Stimulation of Anti-*Brucella* Vaccination in Mice by Tetramisole, a Phenyl-Imidothiazole Salt

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The effect of tetramisole (hydrochloride of racemic 2, 3, 5, 6-tetrahydro-6-phenyl-imidazo [2, 1-b] thiazole) on immunization was investigated in mice vaccinated by killed *Brucella melitensis* cells suspended in incomplete adjuvant. Immunity to *Brucella abortus* challenge was estimated by the reduction in number of *B. abortus* colonies per gram of spleen in those mice which escaped full immunization and also by calculation of mean infective doses for each group of mice. All tetramisole treatments significantly reduced the number of *B. abortus* live cells in spleen from infected mice. Tetramisole, injected twice (at the time of vaccination and 48 h later), induced a significant 3.5-fold increase of the protection brought about by *Brucella* vaccine alone. A single injection of 1.25 mg/kg at the time of vaccination resulted also in a significant increase of the immunity given by vaccination. No modifications of the vaccine potency were observed if tetramisole administration preceded vaccination. In such a mouse assay, tetramisole-induced stimulation was not accompanied by specific antibody increases, although measured by three serological tests.

Two phenyl-imidothiazole salts, tetramisole and levamisole, are synthetic nontoxic chemicals that exert a stimulatory effect on T lymphocytes and macrophages (7-9). Tetramisole interferes with antigenic competition and non-specific immunity and is effective in restoring an immunization that had been impaired after a prior unrelated infection (10).

Tetramisole and levamisole may be of practical usefulness as vaccine adjuvants. The following experiment was designed to investigate the role of a single or of multiple tetramisole doses in modifying the immune response of mice to a bacterial vaccination. A *Brucella* vaccine and a *Brucella abortus* challenge were selected to avoid the remote possibility of false responses that may be due to preliminary inescapable contact of normal animals with antigens or cells of ubiquitous gram-negative organisms by inhalation or ingestion (2). A good vaccine stimulation was obtained when tetramisole was injected at the time of vaccine, and an even more marked adjuvant action was observed following tetramisole administration 2 days after vaccination.

MATERIALS AND METHODS

Vaccine. *Brucella melitensis* H 38 vaccine, a suspension of Formalin-killed cells in incomplete adjuvant, was used throughout this study at a concentration of 15×10^8 cells/ml. This *B. melitensis* killed vaccine is of known potency against a *B. abortus* challenge (3).

Tetramisole. Tetramisole, hydrochloride of racemic 2, 3, 5, 6-tetrahydro-6-phenyl-imidazo [2, 1-b] thiazole, was first known as a nontoxic and powerful antihelminthic drug (13). The dry powder, obtained through the courtesy of P. Janssen (Janssen Pharmaceutica, Beerse, Belgium), was dissolved in sterile, pyrogen-free water to obtain a 1.25 mg/kg concentration adjusted in a volume of 0.1 ml to individual mouse weight.

Vaccination. Groups of 7- to 8-week-old female CD-1 mice (caesarean-delivered, Charles River, Elbeuf, France) weighing 21 ± 1 g were used. Vaccination consisted of a single subcutaneous (s.c.) injection of 0.2 ml of H 38 vaccine alone (control group 2) or associated with tetramisole (treated groups 3 to 6). In group 3, mice were treated three times—48 h before, at the time of, and 48 h after vaccination. Tetramisole was administered twice in group 4, one injection 48 h before and the second at the time of vaccination. In group 5, mice were also treated by two injections, one

simultaneously with vaccination and the second 48 h afterwards. Vaccine and tetramisole were injected simultaneously, albeit separately, to mice of group 6.

Challenge. Untreated and unvaccinated mice, which comprised group 1, were divided in subgroups of 10 animals, challenged, respectively, by 5×10^2 , 1×10^3 , 2.5×10^3 , and 5×10^3 live cells of *B. abortus* strain 544. Subgroups of 10 mice in groups 2 to 6 were challenged 42 days after vaccination by 5×10^4 to 5×10^6 cells of *B. abortus* strain 544 that were contained in a 0.2-ml s.c. injection. All mice were killed 11 days after challenge.

Tests for appraisal of vaccine and tetramisole activities. The modifications due to vaccination and to tetramisole treatments were evaluated by two different means: the calculation of mean infective doses (ID_{50}), and the average numbers of *B. abortus* colonies developed in 1 g of spleen from the mice that were infected after a 5×10^6 challenge. Each spleen, aseptically removed, was weighed and then frozen at -20°C for 2 or 3 days. After thawing, spleens were ground in a potter apparatus. This procedure of organ freezing and thawing increased, by cellular disruption, the number of recoverable *Brucella* (4).

Each spleen homogenate was thoroughly suspended in sterile saline to obtain 10^{-1} to 10^{-4} (wt/vol) dilution. Known amounts of undiluted and diluted spleen tissue were seeded through a calibrated microsyringe, at the end of which was a platinum tubing that delivered exactly 50 drops per ml. Ten drops from each dilution were plated onto Trypticase soy agar (BD-Mérieux, Lyon, France). Two similar dishes were prepared for each dilution. Developed microcolonies were counted with the aid of a binocular lens after 2 days of incubation at 37°C in a 10% carbon dioxide atmosphere. Control counts were made again at the 4th day of incubation. Correct dilution and plating techniques resulted in drop diameters of about 12 mm and in easy readings of those suspensions that provided up to 15 microcolonies per drop. Comparison by *t* tests of the number of colonies in drops for two plates from a given spleen tissue dilution permitted an appraisal of the accuracy of plate counts and the calculation of the number of *B. abortus* colonies developed from each mouse spleen. Also, 0.2 g each of spleen and liver were plated onto Trypticase soy agar, and the results were scored as being positive or negative for *B. abortus*. All infected mice, whatever the number of recovered *Brucella* organisms, were used to calculate the ID_{50} of treatment for each group (1).

Serological tests for *Brucella* antibodies. Three serological tests were employed. Tube agglutination tests and complement-fixation tests followed techniques already described (5). Passive hemagglutination tests involved the coating of a soluble *B. abortus* antigen onto sheep red blood cells through chromium chloride (5).

Blood samples were obtained by aseptic transorbital puncture of the sphenoid sinus before autopsy.

RESULTS

Effects of tetramisole treatments in modifying mouse ID_{50} after *B. abortus* chal-

lenge. Tetramisole administered simultaneously with, or 48 h after, vaccination exerted a stimulatory effect on anti-*Brucella* vaccination. Indeed, H 38 vaccine alone (group 2) induced an immunity in mice so that the ID_{50} was 333 times (confidence limits from 2×10^5 to 8×10^5) higher than the ID_{50} of control mice (group 1), where confidence limits varied from 530 to 4,200 with a mean value of 1.5×10^3 live cells (Fig. 1).

A significant difference was observed, at $P = 0.05$, between mice submitted to a single tetramisole treatment and mice immunized by H 38 alone. Indeed, 1.25 mg of tetramisole per kg given at the time of vaccination (group 6) multiplied by 833 the number of *B. abortus* organisms needed to infect 50% of the mice; in this group, confidence limits consisted of between 9×10^5 and 1.5×10^6 . The same amount of tetramisole given twice, a first injection at the time of vaccination and a second 48 h later, resulted in a better immunizing process so that the ID_{50} for these mice, group 5, was increased to 2.0×10^6 (confidence limits from 1.6×10^6 to 2.5×10^6), a figure which significantly differed from all other findings in this experiment. The time schedule of injections of tetramisole to mice exerted a striking influence upon stimulation of *Brucella* vaccine activity by this drug. Indeed, tetramisole injected three times (group 3) or administered twice, a first injection 48 h before and a second simultaneously to vaccination (group 4), did not significantly modify the immunization brought about by H 38 vaccine alone, as demonstrated by the overlapping of confidence limits (Fig. 1).

Effects of tetramisole treatments upon dose response curves. The dose response curve in group 2 mice immunized by H 38 vaccine alone did not parallel the curve obtained from unvaccinated, control mice (Fig. 1). Such a discrepancy could be attributed to heterogeneity of the random-bred mice used in this study. One tetramisole treatment at the time of vaccination (group 6), or two tetramisole injections (groups 4 and 5) tended to suppress that host-response heterogeneity to vaccination. However, treatment of mice by three separate tetramisole injections (group 3), the first 2 days before vaccination, the second at the time of immunization, and the third 2 days later, resulted in a sharp accentuation of mouse heterogeneity in response to vaccination.

Effects of tetramisole treatments upon *B. abortus* recovery from spleen cultures. A challenge by 5×10^3 *B. abortus* cells resulted in a 10/10 *Brucella* infection of control, untreated, unvaccinated mice (group 1, Table 1). After a challenge by 5×10^6 *B. abortus* organisms, 8 out

FIG. 1. Percentage of infected mice and confidence limits (horizontal rods) plotted on log-probit paper by the method of Bonet-Maury *et al.* (1) after inoculation of *B. abortus*, strain 544, 42 days after H 38 vaccination. Group 1: unvaccinated and untreated-control mice; 2: H 38 vaccine alone; 3: three tetramisole treatments—48 h before, simultaneously with, and 48 h after H 38 vaccination; 4: two tetramisole treatments—48 h before and simultaneously with H 38 vaccination; 5: two tetramisole treatments—at the time of and 48 h after H 38 vaccination; 6: one tetramisole treatment simultaneously with H 38 vaccination. Each tetramisole treatment was 1.25 mg/kg, subcutaneously administered.

of 10 mice in every vaccinated group or vaccinated and treated group, demonstrated *B. abortus* colonies in their spleen cultures. The number of live *B. abortus* in the spleen of those mice which escaped full immunization after vaccination and tetramisole treatments could also serve as a tool to estimate vaccinal protection of these animals. Indeed, after a thousand-fold-higher challenge than in normal, unvaccinated mice, H 38-vaccinated mice (group 2) harbored 38,540 *B. abortus* cells per g of spleen, about 6% of the number (628,150) of *Brucella* found in 1 g of spleen of normal mice (Table 1). In vaccinated and tetramisole-treated mice submitted to a similar 5×10^6 challenge, the numbers of *B. abortus* colonies in 1 g of spleen were again lowered, regardless of the schedule of tetramisole administration and even if such treatments had not modified the corresponding ID_{50} (groups 3 to 6). Besides, two treatments by 1.25 mg of tetramisole per ml, one at the time of vaccination and the second 2 days later, resulted in *B. abortus* colony counts as low as 1,556 live cells in 1 g of spleen (group 5). This figure represented 4% of the colonies in 1 g of spleen from H 38-vaccinated mice, after the same challenge, and was significantly different at $P = 0.01$. Similar differences in the number of viable *B. abortus* cells recovered from positively infected mice were also found in subgroups that were exposed to lower challenge

inocula (Table 1). Spleen weights were not influenced by tetramisole treatments 40 to 44 days before autopsy. Indeed, the mean weight (in milligrams $\pm$ SE) for each group of mice ranged from 297 ± 20 in control, untreated mice (group 1) to 345 ± 39 in mice treated by tetramisole simultaneously with vaccination (group 6).

Effects of tetramisole treatment upon anti-*Brucella* circulating antibodies. Two injections of *Brucella* antigens, vaccination and challenge, resulted in a modest antibody production in sera sampled 11 days after the second injection and tested by three serological tests (Table 1, group 2). Tetramisole treatments in H 38-vaccinated mice did not significantly modify the production of free *Brucella* antibodies (Table 1, groups 3 to 6). By comparing culture results and serological tests, we found that there is no relationship between mouse anti-*Brucella* protection and the presence of circulating antibodies. In addition, in mice treated by tetramisole at the time of vaccination and 2 days later, the best anti-*Brucella* immunization was accompanied by the lowest free *Brucella* antibody production (Table 1, group 5).

DISCUSSION

Tetramisole, if given simultaneously to, or after, antigen, clearly stimulates the immune responsiveness of random-bred mice. The same

drug administered before an antigenic stimulus has reduced stimulating activities, when vaccine potency is measured by relative increases of ID₅₀.

A 2.5-fold enhancing effect of anti-*Brucella* immunity is observed after a single injection of 1.25 mg of tetramisole per kg at the time of *Brucella* vaccination. The immunizing potency of H 38 vaccine significantly increases 3.7-fold when a first injection of tetramisole is followed by a second injection 48 h later. These findings extend previous data on the effects of tetramisole upon hemolytic plaque-forming spleen cell production in mice immunized by sheep red blood cells (SRBC). Although an obvious stimulation was observed after simultaneously injecting SRBC and tetramisole, this effect was markedly increased if mice were treated by tetramisole 6 h after the injection of antigen (7).

Two tetramisole injections, a first given 2 days before and a second at the time of vaccination, do not modify the immune response brought about by H 38 vaccine alone. A similar finding is obtained after three tetramisole treatments, the first occurring 2 days before vaccination.

Bacterial counts for residual infection, after challenge, display an aspect of immunization that is different from ID₅₀ calculations. The increase of the number of pathogens needed to infect 50% of the mice may reflect the value of the shield which prevents the entry of invaders. This preventive shield is established through the activities of the humoral as well as the cellular arms of immunity. Counts of the re-

maining intracellular bacteria may be a reflection only of cellular immunity.

Addition of a tetramisole treatment to anti-*Brucella* vaccination strikingly reduces the number of *B. abortus* colonies developed from 1 g of spleen from mice which escaped full immunization, even in those groups of mice where tetramisole was unable to modify the ID₅₀. Since tetramisole was injected 40 to 44 days before challenge and because this drug seems to be devoid of in vitro antibacterial action (13), the reduction in the number of pathogens in treated mice cannot be attributed to a direct effect of tetramisole on *Brucella* cells.

The stimulatory effect of tetramisole upon immunization is attained without modification of *Brucella* antibody production that could be demonstrated as residual antibodies at the time of autopsy, although antibodies were measured by agglutination of the whole cells, by complement fixation, and by hemagglutination of red cells coated with a soluble antigen. A similar situation is frequently found in man and animal brucellosis, where a correlation seems to exist between a serious illness and high antibody titers. Host heterogeneity in response to antigenic stimulus (12) may be related to genetic differences within the members of a random-bred populations of mice. Interestingly enough, one or two tetramisole treatments suppress mouse heterogeneity to *Brucella* antigens. In contrast, current studies have shown that tetramisole and levamisole may, under some experimental conditions, increase host heterogeneity to SRBC antigens. These observations led

TABLE 1. Effects of tetramisole treatments and of H 38 *Brucella* vaccine upon *B. abortus* recovery from mouse spleens after challenge, and on anti-*Brucella* antibody production

Group no. ^a	Avg. no. of <i>B. abortus</i> colonies ± SE			<i>Brucella</i> antibodies ^c		
	5 × 10 ⁸ ^b	5 × 10 ⁹	5 × 10 ⁴	TA	CF	PH
2	38,548 ± 4,300	2,075 ± 322	5	26 (– to 80)	35 (– to 160)	110 (– to 320)
3	11,354 ± 1,721	598 ± 48	532 ± 44	45 (– to 80)	80 (– to 160)	160 (– to 1,280)
4	3,034 ± 289	183 ± 15	0	60 (– to 160)	65 (– to 640)	310 (– to 1,280)
5	1,556 ± 133	41 ± 5	0	4 (– to 40)	25 (– to 40)	
6	19,355 ± 2,010	133 ± 15	0	35 (– to 160)	38 (– to 160)	200 (– to 1,280)

^a Group 1: at a challenge of 5 × 10⁸, 628,150 ± 47,000 (average number of *B. abortus* colonies ± SE); 2.5 × 10⁸, 121,180 ± 12,350; 1 × 10⁸, 1,030 ± 98. Group 1: unvaccinated and untreated control mice; 2: H 38 vaccine alone; 3: three tetramisole treatments—48 h before, simultaneously with , and 48 h after H 38 vaccination; 4: two tetramisole treatments—48 h before and simultaneously with H 38 vaccination; 5: two tetramisole treatments—at the time of and 48 h after H 38 vaccination; 6: one tetramisole treatment simultaneously with H 38 vaccination. Each tetramisole treatment was 1.25 mg/kg, subcutaneously administered.

^b Challenge.

^c Sera sampled 53 days after immunization. *Brucella* antibodies measured in mean values for 10 mice after a challenge of 5 × 10⁸ *B. abortus* in untreated group 1, and a challenge of 5 × 10⁹ in groups 2 to 6. Figures between brackets are the lowest and the highest titer observed. Abbreviations: TA, tube agglutination test; CF, complement fixation test; PH, passive hemagglutination test. Final titers are expressed in International Units for TA and in reciprocal of final dilutions for CF and PH.

to a study in progress to demonstrate a possible genetic control of the stimulating activities of tetramisole.

Tetramisole, as well as its levogyre isomere, levamisole, inhibits succinate dehydrogenase activity (13). This drug is devoid of blastogenic effects and inhibits alkaline phosphatase activity of various cell lines (unpublished data). All available data are suggestive for two different points of impact of tetramisole activities in immunocompetent cells (11). We are well aware that basic studies are still needed to elucidate the mechanisms of tetramisole, or levamisole, activities and the reasons for their unique ability to induce a specific immune enhancement more markedly and without any granuloma-producing effect if injected late after an antigenic stimulus.

To our knowledge, present data are the first to reveal a simple synthetic compound which could be able to induce an immune enhancement, especially if administered late after antigen. It may be possible to employ these phenylimidothiazole salts as nontoxic, chemically defined adjuvants to improve the usefulness of most vaccines.

LITERATURE CITED

1. Bonet-Maury, P., A. Jude, and P. Servant. 1954. La mesure statistique de la virulence et de l'immunité. Application à l'étude de la virulence du bacille typhique et à la mesure du pouvoir immunisant des vaccins antityphoïdes. *Rev. Immunol.* **18**:21-50.
2. Freedman, H. H., and A. E. Fox. 1969. Host reactivity to bacterial endotoxin, p. 269-284. *In* L. Chedid (ed.), *La structure et les effets biologiques des produits bactériens provenant des germes gram-négatifs*. Centre National de la Recherche Scientifique, Paris.
3. Renoux, G. 1962. Immunisation des chèvres et des brebis contre la brucellose au moyen de microbes tués incorporés à une huile minérale. *Ann. Inst. Pasteur* **103**:786-801.
4. Renoux, G. 1964. Recherche des microorganismes dans les tissus animaux après broyage en masse des échantillons. Application aux *Brucella*. *Ann. Inst. Pasteur* **106**:126-131.
5. Renoux, M., M. P. Dubois, and G. Renoux. 1968. Hémagglutination après couplage par le chlorure de chrome d'une glycoprotéine bactérienne; échec de la benzidine. *Ann. Inst. Pasteur* **115**:978.
6. Renoux, G., and G. R. Gaumont. 1966. Méthodes de diagnostic biologique des Brucelloses. Centre National de la Recherche Scientifique, Paris.
7. Renoux, G., and M. Renoux. 1972. Action stimulante de dérivés du phenylimidothiazole sur les cellules spléniques formatrices d'anticorps. *C. R. Acad. Sci.* **274 D**:756-757.
8. Renoux, G., and M. Renoux. 1972. Restauration par le phenylimidothiazole de la réponse immunologique des souris âgées. *C. R. Acad. Sci.* **274 D**:3034-3035.
9. Renoux, G., and M. Renoux. 1972. Action du phenylimidothiazole (tetramisole) sur la réaction du greffon sur l'hôte. *C. R. Acad. Sci.* **274 D**:3320-3323.
10. Renoux, G., and M. Renoux. 1972. Antigenic competition and non-specific immunity after a prior rickettsial infection in mice. Restoration of antibacterial immunity by phenyl-imidothiazole treatment. *J. Immunol.* **109**:761-765.
11. Renoux, G., and M. Renoux. 1973. Liens entre les événements immunologiques et le contrôle génétique de l'activité enzymatique des macrophages et des lymphocytes. *C. R. Acad. Sci.* **276 D**:861-864.
12. Shortley, G., and J. R. Wilkins. 1965. Independent-action and birth-death models in experimental microbiology. *Bacteriol. Rev.* **29**:102-141.
13. Thienpont, D., O. F. J. Vanparijs, A. M. H. Raeymaekers, J. Vandenberck, P. J. A. Demoen, F. T. N. Allewijn, R. P. H. Marsbroom, C. J. E. Niemegeers, K. H. L. Schellekens, and P. A. J. Janssen. 1966. Tetramisole, a new, potent broad spectrum anthelmintic. *Nature (London)* **209**:1084-1086.
14. Van den Bossche, H., and P. A. Janssen. 1969. The biochemical mechanism of action of the antinematodal drug Tetramisole. *Biochem. Pharmacol.* **18**:35-42.