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A comparison of methods of measuring the persistence of neutralizing and haemagglutinin-inhibiting antibodies to louping ill virus in experimentally infected sheep

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In their preliminary study in 1960–1 on Camlarg Farm, Smith *et al.* (1964) reported that 25% of hoggs (yearling sheep) had lost haemagglutinin-inhibiting (HI) antibody less than 12 months after natural infection with louping ill, yet none had lost neutralizing antibody. The same authors (unpublished) found that among the hoggs exposed to louping ill infection between spring and summer of the years 1961–4, 20% of sera collected in the autumn had both neutralizing and HI antibody and a further 14% neutralizing antibody only. Williams & Thorburn (1961) showed that all of ten sheep had developed HI antibody by 8 weeks after experimental infection with louping ill virus. Thus the 14% of hoggs with only neutralizing antibody in the autumn had probably developed both types of antibody following infection but had lost HI antibody during the summer.

In surveys of sheep populations for antibody to louping ill, it is commonplace to find a higher proportion with neutralizing than HI antibody, presumably because the former is more persistent. For example, Smith *et al.* (1964) surveyed two flocks comprising 132 ewes and found that in March 68% had neutralizing but only 33% HI antibody; in June the percentages were 86 and 46 respectively. Findings like these present considerable difficulties in epidemiological interpretation.

In order to get a clear understanding of the fate of the neutralizing and HI antibodies, an experiment was designed, first, to determine the duration of the neutralizing and HI antibodies after experimental infection with louping ill virus and, second, to establish the effect of re-infection on the antibody pattern. Because this study could not be undertaken in an area where louping ill is enzootic as natural infections would confuse the results, it was carried out at the Wellcome Veterinary Research Station at Frant, Kent.

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MATERIALS AND METHODS

Viruses

The strain of louping ill virus used originated from the Moredun Institute, Edinburgh. The sheep were infected with virus which had been maintained by serial intracerebral inoculation of sheep, and the virus used in the neutralization and HI tests had been passaged 35–40 times in the brains of adult mice.

Sera

Sera were inactivated at 56° C. for 30 min. and then stored at −20° C. Recent work (Verani & Gresikova, 1966; Gresikova & Sekeyova, 1967) has shown that heating of serum before kaolin extraction for non-specific haemagglutinin-inhibitors may generate an inhibitor not removed by kaolin. However, it does not appear to have caused any noticeable problem in this study.

Neutralization tests

These were carried out by two methods in mice 3 to 4 weeks old. In the constant virus/variable serum method (NAbD) dilutions of sera were mixed with an equal volume of about 100 mouse ICLD50 of virus diluted in fresh guinea-pig serum. The mixtures were incubated for 90 min. at room temperature and then inoculated intracerebrally (IC) into groups of six mice. The virus was titrated at $\sqrt{10}$-fold dilutions in 50% normal sheep serum. The results were computed using survival times by the method of Smith & Westgarth (1957). Serum dilutions showing a significant difference from the controls at the 5% level were recorded as positive. In the constant serum/variable virus method (NAgD) the tests were similar except that undiluted sera were mixed with equal volumes of four tenfold dilutions of virus in fresh guinea-pig serum. The log neutralizing index was the difference between the log ICLD50 of the virus after incubation with test sera and the log ICLD50 of the virus after incubation with antibody-free serum. The end-points were calculated by the method of Reed & Muench (1938).

Haemagglutinin-inhibition (HI) tests

Haemagglutinating antigens were prepared by the sucrose–acetone method (Clarke & Casals, 1958) from the brains of moribund baby mice, infected with the Moredun strain of virus. Using the microtitre equipment (Cooke Eng. Co., Alexandria, Va.) and the method of Sever (1962), the test was carried out in two ways, comparable to the neutralization tests. The sera were extracted with kaolin (Clarke & Casals, 1958), heated at 56° C. for 30 min., and then absorbed with goose erythrocytes before testing since high titres of goose agglutinins are quite common in sheep sera (Smith,1967). After the serum–antigen mixtures were incubated at room temperature for 60 min., 0·25% goose erythrocytes were added in phosphate buffer, which adjusted the reaction to pH 6·3. Haemagglutination was allowed to occur at room temperature (Clarke & Casals, 1958). In the constant antigen/variable serum method (HAbD) twofold dilutions of extracted serum were tested against

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8–16 units of haemagglutinin as described by Smith *et al.* (1964). In the constant serum/variable antigen method (HAgD) 1/10 extracted serum was tested against a twofold dilution series of antigen concentrations from 64 to 1 unit.

Statistical analysis of antibody titres

This was carried out by Mr S. Peto and Mr B. Maidment of the Microbiological Research Establishment, to whom we are greatly indebted. Correlation coefficients between HAbD and HAgD, and between NAbD and NAgD, were calculated and their significance levels determined. Regression coefficients of log titre on both time and log time were obtained by computer; in general a better fit on log time was found for HAbD and HAgD while no clear-cut preference could be demonstrated for NAgD and NAbD. Log time was therefore adopted as it provided the best opportunity for a consistent comparison of the eight curves. However, the die-away with NAbD and HAbD following infection was markedly steeper in the earlier than the later stages and a hyperbolic curve (log titre *v.* reciprocal time linear) would have fitted these two much better. The weighted means of the regression coefficients were calculated and adapted for heterogeneity. Combination of these weighted means was justified only for HAbD and HAgD. The variability in response between animals was studied by comparing slopes and by plotting $\log_2$ titres *v.* time and superimposing results following infection on results following re-infection.

Animals

In May 1962, five Dorset Horn hoggs received a dose of louping ill vaccine* after blood sampling. They were bled 21 days later when they were infected subcutaneously (SC) with 10^5 mouse ICLD 50 of louping ill sheep brain virus. Subsequently they were bled at intervals of approximately 4 weeks. Two months after the vaccination of the five hoggs, a further nine hoggs were treated similarly. Three sheep died from causes other than louping ill in April 1963.

In February 1964, all the surviving animals were infected SC with the same dose and strain of virus that they had received 19–21 months previously. One died of a metabolic disease in December 1964.

A number of these sheep lambed once, twice or three times during the course of the experiment and an opportunity was thus afforded to study the fall of maternal antibody in the lambs.

RESULTS

No sheep had either neutralizing or HI antibody before or 21 days after vaccination. Detailed analysis is limited to 11 animals who survived the entire experiment and whose sera have been fully tested.

Neutralizing antibody

Two recognized methods of testing for neutralizing antibody were used. As many as possible of the sera from a single sheep were tested at the same time.

* Burroughs Wellcome and Co., London.

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Constant serum/variable virus method (NAgD)

The geometric mean titres and ranges in sera from the 11 sheep are shown in Fig. 1. Regression analysis showed that the weighted means of regression co-efficients adapted for heterogeneity were $+0{\cdot}25 \pm 0{\cdot}065$ following infection and $-0{\cdot}11 \pm 0{\cdot}054$ following re-infection. Thus there was no fall in NAgD antibody during the 81 weeks following infection (in fact a small gain) and a very little, if any, fall following re-infection. No real change was detected in NAgD antibody from about 4 weeks after infection until the end of the experiment.

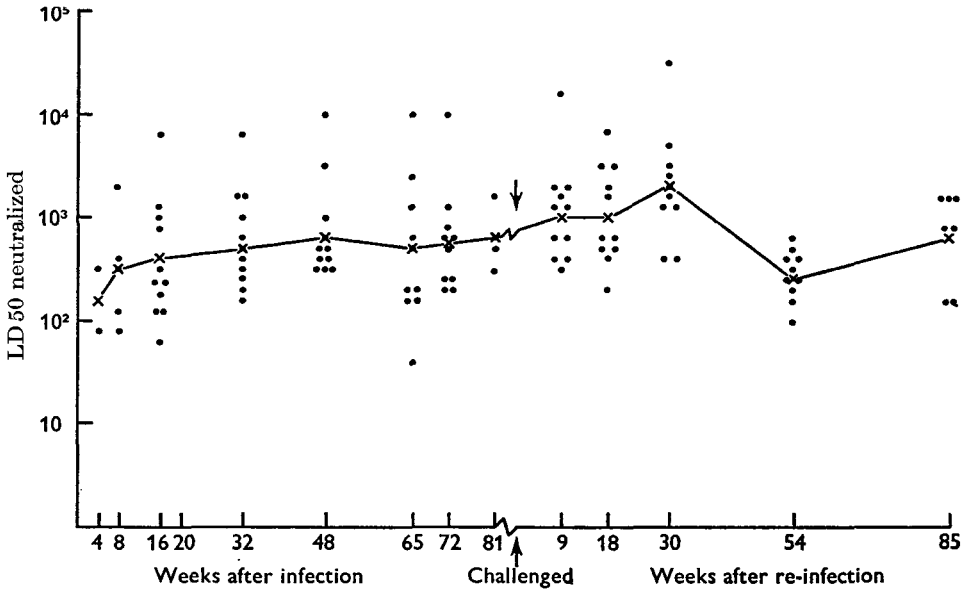

Fig. 1. NAgD. The geometric means and ranges of log neutralizing indices of undiluted sera against louping ill virus.

Constant virus/variable serum method (NAbD)

Most of the same sera were retested by this method (Fig. 2). The corresponding mean regression co-efficients were $-0{\cdot}39 \pm 0{\cdot}069$ following infection and $-0{\cdot}81 \pm 0{\cdot}10$ following re-infection. The geometric mean titres show that there was a rapid initial fall of rather more than fourfold in the first 20 weeks after infection and relatively little change thereafter. Following re-infection the geometric mean titre rose to the level following infection but fell to an ultimately lower level.

The highest titres of NAbD antibody following infection were 1/80 in four animals, 1/20 in two and 1/5 in three. One animal failed to develop significant NAbD antibody, although it had consistent NAgD titres of $> 2{\cdot}0$ log throughout and another similar animal had a barely detectable NAbD titre only on the 68th week after infection. By 64–68 weeks after infection, five of the nine animals with initial NAbD antibody had shown no fall in titre; three had fallen fourfold and and one 16-fold. By 73–81 weeks, seven of the nine had fallen fourfold from the highest level and one 16-fold; only one of the nine no longer had detectable NAbD

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antibody. Nine weeks after re-infection six of the nine animals had reverted to their highest titre following first infection, two had higher titres and one lower. The animal with only a barely positive titre following infection showed a rise to 1/20 following re-infection. The remaining animal was not re-infected and is not considered further. Fifty-four weeks after re-infection, two animals had no loss of titre, five had a fourfold loss and two a 16-fold loss. By the 85th week antibody was no longer detectable in two of the six animals tested.

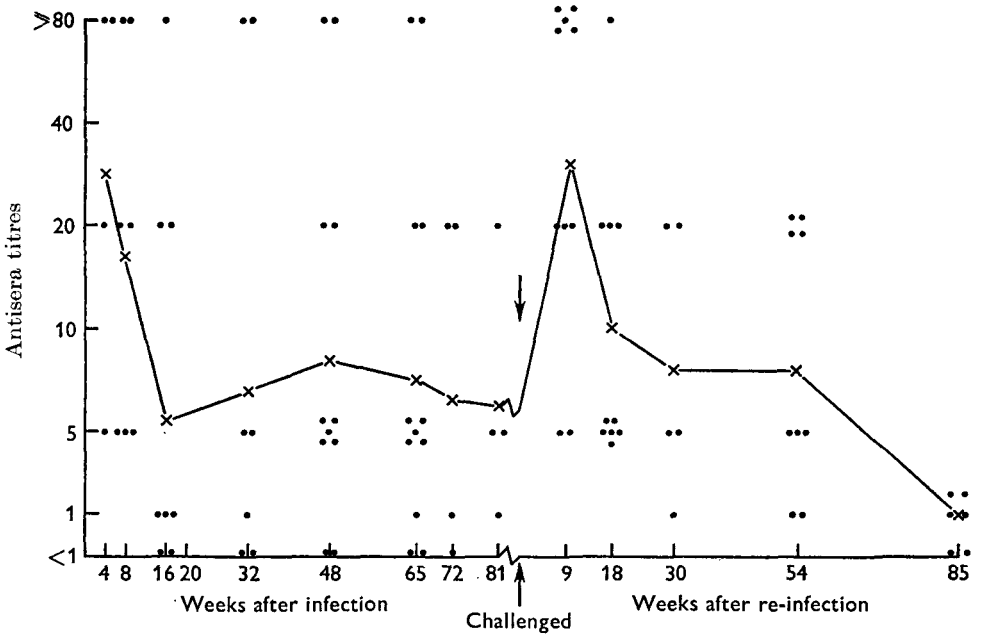

Fig. 2. NAbD. The geometric means and ranges of maximum serum dilutions neutralizing 100 mouse ICLD 50 of louping ill virus.

Haemagglutinin-inhibiting antibody

Constant virus/variable serum method (HAbD)

Three to eight weeks after infection all except one sheep had significant (> 1/10) HAbD antibody (Fig. 3). This sheep developed no HAbD until after re-infection although it had all three other antibody types. The weighted mean regression coefficient following infection was $-1{\cdot}25 \pm 0{\cdot}19$. The geometric mean titres show a rapid initial fall of about $3\frac{1}{2}$-fold during the first 20 weeks and a much slower fall thereafter. At the 32nd week only five sheep had significant antibody titres which were on average fivefold lower than the corresponding titres of these sheep at the 8th week. By the 72nd week after infection only one sheep had detectable HAbD antibody, but 9–14 weeks after re-infection all but one sheep had HAbD antibody. Of nine sheep in which titres 8 weeks after infection could be compared with titres 9 weeks after re-infection, four had similar titres on both occasions, five had titres after re-infection lower by at least fourfold, and the animal with no HAbD antibody following infection developed a titre of 1/20. By 30 weeks after

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re-infection, HAbD antibody had become undetectable in two of the animals and, between 54 and 86 weeks, three more animals became negative. At 86 weeks, four animals had titres which had been unchanged from the 9th week after re-infection. The regression coefficient following re-infection was $-0{\cdot}40 \pm 0{\cdot}10$, indicating a much slower rate than following infection.

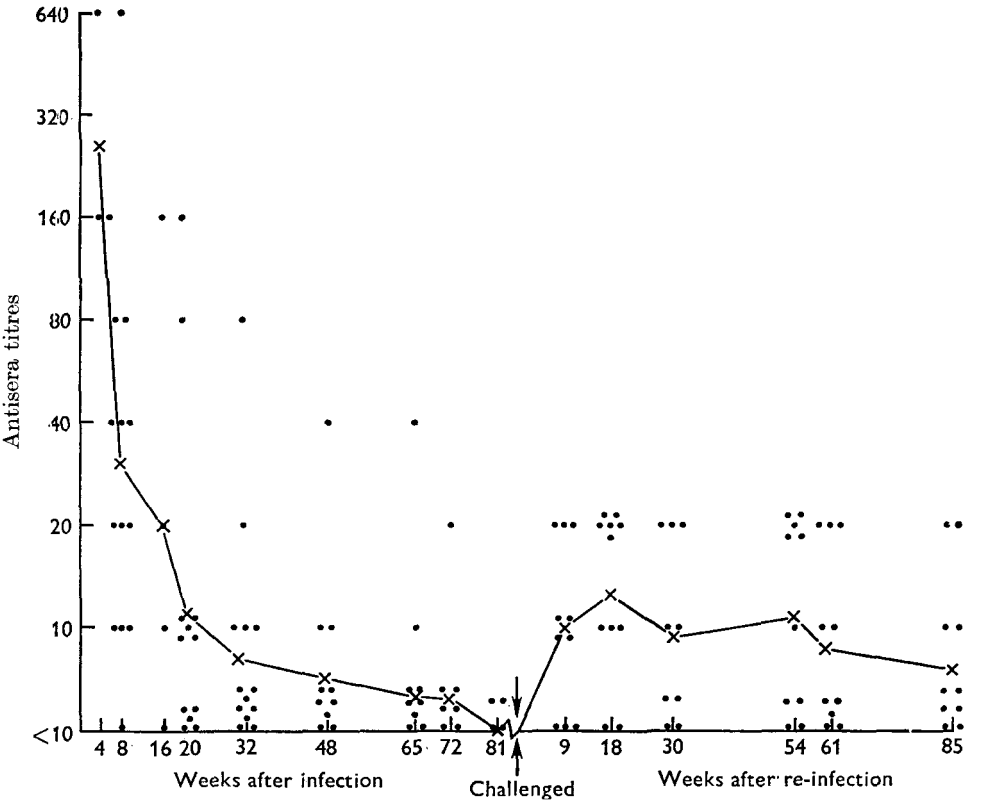

Fig. 3. HAbD. The geometric means and ranges of maximum serum dilutions inhibiting 8–16 units of haemagglutinin.

Constant serum/variable virus method (HAgD)

All sera from animals 3 weeks after infection inhibited at least 64 haemagglutinin (HA) units, and all at 8 weeks at least 16 HA units (Fig. 4). The regression coefficient following infection was $-1{\cdot}06 \pm 0{\cdot}12$. The geometric mean titres show a roughly linear fall of about eightfold during the 81 weeks. At the 72nd week after infection, sera from only two animals failed to inhibit at least 4 HA units. Nine weeks after re-infection, all the sera except two inhibited more than 16 HA units and none less than 8 HA units. Compared with titres 8 weeks after initial infection, the titres 9 weeks after re-infection differed by more than twofold in only two animals. By 86 weeks after re-infection all animals still had significant HAgD antibody; sera of four inhibited 4–8 units and sera of five inhibited 16–32 HA units. The regression co-efficient following re-infection was $-0{\cdot}61 \pm 0{\cdot}10$, indicating a slower rate of fall than following infection.

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Relationship between the tests

The correlation coefficient between the results of the two HI tests were highly significant (0·79 following infection with 75 degrees of freedom (D.F.); 0·70 following re-infection with 72 D.F.) and combination of their corresponding weighted means

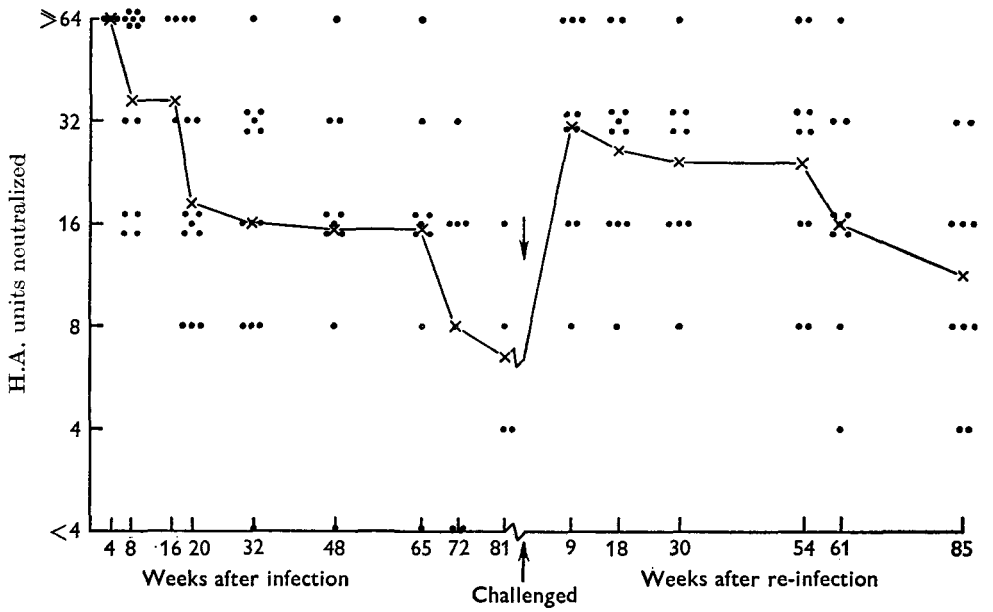

Fig. 4. HAgD. The geometric means and ranges of the maximum number of HA units neutralized by 1/10 dilution of sera.

Table 1. *Variability to responses illustrated by the slopes of the regressions of titre on log time*

Antibody	Following infection or re-infection	No. of slopes		
		Positive	Zero	Negative
HAbD	Inf.	0	1	10
	Re-inf.	3	2	6
HAgD	Inf.	1	1	9
	Re-inf.	0	2	9
NAbD	Inf.	1	2	8
	Re-inf.	0	1	10
NAgD	Inf.	7	0	4
	Re-inf.	3	0	8

of regression coefficients showed $-1{\cdot}17 \pm 0{\cdot}11$ following infection and $-0{\cdot}48 \pm 0{\cdot}07$ following re-infection. Thus, by both tests the rate of fall in HI antibody following first infection was significantly faster than following re-infection. No significance could be attached to the correlation coefficients between the results of the neutralization tests.

There was considerable variation between individual sheep as shown (Table 1)

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by the distribution of positive and negative slopes of these ranges. A high proportion of positive slopes was seen only with NAgD. Four sheep had notably higher HI titres following infection than re-infection at corresponding times but the reverse was true for two other sheep.

Maternal antibody

Lambs born to the ewes in this experiment were bled after birth and at intervals where possible. The mothers were also bled on or near the date of the birth of the lamb.

The HAbD and NAbD antibody titres of sera taken from 12 lambs within 6 days of birth were greater than the corresponding maternal antibody titres in 11 with HAbD and six with NAbD (Table 2).

Table 2. *Relationship of lamb antibody titres at* 1 *week after birth and ewe antibody titres at or near parturition*

Ewes			Lambs			
	Antibody titres			Antibody titres		
Number	HAbD	NAbD	Date of birth	HAgD	HAbD	NAbD
31	< 10	5	Nov. 1964	8	10	*
83	< 10	5	Nov. 1964	† 16	20	20
				16	20	20
85	< 10	5	Sept. 1963	† 64	20	5
				64	20	20
33	< 10	20	Nov. 1964	† 64	20	20
				32	20	80
34	10	5	Oct. 1964	32	20	> 20
32	20	20	Nov. 1964	† 64	20	20
				> 64	40	> 20
85	20	20	Nov. 1964	32	80	5
90	40	20	May 1964	NT	80	20

* Antibody present in undiluted serum.
† Twin lambs. NT = Not tested.

The decay pattern of the antibodies in the ewe and lamb sera appeared to be similar; NAbD antibody in lambs persisted longer than HAbD antibody in 14 of 21 animals examined. However, NAbD antibody persisted longer than HAgD antibody in only five of 13 animals. HAgD antibody lasted longer than HAbD antibody in seven of the 13 lambs but neither type of HI antibody was found after the disappearance of NAgD antibody.

DISCUSSION

In sheep vaccinated, then infected, with louping ill virus, neutralizing antibody is produced and, if measured by the constant serum/variable virus (NAgD) method, persists without significant change of titre for at least 1·5 years. Re-infection at this stage causes no significant change in NAgD antibody levels, which persist for

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at least another 1·5 years. If the measurement is by the constant virus/variable serum (NAbD) method the antibody falls in titre after reaching its peak, so that on the average the titre is fourfold less at the 16th week and thereafter remains constant until at least 1·5 years after infection. One animal failed, and one almost failed, to develop detectable NAbD antibody, although the three other tests demonstrated antibody. After re-infection the NAbD antibody levels rose to those following infection and the animal which had almost failed to produce antibody after infection responded after re-infection. NAbD titres fell significantly more rapidly following re-infection than following infection but at least two-thirds of the animals still had significant antibody about 1·5 years after re-infection.

Although HAbD titres fell more rapidly than HAgD titres following infection no significant difference in the rate of fall in titre between the two HI tests could be established. Both types of titre fell significantly more rapidly than the neutralizing antibody titres following infection; and the HI titres fell more than twice as rapidly following infection than following re-infection. However, because of the greater sensitivity of the HAgD test, significant antibody persisted longer; 7 months after infection HAbD was detectable in only half the animals, while nearly all still had HAgD antibody; after 1·5 years only about one-third had detectable HAbD antibody but 90 % had HAgD antibody. After re-infection 70 % showed an antibody response to either test; 1·5 years later about half the animals had detectable HAbD antibody but all had detectable HAgD antibody.

These results can be compared with those of Williams & Thorburn (1961), who inoculated five sheep from a flock free from louping ill with approximately 10^6 mouse LD 50 of louping ill virus. None developed detectable HI antibody, yet ten other sheep which were inoculated with vaccine and virus produced antibody. This might suggest that the vaccine was necessary for the stimulation of this antibody. However, Smith *et al.* (1964) failed to find antibody following the use of vaccine and in their preliminary study at Camlarg Farm reported that unvaccinated but naturally infected hoggs did produce HI antibody. O'Reilly *et al.* (1965) found that in sheep inoculated with Langat virus the presence or absence of HAbD antibody to Langat virus did not influence the development or titre of louping ill HAbD antibody when these sheep were later exposed to natural louping ill infection.

Of the ten sheep which Williams & Thorburn vaccinated and infected, three lost their HI antibody within 4 months of the infection. During this same period these sheep were each inoculated with a total of six injections of louping ill virus. None of these sheep which lost their HI antibody regained it as did 70 % of those reported here. However, there were differences in the technique of the HI test used.

Previously in work with louping ill, the NAgD method has been generally used for measurement of neutralizing antibody and the HAbD method for measurement of HI antibody. In epidemiological studies this has, for now obvious reasons, led to the common finding of higher prevalences of neutralizing than HI antibody in enzootic areas.

A comparison of the loss of HAbD antibody demonstrated in hoggs between September 1960 and March 1961 following an epizootic in spring 1960 (Smith *et al.*

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1964) with the experimental results reported here shows reasonable agreement ($P = 0{\cdot}20$). In the same study, the HI antibody found in March in 4- to 6-year-old sheep (before infection in that year) probably indicated repeated exposure to louping ill in the preceding years. For the detection of the maximum number of long-past infections there is no doubt that the NAgD type of neutralization test is best, but for the detection of recent infections the HAbD type of HI test is probably most suitable. For diagnosis by demonstration of the largest rise in antibody during the course of a louping ill infection in sheep, reasons of economy recommend an HI test and either could be used. However, the HAgD test appears to be more sensitive. If diagnosis is to be attempted by detection of a falling titre, either of the antibody dilution tests would suffice.

Most of the lambs 6 days after birth had higher titres of antibody than their mothers; 10 of 12 had at least twofold more HAbD and 6 of 12 at least fourfold more NAbD than the ewes. These findings are in agreement with those of Sterne *et al.* (1962), who stated that newborn lambs may have two to four times more *C. welchii* β- and ϵ-antitoxins than the ewes. This phenomenon was explained by Howie, Barr & Glenny (1953), who reported that there is almost a tenfold concentration of diphtheria antitoxin in the colostrum of ewes.

SUMMARY

Sheep, after infection with louping ill virus and after re-infection with the same strain of virus 19–21 months later, were bled at intervals and their sera examined for neutralizing and haemagglutinin-inhibiting antibodies. Each antibody type was measured by the constant serum/variable virus and constant virus/variable serum methods. The persistence of each type of antibody and its significance in epidemiological studies is discussed. The relationship of antibody levels in ewes and their lambs was also examined.

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