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Antigenic Evaluation of *Mycobacterium vaccae* in Relation to *Mycobacterium leprae*[1,3]

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The inability of *Mycobacterium leprae* to grow *in vitro* has of necessity directed efforts toward studying the antigenic composition of other mycobacteria with a view to determining the antigens that these organisms may share with *M. leprae*. This may permit the recognition of an antigenically closely associated mycobacterium(ia) that could be used for studies directed toward vaccine preparation. In an effort to elucidate the antigenic mosaic of *M. leprae*, Abe [1] and Abe, *et al.* [2] and Navalkar [9, 10] and Navalkar and associates [13] were able to demonstrate *M. leprae* specific antigen(s) as well as antigens shared by the leprosy bacillus with other mycobacterial species [9, 10, 11, 12, 13, 14] in cell extracts prepared from lepromatous nodules. Subsequent studies by other investigators [3, 7, 8] have also demonstrated such shared mycobacterial antigens. A few years ago, Stanford and associates [18, 19] presented evidence of immunological linkage between *M. leprae* and *M. vaccae*, a Runyon Group I rapid growing mycobacterium, on the basis of which they proposed that *M. leprae* was more closely associated antigenically with *M. vaccae* than with other mycobacteria. Since then, this organism has been studied by several workers [5, 22] to establish such a relationship. These studies were primarily directed toward the evaluation of cell-mediated immune response in animals sensitized with *M. vaccae* or *M. leprae* and skin tested with homologous and heterologous antigens and also to determine the possibility of an antileprosy vaccine.

The studies reported here are concerned with the antigenic evaluation of *M. vaccae* and the determination of those antigens that are shared by *M. vaccae* and other mycobacteria, including *M. leprae*.

MATERIALS AND METHODS

Mycobacterial strains. The strains used were *M. lepraemurium* (Hawaii), *M. leprae* (human and armadillo derived), *M. paratuberculosis* (*M. johnei*), *M. smegmatis* (no. 8159), *M. kansasii* (no. 24178), *M. marinum* (no. 691), ICRC bacillus, *M. tuberculosis* (no. $H_{37}Rv$), *M. vaccae* (SN920, 923, 931, and 956), *M. avium* (no. 724), *M. intracellulare* (no. 146), *M. fortuitum* (no. 1529), *M. cheloni* (no. 1544), *M. phlei* (no. 1523), *M. gordonae* (no. 1324), *M. scrofulaceum* (no. 1316), and Boone-battey bacillus (no. 1403). The first three organisms listed were separated from infected tissue, and the remainder were grown *in vitro*.

Sera. Thirty-three sera from leprosy patients and four sera from normal healthy individuals were used. Of the 33 leprosy sera, 16 were from lepromatous cases, eight from tuberculoid cases, and nine from borderline cases.

Preparation of cell extract (CE). Cell extracts from mechanically disrupted cells were obtained for all strains execept *M. tuberculosis* (strain $H_{37}Rv$). The $H_{37}Rv$ culture filtrate system was supplied by Trudeau Institute, Inc., Saranac Lake, New York, U.S.A.

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[3] Part of this work was presented at the XI International Leprosy Congress, Mexico City, Mexico, November 1978.

For the preparation of extracts of mycobacterial strains, the bacilli were grown *in vitro* on Sauton ([20]) medium for a period of 6 to 8 weeks. The cells were separated from the medium by means of Buchner funnels and washed three times in 0.15 M sodium chloride. After the third washing the pellet was weighed, and the moist cells were resuspended in isotonic saline at a concentration of 12 mg per ml. The resulting suspension was homogenized in a Teflon grinder and then disrupted in a refrigerated cell fractionator (Sorvall) at 45,000 p.s.i. The material was then stored at −20°C after addition of 1:10,000 merthiolate, until used. For the *M. vaccae* strains, the cells were collected from growth on Löwenstein-Jensen medium since none of the strains showed adequate growth on Sauton medium, either under static or shake culture conditions.

A similar method was used to prepare CE from *M. lepraemurium*, *M. paratuberculosis*, and *M. leprae* grown *in vivo*. The method for the separation of *M. leprae* from infected tissues has been described previously ([9]). *M. lepraemurium* and *M. paratuberculosis* were obtained from Dr. A. M. Dhople and Dr. R. Merkal, respectively. *M. paratuberculosis* were separated from bovine intestinal mucosa, and *M. lepraemurium* were obtained from infected rat livers.

Preparation of reference antisera. Antisera were prepared against the CE of the mycobacterial strains grown or processed in our laboratories. After the collection of a serum sample, male rabbits, weighing 1.5 to 2 kg, were inoculated with 1 ml of a mixture of equal volumes of CE with Freund's incomplete adjuvant. The animals were given weekly subcutaneous injections for 6 weeks, and in some instances, up to 8 weeks. Three rabbits were used for each preparation. One week after the last injection, serum was collected to determine the level of antibodies in each rabbit by immunodiffusion analysis. Only rabbits that showed satisfactory and identical antibody responses to a given immunizing preparation were used to supply serum. The animals were exsanguinated a week after the preliminary sample had been taken and their serum pooled. Serum was stored at −20°C in desired aliquots after the addition of merthiolate. Only *M. vaccae* SN920 was used to prepare a homologous antiserum. No antisera were raised against the other *M. vaccae* strains.

Serological tests. The comparative immunodiffusion (CID) technique of Ouchterlony ([17]), as modified by Hansen ([6]), was used initially to analyze the various sera and to determine the antigenic constituents. Subsequently, the immunoanalysis was carried out using the fused rocket immunoelectrophoresis (FRIEP) technique, as described by Svendsen ([21]) and modified by Chaparas, *et al.* ([4])

RESULTS

As a first step toward recognizing the various antigens of *M. vaccae* (SN920), the CE was tested by immunodiffusion against the corresponding antiserum prepared in rabbits. Nine lines of precipitation were observed, indicating at least nine antigens. The three other strains of *M. vaccae* (SN923, 931, and 956) showed identical antigenic composition as *M. vaccae* (SN920). The next step was to determine by CID analysis the number of antigens shared by *M. vaccae* and other mycobacterial species.

Fig. 1 summarizes the results of the CID studies. *M. vaccae* shared one of its antigens with *M. marinum*, which was grown at 37°C, and with *M. gordonae*; two of its antigens with *M. marinum* grown at 30°C, the ICRC bacillus, and *M. lepraemurium*; three each with *M. paratuberculosis* and *M. kansasii*; four each with *M. leprae* derived from both human and armadillo tissues, *M. phlei*, *M. smegmatis*, and *M. tuberculosis* (strain $H_{37}Rv$); five each with *M. scrofulaceum* and *M. cheloni*; and six each with *M. avium* and *M. intracellulare*. The results given are those obtained with the cell extracts of all strains, except *M. tuberculosis* (strain $H_{37}Rv$) where concentrated culture filtrate was used as the antigen. Fig. 2 is a photograph of a CID analysis between *M. vaccae* and *M. intracellulare* systems.

The Table summarizes the results of the antigenic evaluation of *M. vaccae* and comparative antigen analysis by fused rocket immunoelectrophoresis (FRIEP). With this technique, using only the antisera against other mycobacteria, it was possible to detect anywhere between 13 and 16 antigens of

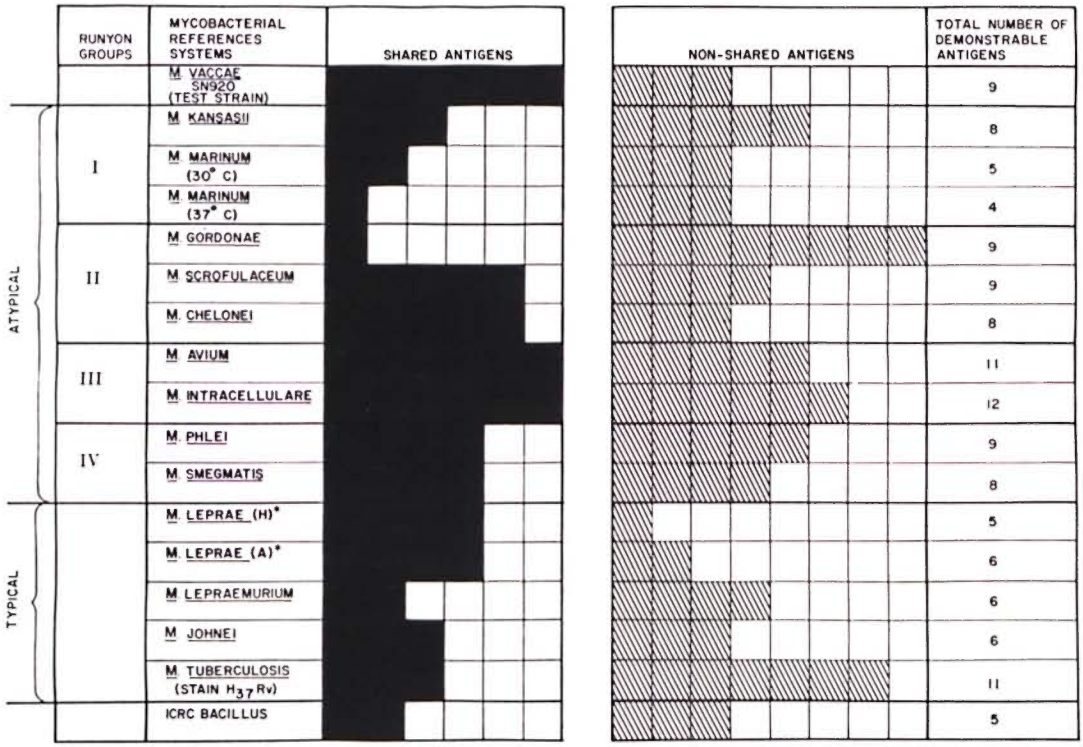

FIG. 1. Antigenic relationships between *Mycobacterium vaccae* and other mycobacteria, as shown by comparative immunodiffusion (CID) analysis. Shared antigens are indicated by dark squares and non-shared by hatched ones. All strains except *M. tuberculosis* (no. $H_{37}Rv$) examined as CE Preparations. (H)* = human derived; (A)* = armadillo derived.

M. vaccae, depending on the strain used. This was more than those detected by immunodiffusion. A comparative analysis of the antigens with other mycobacterial antisera also showed increased numbers of shared antigens. For example, the maximum number shared was with the Battey bacillus and *M. phlei* (12 each), *M. smegmatis* (11), and *M. scrofulaceum*, *M. cheloni*, *M. avium*, and *M. leprae* (10 each). *M. intracellulare* (9), *M. fortuitum* (8), and *M. kansasii* (7) fall into an intermediate group as far as sharing is concerned whereas *M. gordonae* (2) and *M. marinum* (2) exhibit the lowest number of shared antigens. Fig. 3 is a photograph of FRIEP analysis of *M. vaccae* against anti-*M. intracellulare*, *M. avium*, *M. scrofulaceum*, *M. leprae*, *M. vaccae*, and *M. smegmatis*.

When the *M. vaccae* system was used against sera from leprosy patients, only the bacillary positive cases showed lines of precipitins. Not all sera, however, from the bacillary positive cases gave positive results. Of the 16 sera from lepromatous patients, eight showed strong lines of at least two precipitins whereas two showed very faint, almost undetectable reactions. Of the nine sera from borderline cases, positive precipitin reactions were seen with only three cases. In our earlier studies ([10, 13]), all of the bacillary positive cases selected had demonstrated the presence of either one or two immunoprecipitates, and some of the bacillary negative cases had exhibited one immunoprecipitate when various mycobacterial reference systems were used for CID assay.

DISCUSSION

Employing two different techniques of immunoanalysis, *M. vaccae* were shown to possess a minimum of nine (ID) and a maximum of 13 (FRIEP) demonstrable antigens. Thus the FRIEP technique of antigen analysis was more sensitive in eliciting the

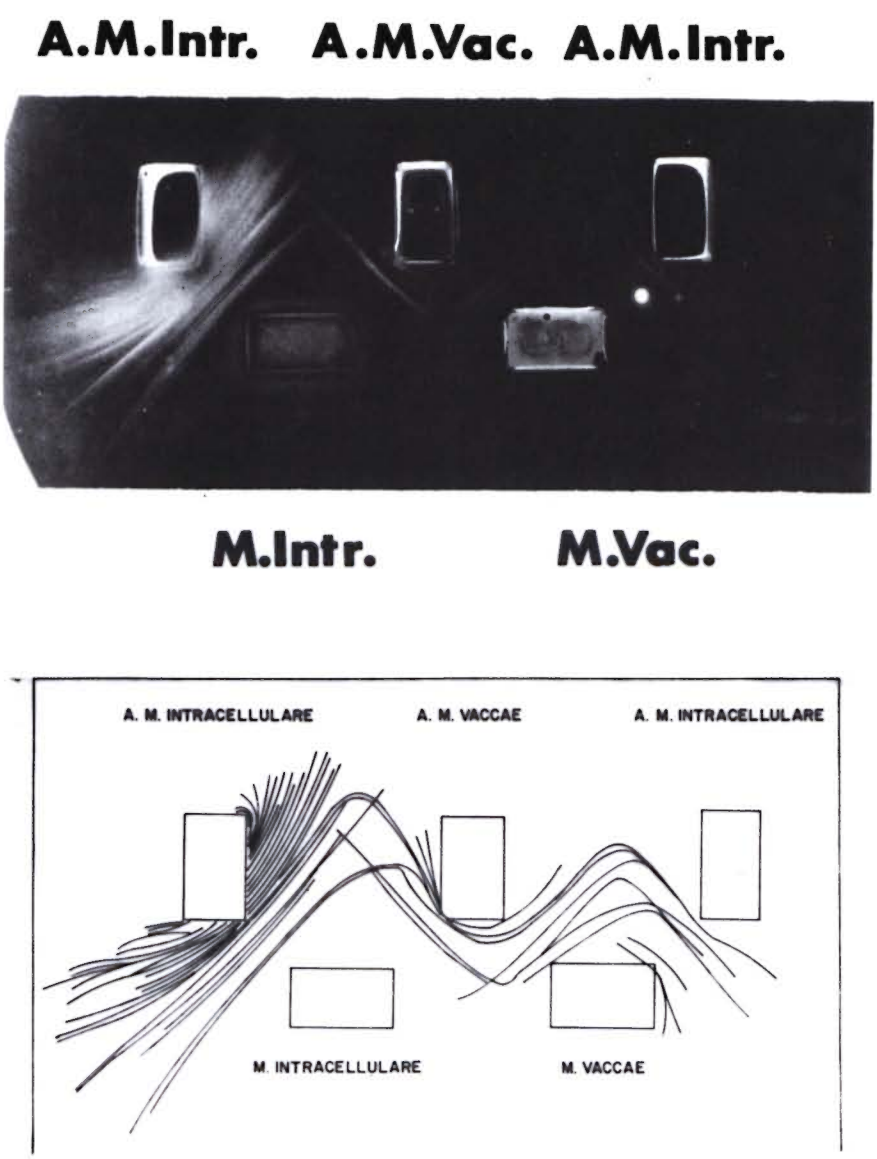

FIG. 2. Comparative precipitation analysis of *M. vaccae* and *M. intracellulare* systems with schematic representation, using the CID technique.

antigenic structure of a given organism. This was also evident in the comparative antigenic studies using other mycobacterial systems. However, with the FRIEP technique it was difficult to establish a reaction of identity between antigens because of the close proximity of the immunoprecipitin bands. These difficulties were not encountered with the five-well comparative ID (CID) method. This facilitated the identification of the two most commonly shared antigens between *M. vaccae* and other mycobacterial species, i.e., the *beta* and *delta* antigens, which we have described previously. Antibodies to these antigens have been shown to be present in the majority of sera from leprosy patients ([10, 12]). Confirmation of the presence of *beta* and *delta* antigens in *M. vaccae* was obtained using leprosy sera. The precipitin positive sera

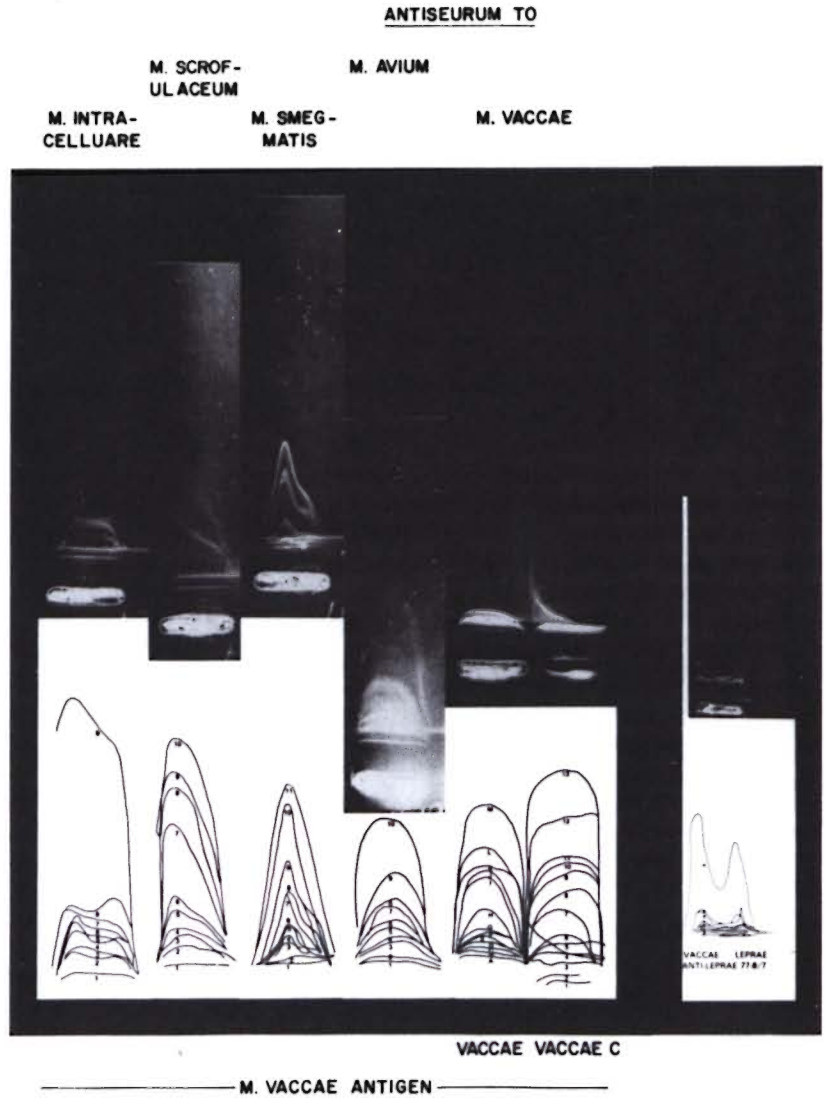

FIG. 3. Precipitation analysis with schematic representation of *M. vaccae* antigen and antisera prepared against various mycobacterial species, using the FRIEP technique.

showed antibodies to these antigens when screened simultaneously against the *M. vaccae* system and *M. smegmatis*, our standard reference system ([10]).

M. vaccae appeared to share some of its demonstrable antigens with other mycobacteria. With the CID technique the maximum number of antigens that were shown to be shared were with the Runyon Group III mycobacteria, particularly with *M. avium* and *M. intracellulare*. *M. gordonae*, which showed a minimal number of shared antigens, either with the CID or the FRIEP technique, was exceptional. In this regard, our data appear to confirm those of Stanford and Rook ([18]). The absence of mycolic acids of the mycobacterial type and the nonreactivity of the skin test agent in leprosy patients described by these authors ([18]) may be the reasons for such limited sharing of antigens with *M. gordonae*.

When one compares the sharing of antigens between *M. vaccae* and other mycobacteria, as demonstrated either by the CID

THE TABLE. *Antigenic sharing of* M. vaccae *(SN920) as demonstrated by the fused rocket immunoelectrophoretic (FRIEP) method, using antisera against various mycobacteria.*

Runyon Group	Mycobacterial antisera	No. of shared antigens	Total no. of demonstrable homologous antigens
I	*M. kansasii*	7	25
	M. marinum 30°C	3	29
	M. marinum 37°C	1	20
II	*M. gordonae*	2	16
	M. scrofulaceum	10	23
	M. cheloni	10	31
III	*M. avium*	10	30
	M. intracellulare	9	22
	Battey boone bacillus	16	22
IV	*M. phlei*	12	22
	M. smegmatis	11	31
	M. fortuitum	8	22
	M. tuberculois ($H_{37}Rv$)	6	24
	M. leprae (human tissue derived)	10	18
	M. leprae (armadillo tissue derived)	11	18
Test strain:	*M. vaccae* (SN920)	—	16

or the FRIEP techniques, one recognizes that such a sharing on a large scale is rather broad based and is not specific to any particular group. The taxonomical significance of this is rather unclear at this time since *M. vaccae* tend to be photochromogenic but exhibit the same general biochemical patterns as *M. phlei* (W. D. Jones, personal communication).

Apparently *M. vaccae* shares a rather limited number of its antigens with either human- or armadillo-derived *M. leprae* or for that matter with any of the typical strains examined regardless of the technique used. Further support for this observation comes from the studies using leprosy sera in which only antibodies against the most commonly shared antigens were detectable. The sera selected for the assays, especially those from the bacillary positive cases, had all shown positive precipitin reactions against the various mycobacterial systems used in our previous studies. Yet in the present study, only a limited number of these sera gave positive results whether they were screened with the *M. vaccae* system alone, with the *M. smegmatis* reference system, or with other mycobacterial reference systems. The sera tested have been from a collection that has been with us over the past 12 years and have been stored at −20°C throughout, except when needed, with minimum frequency of thawing and refreezing. It is likely that such prolonged storage could have led to the discrepancies in our present results and those observed previously.

Although *M. vaccae* is a rapid growing photochromogenic organism, it appears to share very few antigenic determinants with any of the two photochromogens used in the screening. This is not unexpected since Norlin [16] has shown that not all species within a given group will demonstrate the maximum number of shared antigens.

The present study has indicated that *M. vaccae* may be considered as a rapid grower, which is antigenically more similar to the *Mycobacterium avium-intracellulare* complex than to the typical group IV mycobacteria. It does not, however, indicate any particularly close relationship with *M. leprae* by the methods we have employed although Stanford and associates [18, 19] have established such an immunological linkage through their studies. Studies by Collins, *et al.* [5] and Watson, *et al.* [22] have also shown such an absence of rela-

tionship. The closeness of skin reactions in leprosy patients and in presensitized animals with skin test antigens prepared from these two mycobacteria observed by Stanford and coworkers ([18, 19]) could have been due to a *M. leprae* antigen that may be shared specifically by *M. vaccae*. This hypothesis is supported by our earlier studies on the antigens of *M. leprae* ([11]) and on the humoral immune response in *M. leprae* infected mice ([15]).

In these studies ([11, 15]), one of the materials we used was designated as Fraction C, which contained a single antigen of *M. leprae* when analyzed by immunodiffusion. This fraction was strongly immunoactive, as evidenced by the delayed-type hypersensitivity in *M. leprae* sensitized guinea pigs, the number of plaque-forming cells elicited, and by the ability to transfer cutaneous anaphylaxis. This fraction also elicited positive reactions in BCG sensitized animals but not in animals sensitized to a variety of other mycobacteria. It is likely that *M. vaccae* possess this antigen which is shared by *M. leprae* and BCG; we have not, however, analyzed Fraction C using the anti-*M. vaccae* antiserum. On the other hand, it is likely that *M. vaccae* may be sharing a different antigen with *M. leprae* that perhaps is not common to other mycobacteria. Most investigators of mycobacterial antigens have as yet failed to demonstrate any antigen found in *M. leprae* only; although some do suggest that they may be dealing with such an antigen ([3]).

From the data presented, we conclude that *M. vaccae* do possess the two most commonly shared antigens between various mycobacterial species, the *beta* and the *delta*-antigens previously described by us ([9, 10, 12]). We have, however, not been able to establish any particularly close immunological relationship between *M. vaccae* and *M. leprae* that would suggest the use of *M. vaccae* as a possible antileprosy vaccine source.

SUMMARY

Immunodiffusion analysis of *Mycobacterium vaccae* indicated the presence of at least nine antigens. Using the technique of fused rocket immunoelectrophoresis, at least 13 such antigens were detected. Comparative analysis of the *M. vaccae* antigen-antibody system with similar systems established for other mycobacterial species showed that *M. vaccae* shared a significant number of antigens with both typical and atypical mycobacteria. These included the already described *beta* and *delta* antigens that are common to the majority of mycobacterial species. Confirmation of the presence of these two antigens was obtained through comparative analysis of sera from leprosy patients, using *M. vaccae* and *M. smegmatis* conjointly.

It has been concluded that *M. vaccae* possess the two most commonly shared antigens among various mycobacterial species, in addition to many others. It has not been possible with the methods employed to establish any particularly close immunological relationship between *M. vaccae* and *M. leprae* that would indicate the use of *M. vaccae* as a possible antileprosy vaccine source although this possibility has been suggested by other investigators.

RESUMEN

El análisis por inmunodifusión del *Mycobacterium vaccae* indicó la presencia de cuando menos 9 componentes antigénicos. Usando una técnica más sensible (''fused rocket immunoelectrophoresis'') se lograron demostrar cuando menos 13 antígenos. El estudio comparativo del sistema antígeno-anticuerpo correspondiente al *M. vaccae* con sistemas similares establecidos para otras especies micobacterianas demostró que el *M. vaccae* comparte un número significante de antígenos con otras micobacterias típicas y atípicas. Estos incluyen los antígenos beta y delta previamente descritos, que son comunes a la mayoría de las especies micobacterianas. La presencia de estos dos antígenos se confirmó por el análisis comparativo de sueros de pacientes con lepra usando simultaneamente los sistemas de *M. vaccae* y *M. smegmatis*.

Se concluyó que el *M. vaccae* posee los dos antígenos más comunmente compartidos por varias especies micobacterianas, además de muchos otros. Sin embargo, con los métodos empleados, no fue posible establecer alguna relación inmunológica particularmente importante entre *M. vaccae* y *M. leprae* que pudiera indicar el uso del *M. vaccae* como fuente de una posible vacuna contra la lepra aún cuando esta posibilidad ha sido sugerida por otros investigadores.

RÉSUMÉ

Les études d'immunodiffusion de *Mycobacterium vaccae* ont révélé la présence d'au moins neuf antigènes. En utilisant une technique plus sensible d'immunoélectrophorèse croisée (''fused rocket''), on a pu mettre en évidence au moins treize antigènes.

Une analyse comparative du système antigènes-anticorps de *M. vaccae*, avec d'autres systèmes semblables établis pour d'autres espèces mycobactériennes, ont montré que *M. vaccae* partageait un nombre significatif d'antigènes, tant avec les mycobactéries typiques qu'avec les mycobactéries atypiques. Ces antigènes comprenaient les antigènes bêta et delta qui ont déjà été décrits et qui sont communs à la majorité des espèces mycobactériennes. La confirmation de la présence de ces deux antigènes a été obtenue par une analyse comparative d'échantillons de sérum obtenus chez des malades de la lèpre, avec des systèmes antigènes-anticorps pour *M. vaccae* et *M. smegmatis* utilisés conjointement.

On en a conclu que *M. vaccae* possède les deux antigènes qui sont partagés le plus souvent par toutes les espèces mycobactériennes, outre de nombreux autres antigènes. Avec les méthodes utilisées, il n'a pas été possible d'établir aucune relation immunologique particulièrement étroite entre *M. vaccae* et *M. leprae*, pouvant suggérer que *M. vaccae* pourrait servir de source pour la mise au point éventuelle d'un vaccin anti-lépreux. Ceci est en contradiction avec les suggestions faites par d'autres chercheurs qui avaient mentionné cette possibilité.

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REFERENCES

1. Abe, M. Studies on the antigenic specificity of *Mycobacterium leprae*. I. Demonstration of soluble antigens in leprosy by immunodiffusion. Int. J. Lepr. **38** (1970) 113–115.
2. Abe, M., Minagawa, F., Yoshino, Y. and Okamura, K. Studies on the antigenic specificity of *Mycobacterium leprae*. II. Purification and immunological characterization of the soluble antigen in leprosy nodules. Int. J. Lepr. **40** (1972) 107–117.
3. Caldwell, H. D., Kirchheimer, W. F. and Buchanan, T. M. Identification of a *Mycobacterium leprae* specific protein antigen(s) and its possible application for the serodiagnosis of leprosy. Int. J. Lepr. **47** (1979) 477–483.
4. Chaparas, S. D., Brown, T. and Hyman, I. Antigenic relationship among species of mycobacterium studied by fused rocket immunoelectrophoresis. Int. J. Syst. Bacteriol. **28** (1978) 547–560.
5. Collins, F. M., Morrison, N. E. and Montalbine, V. Immune response to persistent mycobacterial infection in mice. Infect. Immun. **20** (1978) 430–438.
6. Hansen, L. A. Immunologic analysis of streptococcal antigens and human sera by means of diffusion-in-gel methods. Int. Arch. Allergy **14** (1959) 279–291.
7. Harboe, M., Closs, O., Bjune, G., Kronvall, G. and Axelsen, N. H. *Mycobacterium leprae* specific antibodies detected by radioimmunoassay. Scand. J. Immunol. **7** (1978) 111–120.
8. Kronvall, G., Closs, O. and Bjune, G. Common antigen of *Mycobacterium leprae*, *M. lepraemurium*, *M. avium* and *M. fortuitum* in comparative studies using two different types of antisera. Infect. Immun. **16** (1977) 542–546.
9. Navalkar, R. G. Immunologic analysis of *Mycobacterium leprae* antigens by means of diffusion-in-gel methods. Int. J. Lepr. **39** (1971) 105–112.
10. Navalkar, R. G. Immunologic studies on leprosy. 2. Antigenic studies of *Myobacterium leprae*. Z. Tropenmed. Parasitol. **24** (1973) 66–72.
11. Navalkar, R. G. and Warick, R. P. Effect of variation in growth temperature on the biochemical activity and antigens of *Mycobacterium marinum*. Zentralbl. Bacteriol. Hyg. (Orig. A) **226** (I. Abt.) (1974) 97–104.
12. Navalkar, R. G., Dalvi, R. R. and Patel, P. J. Antigenic evaluation of *Mycobacterium lepraemurium*. J. Med. Microbiol. **8** (1975) 177–181.
13. Navalkar, R. G., Norlin, M. and Ouchterlony, O. Characterization of leprosy sera with various mycobacterial antigens using double diffusion-in-gel analysis—II. Int. Arch. Allergy **28** (1965) 250–260.
14. Navalkar, R. G., Patel, P. J. and Dalvi, R. R. Immunological studies on leprosy: Separation and evaluation of the antigens of *Mycobacterium leprae*. J. Med. Microbiol. **8** (1975) 319–324.
15. Navalkar, R. G., Patel, P. J., Dalvi, R. R. and Levy, L. Immune response to *Mycobacterium leprae*: plaque-forming cells in mice. Infect. Immun. **10** (1974) 1302–1306.
16. Norlin, M. Unclassified mycobacteria. A comparison between a serological and biochemical classification method. Bull. Un. Int. Tuberc. **36** (1965) 25–35.
17. Ouchterlony, O. Diffusion-in-gel methods for immunological analysis. II. Prog. Allergy **6** (1962) 30–154.
18. Stanford, J. L. and Rook, G. A. W. Taxonomic studies on the leprosy bacillus. Int. J. Lepr. **44** (1976) 216–221.
19. Stanford, J. L., Rook, G. A. W., Convit, J., Godal, T., Kronvall, G., Rees, R. J. W. and Walsh, G. P. Preliminary taxonomic studies on the leprosy bacillus. Br. J. Exp. Pathol. **56** (1975) 579–585.

20. SAUTON, M. B. Sur la nutrition minérale du bacille tuberculeux. C. R. Acad. Sci. **155** (1912) 860.
21. SVENDSEN, P. J. Fused rocket immunoelectrophoresis. In: *A Manual of Quantitative Electrophoresis*. Axelsen, N. H., Krøll, J. and Weeke, B., eds. Oslo: Universitetsforlaget, 1975, pp. 69–70.
22. WATSON, S. R., MORRISON, N. E. and COLLINS, F. M. Delayed hypersensitivity responses in mice and guinea pigs to *Mycobacterium leprae*, *Mycobacterium vaccae* and *Mycobacterium nonchromogenicum* cytoplasmic proteins. Infect. Immun. **25** (1979) 229–236.