J. Hyg., Camb. (1969), **67**, 269
Printed in Great Britain

Dose-response relationships of lymphocytic choriomeningitis viruses in mice and L cell tube cultures

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(*Received* 14 *September* 1968)

INTRODUCTION

Knowledge concerning the relationship between the dose of a virus and the response of the inoculated host is not only of importance for choosing the appropriate method for the estimation of the number of infectious units (IU) in a preparation; it also may aid in the understanding of the relation between infectious agent and its host. As part of our study of the virus of lymphocytic choriomeningitis (LCM) the dose–response relationships between two virus strains, WE_3 and Armstrong, in two hosts, L cell tube cultures and mice, were determined and analysed. If infectivity was taken as the response, its connexion with the dose was found to be compatible with the assumption that one and only one IU was capable of initiating infection. In contrast, if death of a mouse was regarded as signifying the response, its relationship with the dose was not so simple.

MATERIALS AND METHODS

Cell cultures

L cells (Earle, 1943; Sanford, Earle & Likely, 1948–49), kindly supplied by Prof. W. Schäfer, Tübingen, were grown routinely in Roux bottles with a growth medium consisting of Eagle's minimal essential medium (Eagle, 1959), supplemented with non-essential amino acids (Lockart & Eagle, 1959) and 5% heated calf serum. Screw-capped culture tubes, 16×125 mm., were seeded with 1 ml. of growth medium containing 3×10^5 or $1{\cdot}5 \times 10^5$ cells. These were incubated in a stationary position at 37° C., the former for 1 day and the latter for 2 days before use.

Mice

Randomly bred white albino mice of both sexes from a local dealer were employed. The average weights of the animals in different experiments will be given together with the experimental details. Five or ten mice were housed in Macrolon cages (Spiegel & Gönnert, 1961) types I and II respectively, and were fed on commercial mouse cubes.

* Dedicated to Prof. W. Scheid, Köln, on the occasion of his 60th birthday in token of my sincere respect and affection.

Viruses

The Armstrong strain of LCM virus (Armstrong & Lillie, 1934), obtained from the American Type Culture Collection, had been passaged approximately 200 times in the mouse brain after its isolation. A pool was prepared in L cells, and was stored in ampoules at − 60° C. The WE_3 strain (Jochheim *et al.* 1957), a descendant of the WE strain (Scott & Rivers, 1936), was supplied by Prof. W. Scheid, Köln, after passages in guinea-pigs, mouse embryo cells *in vitro*, and mice. Virus was prepared as 2nd passage in L cells, and was stored in ampoules at − 60° C.

Principal design of the experiments

In the case of mice, serial dilutions of the virus were made with balanced salt solution (BSS) (Hanks & Wallace, 1949) containing antibiotics and 1% heated CaS. Employing semi-automatic syringes, 0·03 ml. were inoculated intracerebrally (IC). Animals which were found dead between the 5th and 21st day after inoculation were recorded as having died from the virus inoculation. Surviving mice were challenged on day 21 with approximately 10^3 LD50 of Armstrong virus and observed for 2 more weeks. The infectivity of a given dose was based on mice now surviving, together with the numbers which had succumbed to the original inoculation.

For the determination of the dose–response relationship in L cells, dilutions of virus were made in maintenance medium (MM) (Lehmann-Grube & Hesse, 1967) and tube cultures were inoculated with 0·1 ml. volumes. After an adsorption period of 15 min. at room temperature with frequent rocking of the culture racks, 1·9 ml. of MM were added to each culture, which were then incubated at 37° C. in a stationary position. Infection was determined by assaying the culture media individually for complement-fixing antigen (CF Ag) 6 and 7 days after inoculation of Armstrong and WE_3, respectively. Details of the procedure have been published (Lehmann-Grube & Hesse, 1967).

Statistical analysis

The statistical evaluation of the results was based on the assumption that the IU in the inocula were distributed at random, and that one unit sufficed for evoking a response. The most probable number for each dilution was estimated with the aid of a formula developed by Halvorson & Ziegler (1933). For the calculation of the goodness of fit between observed and expected values the χ^2 method was employed, in accordance with the principles laid down by Haldane (1939). The 50% lethal doses (LD50) and the 50% infectious doses (ID50) were estimated according to Fazekas de St Groth (1955).

RESULTS

Preliminary experiments

For obvious technical reasons, it was not possible to keep single mice. Thus, the possibility had to be considered that infectious spread between cage mates might

influence the results. In order to rule out this possible source of error the following experiments were performed. A total of 120 mice were distributed into 20 cages. Two of each group of six mice, which were to be kept together, received IC approximately $10^{2.5}$ LD 50 of Armstrong or WE_3 virus. All inoculated mice died with typical symptoms; all non-infected animals remained healthy. On day 21 these mice were challenged with approximately 10^3 LD 50 of Armstrong virus. One mouse of 40 which had been housed together with Armstrong-infected animals survived. The 40 companions of WE_3-infected animals died. Because of its significance for our work, this experiment was repeated with some modifications. The total number of mice was increased to 200. Of five mice in each container, three were infected IC with either approximately 10 LD 50 of Armstrong or approximately 200 LD 50 of WE_3 virus. Of 60 mice thus infected with Armstrong, three survived and proved resistant to challenge. None of the 40 non-infected cage mates died or was immune to challenge infection on day 21. In the case of WE_3 the situation was comparable; of 60 infected mice, 58 died with typical symptoms. The two survivors were resistant to challenge. All 40 non-infected controls succumbed to the IC inoculation of 10^3 LD 50 of Armstrong virus. Thus of 160 mice, which, in these two experiments, had been kept together with infected animals, only one resisted later challenge with a deadly dose of Armstrong virus, presumably as a result of contact infection, and it can be concluded that infectious spread within cages can be neglected as a source of experimental error.

Table 1. *Relationship between dose of LCM virus, strain Armstrong, and response in mice (death)*

Relative virus dose (log_{10})	Response (death) Observed No.	Observed %	Expected (%)
0·0	106/109*	97·25	> 99·99
−1·0	108/108	100·00	> 99·99
−2·0	110/110	100·00	> 99·99
−3·0	110/110	100·00	> 99·99
−4·0	110/110	100·00	> 99·99
−5·0	110/110	100·00	> 99·99
−6·0	63/109	57·80	60·57
−7·0	14/108	12·96	8·89
−8·0	1/110	0·91	0·93

* Number of mice dead over number inoculated.

Armstrong virus

Dose–response in mice

Each dilution of a decimal series was inoculated into 110 mice, 55 of either sex. For control purposes a group of 110 mice was inoculated with diluent only. The average weights, determined at the time of inoculation, were 24·7 and 22·8 g. for 50 male and 50 female animals, respectively. Mortality was compared with expected values (Table 1). The apparently good agreement between observed and calculated figures could be substantiated by the computation of χ^2 which was found

to be 2·568, corresponding to $0{\cdot}3 > P > 0{\cdot}2$ (2 degrees of freedom). Unexpectedly, three mice, all inoculated with undiluted virus, survived. (They were not included in the estimation of χ^2.) What is more, they not only came from one group, but had been housed in one cage. Of 660 mice, inoculated with dilutions of virus ranging from 10^{0} to 10^{-5}, these were the only survivors and the probability that this could have happened as a chance event must be considered exceedingly small. The possibility that they had not been inoculated has been ruled out; at the time of challenge (21 days after the original inoculation) two of these mice were killed, the brains were homogenized and titrated in mice. Each brain contained a minimum of 10^{6} ID 50. The third mouse of this group was challenged and proved to be immune.

Table 2. *Relationship between dose of LCM virus, strain Armstrong, and response in mice (infection)*

Relative virus dose ($\log_{10}$)	Response (infection) Observed No.	Observed %	Expected %
0·0	109/109*	100·00	> 99·99
−1·0	108/108	100·00	> 99·99
−2·0	110/110	100·00	> 99·99
−3·0	110/110	100·00	> 99·99
−4·0	110/110	100·00	> 99·99
−5·0	110/110	100·00	> 99·99
−6·0	103/108	95·37	97·07
−7·0	37/106	34·91	29·74
−8·0	4/109	3·67	3·47

* Number of mice infected over number inoculated.

Of the 110 control mice, inoculated with diluent, one died on day 8 without apparent cause.

As regards infection, the close agreement between observed and expected proportions is again evident from the values (Table 2). χ^2 was estimated as 2·464, which, with 2 degrees of freedom, corresponds to $0{\cdot}3 > P > 0{\cdot}2$. Of the 109 control mice, none was resistant to IC challenge on day 21.

A repetition of this experiment with an Armstrong virus, prepared as 2nd passage in monkey kidney cells, led to essentially the same results.

WE_3 virus

LCM virus, strain WE_3, was diluted serially tenfold. Each dilution was inoculated into 110 mice, equal numbers of either sex. The mean weights, based on 50 male and 50 female animals, were found to be 23·4 and 22·3 g., respectively. The results, shown in Fig. 1, clearly show that the mortality at any given dose deviated significantly from expectation. With highest doses ($10^{6{\cdot}73}$ ID 50 per mouse) many animals survived. Upon successive reductions, increasing numbers died until a maximum (91·5 %) was reached at approximately 50 ID 50. Thereafter, deaths decreased again. By way of contrast, the infectivities were found to agree well with the expectation based on the Poisson distribution (Fig. 1); χ^2 was estimated

to be 3·246, which corresponds to $0{\cdot}2 > P > 0{\cdot}1$ (2 degrees of freedom). In order to obtain information on the question whether mice, which had survived the initial high WE_3 doses, did not respond to the challenge with Armstrong virus 21 days later because they had acquired active immunity or because they had become persistently infected carriers, a number of them were killed 80 days after the challenge inoculation, their brains were homogenized to 10 % suspensions and these were then inoculated into three mice each to test for infectivity. The results in Table 3 shows that most mice still had virus in their brains. However, from the proportions responding, the virus contents of the brains could be roughly estimated and were found to be very low, i.e. 10 ID 50 or less in most cases.

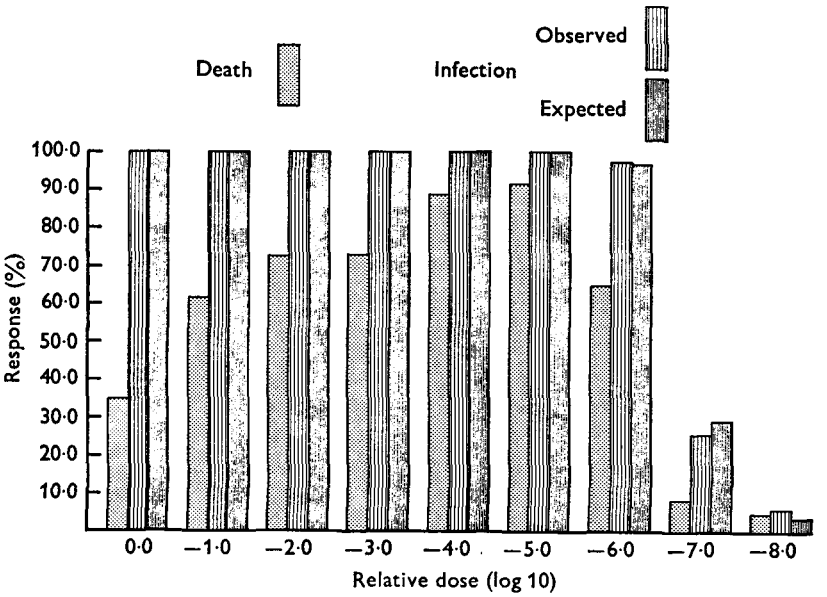

Fig. 1. Dose–response relationship between LCM virus, strain WE_3, and mice.

Table 3. *Detection of virus in brains of mice which had survived high doses of LCM virus, strain WE_3*

Original inoculum ($\log_{10}$ ID 50)	*Number of mice**	
	Tested	Positive
6·73	15	13
2·73	11	8

* Challenged intracerebrally with approximately 10^3 LD 50 of Armstrong virus 80 days before sacrifice.

Armstrong virus *Dose–response in L cultures*

In a preliminary experiment, L cell tube cultures were inoculated with serial tenfold dilutions of Armstrong virus and assayed individually for CF Ag following 6 days' incubation at 37° C. The results, given in Table 4, did not reveal zone phenomena at higher virus concentrations, as had been seen in WE_3-infected mice.

https://doi.org/10.1017/S002217240004167X Published online by Cambridge University Press

Table 4. *Dose–response relationship between LCM virus, strain Armstrong, and L cell tube cultures*

Relative virus dose (log_{10})	Observed response (infection)	
	No.	%
0·0	110/110*	100·00
−1·0	110/110	100·00
−2·0	110/110	100·00
−3·0	110/110	100·00
−4·0	110/110	100·00
−5·0	107/110	97·27
−6·0	31/110	28·18
−7·0	5/110	4·55

* Number of cultures infected over number inoculated.

Table 5. *Dose–response relationship between LCM virus, strain Armstrong, and L cell tube cultures*

Relative virus dose (log_{10})	Response (infection)		
	Observed		Expected
	No.	%	(%)
−4·0	109/109*	100·00	> 99·99
−4·5	109/109	100·00	> 99·99
−5·0	109/109	100·00	97·28
−5·5	77/109	70·64	68·01
−6·0	35/109	32·11	30·27
−6·5	6/110	5·45	10·77
−7·0	3/110	2·73	3·54
−7·5	1/109	0·92	1·13
−8·0	0/108	0·00	0·36

* Number of cultures infected over number inoculated.

Table 6. *Dose–response relationship between LCM virus, strain WE_3, and L cell tube cultures*

Relative virus dose (log_{10})	Observed response (infection)	
	No.	%
0·0	105/105*	100·00
−1·0	105/105	100·00
−2·0	105/105	100·00
−3·0	105/105	100·00
−4·0	105/105	100·00
−5·0	105/105	100·00
−6·0	105/105	100·00
−7·0	23/105	21·90
−8·0	2/105	1·90
−9·0	2/105	1·90

* Number of cultures infected over number inoculated.

https://doi.org/10.1017/S002217240004167X Published online by Cambridge University Press

The dose–response relationship was then more accurately determined by diluting the virus with the factor 3·162. Starting with $10^{-4.0}$, 109 cultures were inoculated with each dilution and checked for infection 6 days later. As can be concluded from Table 5, observed and expected values agree well; χ^2 was found to be 4·013, which, with 4 degrees of freedom, corresponds to $0{\cdot}5 > P > 0{\cdot}3$.

WE_3 virus

With the WE_3 strain of LCM virus, again two large experiments were performed. As before, zone phenomena could not be detected (Table 6). In a further experiment (Table 7) the dilution factor was reduced to 3·162. Again, the observed values did not deviate significantly from expectation; χ^2 was estimated as 0·628, which corresponds to $0{\cdot}98 > P > 0{\cdot}95$ (4 degrees of freedom).

Table 7. *Dose–response relationship between LCM virus, strain WE_3, and L cell tube cultures*

Relative virus dose ($\log_{10}$)	Response (infection)		
	Observed		Expected
	No.	%	(%)
−5·0	102/102*	100·00	> 99·99
−5·5	102/102	100·00	99·98
−6·0	95/102	93·14	93·11
−6·5	58/102	56·86	57·09
−7·0	26/102	25·49	23·48
−7·5	7/102	6·86	8·11
−8·0	2/102	1·96	2·64
−8·5	0/102	0·00	0·84
−9·0	0/102	0·00	0·26

* Number of cultures infected over number inoculated.

DISCUSSION

Dose–response curves in virology are, with few exceptions, adequately characterized by the zero term of the Poisson distribution, which is the same as saying that single infectious units act independently and hence one IU may elicit a response. The hosts have been found to contribute little to the effects in most instances (Meynell, 1957).

As regards infectivity our results fully agree with this general experience. In mice as well as in cell cultures the dose–response curves of both LCM prototype strains, Armstrong and WE_3, were fitted by single hit curves, based on the function e^{-xd}, where x is the number of IU per inoculated volume of the original material and d the dilution. The same type of response was found in the case of Armstrong virus with regard to the death of the mice. In contrast, the shape of the curve relating doses of WE_3 virus with deaths of mice was found to be quite different. Here, a maximum of lethality was found at approximately 50 ID 50; with higher, as well as with lower doses, the proportions of mice responding decreased.

Those who work with LCM viruses are well aware of the fact that high virus

concentrations frequently are less effective in killing mice than are lower ones. It is tempting to speculate with Hotchin & Benson (1963) that this sparing effect is caused by a mechanism which is akin to immunological paralysis in adult mice and is effected by the mass of antigen administered. There are, however, certain reservations to be made. In spite of the continuous multiplication of the antigen, this paralysis appears to be of short duration. A hundred and one days after the first IC inoculation and 80 days after a challenge with approximately 1000 LD 50, the brains of 5 of 26 mice which had survived high doses were found to be free of infectious virus, and in most of the 21 positive animals, little virus was demonstrated (10 ID 50 per brain or less). Furthermore, one is forced to ask why the two strains used here behave so differently, although their rates of multiplication in the brains of infected mice have been found to be indistinguishable (Lehmann-Grube, 1964*a*). However, as there can hardly be any doubt that a mechanism similar to immunologic tolerance protects newborn or unborn mice (Volkert & Hannover Larsen, 1965), it may well be that the administration of excessive amounts of antigen paralyses adults. Hannover Larsen (1968) has found adult mice protected when virus in high doses was injected frequently, which could be explained along similar lines. Certainly, our results do not confirm the opinion of others that LCM strains which exhibit such dose effects are of the 'docile' category, as defined by Hotchin, Benson & Seamer (1962). Both strains are 'aggressive' (Lehmann-Grube, 1964*b*), yet WE_3 spares mice at higher concentrations and Armstrong does not.

The survival of three mice from one cage which had been infected with high doses of Armstrong virus was contrary to expectation and needs some comment. We have on other occasions observed LCM-infected mice to live significantly longer, namely when suffering concurrently from an additional disease, such as a bacterial diarrhoea. Very probably this phenomenon is related to the sparing effect of X-rays (Rowe, 1956; Hotchin & Weigand, 1961) or antimetabolites (Haas & Stewart, 1956), and may indicate the animal's inability to mount an immune response to the virus resulting in an immunological conflict which is thought to be the mechanism of the LCM disease (Hotchin, 1962). It appears likely that these three mice survived longer because they were suffering from an unknown disease, infectious or not, which protected them from death due to LCM.

Much of the recent progress in animal virology can be ascribed to the introduction of accurate methods for the assay of infectivity, e.g. plaque titrations on monolayer cell cultures. Unfortunately, in the case of LCM, simple quantitative procedures are not generally available, and the titration of this virus still rests on the principle of quantal responses in dilution assays. The results presented here confirm our experience, reported previously (Lehmann-Grube & Hesse, 1967), that in cell cultures as well as in mice infection is a useful criterion for the assay of LCM viruses. Both the median effective dose (MED) as well as the most probable number (MPN) may be calculated from the data and provide an estimate of the concentration of infectious units in a given preparation. (It should be stressed that, while the MPN is a correct estimate and furthermore can quickly be looked up in tables, the MED is biased and requires calculations; its only advantage is its widespread use.)

In contrast to infectivities, deaths of mice cannot generally be used to indicate a response to LCM viruses. If a strain behaves like Armstrong, the MED or preferably the MPN may legitimately be calculated from the proportion of animals which succumb to the disease. A dose–response curve, however, as seen with WE_3 where death has been taken to indicate the effect, precludes the correct calculation of either value. Indeed, to my knowledge there is no method available to estimate the concentration of IU in a quantal assay if the underlying dose–response curve is as asymmetrical as the one just discussed.

SUMMARY

The dose–response relationships between two strains of lymphocytic choriomeningitis virus (WE_3 and Armstrong) and two hosts (mice and L cell tube cultures) were determined. The statistical analysis showed that, if infection was regarded as the response, and in the case of Armstrong virus in mice also death, the shapes of the empirical dose–response curves did not deviate from expectation which was based on the zero term of the Poisson distribution. Hence, the hypothesis that individual infectious units are capable of initiating infection and that co-operation is not required, was not contradicted. Furthermore, the units of assay were found to be equally susceptible under the experimental conditions applied.

By way of contrast, the relationship between WE_3 virus and mice dying after intracerebral inoculations was found to be more complex. In this case the empirical curve did not run the expected sigmoid course at all, but rather was bell-shaped with a maximum of mortality (92 %) at approximately 50 ID 50.

The work was supported by a research grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

Armstrong, C. & Lillie, R. D. (1934). Experimental lymphocytic choriomeningitis of monkeys and mice produced by a virus encountered in studies of the 1933 St Louis encephalitis epidemic. *Publ. Hlth Rep., Wash.* **49**, 1019.

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. *Science, N.Y.* **130**, 432.

Earle, W. R. (1943). Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. natl. Cancer Inst.* **4**, 165.

Fazekas de St Groth, S. (1955). Production of non-infective particles among influenza viruses: Do changes in virulence accompany the von Magnus phenomenon? *J. Hyg., Camb.* **53**, 276.

Haas, V. H. & Stewart, S. E. (1956). Sparing effect of a-methopterin and guanazolo in mice infected with virus of lymphocytic choriomeningitis. *Virology* **2**, 511.

Hanks, J. H. & Wallace, R. E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. exp. Biol. Med.* **71**, 196.

Haldane, J. B. S. (1939). Sampling errors in the determination of bacterial or virus density by the dilution method. *J. Hyg., Camb.* **39**, 289.

Halvorson, H. O. & Ziegler, N. R. (1933). Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. *J. Bact.* **25**, 101.

Hannover Larsen, J. (1968). Studies on immunological tolerance to LCM virus. 9. Induction of immunological tolerance to the virus in the adult mouse. *Acta path. microbiol. scand.* **73**, 106.

HOTCHIN, J. (1962). The biology of lymphocytic choriomeningitis infection: Virus-induced immune disease. In Basic Mechanisms in Animal Virus Biology. *Cold Spring Harb. Symp. Quant. Biol.* **27**.

HOTCHIN, J. & BENSON, L. (1963). The pathogenesis of lymphocytic choriomeningitis in mice: The effects of different inoculation routes and the footpad response. *J. Immun.* **91**, 460.

HOTCHIN, J., BENSON, L. M. & SEAMER, J. (1962). Factors affecting the induction of persistent tolerant infection of newborn mice with lymphocytic choriomeningitis. *Virology* **18**. 71.

HOTCHIN, J. & WEIGAND, H. (1961). The effects of pretreatment with X-rays on the pathogenesis of lymphocytic choriomeningitis in mice. I. Host survival, virus multiplication and leukocytosis. *J. Immun.* **87**, 675.

JOCHHEIM, K.-A., SCHEID, W., LIEDTKE, G., HANSEN, I. & STAUSBERG, G. (1957). Komplementbindende Antikörper gegen das Virus der lymphozytären Choriomeningitis im Serum von Versuchstieren und Beobachtungen zur Immunität. *Arch. ges. Virusforsch.* **7**, 143.

LEHMANN-GRUBE, F. (1964*a*). Lymphocytic choriomeningitis in the mouse. I. Growth in the brain. *Arch. ges. Virusforsch.* **14**, 344.

LEHMANN-GRUBE, F. (1964*b*). Lymphocytic choriomeningitis in the mouse. II. Establishment of carrier colonies. *Arch. ges. Virusforsch.* **14**, 351.

LEHMANN-GRUBE, F. & HESSE, R. (1967). A new method for the titration of lymphocytic choriomeningitis viruses. *Arch. ges. Virusforsch.* **20**, 256.

LOCKART, R. Z. & EAGLE, H. (1959). Requirements for growth of single human cells. *Science, N.Y.*, **129**, 252.

MEYNELL, G. G. (1957). Inherently low precision of infectivity titrations using a quantal response. *Biometrics* **13**, 149.

ROWE, W. P. (1956). Protective effect of pre-irradiation on lymphocytic choriomeningitis infection in mice. *Proc. Soc. exp. Biol. Med.* **92**, 194.

SANFORD, K. K., EARLE, W. R. & LIKELY, G. D. (1948–49). The growth *in vitro* of single isolated tissue cells. *J. natl. Cancer Inst.* **9**, 229.

SCOTT, T. F. M. & RIVERS, T. M. (1936). Meningitis in man caused by a filterable virus. I. Two cases and the method of obtaining a virus from their spinal fluids. *J. exp. Med.* **63**, 397.

SPIEGEL, A. & GÖNNERT, R. (1961). Neue Käfige für Mäuse und Ratten. *Z. Versuchstierk.* **1**, 38.

VOLKERT, M. & HANNOVER LARSEN, J. (1965). Immunological tolerance to viruses. *Prog. med. Virol.* **7**, 160.