J. Anat. (1997) **191**, pp. 347–353, with 2 figures Printed in the United Kingdom

Total number and size distribution of motor neurons in the spinal cord of normal and EMC-virus infected mice—a stereological study

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(*Accepted 6 May 1997*)

ABSTRACT

The encephalomyocarditis virus of the diabetogenic M-strain (EMC-M) is known to cause diabetes in mice. The EMC-M virus has also been shown to cause paresis in some of the infected animals. The clinical features include an acute ascending predominantly motor paralysis, developing within days. This resembles acute idiopathic polyneuritis. The alpha motor neurons would be a possible target for the virus, so two parameters, the total number and the size distribution of motor neurons, were therefore selected for further investigation in 6 mice with neurological involvement and compared with 6 control mice. The optical fractionator method was applied for estimating the total number of motor neurons and the 3D size distribution was estimated using the rotator method in a vertical design. No difference was found in the total number of motor neurons and the size distributions were similar in the 2 groups. This design can be used as a model for the estimation of the total number of motor neurons and their size distribution in other experimental animal models.

Key words: Encephalomyocarditis virus; acute idiopathic polyneuritis; Guillain-Barré syndrome; motoneuron size; stereology; optical fractionator.

INTRODUCTION

The encephalomyocarditis virus of the diabetogenic M-strain (EMC-M) is known to cause diabetes in mice (Craighead & McLane, 1968). The EMC-M virus has been shown also to cause a reversible paresis in some of the infected animals, most often affecting the hind limbs and occasionally the forelimbs (Buschard et al. 1983). The aim of this study was to investigate whether the encephalomyocarditis virus causes morphological changes in alpha motor neurons in the spinal cord of mice with paresis and to obtain an estimate of the number and size distribution of motor neurons in normal mice for later animal studies. Using stereological methods, the optical fractionator (West et al. 1991), and the rotator in a vertical design (Baddeley et al. 1986; Jensen & Gundersen, 1993), we estimated the total number and the size distribution of the alpha motor neurons in 6 mice inoculated with EMC-M virus and 6 noninoculated control mice.

The anatomy of the mouse spinal cord

The spinal cord consists of several different neuron populations clustered together in groups. Ten layers can be recognised in columns throughout the whole length of the spinal cord. Motor neurons are found in lamina IX. Lamina IX consists of columns of neurons located in the ventral horn and embedded in laminae VII and VIII. The alpha motor neurons supply the ordinary extrafusal fibres of the striated skeletal musculature, while the gamma motor neurons, which are smaller and less numerous, supply the intrafusal fibres of the neuromuscular spindles. The motor

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neurons supplying the axial musculature are located in the medial part of the ventral horn, those supplying the limb musculature being located more laterally. The exact ratios between alpha and gamma motor neurons and interneurons in the ventral horns are not known (Bjugn, 1993). The motor neurons in the gamma size range have, however, been estimated to about 30% of the horseradish peroxidase labelled motor neurons. Not all motor neurons are confined to the ventral horns. Retrograde labelling by horseradish peroxidase has identified motor neurons in the middle part of the grey matter and occasionally even in the most basal parts of the dorsal horns (Hashizume et al. 1988).

According to Bjugn (1993), the grey matter in the mouse spinal cord contains approximately 4 million neurons with 8–9% located in the ventral horns. Anterior horn tissue contains both small motor neurons and interneurons as well as large, classical motor neurons. Motor neurons are the largest of the multipolar cells, characterised by a predominantly spherical nucleus containing a well developed, deeply stained nucleolus near the centre of a clear nucleoplasm (Mayhew & Momoh, 1974). Clear rounded vacuoles are sometimes seen in the nuclei. The nuclei are more regular than those of small neurons, and the nuclear membrane occasionally presents a wrinkled appearance. The abundant cytoplasm is characterised by substantial amounts of Nissl substance arranged in polygonal clumps.

The size of the cell bodies of the motor neurons varies. Those with a long axon have large cell bodies, whereas those with shorter axons as well as gamma motor neurons have smaller cell bodies. Since alpha motor neurons are substantially larger than most gamma and interneurons, it is possible to identify lamina IX at low magnification. A pilot study indicated that the lower limit of the motor neuron volume was well above 5000 μm^3. The low cut-off point would therefore detect a virus-induced atrophy of the motor neurons. When observing the sections at low magnification ($\times 130$), clusters of motor neurons in lamina IX are easily observed and encircled. When measured at a higher magnification ($\times 1960$) in a pilot study most of the alpha motor neurons appeared to be above 10000 μm^3 and the remaining between 5000 and 10000 μm^3. Cells smaller than 5000 μm^3 were therefore excluded as they were considered to be either other types of neurons or glia cells. Neurons outside lamina IX were seldom above 10000 μm^3. The neurons above 10000 μm^3 and positioned in the encircled area are considered to consist mainly of alpha motor neurons.

MATERIAL

Mice

Eighty five 8-wk-old male BALB/c/BOM mice, also part of a diabetogenic study (to be published) were used in this study; 75 mice were inoculated with EMC-M virus by a single subcutaneous injection as described below, 10 mice were injected with virus-free vehicle. All mice were purchased from Gl. Bomholtgaard Laboratory Animal Breeding and Research Centre, Ry, Denmark. The mice were kept in single-mouse cages and allowed free access to chow and water during the trial. Occurrence of glycosuria as well as paresis was recorded.

Six virus-inoculated mice with paresis described in the Table and 6 mice injected with virus-free vehicle were used for this study.

Virus, virus assays and virus inoculation

EMC-M virus was used. A 6th passage batch of virus was titrated in L-cells (established cell line of mouse fibroblasts) to a titre of 10^{55} $TCID_{50}$ (tissue culture infective doses)/ml. All mice were inoculated subcutaneously with 0.2 ml vehicle, while the control mice were injected with the suspension medium alone.

METHODS

The mice were killed 1–2 d after the development of paresis. They were anaesthetised with ether and exsanguinated. Immediately thereafter the pancreas, heart and spleen were removed and the rest of the

Table. *Clinical state of animals at killing and length and weight of spinal cord*

Clinical state	Length of spinal cord (mm)		Weight of spinal cord (g)	
Exposed	Exposed	Controls	Exposed	Controls
Medium paresis in 4 legs	43	47	0.165	0.153
Severe paresis in left hind leg	50	45	0.168	—
Severe paresis in right hind leg	49	46	0.143	0.142
Paralytic left hind leg	46	45	0.107	0.130
Severe paresis in left hind leg	39	40	0.121	0.099
Severe paresis in both hind legs	40	—	0.134	0.152
Mean	45	45	0.140	0.135

animal was fixed full-body in formalin before the spinal cord was removed. Shrinkage of cells due to delayed fixation could not be detected in the final estimation of cell size and might affect the ratio large/small neurons. The spinal cord was removed in toto before being cut into smaller pieces and the cell counts and size measurements were performed uniformly over the entire spinal cord. Data were not collected separately from different levels. The spinal cord was cut horizontally into 2 or 3 parts, and all parts were rotated randomly around their vertical axis (the rostrocaudal orientation of the spinal cord), and fixed in 7% agar. All tissue was then embedded in Historesin (LKB) and cut exhaustively into 35 μm sections, providing approximately 90 sections per specimen. Every 8th section was sampled systematically with a random start between 1 and 8 and stained with a modified Giemsa stain.

Estimation of total number of neurons using the optical fractionator

In many parts of the central nervous system the number of neurons is in the order of thousands or millions. It is not necessary, however, to count all the neurons in a region to obtain data useful in descriptive and comparative studies. Estimates of the total number are sufficient, as long as they have a sufficient amount of precision and are estimates of the true total number, i.e. are based on unbiased counting and sampling principles (Sterio, 1984). To obtain an unbiased estimate of the total number of motor neurons this study used the optical fractionator (West et al. 1991; Korbo et al. 1996; Mayhew & Gundersen, 1996). The optical fractionator is a combination of the optical disector and a fractionator sampling scheme (Gundersen, 1986; Gundersen et al. 1988; Pakkenberg & Gundersen, 1988). With the optical fractionator the neurons (Q^-) are counted that come into focus inside an unbiased counting frame in a known height of the optical disector in a uniform systematic sample that constitutes a known fraction (F) of the volume of the region being analysed. The optical disector setting consists of a modified BH-2 Olympus microscope connected to a videocamera which transmits the microscopic image to a TV screen. A computer connected to the monitor generates an unbiased counting frame on the screen using a CAST-GRID software system (Olympus, Denmark). A Haidenhain MT2 microcator is used to measure the movements in the z-direction with an accuracy of 0.5 μm. Two sets of motors are connected to the microscope to move the section at known distances in the x and y directions.

The fraction, F, is calculated as follows:

$$F = SSF \cdot ASF \cdot TSF$$

where SSF is the section sampling fraction (i.e. the fraction of sections used), ASF is the area sampling fraction (i.e. the fraction of the sectional profile area of the region being analysed), and TSF is the thickness sampling fraction $= h/t$. (For more details on the stereological method, see West et al. 1991). An average of 140 neurons was counted in an average of 1130 disectors in an average of 10 sections per animal.

The total number of neurons can now be estimated unbiasedly from:

$$N = \Sigma Q^- \cdot 1/F$$
$$N = \Sigma Q^- \cdot 1/SSF \cdot 1/ASF \cdot 1/TSF.$$

Example:

$$\begin{aligned} N &= 145 \cdot 1/F \\ &= 145 \cdot 1/(1/8) \cdot 1/(2400\ \mu m^2/200 \cdot 200\ \mu m^2) \\ &\quad \cdot 1/(15\ \mu m/24\ \mu m) \\ &= 145 \cdot 8 \cdot 16.7 \cdot 1.6 \\ &= 30995 \end{aligned}$$

where 145 is the total number of counted neurons, 8 is the reciprocal of the sampling fraction of every 8th section, $200 \cdot 200\ \mu m^2$ are the x–y step lengths, 2400 μm^2 is the area of the frame, 15 μm is the height of the disector, and 24 μm is the average section thickness in that animal.

Estimation of size distribution of the neurons

An estimate of the volume of 3-dimensional objects as a neuron can be obtained where only 2-dimensional profiles are available for analysis if the tissue is randomly rotated (Jensen & Gundersen, 1993). In a so-called vertical design first described by Baddeley et al. (1986) the vertical axis can be selected arbitrarily, but all sections must be made parallel to the axis and all subsequent measurements must be made with respect to the axis, which must therefore be identifiable in the sections. In this study the rostrocaudal orientation of the spinal cord was the obvious vertical axis and could be recognised easily on all sections. The major principle for obtaining an estimate of a sampled motor neuron is the vertical rotator shown in Figure 1. Intersections between the lines and the cell boundary are defined manually by the operator, and the computer generates an unbiased estimate of the cell body volume.

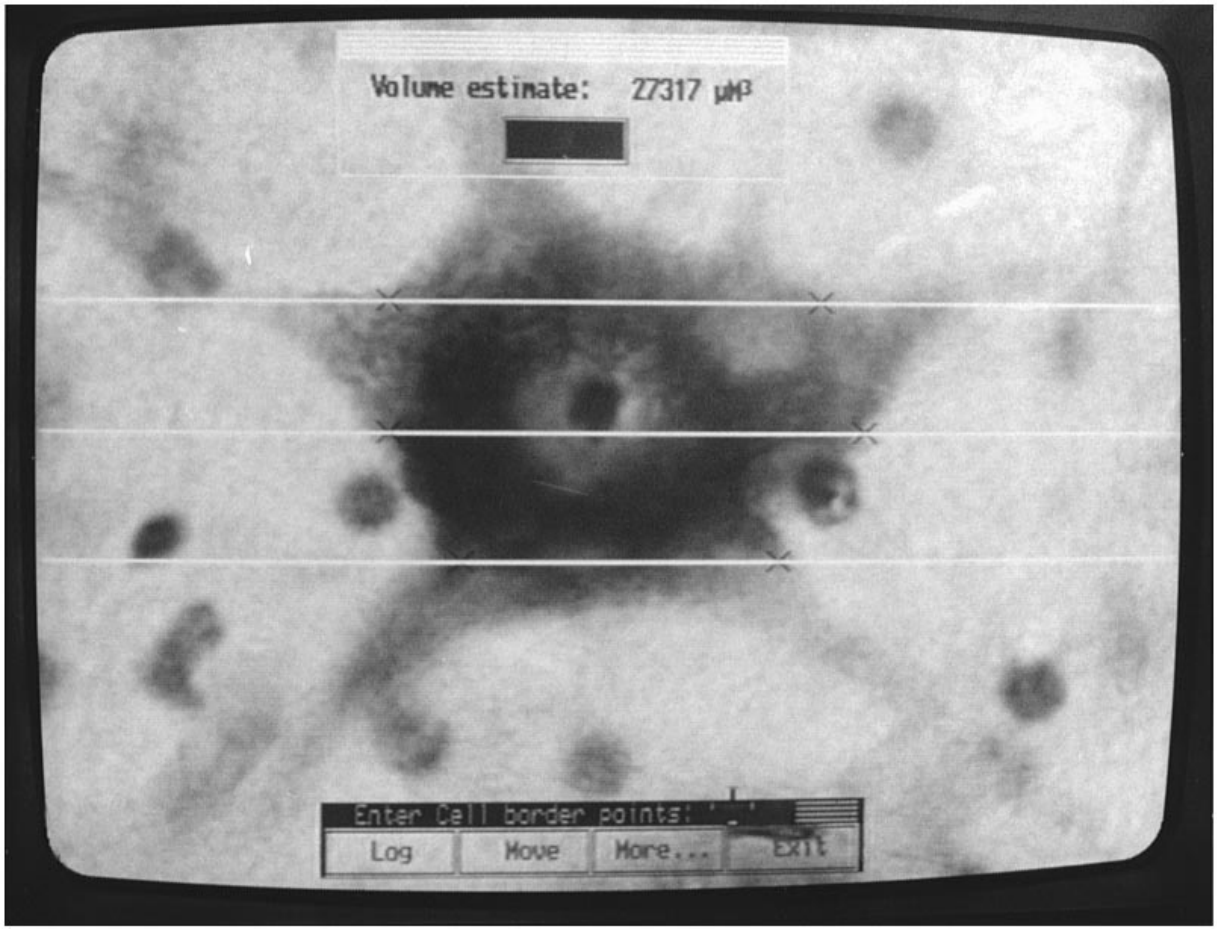

Fig. 1. The stained sections are oriented in the microscope so that the vertical axis is parallel to the *y*-axis on the monitor. The nucleolus is used as a reference point. Top and bottom boundary points are defined by the operator, and the computer then generates 3 lines with a constant distance in a uniform position perpendicular to the vertical axis. The distance between the lines is $t = h/3$, where h is the height of the profile projected on the vertical axis. Intersections between the lines and the cell boundary are defined by the operator, and the computer then generates an unbiased estimate of the cell body volume from the distances between intersections on each of the 3 lines.

To increase precision in the estimate, each part of the spinal cord was rotated separately, providing 3 different orientations of the tissue. The volume estimate is calculated from:

$$\Sigma l_i^2 \cdot \mathrm{t} \cdot \pi$$

where values of l_i are the individual distances between the intersections and the vertical axis, and $t = h/3$, where h is the height of the profile projected on the vertical axis (Fig. 1). As previously described, the neurons are divided into classes depending on their size; because of right-skewed distributions a logarithmic scale was chosen. The first class starts at 5000 µm³ and goes to 6300 µm³. The upper limit of each class is found by multiplying the upper limit of the previous class by a constant = 1.26. (1.26 is chosen as on a logarithmic scale this constant will divide a decade into 3 equidistant classes). The largest observed neurons were 40000–65000 µm³ in both groups.

Statistics

Differences of total numbers between groups were judged by 2-tailed Student's unpaired *t* test employing a significance level of 0.05. Coefficient of variation (CV = SD/mean) is reported throughout in parentheses after group mean values.

The fact that the size distributions turned out to be markedly right-skewed had consequences only for details of the statistics, not for the interpretation. All mean values and most statistical tests were performed on logarithmically transformed individual estimates of motor neuron perikaryon volumes. Geometric mean, $v_N = \mathrm{antilog}[1/n(\Sigma \log(v_i))]$, where N indicates number-weighting due to disector sampling of individual neurons with estimated volume v_i, and n is the number of cells studied in 1 animal. The mean values reported are thus so-called geometric mean values, and accordingly the appropriate measure of absolute variation within an individual is the tolerance factor (TF = antilog(SD(log x)). Since a measure of relative

variation within a group is more meaningful, we report the coefficient of variation, CV = TF − 1, which may be considered analogous to the usual CV = SD/mean for nontransformed values.

Precision of estimates of total numbers

The mean coefficient of error = CE = SEM/mean was estimated from:

$$CE_{total}(N) = \frac{CE^2_{contr\,(N)} + CE^2_{exp\,(N)}}{2} = \frac{(0.16)^2 + (0.13)^2}{2}$$
$$\simeq 0.15 = 15\%,$$

since it evidently did not differ markedly in the two groups. For the control animals the true biological variance was estimated from:

$$OCV^2 = CV^2_{biol} + CE^2$$
$$CV^2_{biol} = OCV^2 - CE^2$$
$$CV^2_{biol} = 0.33^2 - 0.15^2$$
$$CV_{biol} = 0.29 \sim 29\%$$

where OCV^2 is the observed variance and CV^2_{biol} is the true biological variance.

RESULTS

Number of neurons

The mean total number of neurons above 10000 μm³ in lamina IX of the control mice was 12766 (0.23) for controls and 12041 (0.17) in exposed mice, a statistically nonsignificant difference (n.s.). The mean total number of neurons in the 5000–10000 μm³ neuron classes in controls was 15847 (0.43) and 16507 (0.16) in exposed mice (n.s.).

A combined swelling of the cells and cell death could influence the total number of neurons above 5000 μm³. The mean total number of all neurons above 5000 μm³ was 28613 (0.33) in controls and the same in exposed mice 28548 (0.13) (n.s.). The mean length of the spinal cord was 45mm in exposed and 45mm in controls, while the mean weight of the spinal cord was 0.140 g in exposed and 0.135 g in controls (Table).

As indicated above, the CE on the estimate of the total number of alpha motor neurons was 0.16 in controls and 0.13 in exposed mice.

Size distribution

The frequency distribution of the ventral horn neuron volume is shown for control mice and infected mice in Figure 2*a*. No statistically significant difference was found between controls and infected mice. The relative distribution is only strictly comparable among groups with the same number of neurons, which happens to be the case in this study wherefore the comparison is permitted. However, knowledge of the total number of neurons in each animal means that it is possible to compute and compare the absolute size distribution. Total number of neurons in control and infected mice is shown in Figure 2*b*. The animals showed very similar size distribution curves. Figure 2*c* shows the frequency difference as a percentage of the neuron

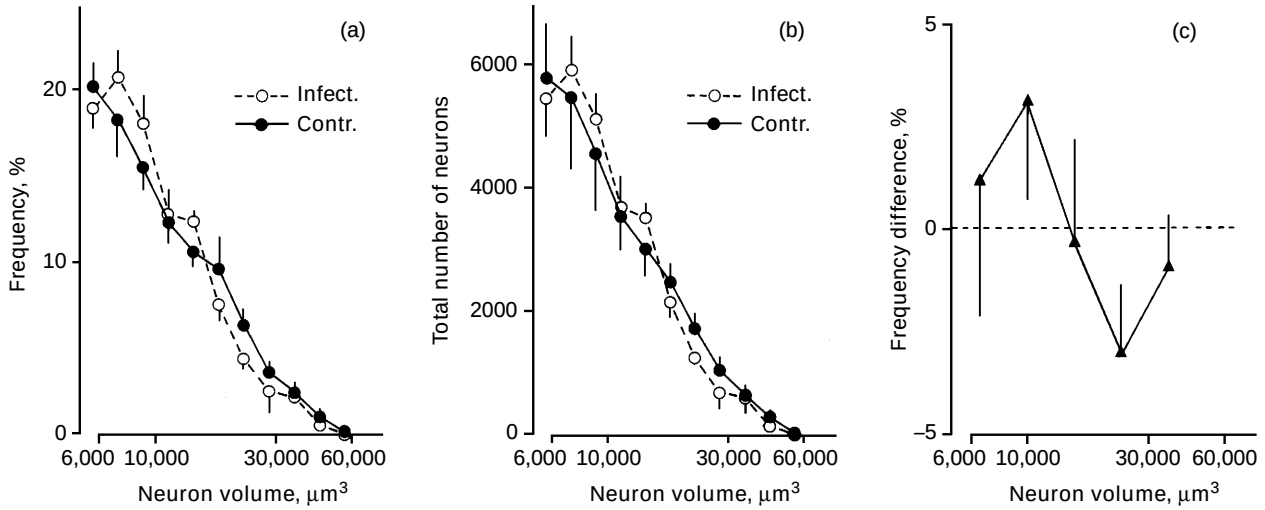

Fig. 2. (*a*) The frequency of ventral horn motor neurons as a percentage is shown on the ordinate and the neuron volume in μm³ on a logarithmic scale is shown on the abscissa. The infected mice are symbolised by an open circle and the controls by a filled circle. No difference was found between controls and infected mice. (*b*) The absolute number of motor neurons in the ventral horns in each class is shown on the ordinate and the motor neuron volume in μm³ on a logarithmic scale is shown on the abscissa. The control mice are shown as filled circles and the infected mice with open circles. No statistically significant difference was found between controls and infected mice. (*c*) The number of motor neurons in the different classes is shown as exposed vs control animals.

volume between the exposed animals compared with controls. None of the 5 differences shown were statistically significant.

The estimate of the true biological variation was 29%, and the impact of the variation of the stereological estimate only gave an increase of the true biological variation from 29 to 33%. This indicated that the precision of the estimated number is well below the biological variation and the design is therefore judged to be sufficient for the purpose, e.g. increasing the number of neurons counted would have no major impact on the precision of the final estimate. The CV for the exposed animals was actually lower (13%) than the CE (15%), which is due to statistical chance in a rather small group ($n = 6$).

DISCUSSION

The virus-exposed and control mice showed virtually identical results in the parameters measured. Thus, despite the clinical diagnosis of a lower motor neuron paresis, the number and size of the motor neuron were not statistically significantly different. There are 2 possible explanations. First, the virus and/or the immunological response might change the alpha motor neuron in a way that would not affect the number or size. Alternatively, the paresis/paralysis might be a result of affection of parts of the nervous system not investigated. The clinical appearance of a flaccid paresis suggests that the roots or peripheral nerves may be the anatomical site of the lesion as in the Guillain–Barré syndrome (GBS) in humans (Honovar et al. 1991), although encephalitic lesions in mice inoculated with EMC-M virus have been described (Navne, 1991). Antisulphatide antibodies have been found in 43% of human cases of GBS (Fredman et al. 1991) and were also found in EMC-M induced disease in mice (K. Buschard et al. unpublished data).

The decrease in number of large neurons and the concomitant increase in the number of small neurons were not statistically significant. Figure 2 shows that point-wise comparison is clearly inappropriate, since the curve is exactly what would be expected if large motor neurons become smaller as a result of the virus infection.

Earlier studies have shown that BALB/c-nude mice are resistant to diabetes and paresis induced by EMC-M virus (Buschard et al. 1987). Thus an immunological mechanism is believed to be involved in the pathogenesis as in human GBS (Honovar et al. 1991).

GBS and the paresis described have several characteristics in common including the rapidity of onset, the reversibility, an infection as the initiator of the immunological reaction, the unaffected motor neurons in the anterior horn of the spinal cord and the presence of antibodies against sulphatide. Whether or not the EMC-M virus induced paresis represents an animal model for human GBS cannot be judged from this study. Further studies should investigate the more peripheral sites of the nervous system to answer this question.

We did not consider a motor neuron in the mouse spinal cord to be difficult to identify when the size was above 10000 μm^3, and also in the classes from 5000 to 10000 μm^3 only those neurons that were located in lamina IX and had the classical appearance of a motor neuron were included in the counts. Problems with identification were similar in the 2 groups. To improve the design further, specific staining methods such as retrograde labelling by horseradish peroxidase could be used to identify the motor neurons with greater certainty.

The design turned out to be very robust, and the precision of the estimate of the variance of the total number of motor neurons was well within the biological variance. The advantage of stereological methods is that the precision can be decided by the investigators and thus be very efficient, and that the basic principles of the estimates are unbiased. It should be mentioned that although the volumes were measured using an unbiased design, it may not necessarily reflect the cell volume in the living animal, since unpredictable factors in histological processing cannot be accounted for. The estimate of the total number is, however, unaffected by any histological handling or shrinkage artefacts. This design can be used as a model for the estimation of the total number of motor neurons and their size distribution in other experimental animal models.

ACKNOWLEDGEMENTS

Hans Jørgen G. Gundersen is sincerely thanked for excellent reviewing and statistical aid. Acknowledged is the financial support provided by the Hartmann Brothers Foundation, the Hede Nielsen Family Foundation, the Foundation of Grosserer L. F. Foght, the Foundation in Memory of Fabrikant Einar Willumsen, and the Foundation of Director E. Danielsen and Wife.

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