

REMOVAL OF TISSUE ANTIGENS FROM BACILLARY SUSPENSIONS OF *M. LEPRAE*¹

CARL E. TAYLOR, M.D.

*Department of Microbiology
Harvard School of Public Health
Boston, Mass.*

and JOHN H. HANKS, Ph.D.

*Department of Pathobiology
Johns Hopkins University School of Hygiene
Baltimore, Md.*

Progress in improving the specificity of lepromin requires as a first step the separation of bacilli from tissue elements. Our previous studies have demonstrated that tissue elements in lepromin are antigenically active (⁵). This report describes three methods of preparing tissue-free bacillus suspensions, and tests in guinea-pigs of their relative antigenicities. The major difference from previously-published methods was further refinement of procedures for obtaining purified bacillus suspensions.

MATERIALS AND METHODS

Sensitization of each of five groups of 13 guinea-pigs with a specific antigen was achieved by two intradermal injections of 0.1 cc. at an interval of 3 weeks. Tuberculin syringes were tested to conform with WHO-BCG campaign standards (⁷). Three weeks after the second injection, cross tests for skin sensitivity in each of the groups were made by giving intradermal injections of 0.1 cc. of a battery of 8 antigens in two parallel rows along the back. Randomization of guinea-pigs and rotation of sites of injection made it impossible for the person reading the reactions to know either the immunization group to which any guinea-pig belonged or the antigen injected at any particular site. Reactions were read by the same person every day for one week and then every other day for one month, with precise measurement of the average diameter of each reaction.

The three procedures for separating bacilli and tissue will be described in detail. Methods of preparing the remaining antigens have already been described (^{4, 6}).

1. *Bile-pancreatin bacillus suspension*.—A solution of 0.5 per cent bile salts (Bacto-Oxgall) and 1 per cent pancreatin (Difco Pangestin 1:75) was passed through a Seitz filter and adjusted to pH 8.0 with 0.5N NaOH. One gm. of autoclaved lepromatous tissue and 20 cc. of bile-pancreatin solution were placed in a glass mill with ground-glass surfaces; grinding was carried out by hand for 10-15 minutes, or with a mechanical stirrer until no particles were visible. Incubation at 40°C for 3 hours with constant stirring with a magnetic mixer was followed by centrifugation at 300 × G for 3 minutes. The sediment was again suspended in bile-pancreatin solution and subjected to an additional three hours' digestion. It was found that this prolonged digestion was necessary to reduce the protein content of the solution to a level where minimal precipitate was formed when aliquots were adjusted to pH 4.6 and boiled for 2 minutes. Gross debris was removed by centrifugation at 150 × G for 3 minutes. The supernatant solutions were pooled and centrifuged at 2000 × G for 30 minutes and the sediment washed in water, recentrifuged and suspended in 0.5 per cent phenol-

¹Supported by Grant No. E-2656(C1), National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

saline. Dilution was carried out until the bacillus content was equivalent to a Mitsuda-Wade lepromin standard prepared by Dr. Wade at the Culion Sanitarium, in the Philippines. The bacilli appeared normal in stained smears, and the suspension was relatively free from visible tissue residue.

2. *Disintegrated bacilli (bile-pancreatin)*.—Aliquots of the bacillus suspension described above were shaken in Mickle vibrator² with carborundum, size 10A. With the arms set for maximum vibration it was possible to break up the bacilli to fragments which appeared as finely granular basophilic material in acid-fast stained smears. One hour of vibration was required to reduce the numbers of *M. leprae* to a level where only rare intact bacilli were seen in smears.

3. *Chloroform bacillus suspension*.—Pieces of autoclaved lepromatous tissue, 1 gm. in weight, were ground in a glass mill for 10 minutes with 20 cc. of saline. Two cc. of chloroform were added and the mixture was shaken end to end vigorously by hand for 15 minutes. After centrifugation at $200 \times G$ for 2 minutes the deposit was resuspended in 10 per cent chloroform-saline and the shaking repeated. The deposit from a second centrifugation at $200 \times G$ for 3 minutes still contained clumps of acid-fast bacilli mixed with tissue debris, but these were discarded. By far the greater proportion of the leprosy bacilli was suspended in the two supernatant solutions, which were combined and centrifuged at $2000 \times G$ for 20 minutes. The relatively clean bacillus deposit was resuspended in 0.5 per cent phenol-saline and diluted to the equivalent bacillus content of the standard lepromin prepared by Wade.

4. *Oil-interface bacillus suspension*.—Such a method of separation has previously been used by Henderson (2). In our work, 1 gm. pieces of autoclaved lepromatous tissue were ground with 10 cc. of saline in glass mill for 10 minutes. The solution was then shaken in a Mickle's vibrator with carborundum (10A) at maximum vibration for 2 minutes, to further break up the tissue cells. The carborundum was separated by centrifugation at $150 \times G$ for one minute, and rinsed with saline which was added to the supernatant. An equal volume of olive oil was added, and the mixture shaken in the Mickle's vibrator without carborundum for 5 minutes. After standing overnight, four layers appeared: (a) the coarse sediment at the bottom contained much cellular debris but also a few clumps of bacilli; (b) the saline solution was milky and contained moderate numbers of bacilli and some cellular debris; (c) a thick flocculent layer at the interface was loaded with bacilli but also contained some debris; (d) the oil layer contained many bacilli but little cellular debris. Separation of layers was achieved either in a burette or by means of Pasteur pipettes with right angle tips. Bacilli were centrifuged from the oil at $2000 \times G$ for 20 minutes and the oil supernatant discarded. The bacilli from the interface and oil layers were resuspended in 0.5 per cent phenol-saline in a dilution equivalent to the standard lepromin.

Decreasing the watability of mycobacteria.—A consistent finding with all of these preparations was that, as purification proceeded, it became increasingly difficult to maintain the bacilli in suspension. They would stick to glassware and clump together in masses, oftentimes around fat crystals or other particles. Suspensions which were washed in distilled water and then centrifuged at high speed were found to have a thick floating layer of bacilli, scattered bacilli on the sides of the test tubes, and a bottom aggregation of bacilli in tissue debris. With each successive washing in water the proportion of floating bacilli

²Manufactured by H. Mickle, Gimshall, Surrey, England. Sold in the U.S.A. by Brinkman, Great Neck, N.Y.

appeared to increase. Testing several wetting agents at various dilutions demonstrated that either Tween 80 diluted 1:1000 or pluronic acid 1:1000 was effective in maintaining bacillus suspensions in a manipulable state.

RESULTS

I. BILE-PANCREATIN BACILLUS SUSPENSION

1. *Effectiveness as an immunizing agent.*—Six hours' digestion with bile-pancreatin reduced slightly the immunizing capacity of the leprosy bacilli. Reactions to bile-pancreatin suspension in guinea-pigs sensitized to the same agent were significantly smaller from the 5th to the 20th days than in groups sensitized either to Mitsuda-Wade lepromin

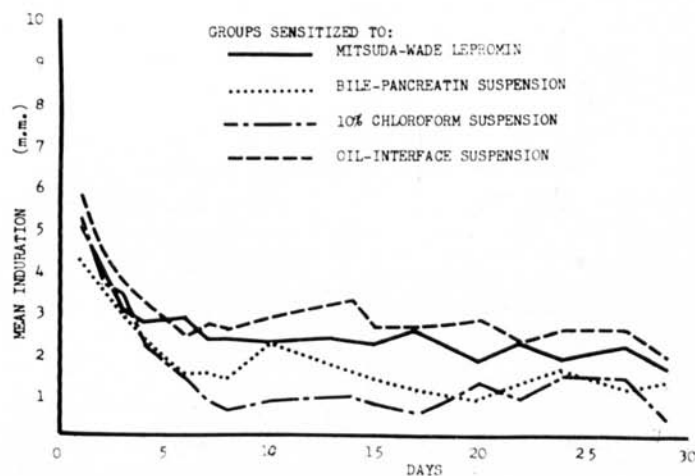


FIG. 1.—Reactions to bile-pancreatin bacillus suspension in guinea-pigs sensitized to various purified suspensions of *M. leprae*.

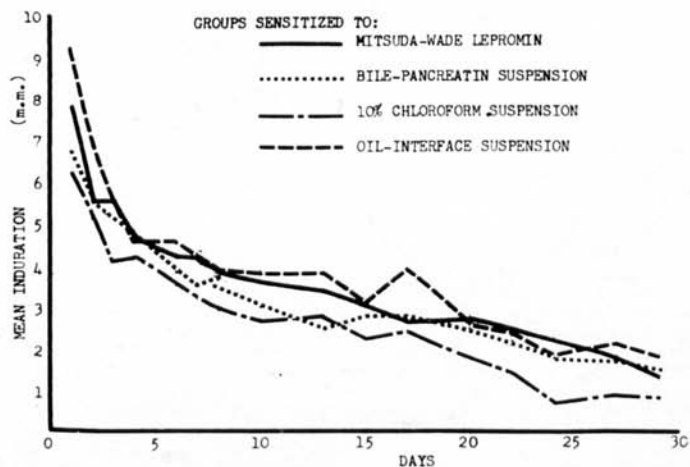


FIG. 2.—Reactions to Dharmendra bacillary antigen in guinea-pigs sensitized to various purified suspensions of *M. leprae*.

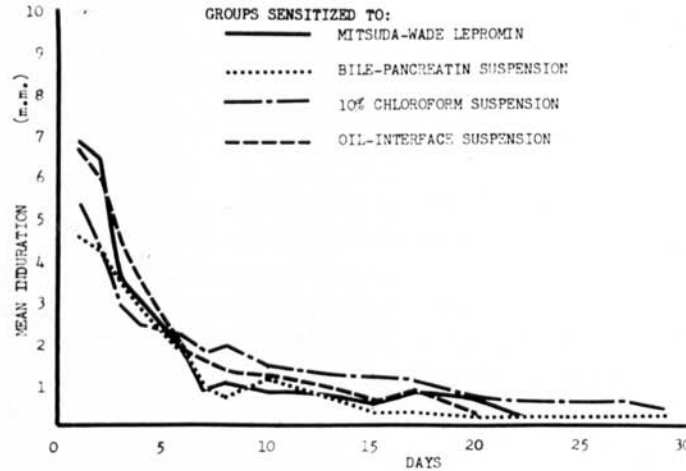


FIG. 3.—Reactions to human spleen antigen in guinea-pigs sensitized to various purified suspensions of *M. leprae*.

or oil-interface suspension, but still slightly larger than reactions in the group immunized to 10 per cent chloroform suspension (Fig. 1). To the Dharmendra bacillary antigen, however, a relatively good response occurred in the group immunized to bile-pancreatin suspension (Fig. 2).

In guinea-pigs sensitized to bile-pancreatin suspension, the relatively small reactions to test preparations containing tissue antigens indicates that almost all of the tissue element had been removed. When tested with human spleen antigen, these guinea-pigs showed significantly smaller reactions during the first 48 hours than did other groups (Fig. 3). Testing with Mitsuda-Wade lepromin in the same group (Fig. 4), also showed reduction of both early and late responses.

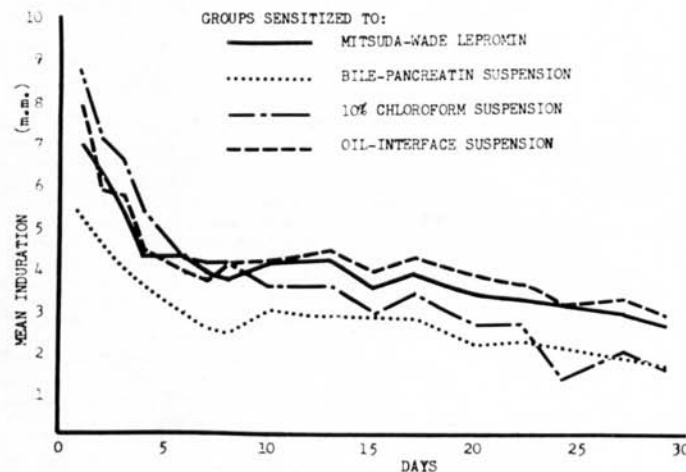


FIG. 4.—Reactions to Mitsuda-Wade lepromin in guinea-pigs sensitized to various purified suspensions of *M. leprae*.

2. *Effectiveness as a test agent.*—Digestion with bile-pancreatin for 6 hours destroyed much of the capacity of autoclaved leprosy bacilli to produce skin reactions. This was clearly demonstrated in each of the immunized groups (Figs. 5, 6, 7, 8), with most of the differences from the mean size of reactions to other bacillus suspensions being highly significant. That this loss of antigenicity probably resulted from the prolonged period of 6 hours digestion is indicated by other experiments in which after one hour of digestion no reduction in the size of reactions was detected (⁶). Although the mean size of the reactions was reduced, they persisted as well as those caused by other bacillus preparations and approximated the Mitsuda-Wade curves at 29 days.

The mechanical disintegration of the leprosy bacilli tended to accentuate the early phase of the reactions (Figs. 5, 6, 7, 8). This is most

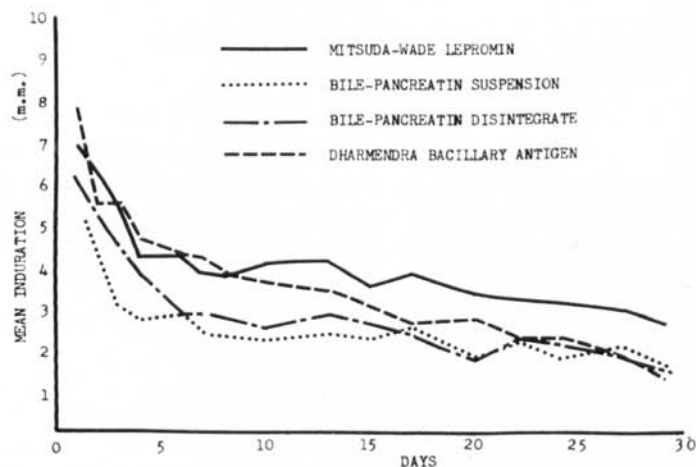


FIG. 5.—Reactions to various preparations of leprosy bacilli in guinea-pigs sensitized to Mitsuda-Wade lepromin.

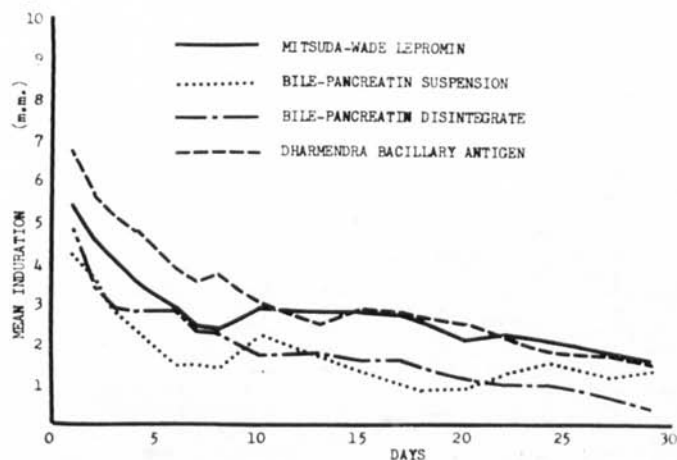


FIG. 6.—Reactions to various preparations of leprosy bacilli in guinea-pigs sensitized to bile-pancreatin bacillus suspension.

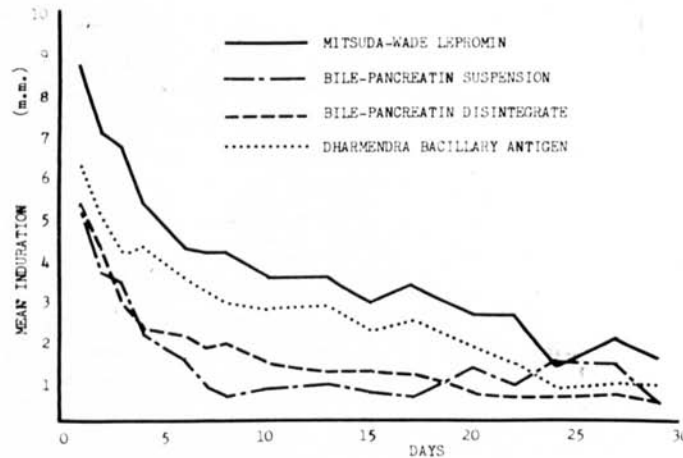


FIG. 7.—Reactions to various preparations of leprosy bacilli in guinea-pigs sensitized to 10 per cent chloroform bacillus suspension.

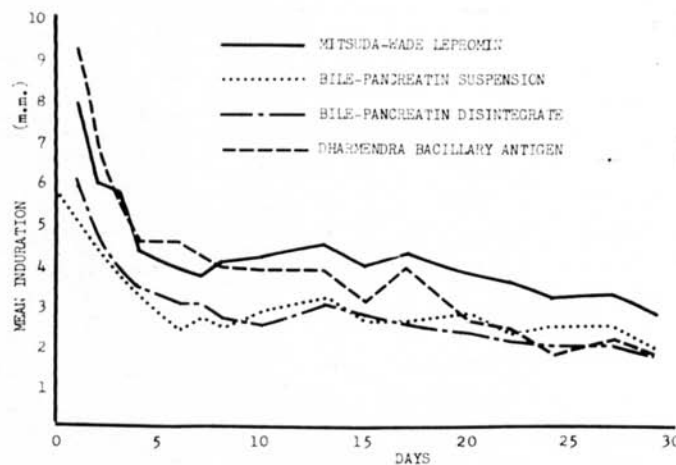


FIG. 8.—Reactions to various preparations of leprosy bacilli in guinea-pigs sensitized to oil-interface bacillus suspension.

evident in the group immunized to the Mitsuda-Wade lepromin, with the difference on the second day being significant at $P > 0.05$. During the latter part of the observation period the reactions produced by disintegrated bacilli were essentially equivalent to those caused by intact bacilli, or in some instances slightly smaller.

II. CHLOROFORM SUSPENSION

1. *Effectiveness as an immunizing agent.*—Guinea-pigs sensitized with the 10 per cent chloroform suspension tended to show more reaction to tissue and less to bacillus antigens than animals sensitized with other purified suspensions of leprosy bacilli (Figs. 1, 3, 4, 9, 10). This is particularly indicated by the poor response to the purest bacillus suspension, prepared with bile-pancreatin digestion (Fig. 1). Also

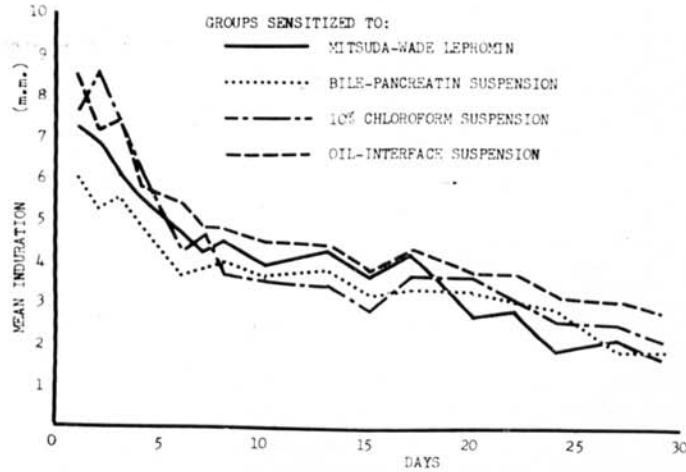


FIG. 9.—Reactions to 10 per cent chloroform bacillus suspension in guinea-pigs sensitized to various mycobacterial suspensions.

indicative of the probability that the relatively large early reactions were partly due to cross reaction with tissue antigen was the strong response to human spleen antigen (Fig. 3). The good early response in these guinea-pigs to all the bacillus suspensions (Figs. 1, 4, 9, 10) was followed by a more rapid decrease in size than in other guinea-pigs sensitized to leprosy bacilli.

2. *Effectiveness as a test agent.*—The relative purification achieved by 10 per cent chloroform separation of bacilli is indicated by the good reactions produced in all groups sensitized to bacillus suspensions (Figs. 11, 12, 13, 14). There was a statistically insignificant tendency for reactions to 10 per cent chloroform suspension to be more persistent than responses to the oil-interface suspension, although the early reactions were weaker. Mitsuda-Wade lepromin produced

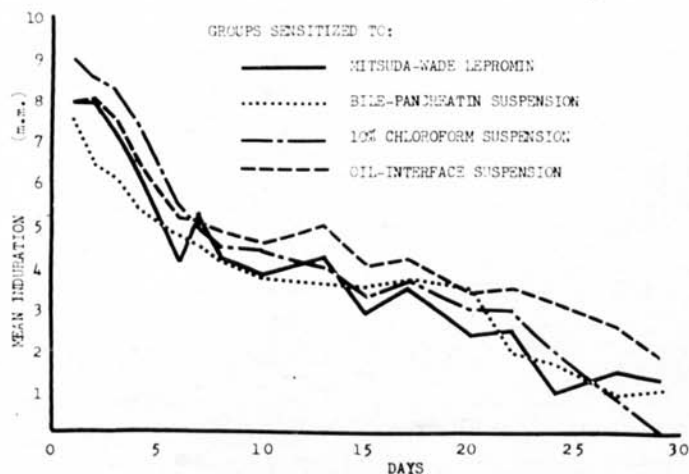


FIG. 10.—Reactions to oil-interface bacillus suspension in guinea-pigs sensitized to various mycobacterial suspensions.

smaller reactions than either the 10 per cent chloroform or the oil-interface suspensions in guinea-pigs sensitized to bile-pancreatin suspension (Fig. 12). Because of the minimal sensitivity in these animals to tissue antigen, it is probable that the larger reactions to purified suspensions were due to improved bacillus response. The 10 per cent chloroform suspension produced more local irritation than other antigens, as indicated by the large initial reactions in the unimmunized control group of guinea-pigs.

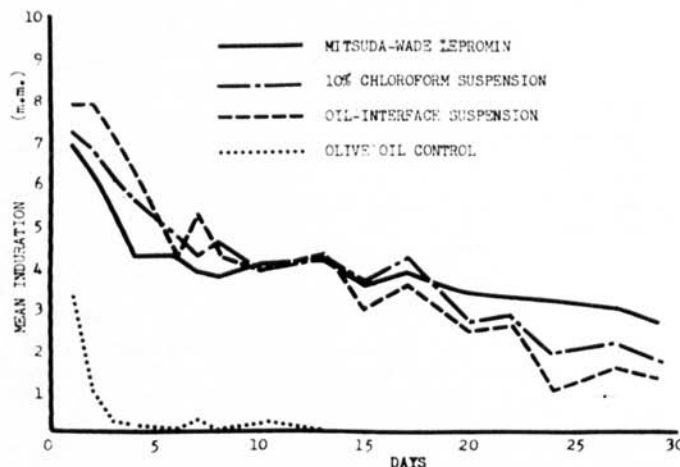


FIG. 11.—Reactions to purified suspension of *M. leprae* in guinea-pigs sensitized to Mitsuda-Wade lepromin.

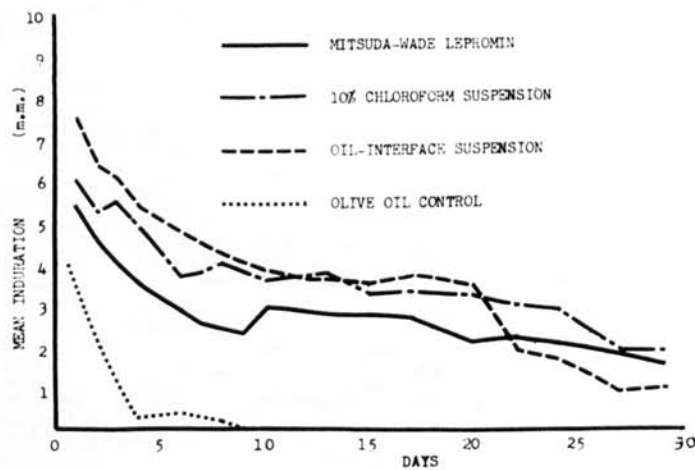


FIG. 12.—Reactions to purified suspensions of *M. leprae* in guinea-pigs sensitized to bile-pancreatin bacillus suspension.

III. OIL-INTERFACE BACILLUS SUSPENSION

1. *Effectiveness as an immunizing agent.*—The oil-interface suspension was the best sensitizing preparation used. Responses to bacillus

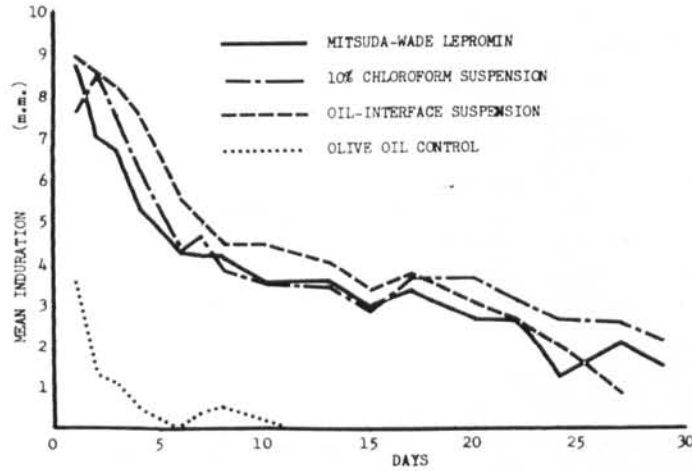


FIG. 13.—Reactions to purified suspensions of *M. leprae* in guinea-pigs sensitized to 10 per cent chloroform bacillus suspension.

suspensions were more persistent in this group of guinea-pigs (Figs. 4, 9, 10) than in the group sensitized to 10 per cent chloroform suspension. The improved bacillary specificity was demonstrated by stronger reactions to the bile-pancreatin suspension than in the other immunized groups (Fig. 1). Also probably indicative of high specific sensitivity are the significantly higher early reactions to the Dharmendra antigen (Fig. 2) than was obtained in other groups. That some tissue antigen does remain in this preparation, however, is indicated by the relatively large response to human spleen antigen (Fig. 3).

2. *Effectiveness as a test agent.*—Good responses were obtained to the oil-interface suspension in all immunized groups (Figs. 11, 12, 13, 14). Rather consistently, these reactions were larger than with other

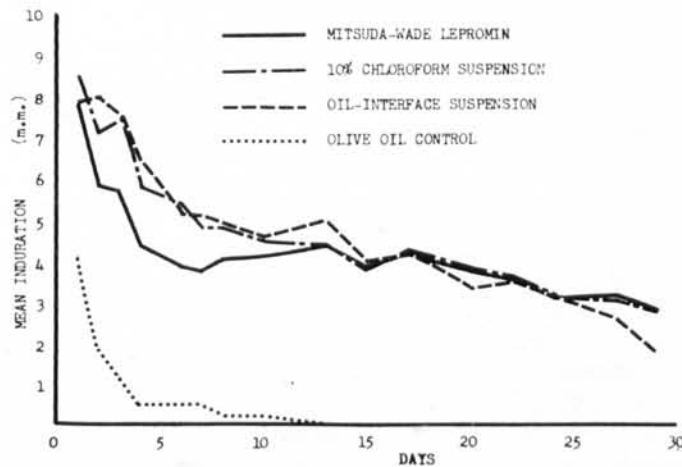


FIG. 14.—Reactions to purified suspensions of *M. leprae* in guinea-pigs, sensitized to oil interface bacillus suspension.

test agents for the first 5 days, but they showed less persistence. Indicative of improved specificity and reduction in tissue antigen were the larger reactions to oil-interface suspension than to the Mitsuda-Wade lepromin in the group immunized to the relatively tissue-free bile-pancreatin suspension (Fig. 12). Some difficulty in interpreting these results is introduced by the observation that, like the chloroform suspension, the oil-interface preparation produced large early reactions in unimmunized control animals while olive-oil control injections produced the expected small reactions.

DISCUSSION

Comparison of the three methods of preparing bacillus suspensions described has provided additional information about the immunologic characteristics of lepromin and leprosy bacilli. The importance of structurally-bound protein within the bacillus is emphasized by the response to bile-pancreatin suspension. Presumably, during the six hours digestion that was used to dispose of tissue antigens, the tryptic enzymes were able to penetrate the lipid protection of the autoclaved bacilli and digest some of the more accessible protein. That bacillary protein was still available to be released during the four weeks observation after injection was indicated by the persistence of skin reactions to this antigen. It was also indicated by the good sensitization produced by the bile-pancreatin suspension, which was more specific for bacillary protein than when other bacillus suspensions were used. Digestion was clearly the best way of eliminating reactivity to tissue proteins.

Disintegration of bacilli by mechanical means made available more antigen for an early response. It did not, however, significantly reduce the late response below that to intact bacilli in the bile-pancreatin suspension. Apparently fragments of the size produced are still large enough to permit slow release of antigen, and the process may, therefore, be more chemical and enzymic than physical. Kitano and Inoue⁽³⁾ treated ordinary Mitsuda lepromin by ultrasonic fragmentation. In 4 healthy volunteers and 7 leprosy patients, they reported a definite acceleration of reaction, the ultrasonic-treated preparations producing maximum reactions on the 3rd day with subsequent fading while reactions to the untreated Mitsuda lepromin were still increasing.

Comparison of the 10 per cent chloroform and oil-interface methods of preparing bacillus suspensions revealed differences in antigenic response which were not statistically significant, but which were consistent. Comparison of capacity to produce reactions as test agents showed that the 10 per cent chloroform suspension produced more persistent reactions than those to the oil-interface suspension. When capacity to sensitize was compared, guinea-pigs sensitized to the oil-interface suspension had the more persistent reactions to all of the

antigens tested. A ready explanation for the greater sensitizing capacity of the oil-interface suspensions is found in its content of adjuvant. Not only was there the possibility that a small residue of olive oil emulsion was carried into the final preparation, but also chloroform separation probably caused considerable loss of normal lipid from the bacilli. The relative accentuation of early-response to oil-interface suspension is less readily explained, unless it is merely due to the greater nonspecific irritating activity that was observed with this preparation in unimmunized controls.

On the basis of these studies a combined procedure for separating bacilli from tissues has been developed. To get a good suspension of intact bacilli with minimal antigenic loss, a preliminary oil-interface separation is followed by one-half hour of pancreatin digestion. Differential centrifugation and suspension in Tween 80, 1:1000, produces a good final suspension. To remove bacillary lipids, washing with chloroform may also be indicated.

SUMMARY

Three procedures for preparing relatively tissue-free suspensions of leprosy bacilli have been compared. Digestion with bile-pancreatin removed tissue antigens most completely, but when continued for six hours it also destroyed the bacillary antigen. Separation by shaking with 10 per cent chloroform resulted in some loss of bacilli with discarded tissue residue, and the final suspension was not completely tissue-free; however, the bacilli had good antigenicity. Separation by Henderson's oil-interface method was somewhat more efficient than the 10 per cent chloroform method in producing relatively clean preparations. The oil had a demonstrable adjuvant effect in immunization, but as a test reagent this preparation produced somewhat less persistent reactions than the other bacillus suspensions.

RESUMEN

Se han comparado tres procedimientos dedicados a la preparación de suspensiones de bacilos leprosos, relativamente exentas de tejido. La digestión con bilis-pancreatina eliminó el tejido casi por completo, pero cuando se continuó por seis horas, destruyó también el antígeno bacilar. La separación por agitación con 10 por ciento de cloroformo hizo que algunos de los bacilos quedaran con residuo de tejido descartado y la suspensión final no quedó completamente exenta de tejido; sin embargo, los bacilos poseían buena antigenicidad. La separación por el método Henderson de aceite-entrecaras fué algo más eficaz que el de 10 por ciento de cloroformo en la producción de preparaciones relativamente despejadas. El aceite ejerció un efecto coadyuvante demostrable, pero como reactivo de prueba esta preparación produce reacciones algo menos persistentes que las otras suspensiones bacilares.

RESUMÉ

On a comparé trois procédés pour préparer des suspensions de bacilles de la lèpre relativement libres de tissus. La digestion avec de la bile-pancréatine débarrasse com-

plètement la suspension des antigènes tissulaires, mais lorsque cette digestion est poursuivie durant six heures, elle détruit également l'antigène bacillaire. L'agitation avec 10% de chloroforme produit une certaine perte en bacilles, ceux-ci étant entraînés avec les débris tissulaires et la suspension finale n'est pas complètement dépourvue de tissus; les bacilles ont cependant dans ce cas une bonne capacité antigénique. La séparation par la méthode de Henderson (oil-interface method) est légèrement plus efficace que la méthode au chloroforme à 10%, et fournit des préparations relativement claires. L'huile possède pour l'immunisation un effet adjuvant qu'il est possible de démontrer, mais lorsqu'elle est utilisée pour les tests, cette préparation produit des réactions quelque peu moins persistantes que les autres suspensions bacillaires.

REFERENCES

1. HANKS, J. H. Enumeration of *Mycobacterium leprae* for the standardization of lepromin. *Internat. J. Leprosy* **27** (1959) 134-140.
2. HENDERSON, H. J. A method for separating intact leprosy bacilli from leprous tissue. *Proc. Soc. Exper. Biol. & Med.* **50** (1942) 92-94.
3. KITANO, H. and INOUE, T. The Mitsuda reaction by vaccines treated with ultrasonic wave. *Internat. J. Leprosy* **9** (1941) 29-38.
4. TAYLOR, C. E. Specificity of Dharmendra lepromin as tested in guinea-pigs. *Internat. J. Leprosy* **30** (1962) 175-190.
5. TAYLOR, C. E., HANKS, J. H., MOSES, H., MITTAL, M. C. and KANT, L. The antigenic components of lepromin as assayed in guinea-pigs. *Internat. J. Leprosy* **28** (1960) 284-299.
6. TAYLOR, C. E., HANKS, J. H., and KANT, L. The effect of partial purification of lepromin on the Fernandez- and Mitsuda-type responses in guinea-pigs. *Internat. J. Leprosy* **28** (1960) 423-427.
7. [WORLD HEALTH ORGANIZATION] Expert Committee on Leprosy; First Report. *Wld. Hlth. Org. Tech. Rep. Ser. No. 71*, Geneva, 1953.