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In vitro method for safety testing of foot-and-mouth disease vaccines

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SUMMARY

The susceptibility of the tissue culture system to small amounts of residual live virus was not influenced by the inactivated antigen present. The depth of inoculum over the cell sheet did not affect results. Negative cultures frequently gave positive first (but not second or later) sub-cultures.

Baby hamster kidney cells were always more sensitive than cattle tongues to infection with any of the strains used.

Confidence in the safety test depends on the number of vaccination doses used; the tissue culture test can be made much more reliable than the cattle test because it is not limited to the 15 ml. of inoculum that restricts the cattle test.

INTRODUCTION

Henderson (1952*b*), when reporting on tests for non-infectivity of foot-and-mouth disease (FMD) vaccines, concluded that the intradermal cattle tongue inoculation test was then the best available. At that time, vaccines were inactivated in the presence of adjuvants, and tissue culture tests could not be used because of the cytotoxic effects of the adjuvants. Now that the virus can be inactivated in the absence of adjuvant, tissue cultures can be used to test for any virus remaining active in the inactivated suspensions.

Because vaccines prepared from virus grown in the baby hamster kidney (BHK 21, clone 13) cell line (Macpherson & Stoker, 1962) are now widely used, it seemed logical to investigate the value of this cell line for innocuity tests. The work described in this paper was directed first to an examination of the factors likely to affect the sensitivity of the tissue culture test and, second, to a series of comparisons between the cattle tongue test and the BHK tissue culture system.

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MATERIALS AND METHODS

Viruses

The viruses used (Table 1) were passaged in BHK cells for the same number of times as were seeds being used to produce antigen in BHK suspension cultures.

Homologous antigens

Virus strains were grown in suspension culture for 48 hr., then harvested and cell debris removed by centrifugation at 6000 rev./min. for 2 hr. at 4° C. The cell-free virus suspension was inactivated with acetylethyleneimine at a final concentration of 0·05 % (v/v) for 30 hr. at 26° C. The inactivation procedure was stopped by adding sodium thiosulphate to a final concentration of 2 % (w/v). The suspension was then tested for live virus by injecting 0·03 ml. intraperitoneally into each of 50 'P' strain mice aged 4–7 days (Skinner, 1951) and also by inoculation of 10 ml. of the suspension onto each of six BHK monolayers.

Table 1. *Viruses used in the investigation, showing passages before use in cattle and in BHK cells*

		Number of passages		
			BHK	
Type	Strain (origin)	Cattle	Monolayer	Suspension
Asia 1	Pak. 1/54	0	13	1
C	B.F.S. 997/53	6	6	1
A	5. Ger. (Eystrup) European vaccine strain	5	5	1
O	1 B.F.S. 1860/67	1	5	1
SAT 1	5. (S.A. 13/61)	2	5	1

Cattle

Two-year-old cross-bred Devon steers were used. These were fully susceptible to FMD. They were housed in loose boxes so designed that the animals could be watered and fed without an attendant having to enter the box. Every precaution was taken to prevent accidental spread of infection during an experiment.

BHK cell monolayers

These were 2-day-old monolayers of BHK 21, clone 13, cells grown in Roux flasks or in 8 oz. medical flats.

RESULTS

(A) *Studies of the* in vitro *tissue culture test*

(1) *The effect of volume of inoculum on sensitivity*

Sets of ten BHK monolayers in 8 oz. medical flats (cell sheet area 55 cm.^{2}) were inoculated with 1, 8 or 20 ml. of a dilution in phosphate-buffered saline (PBS) of

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a suspension of Asia 1 virus. Two experiments were done: in the first there were four ten-fold dilutions from 10^{-7} to 10^{-10}; in the second there were five three-fold dilutions from 10^{-7} to 10^{-9}.

The results (Table 2) were analysed using the logit transformation of the proportions of monolayers in which CPE was seen and constants were fitted for a linear effect of log. (dilution), a linear effect of log. (volume of inoculum), and for the mean difference between experiments. The statistical method is an iterative one and the computer programme for this type of analysis was described by Lewis (1968). The linear effects are the slopes of the two log. dose–response relationships averaged over the two experiments. If increased CPE associated with increased volume of inoculum were caused solely by the extra amount of virus, the slope of the dose–response line for volume of inoculum should equal that for dilutions. The values obtained were $1{\cdot}39 \pm 0{\cdot}22$ and $1{\cdot}40 \pm 0{\cdot}18$, respectively.

Table 2. *Numbers of BHK monolayers (out of 10) showing cytopathic effect due to Asia virus in relation to dilution of virus suspension and volume of inoculum*

	Volume of inoculum (ml.)					
	First experiment			Second experiment		
Log$_{10}$ dilution of virus	1	8	20	1	8	20
−7·0	4	7	10	8	10	10
−7·5	—	—	—	5	10	10
−8·0	1	7	6	2	9	10
−8·5	—	—	—	2	5	7
−9·0	0	1	0	0	0	4
−10·0	0	0	0	—	—	—
Total	5	15	16	17	34	41
CPE (%)	12·5	37·5	40·0	34·0	68·0	82·0

Consequently, the increases in percentage of monolayers showing CPE associated with increasing volume of inoculum (Table 2) are accurately predictable from the changes associated with the dilution series. Thus, although the average depth of inoculum over the cell sheet area of about 55 cm.^{2} ranged from about 0·18 mm. for 1 ml. inoculum to 3·64 mm. for 20 ml. inoculum, this seemed not to modify the effect of the virus.

The expected mean percentage of monolayers showing CPE, calculated after fitting the three constants mentioned above, was 36·3 for the first experiment and 56·3 for the second. If the potency of the virus is assumed constant, this demonstrates significant variation in the sensitivities of different batches of BHK cells.

(2) *Effect of the presence of large amounts of inactivated homologous antigen*

The possibility that inactivated antigen might prevent the detection of small amounts of infective virus by a cellblocking or interference effect was examined. Serial dilutions of the viruses of types C, A and SAT 1, detailed in Table 1, were made in PBS and compared by tissue culture on sets of ten BHK monolayers, with

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dilutions of the same viruses in their homologous antigens. Type A dilutions in homologous antigen produced significantly greater CPE than the corresponding dilutions in PBS, whereas types C and SAT 1 did not. The experiment with type A was repeated twice, with results similar to the first experiment. Table 3 summarizes the results of these experiments.

The essential conclusion relevant to innocuity testing is that there was no indication that homologous antigen might appreciably inhibit the detection of live virus on BHK monolayers. The results with type A virus, although inconsistent with those for the other types, were consistent with this conclusion.

Table 3. *Comparison of the CPE on BHK monolayers of C, A and SAT 1 viruses when diluted in homologous antigen, with their CPE when diluted in phosphate-buffered saline (PBS)*

(Data are numbers of monolayers (out of 10) showing CPE, except where indicated.)

Log$_{10}$ dilution of virus	C		A (total of 3 experiments. No. of monolayers out of 30)		SAT 1	
	Antigen	PBS	Antigen	PBS	Antigen	PBS
−6·0	10	10	—	—	—	—
−6·5	—	—	30	28	—	—
−7·0	7	7	30	18	5	6*
−7·5	—	—	26	8	2	7
−8·0	1	0	18	3	0	0
−8·5	—	—	6	1	1	1
−9·0	0	0	2*	0*	—	—
Total	18	17	112	58	8	14
CPE (%)	45·0	42·5	70·5	36·3	20·0	35·9

* Out of 9 monolayers.

(3) *The effect of sub-culture*

Transfer of fluids from incubated cultures with no evidence of virus to fresh cultures may improve the efficiency of virus detection.

Dilutions of types A, O, C and SAT 1 were made in PBS and 8 ml. of each dilution inoculated onto ten (occasionally nine) BHK monolayers. Virus was adsorbed at room temperature for 30 min., 40 ml. maintenance medium were added and the monolayers incubated at 37° C. After 48 hr. incubation, 8 ml. of the supernatant medium was withdrawn from all cultures apparently still free of CPE and used to inoculate a fresh monolayer. These sub-cultures were treated similarly and fluids from those apparently free from virus were transferred to a further set of sub-cultures. Both primary cultures and sub-cultures were examined for CPE up to 72 hr. after inoculation. An additional test, following this scheme, was done with type C virus diluted in homologous antigen.

Table 4 shows that the first sub-culture revealed additional evidence of virus in all five experiments. This indicates that some of the original cultures received

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Table 4. *Results of titrating five virus suspensions on BHK monolayers and of sub-culturing from monolayers showing no CPE at the primary culture*

(Monolayers with CPE/monolayers tested.)

Log_{10} dilution	Type C (antigen)		Type C (buffer)		Type SAT 1 (buffer)		Type O (buffer)		Type A (buffer)	
	Parent	First passage	Parent	First passage	Parent	First passage	Parent	First passage	Parent	First passage
−5	10/10	—	—	—	—	—	—	—	—	—
−6	10/10	—	10/10	—	10/10	—	7/10	2/3	—	—
−6·5	—	—	—	—	—	—	—	—	8/10	2/2
−7	6/10	1/4	5/10	5/5	9/10	0/1	0/10	2/10	6/10	2/4
−7·5	—	—	—	—	—	—	—	—	1/10	0/9
−8	1/10	0/9	0/10	0/10	1/9	1/8	1/10	0/9	0/10	1/9
−8·5	—	—	—	—	—	—	—	—	0/10	0/10
−9	0/10	0/9	0/10	1/10	0/9	0/9	0/10	0/10	0/10	0/10
−10	—	—	0/10	0/10	0/9	0/9	0/10	0/10	—	—

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too little virus to cause obvious CPE but enough to cause infection and by the first sub-culture there was sufficient virus to infect enough cells to produce visible CPE. The second sub-culture never showed evidence of additional virus.

The practical conclusion, from the point of view of innocuity tests, is that a single sub-culture of BHK monolayers would help to detect minimal quantities of live virus.

(B) *Comparison of cattle tongue inoculation with BHK tissue culture tests*

Two series of investigations were made. The first examined the ability to detect live virus (types Asia 1, C, A and O) in tissue culture at dilutions at or beyond the extinction point for infectivity in cattle. The second compared the ID 50 for cattle and for BHK monolayers.

(1) *The response of tissue cultures to virus causing low levels of response in cattle*

The object of this work was to determine whether evidence of virus could be obtained from tissue cultures at dilutions that caused no lesions in cattle.

The dilution expected to cause 50 % cattle tongue lesions was taken as a starting point and a stepwise series of larger dilutions prepared, using inactivated homologous antigen as the diluent. Each dilution was inoculated intradermally into 25 sites on each of four cattle, using 0·1 ml./site. The series was extended until a dilution was reached that caused no lesion in any of the 100 sites injected. The full series of dilutions was cultured simultaneously on BHK monolayers. For virus types Asia 1, C and O, 20 ml. of each dilution were added to each of two 4-day BHK monolayers in Roux flasks. After 30 min. at room temperature, 80 ml. of maintenance medium were added and the flasks incubated at 37° C. for 48 hr. 20 ml. of medium from each negative culture were then transferred to a further two Roux flasks which were treated in the same way as the parent flasks. All cultures were examined daily for CPE for 3 days. Type A dilutions were added to 48 hr. BHK monolayers in 8 oz. medical flats, using 8 ml. on each of 10 monolayers/dilution. These were not sub-cultured.

Tissue culture fluid from all monolayers that showed CPE and from all control cultures that received inactivated antigen alone was injected intraperitoneally into 4–7-day-old '*P*' strain mice. Tissue homogenates from mice dying were tested for virus by micro-complement fixation.

All the dilutions used in the cattle tests produced CPE in BHK monolayers, either in parent cultures or first sub-cultures, and the presence of virus was confirmed by complement fixation. CPE was therefore observed at dilutions 10 to 100-fold greater than any that produced a lesion in cattle.

The cattle results (Table 5) being sufficiently extensive, the opportunity was taken to estimate the concentration of cattle infectious units for each virus, assuming the Poisson distribution, and to compare the observed with the expected numbers of lesions at each dilution.

If we denote the concentration in the parent suspension by m, then the concentration in any ten-fold dilution is $m_d = m/10^x$. The expected number of lesions

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Table 5. *Observed numbers of lesions (out of* 100) *in cattle tests and expected numbers calculated on the assumption of a Poisson distribution of the frequency of infective doses in small samples*

Cattle

Log_{10} dilution	Observed number	Expected number	χ^2
(*a*) Type Asia 1 virus			
−4·25	40	54·8	8·8
−4·5	33	36·0	0·3
−5·5	17	4·3	99·6
−6	14	1·4	
−6·5	0	0·4	
−7	0	0·1	
(*b*) Type C virus			
−3·35	54	72·5	17·1
−3·6	28	51·6	22·3
−4·6	21	7·0	30·1
−5·1	10	2·2	559·5
−5·35	14	1·2	
−5·6	3	0·7	
−6·1	28*	0·5	
−7·1	1	0·0	
−8·1	0	0·0	
(*c*) Type O virus			
−4·7	19	22·7	0·8
−5·7	7	2·5	6·3
−6·7	0	0·3	
−7·7	0	0·0	
(*d*) Type A virus			
−6·2	19†	26·0	2·9
−7·2	13	4·1	15·4
−8·2	0	0·4	
−9·2	0	0·0	

* Out of 200.

† Out of 75.

(Bracketed expected values in each section are merged; the single χ^2 shown applies to the merged group.)

out of n sites is then $n(1-e^{-md})$. If r is the observed number of lesions and R the expected number, goodness of fit can be tested by

$$\chi^2 = \frac{n(r-R)^2}{R(n-R)},$$

summed over all dilutions after merging neighbouring results with expectations less than 5 or greater than 95.

These calculations (Table 5, last column), based on maximum likelihood estimates of m, show gross deviations from the expected numbers of lesions in cattle tests. There were far too few lesions at the smaller dilutions and far too many at the larger.

Probably several factors contributed to the gross discrepancy from Poisson

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expectation in the cattle test: (1) the dose remaining at the injected site after the needle has been withdrawn may vary considerably; (2) the sensitivity of the cattle to the virus may vary; (3) observers may tend to underestimate the number of independent reactions when lesions from many sites coalesce.

None of the tissue culture experiments produced suitable results for testing goodness of fit to the Poisson expectation, partly because the numbers of monolayers at each dilution were small and partly because dilutions were too widely spaced. However, the results of the second experiment of Table 2 were used and the total χ^2 (with 10 D.F.) for deviations from Poisson expectation was 6·37. This gives no indication of departure from Poisson expectation and we may therefore expect the theoretical relationship of size of test to probability of failure to detect live virus to correspond more closely to the practical situation in tissue culture than in cattle tests.

(2) *Comparative infectivity assays using cattle and tissue culture tests*

Five dilutions of types Asia 1, C, O and A virus were prepared in PBS and 0·1 ml. of each dilution was injected into five sites on the tongue of each of four cattle,

Table 6. *Comparative assays of four virus types on cattle and on BHK monolayers*

(*a*) Number of lesions (out of 25) on tongues of each animal in cattle test

Virus type	Dilution series (steps of 10^{-1})	Cattle 1	2	3	4	Total	$\log_{10}$ ID 50/ml.
Asia 1	10^{-3} to 10^{-7}	3	11	15	15	44	5·72
C	10^{-2} to 10^{-6}	13	11	11	1	36	4·23
O	10^{-2} to 10^{-6}	5	12	7	12	36	4·30
A	10^{-2} to 10^{-6}	17	14	16	17	64	5·71

(*b*) Numbers of BHK monolayers (out of 60) with CPE in tissue culture tests

Virus type‡	Dilution series (steps of $10^{-0.3}$)	Cell batches 1	2	3	4	5	$\log_{10}$ ID 50/ml.
C	10^{-7} to $10^{-8.5}$	14	12	5	18*	20*	6·05
O	$10^{-6.3}$ to $10^{-7.8}$	19	26	15	18	—	5·77
A	$10^{-6.7}$ to $10^{-8.2}$	13	24	27	20†	41†	6·51

* Dilution series steps of 10^{-1} from 10^{-6} to 10^{-9}; responses out of 40.
† Dilution series steps of $10^{-0.5}$ from $10^{-6.5}$ to 10^{-9}.
‡ Data for the Asia 1 type are given in Table 2.

(*c*) Comparison of $\log_{10}$ ID 50s estimated from cattle and tissue culture tests

Virus type	Log ID 50/ml. Cattle	Tissue culture	Ratio of ID 50s (Tissue culture/cattle)
Asia 1	5·72	7·24	33
C	4·23	6·05	67
O	4·30	5·77	30
A	5·78	6·51	6
			Weighted mean 40

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making 25 injections/tongue (Henderson, 1949). This arrangement shows the dose–response relationship within animals and allows a form of analysis that takes account of sensitivity variation from animal to animal.

Dilutions in PBS of the same viruses were assayed on batches of BHK monolayers, using 10 monolayers/dilution and 8 ml./monolayer (the data of Table 2, when 1 and 20 ml. inoculum were also included, are used for the Asia 1 assay).

Each assay of each virus was analysed by fitting constants for animals (in the cattle tests) or for batch of BHK cells and for a linear log. dose–response relationship, using the logit transformation (Lewis, 1968). Results (Table 6) were converted to $\log_{10}$ ID 50/ml. and show that, in these experiments, the tissue culture assay was from 6 to 67 times as sensitive as the cattle assay.

Hyslop & Skinner (1964) provide evidence that the cattle tongue test grossly underestimates the amount of virus in a suspension because material is lost from the injection site. They found that responses to 0·005 ml. were similar to responses to 0·1 ml. The ratio of these doses is of the same order as the ratio of the cattle to BHK infectivity assay results. This suggests that there may be little difference between the sensitivities of cattle tongue epithelium and BHK cells; the difference in sensitivity between the systems probably results mainly from defects in the mechanics of tongue injection.

Because the sensitivities both of cattle and of batches of BHK cells vary, differences in the results obtained by the two methods depend in part on the particular cattle and cell batches used in the comparison. However, the variation between cattle was much greater than the variation between cell batches; nevertheless, the variation between cell batches is large enough to indicate that in virus assays the inclusion of a standard preparation would be advantageous.

(C) *Calculation of an appropriate volume of vaccine for innocuity testing on BHK monolayers*

In practice, a tangible risk must be taken because the volume of vaccine tested must be a small proportion of the amount produced in any batch. Given the volume of a batch (N ml.), an assumed number of infective ml. doses in the batch (m) and an acceptable probability of failing to detect this amount of infection (P), the amount to be tested (n ml.) can be calculated by solving the equation:

$$\frac{(N-m)!\,(N-n)!}{N!\,(N-m-n)!} = P.$$

Solutions can be obtained, using Stirling's approximation to the logarithms of the factorials, for given values of N, m and P. Tables containing 96 solutions (Table 7) have been obtained by means of a computer programme that starts with trial values of n and rapidly converges to the solution. The columns of the Table give solutions for fixed values of $p = m/N$, which are assumed proportions of infective doses. When the sample for testing is a small proportion of the batch (say 1 % or less), the values in Table 7 are very close to $-(\log_e P)/p$, which is the solution for n in the formula $e^{-np} = P$; this formula assumes Poisson distribution of infective doses in samples of size n when the sampling fraction is negligible.

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Table 7. *Size of sample (ml.) from a batch of N l. of vaccine required for probability P of failing to detect a proportion p of 1 ml. infective doses*

									N(l)
$P = 0.10$									
	45	75	223	434	535	697	1000	1750	4
	45	76	228	457	570	759	1134	2236	40
	45	76	229	459	574	765	1147	2288	200
	45	76	229	459	574	766	1150	2300	2000
$P = 0.05$									
	58	97	287	555	682	882	1248	2108	4
	58	98	297	593	740	985	1469	2885	40
	58	98	298	597	746	995	1491	2972	200
	58	98	298	598	747	997	1496	2992	2000
$P = 0.01$									
	89	148	433	821	999	1273	1749	2734	4
	90	151	456	908	1133	1504	2235	4348	40
	90	151	458	917	1146	1527	2287	4550	200
	90	151	458	919	1149	1532	2299	4598	2000
	p 0·05	0·03	0·01	0·005	0·004	0·003	0·002	0·001	—

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Inspection of Table 7 suggests that with a sample of 500 ml. there will be a reasonably small risk of failing to detect live virus. It can be seen that interpreting the results of such a test (in which no virus was detected) involves a statement about the values of both P and p, and that different pairs of values can be attached to the same test. Since $p = -(\log_e P)/500$ closely approximates the relationship of P to p, it is clear that whatever value of p is selected, there is a corresponding value of P, so that to fix either p or P is arbitrary. Table 8 shows

Table 8. *A selection of permissible interpretations of a negative innocuity test with 500 ml. of vaccine in terms of the probability of failing to detect a proportion of infectious doses*

Probability of failing to detect infection	Proportion of infectious 1 ml. doses/l.
0·2	3·2
0·1	4·6
0·05	6·0
0·02	7·8
0·01	9·2

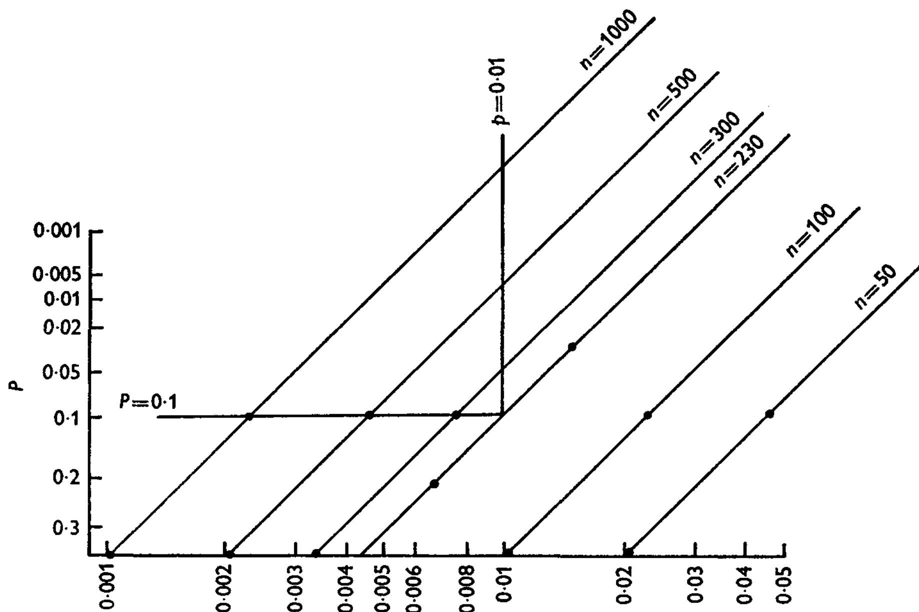

Fig. 1. Relationship of sample size (n) to probability (P) of failing to detect a proportion (p) of infective doses.

some corresponding values, with p converted to infectious doses/l. Thus, when a test of 500 ml. shows no evidence of virus, there is a 20% chance that the batch contained 3·2 infectious doses/l., or a 10% chance that it contained 4·6, and so on. Having fixed n, we can draw a curve relating P and p, but the choice of any point on the curve as an interpretation of the test is quite arbitrary. To simplify statements about the degree of confidence in a test, it may be useful to set a low limit

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of acceptability at $P = 0{\cdot}1$ and $p = 0{\cdot}01$. A test of $n = 230$ satisfies this requirement. The ratio of any selected value of n to 230 is then a measure of the degree of confidence in the selected test relative to the arbitrary standard. The interrelationships of n, P and p are shown in Fig. 1, where equal intervals on the ordinate are $\log_e(-\log_e P)$ and those on the abscissa are $\log_e p$.

DISCUSSION

For a given number of units used in an innocuity test, the relation of P to p is calculated from a mathematical model, assuming that batches showing any evidence of virus will be rejected. We have selected a hypergeometric model as the most realistic, partly because it takes into account the size of batch being sampled but also because it assumes that the material consists of a set of units indistinguishable from each other except that some will infect the test system and some will not. The latter assumption is also implicit in the binomial model used by Henderson (1952*b*). Poisson-type models assume that the material contains a number of randomly distributed dimensionless infective particles. The Poisson model is a reasonable representation of contaminating virus particles in a vaccine; in the present context the particles are merely notional, for the test systems provide no means of counting them. For small p, calculations from the present hypergeometric model differ appreciably from those given by binomial or Poisson models only when the size of sample to be tested is an appreciable proportion of the batch.* Only a very special situation could justify such a large sample.

In most practical situations, calculations based on the Poisson model $n = -\log_e P/p$, as represented in Fig. 1, are sufficiently correct. For the cattle tongue test, we (following Henderson, 1952*b*) take p to represent a proportion of infective 0·1 ml. doses that a test of n doses of 0·1 ml. might fail to detect, with probability P. To find the number of 0·1 ml. doses required to give probability P of failing to detect the same proportion of vaccination doses, each consisting of $k \times 0{\cdot}1$ ml., we merely substitute kp for p in the formula, to get $n = -\log_e P/(kp)$. This is a well known result in Poisson theory. Thus, $n' = nk$ is the number of 0·1 ml. doses to be tested and, if the vaccination dose is 1 ml., $n' = 10n$. In general, if theoretical predictions are to be stated in terms of a proportion p of infected vaccination doses, then $n = -\log_e P/p$ is the number of vaccination doses to be included in the test. This is a simple generalization of results presented in tabular form by Henderson (1952*b*, Tables 7, 8).

The cost of testing more than 15 ml. of vaccine on cattle is prohibitive. This size of test corresponds to a 1/20 chance that 18 % of 1 ml. vaccination doses might contain amounts of live virus detectable on cattle tongues. Although no outbreaks of infection following the use of vaccines that passed the cattle test have been

* A referee has pointed out that $P = (N - n^m/N)$ gives very similar results to the hypergeometric model and is readily solved by taking logarithms. This relationship was the basis of our method of solving the hypergeometric equation but we overlooked the fact that in itself it is a valid solution to the problem. Our attention has also been drawn to a paper by Peto & Maidment (1969) dealing with a very similar problem, in which they use a formula very similar to the formula of this footnote.

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reported, the theoretical risk is far from negligible. With the tissue culture method, the amount of vaccine tested can be selected to make the theoretical risks very small. The method cannot be used after the vaccine has been prepared for field use, because of the cytotoxic effect of certain adjuvants; at this stage, there may be a case for a cattle test to check against accidental gross contamination with live virus of batches that passed the tissue culture test.

The results of Hyslop & Skinner (1964) suggest that the apparent difference in susceptibility between cattle tongue epithelium and BHK cells (Table 6) may be mainly a consequence of the loss of injected virus from sites injected in cattle tongues. Variation in this loss should be one of the factors causing variation in the relative sensitivity of the two assay methods shown in Table 6 (*c*). The loss would also affect calculations of the relative sensitivity of the intradermal tongue and subcutaneous routes of injection made by Henderson (1952*a*). Thus, the subcutaneous route may well have about a tenth of the sensitivity (relative to cattle tongue epithelium) previously supposed. This becomes an additional safety factor in subcutaneous vaccination.

The demonstration in Section B 1 that responses to the cattle test showed gross deviations from Poisson expectation, whereas responses to the tissue culture test did not, implies that theoretical calculations of probabilities of failing to detect infection should be much nearer the truth for tissue culture than for cattle tests.

Our results show that BHK tissue cultures are satisfactory for use in detecting small amounts of infective virus remaining in incompletely inactivated FMD virus suspensions. The inactivated antigen does not interfere appreciably with the detection of residual virus, though at least one sub-culture of the material under test is needed to be certain of obtaining visible CPE. A reasonable volume of inoculum can be applied to each cell sheet, so that fairly large volumes of antigen can be tested on a manageable number of BHK monolayers. This ability to test large sample volumes is the real advantage in the use of a tissue culture test and gives greatly improved levels of confidence compared with the cattle test.

Gard (1960) has argued that there is an implicit advantage in the safety testing of material at various stages of production. This was too costly with the cattle test but it becomes practicable with a tissue culture test. Such tests could be done on each batch of antigen produced and again on multivalent mixtures of antigen. Antigens passing these tests could then be removed to a 'clean' area for final formulation. Tissue culture tests cannot be used after this stage because of the cytotoxic effects of formulation adjuvants.

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