

Evolutionary changes in myxoma virus in Britain

An examination of 222 naturally occurring strains obtained from 80 counties during the period October–November 1962

BY FRANK FENNER*

*Department of Microbiology, John Curtin School of Medical Research,
Australian National University, Canberra, A.C.T., Australia*

AND PAUL J. CHAPPLE†

*Ministry of Agriculture, Fisheries and Food, Infestation Control Laboratory,
Worplesdon, Surrey, England*

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INTRODUCTION

Myxomatosis evolved in association with *Sylvilagus* in the Americas, but since 1950 it has become established as an enzootic–epizootic disease in the populations of European wild rabbits (*Oryctolagus cuniculus*) of three continental areas, Europe, Australia and Chile (Fenner & Ratcliffe, 1965). The disease was established in the wild rabbits of Australia in 1950, and in those of Europe in 1952. The first outbreak in Britain occurred at Edenbridge in Kent, in September 1953 (Armour & Thompson, 1955), and the virus (England/Kent/10–53/1 of Fenner & Marshall, 1957) was shown to have the same high virulence as the Lausanne strain used to initiate the European outbreak in France 15 months earlier.

In Australia the virus employed was the highly virulent standard laboratory strain of myxoma virus, originally recovered in Brazil by Moses (1911) and passaged for many years in laboratory rabbits. Not only was it used on a substantial scale in 1950, but each year since then the same virus (or a closely related derivative of it) has been used on a large scale in mass inoculation campaigns (Fenner & Ratcliffe, 1965). Annual examinations of naturally occurring strains of virus, obtained from many localities in Australia for the first 9 years of the epizootic (1951 until 1958–59), revealed the early occurrence in many parts of Australia, and the subsequent dominance throughout the continent, of less virulent strains (Fenner & Marshall, 1957; Marshall & Fenner, 1960).

Nowhere in Europe was deliberate inoculation of wild rabbits with highly virulent virus encouraged, and in Britain deliberate spread of the disease was made illegal in 1954. The continued occurrence of myxomatosis in Britain may be ascribed to the spread of the virus introduced into Kent in 1953. The occasional occurrence of attenuated strains (case mortality rates of 90% or less) was

* Assisted by a grant from the Wool Research Trust Fund of the Commonwealth of Australia.

† Present address: M.R.C. Common Cold Research Unit, Harvard Hospital, Salisbury, Wilts.

recognized in 1955 (Hudson & Mansi, 1955; Fenner & Marshall, 1955; Jacotot, Vallée & Virat, 1955), and two attenuated strains were obtained from Sussex in September and October 1954 (Fenner & Marshall, 1957). Strains recovered from the counties of Brecknockshire and Durham in 1961 were found by Chapple & Bowen (1963) to be very similar in symptomatology and virulence to the common Australian strains of the late 1950s.

However, no attempt had been made in Europe to determine the overall position as far as virus virulence or the genetic resistance of the rabbit were concerned. The former, in particular, was a matter of considerable interest, since not only was a different virus used to initiate the European outbreak, but in England the disease was transmitted by the rabbit flea and not to any extent by mosquitoes (Andrewes, Thompson & Mansi, 1959), and since inoculation of virulent virus was illegal the situation was not complicated by re-introductions.

This paper reports the results of a study of the virulence of 222 strains of myxoma virus obtained from 80 of the 85 counties of Britain during October–November 1962.

MATERIALS AND METHODS

Capture of rabbits and collection of infected tissues

Wild rabbits, killed when obviously suffering from myxomatosis, were collected by field officers of the Ministry of Agriculture, Fisheries and Food and the Department of Agriculture and Fisheries for Scotland. They were caught by a variety of methods: shooting, snares, by dog or ferret, by hand, or killed with a stick. As soon as they were caught they were placed in insect-proof linen bags and immediately dispatched to the Infestation Control Laboratory at Worplesdon. Here they were processed by one of us (P. J. C.), who weighed and sexed the rabbits, collected ectoparasites, and obtained specimens of skin lesion material, blood clot, and lung. A fragment of skin lesion material was placed in 50% glycerol-saline in a plastic tube, and stored at 4° C. until enough tubes had accumulated for dispatch by air to Canberra. Here it was processed as described below, and the resulting virus suspension was eventually tested for virulence by a standard procedure. Other portions of the skin lesion, lung and blood clot were tested for antigens and antibodies by gel diffusion tests; a procedure which had been used for some years by the Central Veterinary Laboratory, Weybridge, for the routine diagnosis of myxomatosis (Mansi & Thomas, 1958; Chapple, Bowen & Lewis, 1963).

Collection and identification of ectoparasites

All fleas and ticks were removed by combing the carcass with a fine comb. They were identified, counted and sexed by Dr A. R. Mead-Briggs and Mr R. J. C. Page at Worplesdon. Lice and mites were obtained by removing a tuft of hair, and mites were identified by Dr A. M. Hughes, Royal Free Hospital School of Medicine, London.

Gel diffusion tests

Slightly different procedures were adopted with material obtained from wild rabbits in England, and from infected laboratory rabbits in Australia. For the

former Oxoid Ionagar No. 2 (1.5 %) buffered to pH 7.2 in 0.01 M sodium barbitone-hydrochloric acid, was used. Preparation of plates, distribution of tissues and antisera in the wells, and the method of recording the results have been described by Chapple *et al.* (1963).

Sera obtained from laboratory rabbits on the eleventh or twelfth day after inoculation were tested for the presence of antibody against an antigen preparation made from skin lesion material obtained from a rabbit infected with the standard laboratory strain, and for the presence of antigen against antiserum from a rabbit which had recovered after infection with the same strain (Woodroffe & Fenner, 1965). Such antisera and antigen give precipitin lines indistinguishable from those obtained with Lausanne virus (Fenner, 1965). These tests were carried out with 0.75 % agar, buffered to pH 8.9; the cups being 4 mm. apart. They were read after 7 days incubation at 35° C. in a humidified incubator.

Preparation of virus for virulence tests

General experience (see Burnet, 1955), and our own recent investigations, have shown that, if stored or aged suspensions which contain a few viable particles and much non-infective virus are used for inoculating rabbits, they suffer a milder disease than if they are inoculated with a diluted, but freshly prepared, suspension of the same virus. This is not due to a genetic change or selection in the virus, as Burnet once suggested, but probably to a combined effect of interferon and antibody response (Fenner & Woodroffe, unpublished results).

As specimens were unavoidably kept at environmental temperatures for several days between their collection in Britain and receipt in Australia, the skin lesion material was passed once in domestic rabbits before the virus was tested for virulence. A slice of the surface of the 7-day-old skin lesion was removed, ground with sand, suspended in diluent, and stored at -60° C. A sample was thawed and assayed on the chorioallantoic membrane before use. The pock count was usually about 10⁶ p.f.u. per ml.

The test for virulence

Although expensive in the numbers of rabbits used, and in space, there is no satisfactory alternative to the inoculation of rabbits for the assay of the virulence of myxoma virus for *Oryctolagus cuniculus* (Fenner & Ratcliffe, 1965). In the present series we followed the procedure outlined by Fenner & Marshall (1957). Groups of six laboratory rabbits (young adult New Zealand whites, purchased from a commercial breeder) were inoculated in the flank with 10 ID₅₀ (approximately 5 pock-forming units, Fenner & McIntyre, 1956) of the virus which had been passed once in domestic rabbits, as described above. High or low environmental temperatures exert a pronounced effect on the course and outcome of myxomatosis, especially with strains of reduced virulence (Marshall, 1959). To avoid this the animal rooms were heated in winter and air-conditioned in summer so that the temperature was maintained at 70° F. ± 2°.

The inoculated rabbits were observed daily, and carefully examined at weekly intervals, and the signs of myxomatosis were recorded. The primary lesion was classified as protuberant, raised or flat. The severity of the disease was assessed,

and the survival times were recorded. The mean survival time is the best index of the virulence of a strain of myxoma virus (Fenner & Marshall, 1957). Samples of virus were allocated to one of six virulence grades according to mean survival time, as shown in Table 1.

Plaque morphology

Myxoma virus produces well-defined plaques on rabbit embryo fibroblasts and rabbit kidney cells, and characteristic differences in plaque morphology have been observed with material obtained from different parts of the world (Woodroffe & Fenner, 1965). Virus obtained from the first passage skin slice was examined on monolayers of rabbit embryo fibroblasts. Methods of producing the monolayers have been described in detail elsewhere (Woodroffe & Fenner, 1965). Plaques were examined after 7 days incubation at 35° C., neutral red in agar having been added on the sixth day.

RESULTS

The complete data relating to each virus strain, and the wild rabbit from which it was recovered, are available on request. Various aspects of these data are analysed below.

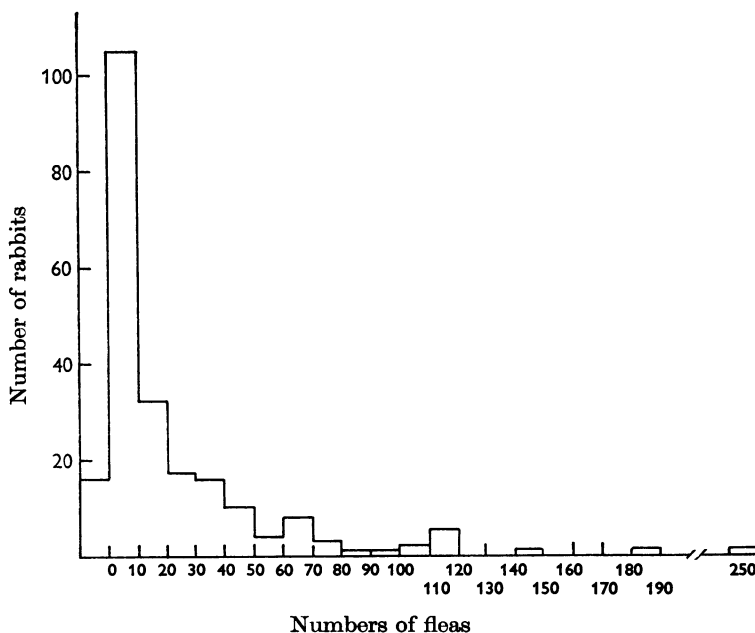


Fig. 1. Histogram showing the frequency distribution of numbers of fleas (*S. cuniculi*) per wild rabbit.

Ectoparasites found on infected wild rabbits

Only 16 (7.2%) of the rabbit carcasses were free of rabbit fleas (*Spilopsyllus cuniculi*). The number of fleas upon each rabbit varied greatly, as shown in the histogram (Fig. 1). Female fleas were usually more common than males (in 68% of the flea-bearing rabbits), the numbers being equal in 8%. Scattered straggler fleas were found, sixteen rabbits being infected with thirty-six specimens of ten

different species of flea. Details of the occurrence and distribution of fleas on these rabbits are published elsewhere (Mead-Briggs, 1964*b*; Mead-Briggs & Page, 1964).

Ticks (usually nymphs of *Ixodes ricinus*) were found on fourteen rabbits, more commonly on those from Scotland. The only other ticks found were single specimens of *I. trianguliceps* and *I. hexagonus*.

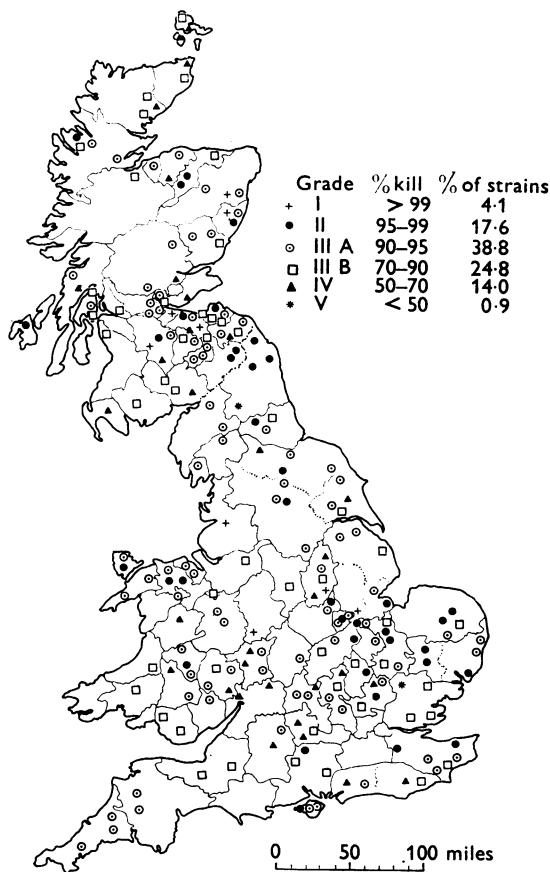


Fig. 2. Map of Britain showing county boundaries, the distribution of samples of myxoma virus collected in October–November, 1962, and their virulence as determined by rabbit inoculation tests.

Classification of virulence by rabbit inoculation tests

The detailed results of the standard inoculation tests are shown in Fig. 2, which shows the virulence and source of each strain of virus examined. The percentages of samples in each virulence grade are shown in Table 1, which also shows the relevant figures for Australia 9 years (1958–59) and thirteen years (1963–64) after the introduction of the virus into that continent. Details of both the Australian groups will be given in a forthcoming paper (Fenner & Woodroffe; to be published). In Britain, as in Australia, there are now to be found a variety of strains of myxoma virus which differ greatly in their virulence for *Oryctolagus cuniculus*.

Symptomatology of rabbits infected with British field strains

The symptomatology of myxomatosis of European rabbits produced by the Lausanne strain of myxoma virus, which was used to initiate the European epizootics, and early English derivatives of this, has been described at length (Fenner & Marshall, 1957). Like all recently recovered South American strains (Fenner, 1965) but unlike the 'standard laboratory strain', which was used in Australia, and its less virulent derivatives, the European viruses produced a florid disease with very large protuberant skin lesions. The strains France/Loiret/4-55/1 and England/Nottingham/4-55/1 (attenuated), which were designated prototype European strains for virulence grades IV and V (Fenner & Marshall, 1957), were associated with these protuberant skin lesions, although they developed this character much more slowly than the virulent strain. However, it was noted that the strain England/Sussex/10-54/1, which was of grade IIIA virulence, produced relatively flat skin lesions. Chapple & Bowen (1963) noted the same characteristics in two attenuated (grade IIIB) strains recovered in England in 1961.

Table 1. *Comparison of the virulence of naturally occurring strains of myxoma virus in Australia and Great Britain, at the time of the first epizootics and at various periods after this. Figures represent the percentages allocated to the virulence grades shown*

Country	Year	Virulence grade						Sample size
		I	II	IIIA	IIIB	IV	V	
		Mean survival time (days)						
		≤ 13	14-16	17-22	23-28	29-50	—	
		Case mortality rate (%)						
		> 99	95-99	90-95	70-90	50-70	< 50	
Australia	1950-51	100	—	—	—	—	—	—
	1958-59*	0	24.6	29.2	26.1	14.0	6.1	130
	1963-64†	0	0	26.4	42.6	25.6	5.4	129
Great Britain	1953	100	—	—	—	—	—	—
	1962	4.1	17.6	38.8	24.8	14.0	0.9	222

* Data from Marshall & Fenner (1960), revised by Fenner & Woodroffe (unpublished).

† Data from Fenner & Woodroffe (unpublished).

In the present survey regular observations were made of the nature of the primary and secondary skin lesions in all inoculated rabbits, and all gradations were found between protuberant (like the European prototype strains of Fenner & Marshall (1957)) and flat (like the Australian prototype strains). In many cases the lesions were intermediate between these extremes, and have been designated raised. The type of response of the six inoculated rabbits was usually uniform and the nature of the primary lesion associated with each strain examined was classified as protuberant, raised or flat. In Table 2 the strains are grouped in virulence grades according to the clinical characters of the primary lesion.

There has clearly been a great change in the type of disease produced as well as in its lethality. Whereas only one of the strains tested from Europe, from collections made in the first three years after release of the virus, did not produce a

protuberant lesion, whatever its virulence, in 1962 only 20% of all strains produced protuberant skin lesions. The majority were classified as flat, although the lesions were often more prominent than those associated with Australian strains, and a substantial proportion of strains produced large raised but not protuberant primary lesions. There was no absolute correlation between type of lesion and virulence, but most of the protuberant skin lesions were associated with more virulent strains (grades I, II and IIIA). However, more of the highly virulent strains were associated with flat skin lesions than with any other type, in striking contrast to the situation in 1953-55, when most strains recovered were of grade I virulence, and all of these were associated with protuberant lesions.

Table 2. *The relation between virulence, as judged by mean survival time, and symptomatology, as determined by the prominence of the primary lesion produced at the site of inoculation. Figures are percentages of 222 strains for the 1962 data, and the absolute numbers for 1953-55 data**

Type of primary lesion		Totals	Virulence grade					
			I	II	IIIA	IIIB	IV	V
Protuberant	1953-55	17	13	0	1	0	2	1
	1962	20.4	0.4	8.4	8.9	2.7	0	0
Raised	1953-55	0						
	1962	34.7	0.9	5.3	10.7	11.6	5.8	0.4
Flat	1953-55	1	0	0	1	0	0	0
	1962	44.9	3.1	4.4	19.1	9.8	7.6	0.9

* Data for 1953-55 strains from Fenner & Marshall (1957).

The type of disease represented by the strain England/Nottingham/4-55/1 (attenuated), i.e. grade V virulence associated with very prominent lumps in the skin, was not found in the present survey, nor did any of the viruses of grade IV virulence produce this type of syndrome.

Samples from each county were deliberately obtained from different areas and different outbreaks of myxomatosis. Not enough were examined from any single county to give a detailed picture, but from the map (Fig. 2) it is apparent that strains of all types were recovered widely over the island. The only strains classified as grade V virulence came from Essex and Northumberland, and strains classed as grade I were collected in Staffordshire, Lincolnshire, Nottinghamshire, Lancashire and from Aberdeenshire, Lanarkshire, Kincardineshire, Midlothian and West Lothian in Scotland. There was a comparable general distribution of the three symptom types, flat, raised and protuberant, throughout Britain.

Gel-diffusion tests for antigen and antibody

Blood and tissues of the wild rabbits forwarded for study were tested for antigens and antibody by the gel-diffusion test, following the routine described by Mansi & Thomas (1958). In these acute cases which furnished material for virulence tests antigen was always found in the local lesion. Serum which exuded from the blood clot sometimes contained antigen, sometimes antibody, and sometimes neither. In

Table 3 the results of this test on the wild rabbits which provided the virus samples are arranged according to the results of the rabbit inoculation (virulence) tests. In this material there is clearly no indication that the results of the gel-diffusion test could have been used to predict the virulence of the virus recovered from the sample; a result which confirms the conclusions drawn by Chapple *et al.* (1963) on more limited material.

Table 3. *The results of gel diffusion precipitin tests for myxoma antigen and antibody in the blood clot of wild rabbits from which virus samples were obtained grouped according to the virulence grade of the viruses recovered from the rabbits. Figures are percentages of the tests on material from 220 rabbits*

Gel diffusion tests with blood showed:	Totals	Virulence grade					
		I	II	IIIA	IIIB	IV	V
Antigen only	32.0	3.1	5.4	11.7	8.6	3.1	0
Antibody only	54.5	0.9	7.7	22.5	12.6	10.4	0.5
Neither antigen nor antibody	13.5	0	4.5	4.5	3.6	0.5	0.5

Table 4. *Correlation between the survival times and presence of antibody and/or antigen in the serum of rabbits 11 or 12 days after they were infected with 10 ID₅₀ of some sixty European field strains. Figures express percentages of the 332 rabbits tested*

Serological findings	Week of disease during which rabbit died				Survivors	Total
	Second	Third	Fourth	Fifth		
Antigen only	9.3	38.3	8.4	3.0	0.3	59.3
Antibody only	0	1.5	3.0	0.3	0.9	5.7
Antigen and antibody	1.8	6.0	4.0	1.2	1.2	14.2
Neither antigen nor antibody	0.3	4.8	6.0	4.2	5.4	20.8

Such wild rabbits were obviously at different stages of the disease when captured. In order to obtain more uniform material for gel-diffusion tests use was made of 332 laboratory rabbits inoculated with some sixty strains of virus, for the virulence tests. Eleven or twelve days after inoculation samples of blood were taken from the ear vein, and the sera thus obtained were tested for the presence of both antigen and antibody, and the results recorded in Table 4. The results have been set out according to the survival time of the individual rabbit from which the sample was obtained. At this stage of the disease (11-12 days after infection) nearly three-quarters of the rabbits showed circulating antigen, whereas only 20% had antibody in their serum, an equal proportion showing neither. In almost all rabbits infected with viruses of grade I or grade II virulence (i.e. those which died late in the second or early in the third week) only antigen was found in the serum, whereas in those which ultimately recovered antigen alone was found in only one of the twenty-six animals, neither antigen nor antibody being found in eighteen.

Production of pocks on the chorioallantoic membrane and plaques on rabbit embryo fibroblast monolayers

The doses used for rabbit inoculations were calculated from the results of pock counts of suspensions of infected skin. Although no particular attention was paid to pock size and morphology any deviation from the appearance usually associated with virulent Brazilian myxoma virus and its derivatives as great as that of either neuromyxoma or Nottingham attenuated (Fenner & Marshall, 1957) would have been noticed. Nothing of this sort was recorded.

All tissue suspensions were also assayed on rabbit embryo fibroblasts, on which all strains of myxoma virus produce well-defined plaques with rather irregular edges (Woodroffe & Fenner, 1965). In all cases the plaque assay was the same as the pock count or slightly higher. Although there was a good deal of variability in the size of plaques on single plates four different types of plaque could be distinguished; large, medium and small clear plaques, all of which had irregular edges, and medium-sized hazy plaques. There was no correlation between plaque type and virulence.

DISCUSSION

This survey shows that in Britain, as in Australia, a variety of strains of myxoma virus now circulate naturally in the wild rabbit population. These differ from one another in several properties: their virulence (lethality) for *Oryctolagus*, the type of lesion they produce in this host, and the type of plaque they produce on rabbit cell monolayers, to list three that have been examined.

Geographically, strains of differing virulence and lesion type are scattered widely throughout Britain, even among these samples, which were collected over a restricted period of time.

The situation in Britain, in regard to myxomatosis, differs in three important respects from that in Australia. There was probably only one introduction of virus, that which started the Bough Beech epizootic in 1953. This strain was indistinguishable from Lausanne, being highly lethal (grade I) and associated with protuberant skin lesions. In Australia the standard laboratory strain produced less prominent lesions, and it has been introduced annually on a large scale during mass inoculation campaigns. The major vector in Britain is the rabbit flea, and not the mosquito, as in Australia.

In spite of these epidemiological differences, moderately attenuated strains of virus are now almost as common in Britain as they were in Australia in 1958-59, although during the last 5 years the level of virulence in Australia has dropped even further, for no grade I or grade II strains have so far been recovered in the 1963-64 tests (Table 1). There are two important contrasts between the situations in Britain and Australia, 9 years after the introductions; strains of grade I virulence were still to be found in Britain, and strains of grade V virulence were less common, in spite of the early appearance of such viruses (exemplified by England/Nottingham/4-55/1 (attenuated)). It has been predicted (Fenner & Marshall, 1957; Andrewes *et al.* 1959) that attenuated viruses would be strongly selected against, if fleas are the major vectors and only leave rabbits when they

die. To some extent this may have happened, for highly virulent strains have persisted in a way not found in Australia, in spite of their annual reintroduction there. However, recent work has shown that the rabbit flea is much less sedentary than was previously believed (Mead-Briggs, 1964*a*; Rothschild, unpublished observations) although not as mobile as mosquitoes, which may help to explain the common occurrence of moderately attenuated strains found in the present survey.

Flea transmission occurs throughout the year, and myxomatosis in Britain does not show the same pronounced seasonal incidences as it does in Australia. During the relatively cold British winter moderately attenuated strains would often cause lethal infections (Marshall, 1959), which could be expected to promote their spread by leading to a dissemination of infected fleas. But the emergence of attenuated strains cannot be explained thus, and must be due to factors in the host-virus balance of which we have little understanding.

The virus introduced into Britain in 1953 was indistinguishable from that introduced into France in June 1952, and produced a disease characterized by very florid symptoms and protuberant skin lesions. In October 1954 a strain of virus was recovered in Sussex which produced flatter skin lesions, not unlike those found in rabbits inoculated with the standard laboratory strain and Australian field derivatives of this. This type of lesion has now become somewhat more common than the original disease with protuberant lesions. In neither of the original situations was the type of lesion correlated with virulence, for all Australian strains produce flat primary skin lesions and the early European strains, which produced protuberant skin lesions, ranged in virulence from grade I to grade V. The data recorded in Table 2 show that in the material examined in this survey there is no correlation between type of lesion and lethality.

Elsewhere it has been shown (Woodroffe & Fenner, 1965) that a plaque mutant of the Lausanne virus obtained from a stock preparation of that virus produced a disease of slightly lower virulence (grade II instead of grade I), which was characterized by raised, and not protuberant, skin lesions. The capacity to produce particular types of lesion, like the virulence, may be controlled by many genes; and because of covariation even single mutational steps may lead to changes in both characters.

SUMMARY

Samples of lesions containing viable myxoma virus were collected from 222 infected wild rabbits captured in 80 counties of Britain during October–November 1962. They were dispatched by air to Australia and passaged once in domestic rabbits before being tested for their virulence by the intradermal inoculation of groups of six laboratory rabbits with small doses of virus.

The results showed that a wide range of viruses of differing properties now co-exist in Britain. Their virulence ranges from very high (99% lethal) to low (about 50%), but the majority of strains fall into the grade III virulence group, with estimated case mortality rates varying between 70 and 95%. The viruses also produce disease of varying symptomatology, skin lesions being very prominent, raised or flat. There was no obvious association between lesion type and virulence.

Tests made on the wild rabbits from which the samples were obtained, and on

inoculated domestic rabbits, showed that virulence could not be predicted by gel-diffusion tests.

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