuscularly with the same strain of virus, and bled out 10 days later. Sera from guinea pigs immunized with the same strain of virus were pooled.

Three type A strains were employed: No. 119, a stock A type bovine strain of British origin, Ca-I, the Canadian 1952 strain, and No. 734, a strain from an outbreak in England in November, 1951, that is about the same time as the initial outbreak in Canada. All three strains had been passaged at least three times in guinea pigs.

The lyophilized, hyperimmune, pooled guinea-pig serum used as "standard" A type, had been produced with the stock guinea-pig strain, GB.

In cattle. Cattle were inoculated with virus at several sites in the tongue with one of four strains of A type virus, A_4, A_5, H2200, or Ca-I. Bleedings were taken before infection and at approximately 5, 7, 10, 14, and 21 days and at 6 weeks after the appearance of the primary lesions on the tongue. All sera were stored in the frozen state and tested within a few days of collection.

Preparation of Antigens

Antigens were prepared from infected bovine epithelium, or infected plantar pads of guinea pigs. The infected tissue was cut into fine pieces with scissors and ground in a mortar with sand. Saline was added slowly during grinding to a final proportion of 6 ml per gram of moist bovine epithelium or of 1 ml per guinea-pig foot pad. The suspension was centrifuged at moderate speed and the supernatant fluid used as antigen.

I. DIRECT COMPLEMENT-FIXATION TESTS

Technique of Tests

Two methods were employed, the first that described in previous papers in this series of studies (25, 26), the second, that used routinely at Pirbright for FMD virus typing (1). Fundamentally, the two techniques are similar in that all titrations are made with serial amounts of complement and all reactions are estimated in relation to the amount of complement required for 50% hemolysis. The Pirbright method differs from the New York State quantitative technique (27) in certain details: (a) a larger volume of each reagent is used, (b) all dilutions of reagents are prepared in veronal buffer containing magnesium and calcium (28) rather than in borate buffer, (c) the readings of the degree of hemolysis are made spectrophotometrically rather than visually, and (d) the amount of complement required for 50% hemolysis is estimated by the probit method rather than by the van Krogh equation.

In the New York State method all reagents are used in a volume of 0.1 ml with a total volume of 0.5 ml. For the Pirbright test, the volumes are as follows: serum 0.4 ml, antigen 0.2 ml, complement 1.0 ml, sensitized sheep red cell suspension 1.0 ml, with a total volume of 2.6 ml. Owing mainly to the larger total volume, the "hemolytic unit" or amount of complement required for 50% hemolysis in titrations in the absence of antigen and antiserum was 3 to 4 times that required in the former test.

The period for fixation in each test was 30 min at 37 C; the period for hemolysis was likewise 30 min at this temperature. All sera were inactivated for 30 min at 56 C before use.

Antigen titration. Each FMD viral antigen was titrated in serial dilutions with a constant and excess amount of the respective guinea-pig antisera in the presence of 5 to 7 different quantities (1 to 6 units). The values of K', the amount of complement required for 50% hemolysis, was estimated for each dilution of antigen and the complement-fixing capacity, in units per ml, determined by extrapolation.

Antibody titration. Similarly, the maximal complement-fixing capacity of each guinea-pig antiserum with each viral antigen was estimated by extrapolation from the K' values obtained in tests of serial dilutions of the respective serum with a constant amount of the particular antigen and varying complement.

The usual antigen, serum, and complement controls were included in all tests.

Results

Guinea-pig antisera. To exemplify the data obtained in *direct* complement-fixation tests, the results of titrations of 4 A type antigens with three A type guinea-pig antisera are presented in Table I. In each case, the quantitative relationships between complement and antigen in the presence of the maximally-reactive dose of a particular antiserum were found to be linear over the range in which it was possible to test them. The relationships between complement and antigen were likewise found to be linear when antiserum had been added in suitable

excess. In other words, the FMD virus systems behaved in a manner analogous to that previously observed with the majority of other viral and bacterial systems investigated earlier (25, 26).

The No. 119 strain antigen fixed more complement than either the Ca-I or 734 antigen with the strain 119 guinea pig antiserum, while conversely the Ca-I and 734 antigens fixed definitely more complement that the 119 strain antigens with the Ca-I and 734 guinea-pig antisera. With the "standard" A type (strain GB) antiserum on the other hand, all three antigens showed a roughly comparable degree of fixation, but considerably less than would have been obtained with a GB strain antigen had it been included in the test.[1] From the slopes of the respective complement-antigen lines, the complement-fixing capacity per ml of each antigen with each of the four guinea-pig antisera was estimated (Table I). The values suggest that the Canadian A type strain was more closely related antigenically to the recently-isolated English strain, No. 734, than to the old stock bovine A strain, No. 119.

When each of the guinea-pig antisera was titrated in serial dilutions for maximal complement-fixing activity with constant amounts of the 119, Ca-I and 734 antigens, these strain differences were again evident (Table I). The 119 antiserum appeared of much higher complement-fixing titer with the 119 antigens than with the antigens of either of the other A type

TABLE I

Complement-fixing capacity, expressed in 50% hemolytic units per ml, of FMD viral antigens and guinea-pig hyperimmune sera, estimated by extrapolation from K' values for serial dilutions of antigen and antiserum respectively

ANTIGEN TITRATION		TITER OF ANTIGEN UNITS/ML†	ANTISERUM TITRATION		TITER OF ANTISERUM UNITS/ML†
Antigen*	Antiserum		Antiserum	Antigen*	
119	119	19	119	119	129
	Ca-I	10		Ca-I	57
	734	13			
	St.A (GB)	24	Ca-I	119	77
				Ca-I	418
Ca-I	119	4	734	119	34
	Ca-I	34		Ca-I	72
	734	24		734	59
	St.A (GB)	28			
			St.A (GB)	119	145
734	119	3		Ca-I	190
	Ca-I	30		734	141
	734	23			
	St.A (GB)	24			

* Prepared from infected guinea-pig foot-pad tissue.

† Tests made by Pirbright method; unit of complement = 0.0020 to 0.0030 ml as compared with 0.0006 to 0.0010 ml in New York State quantitative method.

[1] The fact that the Ca-I and 734 strain antigens appeared of almost as high complement-fixing titer with the "standard" hyperimmune A type serum as with their homologous serum brings out a point which has been emphasized previously (1), namely, that homologous antigens and antisera must always be included in evaluations of the antigenic relationships of strains within types. Thus, when a new strain of virus is being tested with a stock-strain antiserum of exceptionally high antibody titer, an antigen prepared from this stock strain should be tested in parallel to indicate the potential complement-fixing capacity of this particular hyperimmune serum. Otherwise, the greater fixation exhibited by the test strain with this than with another antiserum of lower potential complement-fixing titer, might be attributed to a strain relationship, rather than to a reaction with antibodies for an antigen or antigens common to the type and present in both the stock and new strains.

strains. Conversely, the complement-fixing titers of the strain Ca-I and the 734 guinea-pig antisera were much higher with the Ca-I and 734 antigens than with the 119 preparations.

Antigen titrations similar to those recorded in Table I, except that the New York State quantitative technique was employed, were also made on the Canadian A type virus when it was first isolated in Canada, using lyophilized guinea-pig antisera for the A, O, and C types FMD virus and for the New Jersey and Indiana types of vesicular stomatitis virus that had been received from Pirbright. Significant fixation was recorded only with the A type FMD antiserum. The complement-fixing capacity of the first antigen preparation tested, which had been prepared from tongue epithelium of a calf injected with suspected material, was estimated to be 94 units per ml as compared with 7.9, 5.0, 4.9, and 5.9 units with the other four guinea-pig antiserum, and 5.9 units with normal guinea-pig serum. In comparing these values with those given in Table I allowance should be made for the difference in the amount of complement that constitutes a "unit" in the New York State and Pirbright techniques. When both sets of values are converted into ml of complement, these differences are largely eliminated.

Bovine Antisera

Canadian series. Serial bleedings collected from four calves before and after infection with the Ca-I strain of virus during diagnostic investigations, were tested for complement-fixing activity with Ca-I bovine epithelial antigens by the *direct* method. No fixation was obtained with these post-infection sera, nor with any of the 34 specimens from the *W* herd described in Section II.

Pirbright series. The serial bleedings from three of the four A type FMD cattle in the Pirbright series also failed to react in *direct* complement-fixation tests with FMD antigens prepared from infected bovine or guinea-pig tissue, that is with antigens that gave satisfactory and typical fixation with A type guinea-pig antisera. Three bleedings from the fourth animal, CBL/15, exhibited weak fixation in lower dilutions. One of a miscellaneous group of A, O, and C types convalescent bovine sera also fixed complement with homologous type antigen (type O).

These findings were in conformity therefore with general experience in the Pirbright and other laboratories that it is only the occasional specimen of heat-inactivated, convalescent bovine FMD serum that fixes guinea-pig complement with FMD viral antigens.

In passing, attention might also be called to the fact that human FMD convalescent sera have been shown to resemble bovine convalescent sera in their irregular complement-fixing behaviour (29). It raises the question of whether such behaviour may characterize other antiviral human sera.

II. INDIRECT COMPLEMENT-FIXATION TESTS

Technique of Tests

In the initial tests made in Canada a serum-dilution technique slightly modified from that described earlier for avian antisera (20, 30) was applied. A constant-serum technique was employed in the Pirbright series of experiments.

Serum-dilution tests. To 0.1 ml amounts of duplicate sets of serial dilutions of bovine serum, 0.1 ml of A type FMD viral antigen was added and the mixtures incubated for 60 min at 37°C; 0.1 ml of the 3-unit dilution of guinea-pig complement was then added to both sets, 0.1 ml of a 1:20 dilution of A type hyperimmune guinea-pig serum to the first set, 0.1 ml of a 1:20 dilution of normal guinea-pig serum to the other. After a further period of 30 min at 37°C to allow for fixation of complement by residual A type antigen and A type guinea-pig antibody, the hemolytic indicator, 0.2 ml of maximally-sensitized sheep red cells (27), was added. The customary period of 30 min at 37°C was allowed for hemolysis. Controls of serum, antigen, and complement were always included.

Constant-serum tests. Diluted or undiluted bovine serum (pre- or post-infection) was mixed with an equal amount of diluted FMD viral antigen, incubated for 2 hours at 37°C, then placed in the refrigerator overnight. Each mixture, undiluted and in 1:2 and 1:4 dilutions, was titrated for residual complement-fixing activity with 0.4 ml of the "standard" guinea-pig antiserum of the corresponding type, using the Pirbright *direct* quantitative method described above. To a duplicate set of each mixture, 0.4 ml of veronal buffer was added instead of the "standard" guinea-pig antiserum to determine its inherent "specific" and "nonspecific" complement-fixing activity.

A mixture of each antigen with an equal amount of buffer solution was included as a control. The titration of this control mixture with buffer solution indicated whether the antigen was anticomplementary; the test of the control mixture with the homologous "standard" guinea-pig serum indicated its "specific" complement-fixing properties.

TABLE II

% hemolysis recorded in indirect complement-fixation tests with an A type FMD viral antigen of sera from the W herd of cattle and from a group of non-contact cattle at the Central Experimental Farm, Ottawa*

NO. OF SPECIMEN	HERD	DILUTION OF SERUM			NO. OF SPECIMEN	HERD	DILUTION OF SERUM		
		1:2	1:5	1:10			1:2	1:5	1:10
6-O	C.E.F.	80	60	30	1-H	*W*	—	95	75
8-O		95	60	45	2-H		—	—	90
11-O		90	60	30	3-H		95	80	60
17-O		80	60	40	4-H		—	—	95
23-O		80	50	30	5-H		—	—	95
26-O		70	40	10	6-H		—	—	95
27-O		90	55	35	7-H		—	—	—
30-O		80	60	35	8-H		—	—	90
33-O		75	40	20	9-H		—	—	95
34-O		90	55	45	12-H		—	—	85
37-O		60	35	20	13-H		—	—	95
43-O		80	55	20	14-H		—	—	95
44-O		60	30	20	15-H		—	—	90
59-O		90	60	30	16-H		—	—	—
61-O		90	60	25	18-H		—	—	80
62-O		80	40	30	19-H		—	—	95
63-O		65	30	10	21-H		—	—	95
65-O		90	60	30	22-H		90	35	20
67-O		85	60	45	23-H		60	45	5
70-O		85	55	30	24-H		95	25	10
71-O		90	60	30	26-H		—	—	—
74-O		90	65	30	28-H		—	—	95
78-O		90	60	40	32-H		—	—	—
82-O		90	60	25	34-H		—	—	—
None†		10			36-H		—	—	—

— = 100% hemolysis.

* Not previously absorbed with sheep red cells to remove natural anti-sheep-red-cell antibody.

† Control, without bovine serum, of complement-fixing activity of the A type viral antigen and the "standard" A type guinea-pig serum added in the second stage of the test.

Results

Canadian series. The first opportunity to determine the value of the *indirect* complement-fixation test in detecting antibody in non-complement-fixing convalescent FMD bovine sera came when a group of blood specimens was received from a herd of cattle on a farm, *W*, where it was suspected the initial Canadian outbreak had occurred. *Indirect* complement-fixation tests of these 34 sera and of sera from 30 normal animals at the Central Experimental Farm, Ottawa, that had never had any contact with the virus were made by the first method described above. The results of these tests, which will be described in greater detail elsewhere (31), will be summarized briefly.

The majority of the normal bovine sera in 0.05 and 0.02 ml amounts showed a slight reducing effect on the fixation by FMD antigen with subsequently added A type FMD guinea-pig antiserum. No inhibition was observed with 0.005 ml or less of any of the normal bovine sera (Table II). By contrast, all but 4 of the

34 *W* herd sera displayed definite inhibition of complement-fixation beyond this range. In 0.1 ml of the 1:5 dilution, 27 of the 34 *W* herd sera produced complete inhibition; with 7 complete or almost complete inhibition was recorded with 0.001 ml of serum.

Since the infection in this *W* herd had been clinically diagnosed as vesicular stomatitis, *direct* and *indirect* tests were also made with VS virus antigens of the New Jersey and Indiana types. No fixation was recorded in the *direct* tests with these antigens, nor in *indirect* tests did these sera reduce the complement-fixing properties of either of the VS antigens with homologous guinea-pig antiserum to a degree beyond that exhibited by normal bovine serum. The evidence obtained in the *indirect* complement-fixation tests therefore supported the view that the outbreak of vesicular disease in this herd had been caused by FMD virus rather than by either of the vesicular stomatitis virus types, as originally surmised.

Sera from the four calves that had been experimentally infected with the Ca-I strain during this period were also tested by the *indirect* serial serum-dilution method. Beginning about four days after infection, a slight increase in inhibitory properties was noted in sera from two of the calves and was maximum at two weeks. In no case, however, was the degree of neutralizing activity at all comparable with that observed in the *W*-herd adult cattle. When the mixtures of serum and antigen were held for one hour at 37°C and overnight in the refrigerator before the addition of complement and FMD guinea-pig antiserum, more definite evidence of neutralization was obtained.

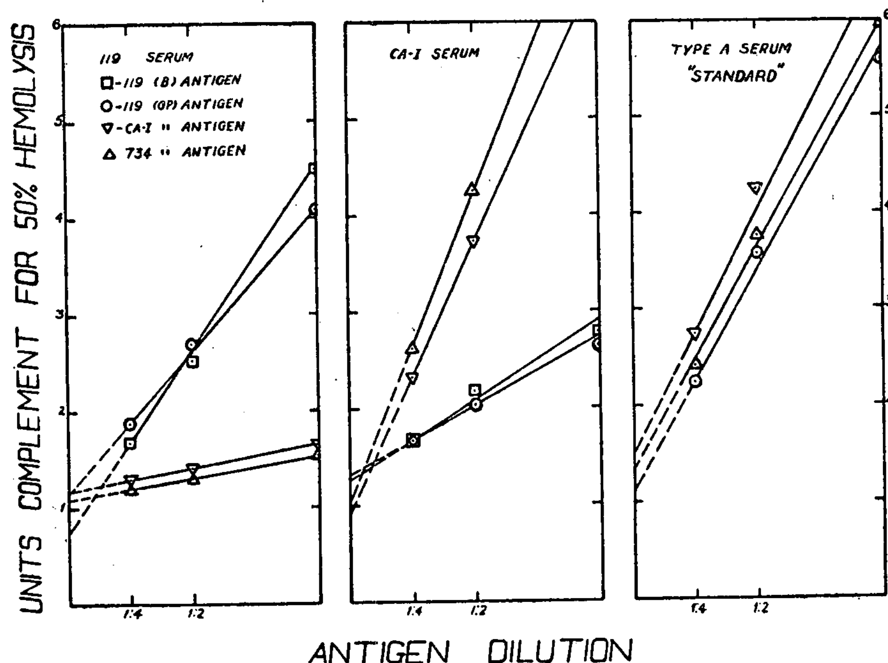

Figure 1. Complement-fixing activity of four A type antigens, two of strain No. 119, one of strain Ca-I, and one of strain No. 734, with type A guinea-pig antisera prepared with strains No. 119, Ca-I, and GB.

Pirbright series. At this point in our investigation the outbreak of foot-and-mouth disease in Saskatchewan had been brought under control, and although our work was being conducted on an island where strict quarantine measures could be enforced, it was no longer considered advisable to continue experimental work with FMD virus in Canada. Arrangements were made with Dr. Galloway for these studies to be continued at Pirbright, England.

The preliminary studies at Pirbright were made with five convalescent bovine sera that had been stored in the frozen state for a consider-

able period; three were of the O type, one of A type, and one of C type. One of the O type sera, as mentioned earlier, showed some fixation in the *direct* test. All five of these antisera, when tested by the constant-serum technique showed definite inhibitory properties in *indirect* tests with FMD antigens and guinea-pig antisera of the homologous type. Preliminary *indirect* tests with FMD antigens and guinea-pig antisera of heterologous types suggested that the inhibitive effect might not be strictly "type specific".

Tests with A-type virus. In the first of these experiments, undiluted sera from each of the four animals were mixed with equal amounts of a strain A_5 antigen prepared from infected bovine tongue epithelium. Only in mixtures of virus with the four pre-infection bleedings could significantly more fixation with A type guinea-pig antiserum be demonstrated than in the control with saline. In other words, all of the post-infection bleedings, including those collected five days after the appearance of lesions completely or

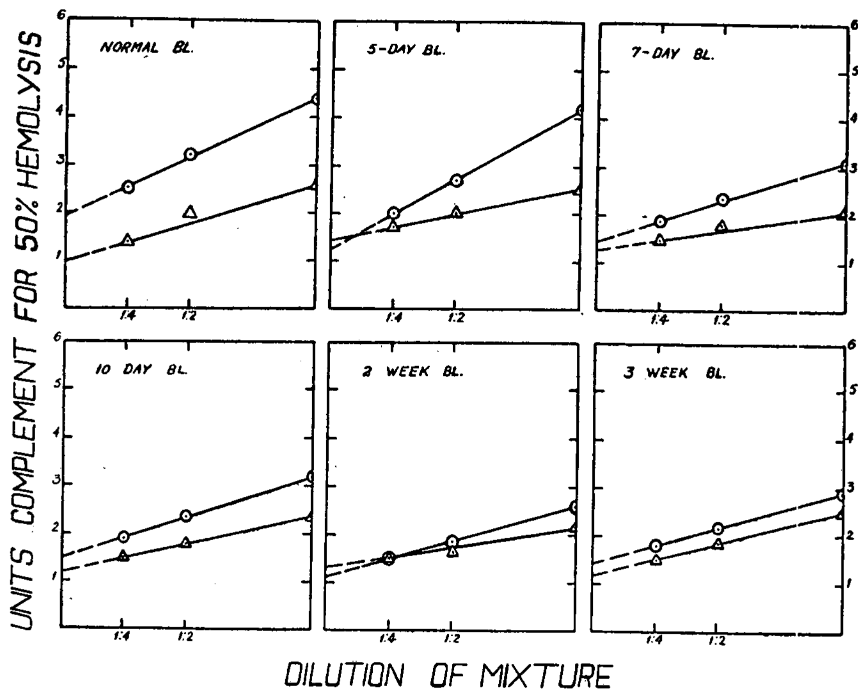

Figure 2. Complement-fixing activity of mixtures of a 1:8 dilution of serial bleedings from C/BL 13 with an equal quantity of type A virus, strain GB, antigen in the presence (upper line), and absence (lower line) of "standard" A type guinea-pig antiserum.

A more extensive investigation was then undertaken with pre-infection and post-infection sera from the four cattle infected with the four A type strains, A_4, A_5, Ca-I and H2200, as described above. The first two of four experiments were designed to determine the time at which neutralizing activity for two A type strains, A_5 and GB, appeared in the serum. The third and fourth experiments were made to determine if inhibitory activities for O or C type strains also developed in the sera of the A-type infected cattle, and whether their appearance was simultaneous with, or later than, that for A type virus.

almost completely neutralized its complement-fixing activity with the subsequently added A type guinea-pig antiserum. All of the sera, both pre-infection and post-infection, showed a weak "nonspecific" fixation[2] with the extracts of infected bovine epithelial tissue.

[2] The "nonspecific" fixation produced by these normal and convalescent sera with tissue antigens, did not appear to augment the "specific" fixation. Had it done so, the K' values for tests of mixtures of A_5 virus and serum with A type guinea-pig serum should have been proportionately greater than the K' values for tests of mix-

In the second experiment with the GB strain of A type virus derived from infected guinea-pig pads, two serum dilutions were used, 1:2 and 1:8. The mixtures of GB virus antigen and serum were tested for residual antigen, undiluted, 1:2, and 1:4. No significant amounts could be detected in mixtures of GB virus with 1:2 dilutions of any of the post-infection bleedings or with 1:8 dilutions of those from C/BL 15 and C/BL 16, the cattle infected with the Ca-I and H2200 strains respectively. The 1:8 dilutions of the 5-day and 7-day bleedings from the other animals, C/BL 13 and C/BL 14, infected with strains A_4 and A_5 respectively, were somewhat less inhibitory than those collected later, which completely or almost completely neutralized the complement-fixing activity of the GB strain antigen with the "standard" A type guinea-pig antiserum. In fig. 2 the results of titrations of the 1:8 bleedings from C/BL 13 are presented graphically.

tures of A_5 virus and buffer with the same A type guinea-pig serum. Rather than being greater, the former values were usually less. This suggested that in the presence of the "specific" antigen-antibody complexes, "nonspecific" fixation of complement by bovine serum and viral antigen did not occur.

Mixtures of the GB viral antigen with higher dilutions of the 10-day and 6-week bleedings from the four cattle were also prepared and inhibitory properties demonstrated in all at the 1:32 dilution and in some even at a dilution of 1:128 (Table III).

Tests with O and C-type virus. Mixtures were prepared of 1:2 dilutions of each bleeding with 1:2 dilutions of extracts of guinea-pig foot-pad tissue infected with the V_1 strain of O type FMD virus or of the GC strain of C-type virus. After the usual period of incubation these various mixtures were tested for residual complement-fixing activity with "standard" types O or C guinea-pig serum respectively. Complete or almost complete removal of these activities from both the O and C antigens was effected by the 1:2 dilutions of most of the post-infection bleedings from all four cattle (Table IV). When the experiment was repeated using a 1:8 dilution of the pre-infection and of three of the post-infection bleedings from each animal, little or no inhibitory effect was noted for either of the heterologous types. Two specimens from each animal, the 10-day and 6-weeks bleeding, were re-tested in 1:8, 1:32, and 1:128 dilutions with O type and C type antigens. The K' values for these several mixtures were comparable with

TABLE III

Residual complement-fixing activity with "standard" A type hyperimmune guinea-pig serum detected in undiluted mixtures of A type, strain GB, viral antigen (g-pig) and serial dilutions of sera from bleedings from two A type-infected cattle

NUMBER OF ANIMAL	DATE OF BLEEDING	DILUTION OF SERUM	K' (UNITS)*		NUMBER OF ANIMAL	DATE OF BLEEDING	DILUTION OF SERUM	K' (UNITS)*	
			+Saline	+Standard A type Serum				+Saline	+Standard A type Serum
	1952					*1952*			
C/BL 14	11/23	1:8	1.8	2.0	C/BL 16	11/26	1:8	2.3	2.1
(A_5)		1:16	1.3	1.9	(H2200)		1:16	1.8	2.3
		1:32	1.0	1.7			1:32	1.4	2.1
		1:64	2.0	3.3			1:64	1.7	2.3
		1:128	2.3	3.7			1:128	2.1	3.3
C/BL 14	12/24	1:8	1.6	2.0	C/BL 16	12/24	1:8	2.2	2.1
		1:16	1.5	2.0			1:16	1.6	2.0
		1:32	1.4	2.2			1:32	1.1	2.1
		1:64	2.0	3.3			1:64	2.0	2.3
		1:128	1.7	3.9			1:128	1.7	2.4
Normal I	—	1:8	1.9	3.2	Saline	—	—	1.4	3.4

* Unit = 0.0020 to 0.0022 ml complement.

the K' values of mixtures of the respective viruses with the 1:8 dilutions of the pre-infection bleedings from the same cattle.

In brief, these *indirect* complement-fixation tests with antigens of the A, O, and C types showed that combining activity with antigens of the heterologous as well as of the homologous type may develop relatively rapidly in the serum of cattle infected with FMD virus of the A type. However, in tests of a small number of the post-infection sera, the combining activity with homologous type antigen was detected in considerably higher serum dilutions than with the heterologous types. This suggested that the *indirect* complement-fixation method with suitably-diluted antisera, might be used not only in detecting FMD antibody but possibly also in determining its type specificity. Further experiments are required to settle the question of type specificity definitely, but could not be completed before the first author's return to Canada. Studies of the value of the *indirect* test are being continued at Pirbright by J. B. B., the results of which will be evaluated in relation to those obtained by other methods of titrating antibody in convalescent bovine FMD sera.

TABLE IV

Residual complement-fixing activity with standard O or C type guinea-pig antisera detected in undiluted mixtures of O or C type viral antigens with 1:2 dilutions of sera from serial bleedings from two A type cattle

NUMBER OF ANIMAL	DATE OF BLEEDING	FMD VIRAL ANTIGEN†	K' (UNITS)*		FMD VIRAL ANTIGEN†	K' (UNITS)*	
			+Saline	+Standard O type Serum		+Saline	+Standard C type Serum
	1952						
C/BL 13	11/11(N)	O Type strain	2.8	6.2	C type strain	2.1	3.6
(A_4)	11/17	V_1	2.7	5.0	GC	1.9	2.5
	11/19		2.7	3.6		1.8	2.3
	11/22		2.2	3.5		1.6	2.5
	11/26		1.8	3.3		1.9	2.5
	12/3		2.4	3.2		2.3	2.6
	12/24		2.6	2.9		1.7	2.0
C/BL 15	11/13(N)	O Type strain	2.0	6.2	C type strain	2.1	4.7
(Ca-I)	11/19	V_1	2.5	2.3	GC	2.6	2.8
	11/21		2.2	3.0		2.9	3.5
	11/24		2.3	3.3		2.5	2.9
	11/27		2.3	2.9		3.3	3.7
	12/5		2.9	2.4		1.9	2.4
	12/24		2.9	3.0		2.4	2.2
Saline	—	—	1.0	5.2		1.5	4.0

* Unit = 0.0020 and 0.0021 ml complement.
† Prepared from infected guinea-pig plantar pads.

DISCUSSION

This preliminary investigation has demonstrated that following infection with foot-and-mouth disease virus substances appear, in the serum of cattle, which combine with FMD viral antigens of the homologous type in such a way that these no longer react with homologous FMD guinea-pig antiserum to form complexes that fix guinea-pig complement. Palacios and Rodriguez briefly reported a very similar observation (13). The inhibitory effects of lower serum dilutions extend to FMD viruses of other types as well as the same type. Such a finding is in accordance with general experience that inhibition tests may be less strictly specific than direct reactions. Inhibition of precipitation, for example, may be obtained not only with homologous antigen but also with antigens containing the reactive groups in other positions or even with compounds showing considerable structural differences (31). Alternatively, it may be postulated

that this cross-inhibition is related to the presence of a "common" or "group" antigen in viruses of the three types, which evokes the production of corresponding "group" antibody in cattle infected with A, O or C type viruses. The demonstration that when sufficient A-type bovine antibody was present, the inhibition of the complement-fixing properties of O or C type viruses with homologous guinea-pig serum was complete, suggested that this hypothetical "group" antigen must be closely associated with the type-specific O or C antigen on the same particle or molecule. Otherwise, were the type-specific and group antigens on different particles, or widely separated on the same particle or molecule, it might be visualized that even after the "group" antibody in the A type bovine antiserum had combined with the "group" determinant, the type-specific O or C determinants would still have been left free to react with the subsequently-added O or C type guinea-pig antiserum.

Aside from theoretical considerations as to the nature of the inhibitory properties of FMD convalescent bovine sera, should further investigation demonstrate that this neutralizing activity is confined to FMD virus antigens, the *indirect* complement-fixation may be developed into a useful method for the detection of evidence of past infection in suspected herds of cattle. That it might have such value was suggested by the results of our preliminary studies with sera from a herd of animals believed to have been implicated in the initial outbreak of the disease in Canada in 1951. Vesicular disease had developed in this herd shortly after the arrival of an immigrant labourer, whom subsequent epidemiological investigation showed had come almost directly from a farm in Germany where foot-and-mouth disease had been present. These *W* herd sera showed inhibitory properties in *indirect* complement-fixation tests with A type FMD virus but not with the New Jersey or Indiana types of vesicular stomatitis virus.

SUMMARY

In *direct* complement-fixation tests, the quantitative behaviour of FMD viral antigens with hyperimmune guinea-pig sera was found to be similar to that noted in a number of viral antigen-antibody systems previously studied. The relative complement-fixing capacity of several FMD antigens with different guinea-pig antisera was estimated from the slope of the complement-antigen regression lines. Such a comparison showed a much closer resemblance in the complement-fixing behaviour of the strain of A type FMD virus isolated during the recent Canadian outbreak and an A type strain isolated in England at about the same time, than either displayed toward a stock A strain, No. 119, that had been maintained for many years by cattle passage.

Sera of only two of the convalescent cattle tested, exhibited fixation of guinea-pig complement with FMD virus antigen. The relatively rapid development of inhibitory or neutralizing activity in the sera of cattle infected with FMD virus could be detected, however, by an *indirect* complement-fixation method with FMD antigens; pre-infection bleedings from the same animals did not show this activity. In lower dilutions, these convalescent sera blocked or neutralized the complement-fixing activities of FMD viral antigens of heterologous as well as of the homologous type. In higher serum dilutions the inhibitory activity extended only to virus of the homologous type.

At the present time, there is an evident need for a satisfactory *in vitro* method of demonstrating antibody in the sera of cattle convalescent from foot-and-mouth disease. The *direct* complement-fixation test has proved of limited value for this purpose since only a small proportion of cattle develop complement-fixing activity with FMD antigens. The preliminary studies presented here suggest that the *indirect* complement-fixation test, particularly if used in parallel with one of the *direct* form, merits further investigation from this practical standpoint.

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REFERENCES

1. BROOKSBY, J. B. The technique of complement fixation in foot-and-mouth disease research. Agricultural Research Council, Report Series No. 12, Lond. (H.M.S.O.), 1952.
2. CUICA, A. J. Hyg., **28:** 325, 1929.
3. TRAUB, E. AND MÖHLMANN, H. Zentralbl. Bakt., I. Orig., **150:** 289, 1943.
4. TRAUB, E. AND RODRIGUEZ, F. M. Zentralbl. Bakt., I. Orig., **151:** 380, 1944.
5. TRAUB, E. AND MÖHLMANN, H. Berl. Münch. tierärztl. Wchnschr., **1:** 1, 1946.
6. BROOKSBY, J. B. Proc. Soc. Exper. Biol. & Med., **67:** 254, 1948.
7. BROOKSBY, J. B., GALLOWAY, I. A. AND HENDERSON, W. M. Proc. Soc. Exper. Biol. & Med., **69:** 70, 1948.
8. BROOKSBY, J. B. The Antibodies in foot-and-mouth disease. Agricultural Research Council, Report Series No. 9, Lond. (H.M.-S.O.), 1949.
9. BROOKSBY, J. B. J. Hyg., **47:** 384, 1949.
10. CAMARGO, F., EICHORN, E. A., LEVINE, J. M. AND TELLEZ-GIRON, A. Proc. Amer. Vet. Med. Assoc., 87th Annual Meeting, p. 207, 1950.
11. ARAMBURU, H. G., Publicacion del Laboratorio de Investigaciones, Ministerio de Agricultura de la Nacion, Instituto Nacional de la Fiebre Aftosa, Buenos Aires, Republica Argentina, No. 5, p. 1, 1949.
12. CUNHA, R., DA SILVA PASSOS, W. AND MONTEIRO DE CARVALHO, J. Arq. Inst. biol., **1:** 1, 1950.
13. PALACIOS, P. AND RODRIGUEZ, R. Bull, Off. Internat. Epiz., **31:** 75, 1949.
14. UBERTINI, B. Bull. Off. Internat. Epiz., **31:** 84, 1949.
15. NAGEL, H. C. Gac. vet. B. Aires, **13:** 147, 1951.
16. FREDERICKS, H. H. J. Tydsche. Diergeneesk., **74:** 839, 1949.
17. SERRA, A. AND GUARINI, G. Clin. vet. Milano, **84:** 321, 1951.
18. RICE, C. E. Canad. J. Comp. Med., **11:** 236, 1947.
19. RICE, C. E. J. Immunol., **59:** 365, 1948.
20. RICE, C. E. Canad. J. Comp. Med., **12:** 130, 1948.
21. WOLFE, D. M., KORNFELD, L. AND MARKHAM, F. S. Proc. Soc. Exper. Biol. & Med., **70:** 490, 1949.
22. KARRER, H., MEYER, K. F. AND EDDIE, B. J. Infect. Dis., **87:** 13, 1950.
23. HILLEMAN, M. R., HAIG, D. A. AND HELMOLD, R. J. J. Immunol., **66:** 115 1951.
24. DOWNIE, A. W. AND MACDONALD, A. J. Path. & Bact., **62:** 389, 1950.
25. RICE, C. E. J. Immunol., **53:** 225, 1946.
26. RICE, C. E. J. Immunol., **56:** 343, 1947.
27. WADSWORTH, A. B. Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Albany, N. Y. Williams and Wilkins Company, Baltimore, 3rd. ed.: 361, 1947.
28. MAYER, M. M., OSLER, A. G., BIER, O. G. AND HEIDELBERGER, M. J. Exper. Med., **84:** 535, 1946.
29. REYN, A. Acta. Path. et microbiol. scandinav., **17:** 145, 1940.
30. RICE, C. E. J. Immunol., **60:** 11, 1948.
31. RICE, C. E. AND BOULANGER, P. Proc. Amer. Vet. Med. Assoc., 90th Annual Meeting, 1953.
32. MARRACK, J. R. The Chemistry of Antigens and Antibodies. Spec. Rep. Ser. med. Res. Coun. London, No. 230, p. 72, 1938.

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