# Evaluation of the Immune Response in Mice Infected With *Mycobacterium marinum* [1,2]

Ramchandra G. Navalkar and Parsottam J. Patel [3]

*Mycobacterium marinum* is a photochromogenic mycobacterium capable of inducing a self-limiting localized infection within the mouse foot pad ([1,4]). Because this infection is analogous to the *M. leprae* infection in the mouse, it has been suggested as an alternative experimental model for studying the role of host immunity to *M. leprae* infection in the mouse foot pad ([12,16,19]). Present studies were undertaken with a view to studying the humoral immune response in mice infected with *M. marinum*. This study is a parallel to previous studies of the immune response in mice infected with *M. leprae* ([11]), and *M. lepraemurium* (unpublished data).

## MATERIALS AND METHODS

**Mice.** Six to eight week old female inbred strains of BALB/c mice were used throughout the experiments. These mice were bred and raised in the laboratory of Dr. L. Levy, USPHS Hospitals, San Francisco.

**Infection.** Mice of one group were inoculated in the right hind foot pad, each with $5 \times 10^3$ *M. marinum* (Runyon strain 691) harvested from six week's growth of the organism on Sauton medium ([14]) maintained at 30°C under static conditions. Another group was used as control. Fifteen days after the primary infection, some of the mice from the first group were injected a second time with the same number of viable cells in the left hind foot pad.

**Collection of splenic cells.** Four mice from each group were sacrificed at intervals beginning the third day after infection and continuing up to day thirty. The spleens were removed, and the splenic cells were teased into Hanks balanced salt solution (HBSS) with sterile blunt forceps. The resulting suspension was passed through sterile 20 to 27 gauge needles until a homogenous, single cell suspension was obtained. The suspensions were centrifuged in the cold at 600 × g for five minutes, and the sediments were resuspended in fresh, sterile HBSS to obtain a cell count of $10^6$/ml. Splenic cell suspensions were stored at 4°C until used.

**Antigen-coated sheep erythrocytes.** Antigen was conjugated to sheep erythrocytes with carbodiimide reagent ([9]) by the modification of the method of Johnson *et al* ([7]), and described by Golub *et al* ([6]). Five ml of 50% sheep erythrocytes (Servecel-Texas Biological Laboratories, Fort Worth, Texas) were washed three times in conjugation buffer and resuspended in the same volume of buffer. To 1 ml of this suspension, 0.5 ml of a solution of 200 mg of 1-ethyl-3-(3-di methyl-aminopropyl) carbodiimide per ml (Ott Chemical, Muskegon, Michigan) and 1 ml of the antigen were added and the mixture was kept at 4°C for one hour. The antigen-coated sheep erythrocytes were washed three times with the buffer, resuspended to obtain a concentration of 50% and stored at 4°C until used. The antigen used in these assays was the cell extract derived from *M. marinum*. The cell extract was prepared by washing the *in vitro* grown cells in sterile isotonic saline three times. After the third washing a known amount of cells were suspended in 0.15 M sterile saline to obtain a concentration of 12 mg/ml. After homogenization in a Teflon grinder, the suspension was transferred to a sterile flask and passed through the Refrigerated Cell Fractionator (Sorvall). The cells were disrupted at 40,000 psi and the resultant cell extract was used for coating of the sheep erythrocytes.

**Hemolytic plaque assay.** The hemolytic plaque assay has been described previously ([11]). In brief, the assays were performed in 10 cm diameter sterile plastic dishes contain-

[1] Received for publication 19 October 1976.

[2] This investigation was supported by the U.S. Leprosy Panel of the U.S.-Japan Cooperative Medical Science Program, administered by the Geographic Medicine Branch, National Institute of Allergy and Infectious Disease (Grant R22 AI-08647).

[3] R. G. Navalkar, Ph.D., Professor, and P. J. Patel, Ph.D. Candidate, Department of Microbiology, Meharry Medical College, Nashville, Tennessee 37208. P. J. P.—Present address: Trudeau Institute, Inc., Saranac Lake, New York 12983.

ing a base agar layer of 2.8% Noble agar mixed with an equal volume of 2x Hanks tissue culture medium (Difco). The overlay agar was prepared at a concentration of 1.4% in distilled water and mixed with an equal volume of 2x Hanks tissue culture medium.

**Direct plaque assay.** One-tenth milliliter of splenic cell suspension was poured onto the base agar layer. Diethylamine ethylene dextran in a concentration of 0.85 mg/ml was added to the melted top agar layer to block any anticomplementary activity of the agar. Antigen-coated sheep erythrocytes were added to the agar in a concentration of 0.1 ml/ml of agar; this mixture was then overlayed on the cell suspension and mixed to form a monolayer. This mixture was allowed to harden, and the plates were incubated at 37°C for one hour. Then the plates were removed and 2 ml of a 1:10 dilution of freshly reconstituted guinea pig complement (Texas Biological Laboratories, Inc. Fort Worth, Texas) was added. The plates were reincubated at 37°C for one to two hours and the resulting clear plaques were counted under a stereo microscope.

**Developed plaque assay.** It is generally accepted that the plaques appearing after the addition of complement indicate cells producing IgM antibodies. Sterzl and Riha [21], and Dresser and Wortis [2] have shown that plaques can be obtained with cells producing IgG antibody if the system is amplified by the addition of antibody to IgG. In the present study, indirect plaques were detected by the addition of commercially obtained antisera to mouse immunoglobulins. The assays were carried out employing the procedure described above, except that 1 ml amounts of 1:40 dilution of the antiglobulin serum were added after the first incubation and the plates were incubated again for one hour prior to the addition of the complement.

**Immunoglobulin assay.** Commercially obtained (Meloy Laboratories, Bethesda, Maryland) radial immunodiffusion plates impregnated with monospecific mouse antiglobulins were used for this assay. A standard reference curve was first drawn using various dilutions of mouse sera with known immunoglobulin concentrations. Wells in each plate were filled with dilutions of standard sera and incubated in a humid atmosphere for 18 hours at room temperature. The diameter of precipitation rings around each well was measured using the standard magnifier and recorded. The results were plotted on a semilog graph paper and the best straight line between the points was drawn. This served as a reference curve for the immunoglobulin assay of the test and control sera.

**Hemagglutination studies.** These studies were carried out to determine the presence of both the IgM and IgG antibodies. The HA titers were assayed by the micro-hemagglutination method. Serial twofold dilutions of the test and control sera were made and an equal amount of antigen coated (50 $\mu$l) sheep erythrocytes were added to each well. The plates were incubated at 37°C for 30 minutes and then maintained in cold at 4°C overnight. The titers were read and recorded thereafter. For the IgG antibody assay, the sera were mixed with an equal volume of 0.2 M 2-mercaptoethanol and left overnight at 4°C. The treated sera were then diluted twofold and tested for agglutinins.

## RESULTS

The experiments were designed to determine the kinetics of the primary and secondary immune response at the humoral level of mice infected with *M. marinum.*

**Plaque forming cell (PFC) assay.** Figure 1 represents the change with time of the number of PFC in the infected and the control mice. Each point in the graph gives the average number of PFC from four animals and for two different experiments. In the primary

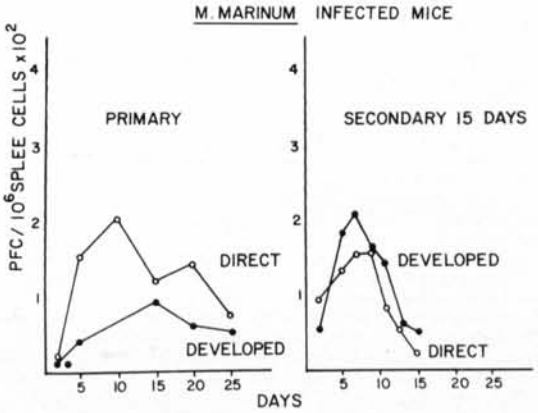


FIG. 1. Primary and secondary immune responses in mice inoculated with $5 \times 10^3$ viable *Mycobacterium marinum* in the right hind foot pad, as measured by the PFC response of spleen cell suspension.

infected group, the direct plaques were detected as early as the third day post-infection with a subsequent increase that peaked on day ten and thereafter declined as infection progressed. The developed plaques on the other hand, although seen at about the same time as the direct plaques, peaked at about the 15th day post-infection and declined thereafter. The magnitude of developed plaques was considerably lower than the direct plaques. The same figure gives the plaques observed in mice given a secondary challenge at 15 days. In this instance, both the direct and developed plaques appeared within a short time but the developed plaques reached a peak in about 6 days after the secondary challenge, which was higher than that observed in the direct plaques. However, the decline in both these at about 12 days after secondary challenge was identical.

**Immunoglobulin assay.** Table 1 gives the immunoglobulin levels in mice infected with *M. marinum* and in normal mice. The table indicates that in the infected mice given only a single challenge, the concentrations of IgM and IgG are considerably higher than those in the normal mouse, whereas the concentration of IgA appears to be constant. In the primary infected animals, both IgG and IgM levels increase appreciably with the course of infection. In the animals given a secondary challenge, the level of IgG is considerably higher than that seen in the primary infected mice, but the level of IgM appears to remain comparatively the same. These data appear to be in concordance with the detection of greater number of developed plaques in those animals that received a secondary challenge.

**Hemagglutination studies.** Table 2 gives the results of the hemagglutination studies which are expressed as the last reciprocal dilution in a twofold dilution system showing a positive reaction. Plasma collected from age-matched normal mice and tested simul-

TABLE 1. *Immunoglobulin levels in mice infected with* M. marinum.[a]

| Days post-infection | Normal mice | | | *M. marinum* infected mice | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Primary infection | | | Secondary infection | | |
| | IgA | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG |
| 3 | 32 | 14 | 230 | 37 | 36 | 560 | 37 | 52 | 690 |
| 5 | 40 | 16 | 235 | 43 | 38 | 580 | 47 | 56 | 700 |
| 10 | 56 | 18 | 238 | 37 | 40 | 560 | 32 | 40 | 605 |
| 15 | 68 | 26 | 250 | 62 | 40 | 610 | 78 | 52 | 780 |
| 30 | 78 | 24 | 224 | 77 | 52 | 760 | — | — | — |

[a]Values expressed in mg%.
— = Not Done
IgA = Immunoglobulin A
IgM = Immunoglobulin M
IgG = Immunoglobulin G

TABLE 2. *Hemagglutinin titers in plasma of* M. marinum *infected mice.*[a]

| Days post-infection | Primary infection | | Secondary infection | |
|---|---|---|---|---|
| | IgM | IgG | IgM | IgG |
| 3 | 8 | 0 | 16 | 64 |
| 5 | 16 | 4 | 16 | 64 |
| 10 | 8 | 32 | 8 | 32 |
| 15 | 16 | 32 | 8 | 32 |
| 30 | 32 | 64 | 8 | 32 |

[a]Results expressed as the last reciprocal dilution in a twofold dilution system showing a positive agglutination reaction. Simultaneously tested plasma from age-matched normal mice gave no visible precipitin reactions.

taneously gave no positive reactions. In the mice given a single challenge, both IgM and IgG antibodies were detected, with the IgM being detected as early as the third day post-infection, whereas the IgG was detected as early as the fifth day post-infection. In the mice given a secondary challenge, both IgM and IgG were detected at an appreciable level, with the IgG being considerably higher in magnitude, an observation that correlates well with the data obtained in PFC and immunoglobulin assays.

## DISCUSSION

The present study was undertaken with a view to comparing the immune response on the humoral level of mice infected with *Mycobacterium marinum* with that observed previously in mice infected with *M. leprae* ([11]). The analogy between *M. marinum* disease and *M. leprae* infections of the mouse depends largely on the self-limited nature of these infectious processes. Based on this analogy, it was expected that the comparative humoral response in the two infections would be parallel if not identical. This is so, especially because it has been shown by Ng *et al* ([12]) that there was a similarity between the two infections in the pattern of protection against homologous and heterologous infections.

The PFC assays are in accord with such a similarity in terms of the first appearance of the immunocytes and the time at which the peak and the decline in the antibody-producing cells is concerned. However, the absence of IgG response as determined by the developed plaque method in the *M. leprae* infected mice ([11]), is in contrast to the observations made in the present study, either during a primary challenge or after a secondary challenge was administered 15 days post-primary infection. The immune response in the animals challenged a second time appeared to be predominantly IgG antibodies.

The hemagglutination studies are in agreement with the PFC studies. The presence of both 2-mercaptoethanol sensitive and resistant antibodies in the *M. marinum* infected mice, indicate that as bacillary multiplication occurs, the animal is capable of responding to the antigenic challenge by producing both IgM and IgG antibodies. Such a situation was not observed in the *M. leprae* infected mice. On the other hand, preliminary studies conducted on mice infected with *M. lepraemurium* (unpublished data), indicated that a high level of IgG antibody production occurs as the bacilli multiply and the disease becomes systemic, whereas in the initial stage, when the bacillary multiplication is rather slow, there is more of IgM than IgG response.

The immunoglobulin assays indicate very clearly and distinctly that of the three immunoglobulin classes, i.e., IgA, IgG and IgM, and IgA response in the infected mice following either a primary or a secondary one, parallels the IgA levels in normal, uninfected mice. There is a definite increase in IgM levels in the infected mice that appears to parallel the PFC assays, but the concentration of this globulin then appears to be stabilized as the infection progresses. On the other hand, the IgG response continues to increase and reaches a level that is higher than that in the normal mouse. Similar studies in *M. leprae* infected mice (unpublished data) indicate no such dramatic shift in the levels of IgG, although the IgM response seemed to be identical to that seen in the *M. marinum* infected mice. In the *M. lepraemurium* infected mice also a similar situation is seen with regard to the IgM production, but the IgG response observed during the later stages of infection was on par with the one observed in marinum infected mice.

It has been suggested by a number of workers ([12, 19]) that the disease process that follows the inoculation of the mouse foot pad with *M. marinum* might serve as a useful model, which could substitute the mouse foot pad infection with *M. leprae* for immunologic studies. The *M. marinum* model has also been used by other workers for evaluation of various antileprosy drugs with the hope that this model could provide information that could then be extrapolated to the *M. leprae* infection. Our studies of the humoral immunologic response as indicated by the three parameters used, argue against the use of this model for such extrapolative studies. Although the growth temperature requirement of the two organisms and their ability to initiate infection in areas of the body that have lower than normal temperature are similar, the type of infection induced in the mouse by each one of these species may disallow the serious consideration of the *M. marinum* infection model. Studies on cell-mediated immune response could possibly present a

picture that may be different from the one observed in the humoral response.

One of the reasons why the *M. marinum* model could, on the basis of cell-mediated immune response (CMI), be considered as a possible model for understanding the host-parasite interactions in the leprosy infection initiated in the mouse, is that the process that limits multiplication of *M. leprae* has been suspected to result from the triggering of the cell-mediated immune response in the mouse. Evidence to this effect has been forthcoming through studies on prior infection of mice with BCG ([3, 15, 17, 18]), *Toxoplasma gondii* ([10]), or *M. leprae* ([8]). These organisms have been shown to inhibit multiplication of *M. leprae* in the foot pads of mice. On the other hand, prior treatment of the mouse with immunosuppressive agents such as cortisone ([20]), thymectomy with whole body irradiation ([13]), and treatment with antilymphocyte globulins ([5]) have been shown to allow multiplication of *M. leprae* beyond the plateau level that is seen in the immunologically intact mouse, thus indicating that the depletion of CMI leads to proliferation of the organism in the mouse host.

The possibility, therefore, that an identical cell-mediated immune response in *M. marinum* infection of the mouse by the foot pad method, as is seen in similar studies on *M. leprae* infected mice in our hands, cannot be overlooked. The work of Ng *et al* ([12]) on protection conferred on *M. marinum* infected mice, through prior infection with several microorganisms including *M. leprae* lends credence to this possibility.

## SUMMARY

Foot pad infection of mice with *Mycobacterium marinum* carried out with a view to comparing the immune response on the humoral level of such mice, with that observed previously in mice infected with *M. leprae,* indicated that there was a similarity in terms of the first appearance and proliferation of immunocytes and the time at which the peak and decline in the antibody-producing cells occurred. The significant difference appeared to be in the immunoglobulin G response, which was absent in the *M. leprae* infected mice, but occurred simultaneously with the immunoglobulin M response at a high level, both during a primary and after a secondary challenge administered 15 days post-primary infection in the *M. marinum* infected mice.

Further confirmation was obtained through additional studies on the specific immunoglobulin levels and determination of both immunoglobulin M and immunoglobulin G antibodies by hemagglutination.

Although the growth temperature requirement of the two organisms and their ability to initiate infection in areas of the body with lower temperatures are similar, it is suggested that the type of infection induced by each one of these species in the mouse may disallow the serious consideration of the *M. marinum* infection model as a possible alternative experimental model for studying the role of host immunity to *M. leprae* infections in mice.

## RESUMEN

Se inoculó *Mycobacterium marinum* en los cojinetes plantares de ratones con la idea de comparar la respuesta inmune humoral de estos animales con aquella observada previamente en ratones infectados con *M. leprae.* Se encontró que hubieron similitudes en cuanto a la primera aparición y proliferación de inmunocitos y en cuanto a los tiempos en los que ocurrieron la máxima respuesta y la disminución en el número de células productoras de anticuerpos. La diferencia importante pareció estar en la respuesta a base de inmunoglobulina G, la cual no ocurrió en los ratones infectados con *M. leprae.* En cambio, esta inmunoglobulina G se produjo simultáneamente con inmunoglobulina M, a niveles elevados, en los ratones infectados con el *M. marinum* tanto durante una respuesta primaria como después de un reto secundario administrado 15 días después de la infección primaria de los ratones con el *M. marinum.* Esto se pudo confirmar determinando los niveles de las inmunoglobulinas específicas y determinando los niveles de los anticuerpos de las clases M y G, usando una técnica de hemaglutinación.

Aunque los requerimientos de temperatura de los dos microorganismos y su habilidad para iniciar una infección en áreas corporales de baja temperatura son similares, se sugiere que el tipo de infección inducido en el ratón por cada una de estas dos especies puede desalentar la consideración del modelo de infección con el *M. marinum* como un posible modelo experimental alternativo de utilidad en el estudio de la inmunidad del ratón a la infección con el *M. leprae.*

## RÉSUMÉ

On a provoqué l'infection du coussinet plantaire de souris avec *Mycobacterium marinum,*

dans le but de comparer la réponse immunitaire humorale, avec celle observée précédemment chez des souris infectées par *M. leprae*. Les résultats démontrent une ressemblance en ce qui concerne la première apparition et la prolifération des immunocytes, de même qu'en ce qui concerne le moment auquel le pic et le déclin des cellules productrices d'anticorps prennent place. Une différence significative est apparue en ce qui concerne la réponse des immunoglobulines G. Cette réponse était absente chez les souris infectées par *M. leprae*, mais se produisait simultanément avec une réponse d'immunoglobuline M, chez des souris infectées par *M. marinum*, et ceci tant au cours du premier challenge qu'au cours du second administré 15 jours après l'infection primitive.

Une confirmation supplémentaire a été obtenue par des études complémentaires concernant les niveaux spécifiques d'immunoglobulines et la détermination par hémagglutination des anticorps, immunoglobulines M et immunoglobulines G.

Les conditions de température qui régissent la croissance de l'un ou l'autre de ces organismes, de même que leur capacité à amorcer le processus infectieux dans des régions du corps témoignant de température peu élevée, sont certes semblables. On suggère cependant que le type d'infection induite par chacune de ces espèces chez la souris ne permet pas de considérer sérieusement que *M. marinum* puisse constituer un modèle infectieux expérimental de rechange pour étudier le rôle de l'immunité de l'hôte aux infections par *M. leprae* chez la souris.

**Acknowledgments.** We extend our grateful appreciation to J. A. Gally and M. V. Kanchana, for their helpful comments and suggestions during the preparation of this manuscript. Our sincere thanks are also extended to L. Levy, USPHS Hospital, San Francisco, California for his invaluable help during the conduct of this research and for the supply of mice. Active participation of Rekha R. Dalvi during the preliminary stages of these studies is sincerely appreciated.

We would also like to thank C. W. Johnson, Dean, School of Graduate Studies for partial financial help to P. J. P. during his tenure as a doctoral candidate in the department of microbiology.

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