The contribution of Charles C. Shepard to leprosy research: from the mouse footpad model to new DNA technology

RJW REES

Division of Communicable Diseases, Clinical Research Centre,
Harrow, Middlesex, England

While appreciating the honour in having been invited to summarise the contributions of Charles C. Shepard to the field of leprosy research, I hope I can do justice to the magnitude of his scientific and personal contributions sustained over the past quarter of a century. Undoubtedly I was strongly influenced in accepting the invitation as a means of conveying my everlasting appreciation of the personal and professional friendship shared throughout with Shep since our first meeting in 1958 at the 7th International Congress of Leprology, Tokyo.

When Dr. Shepard died unexpectedly in February 1985, at the age of 70, he was still very actively engaged in research, as he had been throughout his scientific career, from the time he entered the United States Public Health Service (US PHS) in 1941. Until 1950, except for a sabbatical year with Dr. Arne Tiselius, Sweden, he worked at the National Institutes of Health from where he moved to the Rocky Mountain Laboratory in Montana, and remained there until 1953. Finally Dr. Shepard joined the Centres for Disease Control (CDC), where he remained until his death, although transferring to the US Civil Service in 1978 following retirement from the US PHS.

His career, research interests and achievements

To relate Dr. Shepard's calendar of postings within the PHS to his research achievements and interests I have set these out against his bibliography, analysed by subject and year from 1941 to date (see Table 1).

By these criteria, based on 88 % (159/181) of the publications, his interests and successful achievements fall into three very different subjects, the timing of which by chance played an important part in determining Shep's entrée into the field of leprosy research. While undoubtedly his first publications after joining the USPHS initially at NIH and subsequently at their laboratory in Montana during the period from 1941 to 1953 on rickettsial diseases was determined by PHS; the high quality and application of his studies to the differentiation and epidemiology of these diseases was impressive. Undoubtedly his sabbatical with Tiselius was awarded on the basis of his achievements, but from which Shep gained a life-long appreciation and application of modern biophysical technology to the fractionation and identification of protein type antigens. This specialised expertise on rickettsial diseases resulted in his laboratory at CDC, Atlanta being retained throughout his career.

Although it is of interest that the first two publications of Shepard in 1941 and '43 were on mycobacteria, based on experimental pulmonary tuberculosis in the dog (1, 2), resulting from studies undertaken at Northwestern University before joining the PHS, it seems unlike-

Table 1. Publications (181) by Shepard 1941–1986 indicating particular interests.

Period (years)	Subject	Number
1945 – 78	<i>Rickettsial diseases</i> including murine and epidemic typhus, Rocky Mountain spotted fever, Q fever	31
1955 – 59	<i>Mycobacteria in tissue culture</i> cells (Hela, amnion, monkey kidney)	10
1960 – 84	<i>Mouse foot-pad studies</i> Applied to <i>M. leprae</i> / immunology (13 on vaccination, 1965 – 83)	44
1962 – 86	Applied to <i>Chemotherapy</i>	55
1968 – 84	<i>Leprosy miscellaneous</i>	12
1977 – 79	<i>Legionnaire's Disease</i> including identification of <i>Legionella pneumophila</i>	7
	Subtotal	159
<i>Important Influences</i>		
1948 – 49	A. Tiselius, during sabbatical in Uppsala	
1950 – 57	Lucius F. Badger, Director Leprosy Section at CDC.	

ly that they resulted in his subsequent devotion to leprosy. However, it is clear from his publications that completely unrelated to his specialization in rickettsial researches, he was already becoming interested in mycobacteria. Thus in the period 1955 – 60 a series of 10 papers were published on the behaviour and growth of several species of saprophytic and atypical mycobacteria and human tubercle bacilli in Hela and other cell cultures. The studies on mycobacteria in cell cultures preceded Shepard's epoch-making papers in 1960 on the first reproducible multiplication of *Mycobacterium leprae* using the mouse foot-pad model (3, 4). Both these new and additional studies were initiated in Montana before he even moved to CDC, and resulted from the compelling influence of Dr. Lucius Badger in stimulating Shepard to apply his expertise to culturing *M. leprae*. During Badger's long and varied career also with APHS from 1925 – 57, his most significant work had been on rickettsial diseases and leprosy. The latter included from the laboratory side, the establishment of the Hawaii strain of *M. lepraemurium* and clinically the first definitive epidemiological analysis of leprosy in the U.S.A. With the mutual respect for each other, the stage was set for Shep to accept the challenge. His initial strategy was to pursue both possibilities – *in vitro*, cultivation of *M. leprae* on a cell-culture system in which to maintain an intracellular environment – *in vivo*, cultivation of *M. leprae* in an available and suitable laboratory animal. Hence the probing studies on the behaviour of various mycobacteria in cell culture, which failed, and the behaviour of *M. leprae* in the mouse foot-pad model which came off trumps! This successful model was carefully but remorselessly exploited by Shepard in his remaining 25 years towards the most effective treatment or control of human leprosy.

While leprosy dominated his research from 1960 on wards, his laboratory continued to be responsible as a service for rickettsial diseases. Shepard's third contribution to medicine arose from his laboratory being called in to help solve the cause of the acute and often fatal outbreaks of pneumonia in Philadelphia in 1976. His extensive studies with Dr. McDade at

CDC led to the successful isolation and identification in 1977 of a new species of bacterium – *Legionella pneumophila* – as the causative organism of the respiratory infection now referred to as Legionnaire's disease. This work resulted in a series of 7 publications in the period 1977 – 79.

The mouse foot-pad model

DEVELOPMENT AND LIMITATIONS

In the 1950's Shepard was attempting to transmit leprosy to animals, his first choice being the mouse as a readily available and standard laboratory species. He also chose the foot-pad as the site of inoculating *M. leprae* in view of Fenner's (5) then recent observation that *M. balnei* and *M. ulcerans*, the causative organisms of two skin infections in man, whose optimum temperature for *in vitro* growth was 30°C, and which only multiplied locally in the foot-pads but not systemically in mice.

We, like Shep, were also using the mouse at that time, albeit using the intraperitoneal or venous routes of inoculation; we were all giving the largest possible dose of *M. leprae*. Likewise, none of us had quantitative evidence of multiplication of *M. leprae* in the foot-pads followed an unusually low dose from a small human biopsy, given by chance rather than by plan. It was on the basis of this chance observation that Shep subsequently established that multiplication of *M. leprae* in the mouse foot-pad resulted from smaller rather than large bacterial inocula.

Another important contribution for the successful foot-pad growth of *M. leprae* also arose by chance. A consignment of skin biopsies from patients in the Philippines for Shep's transmission studies in mice were unexpectedly shipped by the U.S. Air Force. However, when none of the *M. leprae* isolated from these biopsies multiplied in the mouse foot-pads, it was revealed that the Air Force had with pride shipped on dry ice rather than wet ice as instructed! From these preliminary and probing studies Shep built up substantial evidence confirming growth of *M. leprae* in the mouse foot-pad.

Therefore, although the development in 1960 of the foot-pad infection provided the first opportunity for the cultivation of *M. leprae* in the laboratory, no growth on artificial media was yet available. Moreover, although by 1986 very substantial extensions and advances in cultivation of *M. leprae* within animal systems have been achieved, including foot-pads of rats and hamsters, nine-banded and other species of armadillos and several species of monkeys, still no artificial media are available, or are likely to be developed in the foreseeable future. Thus, the experimental animals, essentially the mouse and armadillo, have both played a unique and critical role in contributing to advances in leprosy research. For leprosy, this requirement of an *in vivo* animal model, rather than the more usual *in vitro* culture medium, has very seriously restricted such essential studies on *M. leprae* to centres with extensive animal facilities and expertise.

The animal dependent culturing of *M. leprae* also limited the search for new anti-leprosy drugs because even the first assay in the mouse foot-pad infection would require 10-15g. of drug, compared with only a few mg for such an assay in the standard *in vitro* method against culturable bacteria.

It may well be that Shep realised that unless he personally continued to play a major role in exploiting the foot-pad method, its full potential in the control and treatment of leprosy might be seriously delayed. In the following sections on the foot-pad model I have summarized and highlighted its major contribution to the biology of *M. leprae*, the development of a vaccine and to the chemotherapy of leprosy.

APPLICATION TO THE BIOLOGY OF *M. LEPRAE*

As had been anticipated the cultivation of *M. leprae*, albeit only *in vivo*, made it possible to determine its biological characteristics for the first time. Some of these characteristics, including those particularly associated with Shep's work, are summarised in Table 2. First and foremost the foot-pad model, both for the inoculum and harvest, is entirely dependent on bacterial numbers, which have laboriously to be counted as acid-fast bacilli (AFB).

Table 2. Biological characteristics of *M. leprae* derived from the mouse foot-pad model infection.

Minimal countable numbers as AFB	5 x 10 ⁴
Routine infecting dose. (Toalt AFB)	5 x 10 ³ – 10 ⁴
Minimal infecting dose.	
(Solid staining AFB)	1 – 10
Temperature for optimum growth	27 – 30°C
Doubling time (logarithmic phase)	12.5 days
Plateau yield (total AFB)	1 x 10 ⁶
Resistance to 0.5 N sodium hydroxide	20. min.
Retention of viability in	
tissues/homogenates at 4°C	7 – 10 days
Cryopreservation in liquid nitrogen	10 % v/v DMSO; cooling rate ≤ 1°C/min over range – 15 to -60°C
Survival in dried nasal discharges:	
At 20.6°C; 43.7 % humidity	Up to 7 days
At 26.7°C; 77.6 % humidity	Up to 14 days
Stability of pathogenicity after 26-42 serial mouse passage (through 16 – 24 years)	Unchanged
Strain differences:	
Drug resistant-	Dapsone, rifampicin, thioureas
Growth pattern-	Faster / high plateau Slower / low plateau

While foot-pad *per se* confirmed a preference for a cooler site, more precise studies showed optimum growth of *M. leprae* in the foot-pad at 27 – 30°C (6), with a doubling time of 12.5 days. Furthermore, from Figure 1 it can be seen that growth was correlated with inoculum dose; also yields seldom exceeded a plateau of 10⁶ AFB and multiplication tailed off as the inoculum dose rose to 10⁶ and ceased altogether above that figure.

To help strengthen the identification of the foot-pad isolates as *M. leprae*, Shep as early as 1963 (7) prepared a Mitsuda type lepromin by the standard method from a series of foot-pad harvests of a fourth passage strain of *M. leprae*. This was then compared with a standard human Mitsuda lepromin in 34 lepromatous and 30 tuberculoid patients. The reactions to the foot-pad Mitsuda exactly matched those to the human in that both were negative in all 34 lepromatous patients and all were comparably positive in all 30 tuberculoid patients (see Figure 2).

Studies on the stability of *M. leprae* were particularly encouraging for ease of handling in the laboratory. Thus it retained good viability (as infectivity) at 4°C for 7 – 10 days either within biopsies or in suspension, which can also be preserved on liquid nitrogen in the pres-

Figure 1.

The relationship between the number of acid-fast bacilli (AFB) inoculated and the number harvested at time of passage. Points falling above the heavy line marked 1X represent decreases in numbers of bacilli; those below, increases. The two marked D were associated with unfavourably delayed harvests. Those numbered 1 through 4 indicate the successive passages of the oldest strain.

ence of dimethylsulphoxide. The leprosy bacillus, like *M. tuberculosis*, is resistant to treatment with sodium hydroxide, which can therefore be used to decontaminate specimens. It was Shep's policy to retain a few strains of *M. leprae* by serial passage in mice, particularly as «standard» strains for drug testing. Some of these had been serially passaged in mice for 16-24 years, without any detectable change in pathogenicity or growth patterns in mice. Finally the stability and survival of *M. leprae* outside the body was assessed using the foot-pad model, on bacilli recovered from dried nasal discharges. Such bacilli retained some viability up to 7-10 days after discharge, with somewhat greater viability under conditions of higher humidity.

Although no differences have been reported on biological characteristics or pathogenicity in mice of *M. leprae* isolates from patients, related to their type of leprosy, race or geographic origin, Shepard observed two related characteristics that did differ among mouse foot-pad isolates. These differences related to the average rate of growth between inoculation and harvest (G) and the number of bacilli per harvest (H). These related differences are plotted in Figure 3 for 59 isolates; where for the «fast» strains the median G was < 25 days and the median H > $10^{6.1}$ and for the «slow» strains the corresponding figures were > 30 days and < $10^{5.6}$ respectively (8).

Figure 2. Skin test readings (Mitsuda, 21 days) in 30 tuberculoid patients. Correlations between antigens A and B, A and C, and B, and C. (A, foot-pad; B, and C Mitsuda preparations at 1.6 , 1.6 and 4.2×10^7 AFB/ml respectively).

Figure 3. Median harvest (H_{med} , Log_{10}) plotted against median generation time (G_{med} , days) of each of 59 strains. Where known, the sensitivity to DDS is also indicated.

The G and H values for the 59 isolated formed a continuous spectrum between the two extremes. While the two characteristics were unrelated to dapsone resistance, geographic origin of the strain (see Table 3) or type of leprosy, they were stable. Thus the particular «fast» or «slow» strain characteristic of the isolate was unchanged after repeated mouse passage and was similar in all isolates from the same patient.

Finally, from all the above biological characteristics now available, the only true strain differences relate to the faster/high plateau and slower/low plateau strains and as will be seen from the chemotherapy section, strains resistant to dapsone, rifampicin or the thioureas.

Table 3. Median generation time of strains according to geographic origin of strain.

Place infection acquired	Median generation time (days/generation)
Puerto Rico	18.2, 21.2, 23.8, 25.8, 28.3, 34.8
Other Caribbean	22.7, 33.8, 38.4, 41.5
Louisiana	23.1, 26.4, 33.0
Texas	23.3, 26.4, 27.2, 29.4, 35.2
Mexico	22.8, 24.4, 27.3, 27.9, 34.2, 37.3, 37.6, 38.8
Other Latin America	29.6, 32.6
Hawaii	26.6, 28.5
SW Pacific	26.6, 31.2
Philippines	19.5, 23.0, 24.3, 25.2, 25.4, 27.0, 28.6, 31.8, 35.2, 37.2, 41.4
SE Asia	17.7, 21.0, 24.9, 27.6, 32.6
Other Asia and Africa	32.6, 31.7, 32.2, 20.3, 22.8

APPLIED TO VACCINE INDUCED IMMUNITY

Even as early as 1965 Shepard was beginning to use the mouse foot-pad model for determining the conditions required by a vaccine for inducing maximum immunity against experimental leprosy infections in the mouse. The mouse foot-pad model would seem to be a particularly suitable one in view of the characteristic limited type of growth of *M. leprae*, which seemed to result from an infection-induced immunity.

The first observation substantiating that immunity to *M. leprae* infection could be induced in the mouse followed the study by Shepard showing that by the time an *M. leprae* infection in one foot-pad had reached plateau, there was complete immunity to the growth of an inoculum of live *M. leprae* into the other hind foot-pad. There followed a large series of «vaccination studies» by Shepard which followed a standard pattern. One foot-pad was used for the challenge dose of live *M. leprae* 28 days after vaccination and the results were determined from counts on vaccinated and unvaccinated animals killed some 6 months and at least 9 months after challenge. The second, later harvest was done to distinguish delayed growth from sustained protective immunity, which it proved to be.

The results are given in Figures 4, 5 and 6, which, summarised, showed:

1. Live (Figs. 4, 5 and 6) (9) but not heat killed BCG (10) (M, Figure 5) gave good protection, whereas live or killed *M. leprae* were equally effective (10) (Figs. 4 and 5) and comparable to BCG.

2. For BCG and *M. leprae* vaccines the intradermal route resulted in significantly better protection than following the subcutaneous route (Figs. 4 and 5).
3. The immunogenicity of *M. leprae* was not only heat stable but a heated *M. leprae* vaccine gave somewhat better protection than an unheated one (Fig. 5) (10).
4. Vaccines prepared from *M. chelonae*, *M. diernhoferi*, *M. nonchromogenicum*, *M. phlei*, *M. smegmatis* and *M. vaccae* failed to give protection (Fig. 6) (11).

Figure 4. Results of vaccination of mice with BCG and *M. leprae*. The *M. leprae* (ML) vaccine dosage was 3×10^6 bacilli; the BCG dosage was 1×10^7 bacilli. The vaccines were given into the foot-pads (FP) or intradermally (ID) 28 days before challenge.

Figure 5.

Results of harvests: - for each vaccine, the left column gives the results at 151 days and the right gives the results at 241 days. F + T = freezing and thawing; H 60 = heating at 60°C for 30 mins; AUTO = autoclaving for 15 mins.

APPLIED TO CHEMOTHERAPY

Table 4 shows how successfully and extensively Shepard was able to apply the mouse foot-pad model to many aspects of the chemotherapy of leprosy, having demonstrated as early as 1962 that the growth of *M. leprae* in the foot-pads was inhibited by dapsone (DDS) and other anti-leprosy drugs administered to mice in their diet (12). In fact Shep's contributions to the field of chemotherapy with a total of 55 publications were even greater than his work on the bacteriology and immunology of *M. leprae* with 44 publications. Although this side of Shepard's research in leprosy is of less relevance to this Symposium, Shep undoubtedly rated it of equal importance.

The mouse foot-pad model was applied and modified to study, on the one hand, the activities and kinetics of known or new potential anti-leprosy drugs or, on the other hand, to measure the efficacy of chemotherapy or emergence of drug resistance in leprosy patients. The former studies on drugs are carried out in mice where the drugs at various doses or intervals are administered, and also where possible the drug serum levels are measured. By measuring the serum levels the minimal effective dose (MED) and minimal inhibitory concentration (MIC) of each drug can be calculated. If instead of administering the drug continuously to mice after infection, it is started later (usually 70-80 days, when some 6 generations of *M. leprae* will have occurred), this so-called kinetic method can be used to distinguish be-

Figure 6.
Harvests of *M. leprae* from mice vaccinated with various mycobacterial cultures. The challenge was with 5×10^3 *M. leprae* 28 days after vaccination. P* values: 0, > 0.05 ; 1, 0.01 to 0.05; 2, 0.001 to 0.009; 3, > 0.001 . B = BCG, Rosenthal; C = BCG, Trudeau; D = *M. chelonae*; E = *M. diernhoferi*; F, G, H = *M. nonchromogenicum* strains 269, 383 and 1481 respectively; I = *M. phlei*; J = *M. smegmatis*; K, L, M = *M. vaccae* 296, 426 and 1526 respectively.

tween bacteriostatic and bactericidal drugs (13). The distinction is made by determining whether plateau growth in the treated mice is reached at the same time or later than in the untreated mice, and if later, whether the delay exceeds the 70 – 80 days. If no delay, the drug is inactive, if up to 70-80 days only it is bacteriostatic or only if significantly beyond 70 – 80 days is it bactericidal. A composite picture of this type of data is shown in Table 5.

Table 4. Shepard's contributions applied to the chemotherapy of leprosy based on 55 publications.

Subject	No. of publications
<i>Methodology</i> : including kinetic method to distinguish bacteriostatic / bactericidal activity; drug resistance.	5
<i>Drug screening</i> : 50 compounds assessed, including kinetic method.	10
<i>Dapsone</i> (DDS)) * MED;	5
<i>Acedapsone</i> (DADDS; repository)) MIC;	10
<i>Rifampicin</i>) Kinetic	5
<i>Clofazimine</i>) assessment	5
<i>Dapsone resistance</i>) blood levels	5
<i>Clinical trials</i> : including clofazimine; rifampicin; DDS in Cebu and San Francisco and particularly DADDS therapeutic (N. Guinea) and prophylactic (Micronesia) trials.	10

* MED = minimal effective dose. MIC = minimal inhibitory concentration.

Table 5. Sensitivity characteristic of *M. leprae* to eight anti-leprosy drugs.

Drug	Minimal effective dose (MED) (% in diet)	Minimal inhibitory concentration (MIC) ($\mu\text{g/ml}$ serum)	Bactericidal activity
Rifampicin	0.003	0.3	High
Dapsone (DDS)	0.0001	0.003	Low
Ethionamide	0.01	0.05	Intermediate
Prothionamide	0.01	0.05	Intermediate
Clofazimine	0.0003	— *	Low
Thiacetazone	0.03	0.2	None
Thiambutosine	0.05	0.2	None
Sulphamethoxy-pyridazine	0.1	30	None

* Estimate of MIC is not possible because of deposition of drug in tissue.

On the other hand the efficacy of a particular anti-leprosy drug or regimen in the treatment of patients can be measured by following the viability (infectivity) of their bacilli from biopsies of skin, by mouse foot-pad inoculation. This type of data is shown in Figure 7 from patients receiving rifampicin, DDS, clofazimine or acedapsone (DADDS). The data particularly show the rapid bactericidal effect of rifampicin (14)

Figure 7.

Comparative duration of therapy before loss of bacterial viability detectable by mouse inoculations. Each line refers to a patient. Results are shown only for the last positive and the next negative specimen. Drug dosages: – Rifampin, 600 mg daily; Dapsone, a dosage ascending to 50 mg daily in a few weeks; Clofazimine, 200 mg daily; Acedapsone, 225 mg every 11 weeks.

In order to make sure that the relevant foot-pad techniques were used to the full in chemotherapeutic trials in leprosy, Shepard and his team actively participated in such trials of rifampicin, clofazimine (15) and especially of the injectable repository sulphone, acedapsone, DADDs (a single 225 mg dose releasing 2.4 mg DDS/day, is administered every 77 days) (16, 17). Shepard's special interest in DADDs was on the basis of its slow but steady release of DDS at a therapeutic level, for which he hoped it could be used for the treatment of patients in remoter areas, obviating frequent visits (in New Guinea) (16) and also as a prophylactic (in Micronesia (16, 17)). While the results of the therapeutic trial were promising, if DADDs was introduced routinely for more remote areas, supervised monthly rifampicin would have to be included. However, as a prophylactic given for 3 years, except during the first 6 months, new cases were reduced to zero. Therefore in a heavily exposed population DADDs prophylaxis together with the energetic treatment of lepromatous cases would be expected very significantly to reduce the incidence of leprosy in an area.

Finally, but no means least of Shep's contributions to the chemotherapy of leprosy was his almost continuous drug screening programme in which he assessed the anti-leprosy activity of some 50 compounds. Many of these were new drugs or new or modified series of

known anti-leprosy drugs arising from the Drug Development Sub-Committee of the Therapy of Leprosy (THELEP) Scientific Working Group (18). This contribution is yet another example of the enthusiastic dedication of Shep for any studies which might advance the treatment and control of leprosy.

Shepard's contribution to the field of DNA technology as applied to *M. leprae*

While no one, however remotely associated with the treatment and prevention of leprosy, could be unaware of Shepard's dominating contributions to these fields, few may be aware of the leading role he played in first stimulating interest in, and then initiating studies on, DNA technology applied to *M. leprae*.

As early as 1977 Shepard in appreciating the revolutionary developments in microbiology resulting through DNA recombination and other methods of genetic exchange with cultivable organisms considered that they might similarly revolutionize studies on the leprosy bacillus, without it being cultured *in vitro*. He was probably encouraged by the then recent progress made in the successful extraction and purification methods providing milligram quantities of *M. leprae* from infected armadillo tissues, which would provide adequate quantities of specific DNA. Anyhow, in order to even investigate these possibilities for *M. leprae*, particularly because there was very little genetic or DNA information even on any cultivable species of mycobacteria, Shep considered that the first step would be to organise a workshop on «Genetics of *M. leprae*», by bringing together authorities on the microbiology and taxonomy of mycobacteria with those in the advancing fields of basic recombinant DNA research. With Shep's enthusiasm and drive a two day workshop was held in September 1977 in Boston under the joint chairmanship of Charles Shepard and Roy Curtiss.

Undoubtedly the calling of this workshop by Shep and its resulting recommendations on the feasibility of applying modern recombinant DNA technology to *M. leprae* in view of the quantities of *M. leprae* DNA which should be obtainable from armadillo derived and purified organisms, led to Roy and Josephine Curtiss being funded jointly by the IMMLEP/THELEP Steering Committees from 1979.

The joint studies by these two were initiated with *M. leprae* derived from armadillos from IMMLEP with Shep's assistance and initially were used to develop methods for the successful isolation of adequate quantities of DNA. From then onwards a series of rapidly advancing technical publications by Roy & Josephine Curtiss and colleagues (19, 22) have resulted on the specific genome size of *M. leprae* distinguishing it from other species of mycobacteria as well as genomic libraries of *M. leprae* resulting basically using *E coli* K-12 as the host vector. It is pleasing to see that one of these publications (22) is dedicated to Shepard.

Finally I learned very recently during a telephone call with Laura Walker, one of Shep's assistants, that as recently as October 1984 he had with great energy and pleasure embarked on a major collaborative research programme with Molly Kelly on the taxonomy of mycobacteria based on DNA-DNA homology studies. Shep looked on this programme as only a beginning to a new phase of interest in his research career!

References

- 1 Shepard CC, Jung RW, Colwell CA, Gunn FD, Barth EA. Complement fixation in tuberculous dogs. *Am Rev Tuberc*, 1941, **43**, 825-38.
- 2 Gunn FD, Mills MA, Shepard CC, Barth EE. Experimental pulmonary tuberculosis in the dog. *Am Rev Tuberc*, 1943, **47**, 78-96.
- 3 Shepard CC. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. *J Exp Med*, 1960, **112**, 445-54.

- 4 Shepard CC. Acid-fast bacilli in nasal excretions in leprosy and results of inoculation of mice. *Am J Hyg.* 1960, **71**, 147-59.
- 5 Fenner F. The pathogenic behavior of *Mycobacterium ulcerans* and *Mycobacterium balnei* in the mouse and the developing chick embryo. *Am Rev Tuberc.* 1956, **73**, 650-73.
- 6 Clark HF, Shepard CC. Effect of environmental temperatures on infection with *Mycobacterium marinum* (balnei) of mice and a number of poikilothermic species. *J. Bacteriol.* 1963, **86**, 1057-69.
- 7 Shepard CC, Guinto RS. Immunological identification of foot-pad isolates as *Mycobacterium leprae* by lepromin reactivity in leprosy patients. *J Exp Med*, 1963, **118**, 195-04. (Reprinted in: *Internat. J. Leprosy* 1963, **31**, 353-62).
- 8 Shepard CC, McRae DH. Hereditary characteristic that varies among isolates of *Mycobacterium leprae*. *Infect. Immun.*, 1971, **3**, 121-26.
- 9 Shepard CC. Vaccination of mice against *M. leprae* infection. *Internat. J. Leprosy*, 1976, **44**, 222-26.
- 10 Shepard CC, Walker LL, Van Landingham R. Heat stability of *Mycobacterium leprae* immunogenicity. *Infect. Immun.*, 1978, **33**, 87-93.
- 11 Shepard CC, Van Landingham R, Walker LL. Searches among mycobacterial cultures for antileprosy vaccines. *Infect. Immun.*, 1980, **29**, 1034-39.
- 12 Shepard CC, Chang YT. Effect of several anti-leprosy drugs on multiplication of human leprosy bacilli in foot-pads of mice. *Proc Soc Exp Biol Med*, 1962, **109**, 636-38.
- 13 Shepard CC, Walker LL, Van Landingham RM, Redus MA. Kinetic testing of drugs against *Mycobacterium leprae* in mice. Activity of cephaloridine, rifampin, streptovaricin, vadrine, and viomycin. *Am J Trop Med Hyg*, 1971, **20**, 616-20.
- 14 Shepard CC, Levy L, Fasal P. Further experience with the rapid bactericidal effect of rifampin on *Mycobacterium leprae*. *Am J Trop Med Hyg*, 1974, **23**, 1120-24.
- 15 Collaborative effort of the US Leprosy Panel (US-Japan Cooperative Medical Science Program) and the Leonard Wood Memorial. Spaced clofazimine therapy of lepromatous leprosy. *Am J Trop Med Hyg*, 1976, **25**, 437-44.
- 16 Russell DA, Worth RM, Scott GC, Vincin DR, Jano B, Fasal P, Shepard CC. Experience with acedapsone (DADDS) in the therapeutic trial in New Guinea and the chemoprophylactic trial in Micronesia. *Internat J Leprosy*, 1976, **44**, 170-76.
- 17 Russell DA, Worth RM, Jano B, Fasal P, Shepard CC. Acedapsone in the prevention of leprosy; Field trial in three high prevalence villages in Micronesia. *Am J Trop Med Hyg*, 1979, **28**, 559-63.
- 18 Shepard CC, Van Landingham RM, Walker LL. Recent studies in antileprosy drugs. *Lepr Rev*, 1983, **54**, 23s-30s.
- 19 Young RA, Mehra V, Sweetser D, Buchanan T, Clark-Curtiss J, Davis RW, Bloom BR. Genes for the major protein antigens of the leprosy parasite *Myco leprae*. *Nature* 1985, **316**, 450-52.
- 20 Clark-Curtiss JE, Jacobs WR, Docherty MA, Ritchie LR, Curtiss R III. Molecular analysis of DNA and construction of genomic libraries of *Myco leprae*. *J Bacteriol*, 1985, **161**, 1093-02.
- 21 Jacobs WR, Barrett JF, Clark-Curtiss R III. In vivo repackaging of recombinant cosmid molecules for analyses of *Salmonella typhimurium*, *Streptococcus mutans* and mycobacterial genomic libraries. *Infect. Immun.* 1986, **52**, 101-09.
- 22 Jacobs WR, Docherty MA, Curtiss R III, Clark-Curtiss JE. Expression of *Mycobacterium leprae* genes from a *Streptococcus mutans* promoter in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA*, 1986, **83**, 1926-30.