THE THYMECTOMIZED-IRRADIATED MOUSE AND THE NEONATALLY THYMECTOMIZED RAT IN THE EXPERIMENTAL CHEMOTHERAPY OF LEPROSY

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Application of the Thymectomized-irradiated Mouse to the Detection of Persisting *Mycobacterium leprae*

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Inoculation, either systemically or locally, of immunologically deficient rodents with *Mycobacterium leprae* results in a greater maximum of multiplication, multiplication after inoculation of a larger number of *M. leprae,* dissemination of the infection, prolonged survival of the organisms, and grossly evident lesions in a variable proportion of the animals studied. Most of the published work has involved the adult-thymectomized, lethally irradiated, and bone-marrow reconstituted (T900R) mouse originally employed by Rees (2).

Virtually all of the work with this experimental animal has been carried out at the National Institute for Medical Research (NIMR) in London. During the early years, the radiation was administered in a single dose of 900 rad; because this dose is sufficient to destroy the functioning bone marrow, it was necessary to transfuse the mice with syngeneic bone marrow immediately after irradiation. In those years, T900R mice survived well in clean, but otherwise conventional, animal-house conditions. In more recent years, T900R mice have not always survived well at the NIMR. To improve survival, the dose of radiation is delivered in five doses of 200 rad each over a period of eight weeks. This fractionated dose of radiation is less destructive to the bone marrow, and "T200X5R" mice do not require bone-marrow transfusion. They are not as immune deficient as T900R mice, a fact that probably accounts for their better survival.

The T900R or T200X5R mouse is most frequently employed for the detection of persisting *M. leprae.* Immunologically intact mice inoculated in the hind foot pad with 5000–10,000 *M. leprae* are not useful for the detection of persisting *M. leprae,* at least early in the therapy of the lepromatous patient. The proportion of viable organisms (persisters) is so small that a larger inoculum ($\geq 10^5$) is required to detect them. In immunologically intact mice, an inoculum of this size might well immunize the animals, so that the viable *M. leprae* contained in the inoculum are unable to multiply. On the other hand, animals that are sufficiently immune deficient are not immunized by the large inocula required for the demonstration of persisters, and should permit multiplication of viable *M. leprae,* even when these represent only a minute proportion of the inoculum.

Preparation of thymectomized-irradiated (TR) mice. In our laboratory, female CBA mice are subjected to thymectomy when the mice are between six and eight weeks of age. The techniques described below are based upon our experience, and modifications may be required when mice of different strains or different ages are employed.

Mice are anesthetized with tribromoethyl alcohol (Avertin®), a stock solution of which is prepared by dissolving the material in amylene hydrate (tertiary amyl alcohol) in a ratio of two volumes of Avertin to one volume of amylene hydrate. This stock solution is stable at room temperature for many months, when it is stored in the dark. For use, 0.1 part of the stock Avertin solution is added dropwise with shaking to a solution of 0.4 parts of ethanol in five parts of physiological saline. The anesthetic is administered intraperitoneally in a dose of 0.01 ml per g body weight; deep anesthesia is obtained within five minutes. If excessive bronchial secretion is encountered during the anesthesia, this can be controlled by the intraperitoneal administration of 0.1 ml atropine. A stock solution of atropine is prepared by adding two parts of atropine sulfate to five parts of saline; the stock solution is diluted 100-fold with saline for use.

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For thymectomy, the anesthetized mouse is restrained on a cork board by elastic bands and pins. The fur of the anterior chest is swabbed with 70% ethanol, and a mid-line incision of the skin is made from below the chin to just below the manubrium. The trachea is then exposed by blunt dissection, and a 1–2-mm incision is made through the manubrium to expose the thymus. After the thymus has been exposed, it is removed by suction, employing a Pasteur pipette, the aperture of which has been enlarged, and a water aspirator. Some trials are required to arrive at the optimal pipette aperture and vacuum. Because the entire gland may be removed *in toto,* or each lobe may be removed separately, it is essential to examine the area to ensure that both lobes of the thymus have been removed. Following surgery, the skin is closed by means of a half-curved no. 19 needle and black braided silk suture R2492 (Sutures Ltd., U.K.).

Two weeks after thymectomy, the mice are irradiated. As noted above, two methods of irradiation have been employed. Total body irradiation may be administered in a single dose of 900 rad from a therapeutic X-ray generator or a gamma ray source, either cobalt (^{60}Co) or caesium (^{137}Cs), of the type employed in radiotherapy. The time of exposure required will depend on the available source and apparatus. Immediately after irradiation, the mice are transfused with syngeneic bone-marrow cells obtained from female CBA mice 6–8 weeks of age. The bone-marrow cells are obtained by cutting the ends off the donor femurs and tibias, and washing out the plugs of marrow with Hanks' balanced salt solution. The plugs are dissociated by pressing them through a sterile wire mesh, and each irradiated recipient mouse is administered $1–2 \times 10^6$ nucleated cells. The bone-marrow cells obtained from one donor are usually sufficient for three recipients. To reduce the risk of intercurrent infection following irradiation, TR mice are administered oxytetracycline (Terramycin®) in a concentration of 125 mg per liter in the drinking water. Oxytetracycline is administered for three weeks, with the solution being renewed daily. After an additional week, during which oxytetracycline-free drinking water is administered, the mice are ready for use.

In spite of the precautions just described, many laboratories have experienced excessive mortality of T900R mice. A satisfactory alternative is the T200X5R mouse. Two weeks after thymectomy, the mice are exposed to total body irradiation in a dosage of 200 rad every two weeks for five doses. Two weeks after the course of irradiation has been completed, the mice are ready for use. Because these mice do not require transfusion of syngeneic bone-marrow cells, outbred mice may be used.

TR mice have been depleted of T cells. Not only are these mice more susceptible to infection with *M. leprae,* they are also more susceptible to infection with other organisms. Therefore, although the requirements for husbandry of TR mice are less stringent than those for nude mice, they should be maintained under the cleanest conditions possible.

Husbandry of TR mice. As has already been described, these animals are all immune deficient to a degree; therefore, their successful husbandry imposes requirements in addition to those for husbandry of good quality immune-competent mice. These additional requirements derive from the need to protect these animals from intercurrent infection by organisms non-pathogenic for normal mice, but capable of producing fatal illness in immune-deficient mice. All of the requirements listed in the discussion of husbandry of the immune-competent mouse must be met. Immune-deficient mice must be derived from SPF if not from germ-free stock. Having started with sufficiently clean animals, one must maintain them in the same condition. It is particularly important that immune-deficient mice be protected from contact with all other rodents, including not only wild rodents, but also those brought in from another laboratory, and even those immune-competent mice raised in the same laboratory, unless one can be assured of their cleanliness.

Not only must the animals be protected from contact with other rodents, but also from infection by other agencies, such as infected animal handlers, air, food, water and bedding. Animal handlers suffering from infections of the skin or respiratory tract should not enter the quarters in which immune-deficient mice are housed. Clothing,

including foot wear, in which one has entered other animal quarters must not be worn in the quarters housing the TR mice.

To prevent infection by air-borne organisms, immune-deficient mice should be housed in rooms in which the air pressure is maintained at a higher level than that in the immediate surroundings, so that entry into the room is not accompanied by intake of air from the surrounding animal quarters. Air should be taken in by a system of ducts and blowers directly from the outside, preferably through a system of filters. Under no circumstances should ventilation be accomplished by an exhaust fan, which creates a vacuum in the room, allowing air to flow in from adjacent rooms and corridors. The risk of air-borne infection can be further reduced by housing the animals in cages fitted with filter caps, or by placing the cages in laminar-flow racks, in which filtered air is directed outward from behind the cages.

The certain means of preventing food-borne infection is to sterilize the food. Sterilization may be accomplished by one of two means—irradiation and autoclaving. If the animal diet can be irradiated, this is preferred. Exposure of the diet to the heating involved in autoclaving is destructive to many of the vitamins, which must be replaced—by addition either to the diet or the drinking water. Needless to say, sterilization must be carried out with the food sealed in plastic (not suitable for autoclaving) or tough paper or cloth bags, which will prevent subsequent entry of vermin during storage. Moreover, because storage at ambient temperature will also result in loss of vitamins, the time between manufacture of the diet and its use must be minimized. When the diet must be stored for longer than a few weeks, storage should be carried out at a temperature below 10°C.

Water should be sterilized by autoclaving. This may be most conveniently accomplished by autoclaving the water in the drinking bottles. Adequate chlorination of the water may suffice as a substitute for autoclaving, and acidification of the sterile water will help to limit contamination of the water during use, which is inevitable unless one is working under germ-free conditions. As an aid in maintaining immune-deficient animals, oxytetracycline may be administered in the drinking water. Moreover, treatment of the animals for periods of a few weeks with drinking water to which have been added mixtures of antibiotics that are not absorbed from the gastrointestinal tract has been proposed ([1]) as a means of decontaminating immune-deficient animals.

Bedding, sealed in vermin-proof paper or cloth bags, should also be autoclaved before use.

Under the conditions prevailing at the NIMR, the mortality among T200X5R mice is 15–20% during the first year.

Detection of persisting *M. leprae*. To demonstrate persisting *M. leprae*, a lesion is biopsied, a bacterial suspension is prepared with minimal dilution, so as to obtain the largest possible inoculum, and 10^5 *M. leprae* are inoculated into the hind foot pads of TR mice. A ten-million-fold increase—i.e., from 1 to 10^7—represents 23 doublings; at an average rate of one doubling per two weeks, 46 weeks (about one year) should be a time sufficient for unequivocal multiplication of any viable *M. leprae* present in the inoculum. Thus, one year or more after inoculation, harvests are performed and the *M. leprae* counted.

Because the demonstration of persisting organisms is commonly attempted after the patient has been treated for some considerable duration (at least one year), by which time the BI has decreased, and only small numbers of *M. leprae* can be recovered from the biopsy specimen, it may be difficult to prepare a concentrated inoculum. To maximize the number of organisms that can be inoculated, recovery of the organisms from the biopsy specimen must be carried out with minimal dilution.

All procedures are carried out using sterile materials in a microbiological safety cabinet. First, the biopsy specimen is trimmed of fat and weighed, to determine the volume of suspending fluid (0.1% BSA in water) required, as shown in Table 1. The tissue is then thoroughly minced with small curved scissors and transferred in its entirety, by scraping up with the scissors, to the lower end of the piston of a tissue grinder that has been immersed in ice water. A grinder of 15-ml capacity is employed for specimens to be suspended in 2 ml. The minced tissues

TABLE 1. *Dilution of biopsy specimens for inoculation of immune-deficient mice.**

Weight of specimen (g)	Volume of suspending fluid (ml)
0.04	0.5
0.05–0.07	0.8
0.08–0.10	1.0
0.11–0.15	1.5
0.16–0.20	2.0
0.21–0.25	2.5
0.26–0.30	3.0

* Adapted from reference no. 3.

are very thoroughly homogenized, and smears are prepared as described as for other biopsy specimens. Although the resulting homogenates are dense, they are adequate, without further dilution, for visualizing AFB in the stained smears, and for inoculation of foot pads. The homogenates are inoculated into mice whether or not they are seen to contain AFB. The inoculum is 0.03 ml per food pad; groups of eight or more mice are inoculated in both hind foot pads. Harvests of *M. leprae* are performed from the foot pads of mice 12 months later.

Deciding whether or not the organisms have multiplied may be difficult, as demonstrated in the examples presented in Table 2. In Example 1, it is obvious that no viable organisms were detected; therefore, the proportion of viable *M. leprae* in the original suspension must have been smaller than 1 in 10^5. In fact, because a total of 16 foot pads were inoculated, a total of 1.6×10^6 organisms was inoculated; thus, one may argue that the proportion of viable organisms was smaller than 1 in 10^6 *M. leprae.*

In the situation depicted in Example 2, there is unequivocal evidence that the *M. leprae* multiplied in two of the 16 foot pads inoculated (i.e., two foot pads show an increase greater than 10-fold the number inoculated).

The problematic situation is that depicted in Example 3. Here, acid-fast bacilli were detected in harvests made from three foot pads. In two of the foot pads, however, the number of organisms detected was smaller than the number inoculated, and, in the third foot pad, the number had increased by only 2.5-fold. In this situation, we usually passage the organisms contained in the suspension providing the largest number of organisms (designated by the * in Table 2) into the hind foot pads of five normal mice, inoculating no more than 10^4 organisms per foot pad. Harvests are performed from the passage mice 12 months later; an increase of bacterial numbers to at least 10^5 per foot pad in at least one foot pad is taken as evidence that viable *M. leprae* were present in original inoculum.

In fact, in the analysis of the mouse footpad data resulting from inoculation of TR mice in the course of the THELEP controlled clinical trials in Bamako and Chingleput, *M. leprae* were determined to have multiplied (i.e., persisters were detected) only if the increase of organisms was at least 10-fold, and at least to 10^5 per foot pad in those instances in which no more than 10^4 organisms had been inoculated, or if organ-

TABLE 2. *Examples of the results of inoculating the foot pads of TR mice with suspensions of* M. leprae *that contain small proportions of viable organisms.*

Mouse no.	Foot	No. organisms per foot ($\times 10^4$)	Mouse no.	Foot	No. organisms per foot ($\times 10^4$)
		Example 1			
1	L	<1	5	L	<1
	R	<1		R	<1
2	L	<1	6	L	<1
	R	<1		R	<1
3	L	<1	7	L	<1
	R	<1		R	<1
4	L	<1	8	L	<1
	R	<1		R	<1
		Example 2			
1	L	<1	5	L	<1
	R	<1		R	<1
2	L	320	6	L	<1
	R	<1		R	<1
3	L	<1	7	L	<1
	R	<1		R	<1
4	L	<1	8	L	<1
	R	<1		R	750
		Example 3			
1	L	<1	5	L	<1
	R	<1		R	<1
2	L	<1	6	L	6.4
	R	<1		R	<1
3	L	24*	7	L	<1
	R	<1		R	<1
4	L	3.2	8	L	<1
	R	<1		R	<1

isms harvested from the foot pads of TR mice multiplied upon passage to the foot pads of normal mice.

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