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Detection of Antigen in *Nocardia caviae* Cross-Reacting with Mycobacterial Antigen No. 21 in *M. leprae* Using a Lepromatous Leprosy Serum Pool as Antibody Reagent[1]

Bjarne Bjorvatn and Göran Kronvall[2]

The crossed immunoelectrophoresis (CIE) technique has been successfully applied to several studies of the antigenic composition of various mycobacterial species including *M. leprae* ([1,3,7,9,10,11,17]). Using rabbit hyperimmune sera against mycobacterial strains, more than 40 antigen components can be defined with this method ([3,8,10]). The antigens can be compared in different bacterial species aiding in serological classification ([3,10,11,17]). Similar comparisons can be made using specific antisera ([3,7,9]). So far, CIE systems employing *M. leprae* antigen preparations against either rabbit antisera or lepromatous serum pools have shown a strikingly low number of precipitin lines, mostly not more than ten ([7,10,12]). Hence, a serological classification of *M. leprae* in relation to other mycobacteria has not been possible using these techniques of antigen component identification. The low number of antigens detected seemed to be due to a correspondingly low number of components in the antigen preparation itself as prepared from armadillo grown bacilli ([7]). A recent report, however, shows that immunization with more concentrated antigen preparations will provide rabbit antisera detecting up to 20 components ([4]).

The detection of antigenic heterogeneity in one single component labeled No. 21 in a *M. smegmatis* reference system indicated another way to make comparisons between different species ([10,12]). Four different groups of antigenic determinants were detected on this antigen. Group 21 A was apparently shared by all mycobacteria, 21 B seemed to be unique for antigen No. 21 of *M. leprae*, 21 C was present in all mycobacteria tested except the leprosy bacillus, and finally, 21 D was confined to two fast growing species tested ([11]). When tested for its taxonomic validity on another noncultivable mycobacterium, *M. lepraemurium*, this species behaved like *M. avium* regarding antigen No. 21 determinants, in accordance with reports by others on its classification ([17]). A separate position of *M. leprae* as compared to other mycobacterial species was therefore concluded ([11]). In the present studies antigen No. 21 comparisons were extended to *Mycobacterium*-related genera with special emphasis on the genus *Nocardia* in a search for species with relationships to *M. leprae*.

MATERIALS AND METHODS

Bacterial strains. The various bacterial strains tested and their origin and designation are given in the Table. Details concerning the methods used have been described previously ([12]).

Antigen preparations. Antigen preparations of *M. leprae* were obtained from bacilli grown in armadillos. The preparative steps included homogenization of armadillo tissue, repeated washings in phosphate buffered saline (PBS), dehydration with acetone and ether, and then extraction of the bacilli using an oil-chloroform mixture ([12]). The centrifuged extracts were washed and ultrasonicated. Insoluble residues were separated by centrifugation from the soluble material that was finally used in the CIE.

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[2] B. Bjorvatn, M.D.; G. Kronvall, M.D.; Armauer Hansen Research Institute (AHRI), P.O. Box 1005, Addis Ababa, Ethiopia. Dr. Bjorvatn's present address is Microbiological Department, Regional Hospital, 9012 Tromsø, Norway; Dr. Kronvall's present address is Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

TABLE. *Designations and origins of 18 nocardia and four other strains under investigation shown together with the number of precipitation lines obtained in CIE with soluble antigen preparations against a pool of lepromatous sera (LSP) and the relation of such precipitates to the No. 21 antigen of* M. leprae.

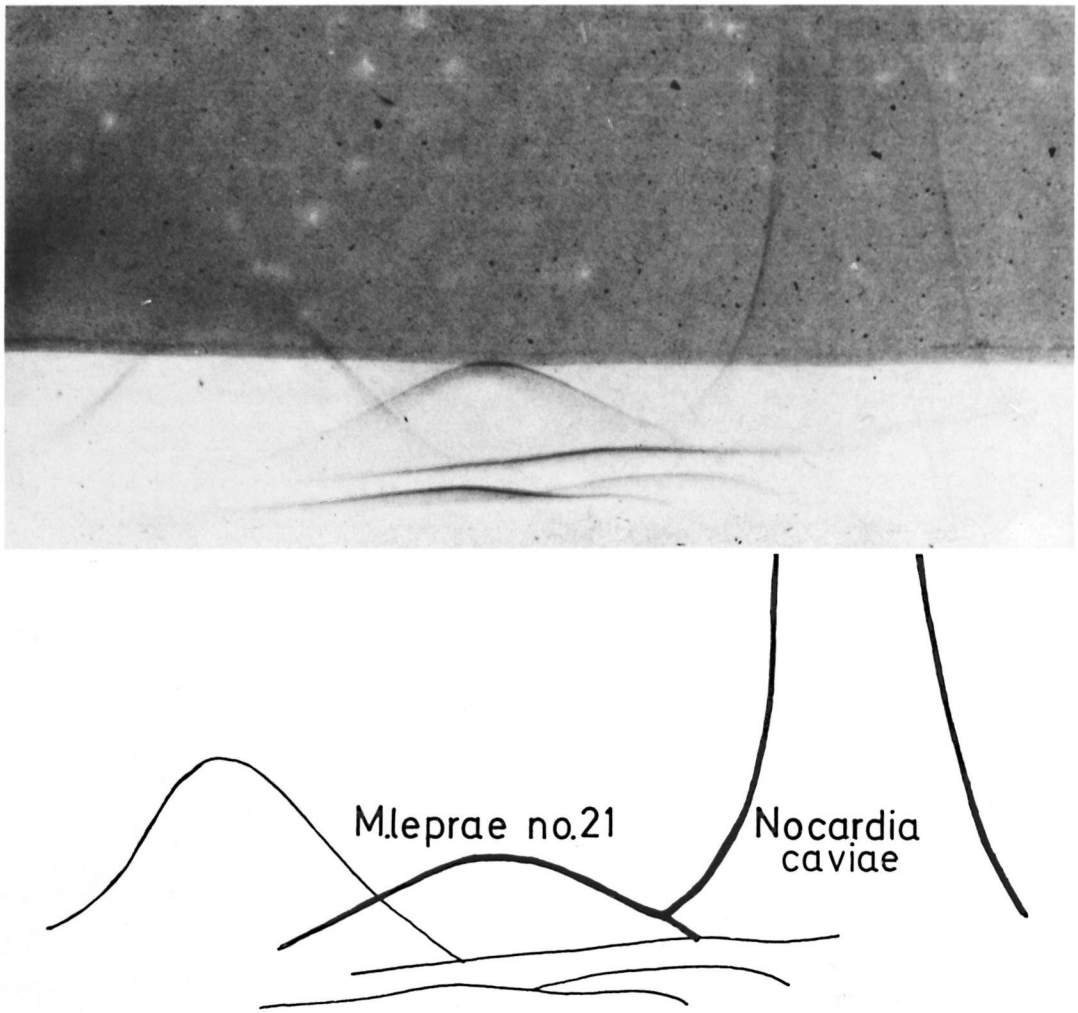
Strain	Supplied by[a]/Origin/Designations	Lines against LSP	Relation[b] to antigen 21 of:		
			M.l.[c]	M.a.i.[d]	M.s.[e]
N. asteroides	B/Olive View Hosp. Los Angeles/SN 5401, 2267, N. 10051	1	0	0	0
N. asteroides	B/Olive View Hosp. Los Angeles/SN 5421, 2270, N. 10048	1	0	0	0
N. asteroides	B/Dr. M. Goodfellow, Newcastle/SN 5422, N. 100, R. Gordon 652	1	0	0	0
N. asteroides	K/Dr. Emmons via Statens Seruminst. Copenhagen/ Emmons 9935, Gordon 404	2	0	0	0
N. brasiliensis	B/Dr. Mariat, Inst. Pasteur, Paris/SN 5501, 700	0	0	0	0
N. brasiliensis	G/Dr. R. Gordon/N 78, 605	1	0	0	0
N. brasiliensis	K/Dr. M. Goodfellow, Newcastle/354, N 318, ATCC 19296	0	0	0	0
N. caviae	B/Dr. Mariat, Inst. Pasteur, Paris/SN 5601, 775	2	X	I	I
N. caviae	B/Dr. Mariat, Inst. Pasteur, Paris/SN 5622, 774	2	X	I	I
N. caviae	B/Dr. Mariat, Inst. Pasteur, Paris/SN 5624, N 231, Dr. Olds CN 749	2	X	I	I
N. caviae	K/Dr. R. Gordon/606, 617	1	X	I	I
N. pellegrino	B/Dr. Szabo, Ntl. Inst. Tuberculosis, Budapest/SN 5101, Pellegrino 327	3	0	0	0
N. pellegrino	B/Dr. Szabo/Ntl. Inst. Tuberculosis, Budapest/SN 5102, Pellegrino 330	3	0	0	0
N. pellegrino	B/Inst. Pasteur, Lille/SN 5103	3	0	0	0
N. rubra	B/Olive View Hosp. Los Angeles/SN 5201, 2295, N. 12000	2	0	0	0
N. rubra	B/Olive View Hosp. Los Angeles/SN 5202, 2296, N. 12001	3	0	0	0
N. rubra	B/Olive View Hosp. Los Angeles/SN 5203, 2299, N. 12005	3	0	0	0
N. rubra	K/Dr. McClung/784, 74 NRRL B-85	1	0	0	0
C. equi (1c)	G/Dr. R. Gordon/R78, 1621, ATCC 25729	2	0	0	0
C. fascians	G/Dr. R. Gordon/R87, 12974, ATCC 25738	2	0	0	0
M. rhodochrous (1a)	G/Dr. R. Gordon/R75, 1095, ATCC 25726	1	0	0	0
M. rhodochrous (1b)	G/Dr. R. Gordon/R72, 1054, ATCC 25723	0	0	0	0

[a] Supplied by B: Dr. I. Tarnok, Forschungsinstitut, Borstel.
K: Dr. M. Magnusson, Statens Seruminstitut, Copenhagen.
G: Dr. A. Lind, Inst. of Med. Microbiol., Univ. of Gøteborg.
[b] X: partial identity I: identity
[c] M.l.: *M. leprae*, [d] M.a.i.: *M. avium-intracellulare*, [e] M.s.: *M. smegmatis*

The cultivable bacterial strains were harvested after maximal growth on Sauton or Sabouraud medium solidified with 1.5% agar. One g (wet weight) of each bacterial strain was suspended in 10 ml of PBS, ultrasonicated, and insoluble residues separated by centrifugation from the soluble antigen preparation.

Antibody source. Serum samples from 20 patients with lepromatous leprosy were pooled and stored at −20°C until used. Each of these sera had been shown to give rise to several precipitation lines against *M. leprae* antigens in the present CIE system.

Antigen reference systems. The precipitation system obtained with *M. leprae* antigen in CIE against the lepromatous serum pool (LSP) was used as a reference for antigenic comparisons. Initial tandem CIE experiments confirmed the partial identity be-



THE FIGURE. Partial identity of No. 21 antigen of *M. leprae* over the corresponding *N. caviae* antigen in tandem CIE against a lepromatous serum pool.

tween the 21 antigen of the *M. leprae* preparation used and the corresponding antigen of the *M. smegmatis* reference system of Kronvall, *et al.* [10].

Crossed immunoelectrophoresis. The Laurell method [14], modified according to Axelsen [2], was used with the tandem technique for studies of antigenic cross-reactivity. To facilitate the comparison of new lines with the reference system the intermediate gel method was used.

RESULTS

In crossed immunoelectrophoretic analysis using a lepromatous leprosy serum pool as the antibody source, most of the bacterial strains tested gave rise to one to three precipitation lines. As shown in the Table, three lines were detected in the *N. pellegrino* antigen preparations (three strains) and in two out of the four antigenic preparations of *N. rubra*. Three *N. caviae* strains showed two lines, and one line was obtained with all four strains of *N. asteroides* except one whereas only one out of the three *N. brasiliensis* strains gave rise to visible precipitation in this system. The two strains of corynebacteria tested (*C. equi* and *C. fascians*) showed two distinct precipitates each whereas only one of the two *M. rhodochrous* strains gave rise to a single precipitation line. In terms of number of antigens detected, there were no major

differences seen among the different strains or bacterial species.

All precipitation lines obtained with the different bacterial antigen preparations against the lepromatous serum pool were subsequently compared directly with the No. 21 antigen of *M. leprae* in tandem experiments. One antigen component shared by all four *N. caviae* strains consistently showed a reaction of partial identity with the No. 21 antigen of *M. leprae*. In all such cases the antigen of *M. leprae* spurred over the corresponding *N. caviae* antigen, indicating that the antiserum used detected more antigenic determinants on the *M. leprae* antigen (The Figure). All other strains of nocardia included in these studies were negative with respect to antigen No. 21 determinants detectable with the lepromatous serum pool.

The *N. caviae* strains were compared in tandem experiments with *M. avium-intracellulare* and with *M. smegmatis* as representatives for slow-growing and fast-growing mycobacterial species, respectively. Both of these mycobacteria gave a reaction of identity with the *N. caviae* strains. The results indicate that antigen determinants are present on *N. caviae* No. 21 antigens which are shared by mycobacterial species but are not detected in the other strains of nocardia tested with the antiserum used.

DISCUSSION

Antigenic relationships between species of nocardia and mycobacteria have been well documented in several studies [5, 6, 7, 9, 13, 15]. Thus, the mycobacterial common precipitinogen alpha has been demonstrated in nocardial species, including strains of *N. asteroides, N. brasiliensis,* and *N. caviae*. The related, so called partial alpha has been found in, e.g., strains of *N. rubra*. Another mycobacterial common precipitinogen designated beta, identical with antigen No. 1 in our present *M. smegmatis* reference system, is known to be shared by nocardial species such as strains representative of *N. asteroides, N. caviae, N. rubra,* and *N. pellegrino* [15]. In recent CIE studies by Harboe, *et al.* [9], *N. asteroides* was shown to share eight antigens with *M. bovis* (BCG). Also, anti-*N. asteroides* serum included in the intermediate gel of an *M. leprae*-anti-*M. leprae* system was found to interfere with six out of seven components of *M. leprae* [7]. It is therefore clear that relatives of *M. leprae* might be sought not only among species of mycobacteria but also among strains of nocardia. Our approach to studies of relationships with *M. leprae* is based on the finding that one major antigenic component, called antigen No. 21, varies slightly in different species [10, 11, 12]. This variation, which can be detected immunologically using simple precipitation systems like CIE, seems to reflect the degree of taxonomic relationship in much the same way as, for instance, hemoglobin variations among mammals. This approach is particularly valuable for studies with *M. leprae* in which a low number of antigenic components are usually detected. The demonstration of cross-reactions between antigens, however, critically depends on the properties of the antibody reagent used. Inability to produce visible precipitates does not exclude the presence of cross-reacting antigens. Lepromatous serum pools as used in the present studies appear to be efficient sources of hyperimmune antibodies against *M. leprae* antigens with some degree of cross-reaction with common mycobacterial antigens [4, 10].

In the present studies, using a lepromatous serum pool as the antibody source, the mycobacterial common antigen No. 21 was consistently found in *N. caviae* strains but in none of the other four nocardial species under study. The No. 21 antigen of *M. leprae* and the corresponding antigen of *N. caviae* showed a reaction of partial identity as demonstrated in tandem CIE experiments using lepromatous sera as the antibody source. This was constant and clear-cut using antigen preparations from all four *N. caviae* strains represented. With the same antibody source, reactions of full identity were obtained with the *N. caviae* strains in tandem with *M. avium-intracellulare* and *M. smegmatis*. These results confirm the molecular heterogeneity of antigen No. 21 in different bacterial species. They also suggest that antigen No. 21 of *N. caviae* is closely related to the mycobacterial antigen No. 21. In studies by Harboe, *et al.* [7, 9], hyperimmune serum against *N. asteroides* used in the intermediate gel included antibodies which interfered with antigen No. 21 equivalents of *M. bovis* (BCG)

and *M. leprae*, designated No. 62 and No. 4 in these two reference systems, respectively. Thus, according to these experiments antigen No. 21 equivalents exist in *N. asteroides*.

Extensive numerical classification studies of *Nocardia* ([16]) have recently indicated that strains generally referred to as *N. asteroides* constitute quite a heterogeneous group and may more correctly be divided into *N. farcinica*, *N. asteroides* A, and *N. asteroides* B. Provided that this heterogeneity has immunologic implications, the choice of *N. asteroides* strains for experimental work may be crucial, and accordingly, results obtained with different *N. asteroides* strains might not be strictly comparable. Since the heterogeneous antigen No. 21 is also present in at least some *N. asteroides* strains ([7, 9]), continued studies along these lines may add to the description of species and subspecies within the genus *Nocardia* in a similar fashion as this antigen is now being exploited for the taxonomy of mycobacteria. However, the availability of potent monospecific hyperimmune sera is a prerequisite for further progress in this field.

Taxonomic investigations of *M. leprae* based on immuno-precipitation techniques have until recently been hampered as a consequence of the few antigenic components demonstrable ([7]). These few components also show wide cross-reactivity within the genus *Mycobacterium* as well as with related genera ([7, 10]). In a recent investigation, Closs, *et al.* ([4]) have shown that armadillo-grown *M. leprae* antigen preparations do contain at least 24 antigens, most of which are apparently in very low concentrations. The possibility that some of these additional antigens may be *M. leprae* specific awaits further confirmation. Studies of antigen No. 21 heterogeneity among mycobacterial and nocardial strains offer a new approach to studies of bacterial relatedness. In view of the previous demonstration of antigen No. 21 equivalents in *N. asteroides* by Harboe, *et al.* ([7, 9]), the inability to detect cross-reactions between this species and *M. leprae* in the present identification system using lepromatous leprosy sera is of interest. The results suggest a very distant relationship between *M. leprae* and strains of nocardia with one exception. *Nocardia caviae* seems to possess an antigen No. 21 which is similar to the mycobacterial antigen No. 21. Its exact relationships among mycobacterial species remains to be determined using other antibody reagents.

Note added in support: In a recent article by Ridell, M., Baker, R., Lind, A. and Ouchterlony, Ö., entitled, "Immunodiffusion studies of ribosomes in classification of mycobacteria and related taxa" (Int. Arch. Allergy Appl. Immunol. **59** [1979] 162–172), the authors conclude (p. 171): "Thus common antigens appearing with and without a spur such as precipitinogen d of the *M. fortuitum* 30 S can be regarded as being more useful in serotaxonomical work than antigens which cannot be serologically differentiated." This statement supports the research approach for *M. leprae* studies introduced by one of the present authors (G.K.) in 1975 ([10, 11, 12]).

SUMMARY

Eighteen strains of nocardia, representing five different species, were studied in crossed immunoelectrophoresis against a lepromatous leprosy serum pool for the presence of antigen No. 21. This mycobacterial antigen shows antigenic heterogeneity with species specific antigenic determinants defined in *Mycobacterium leprae*. All four strains of *N. caviae* were found to share antigen No. 21 determinants with mycobacteria. All other strains of nocardia were negative in these direct immunoprecipitation tests. When compared with *M. leprae* antigen No. 21, the *N. caviae* antigen gave a reaction of partial identity in the same way as all strains of other mycobacteria tested previously, i.e., with spurring by the *M. leprae* antigen. There was a reaction of complete identity between *N. caviae* and *M. avium-intracellulare* and *M. smegmatis*, respectively, with the lepromatous leprosy serum pool used as the antibody source. The results suggest that *N. caviae* antigen No. 21 is more closely related to the corresponding antigen of the genus *Mycobacterium* than to the antigen No. 21 equivalent of other nocardial species tested.

RESUMEN

Se estudiaron 18 cepas de nocardia, representando a cinco especies diferentes, por inmunoelectroforésis cruzada en contra de una mezcla de sueros lepromatosos y en presencia del antígeno No. 21. Este antígeno micobacteriano muestra heterogeneidad antigénica con determinantes antigénicos especie-específicos presentes en el *Mycobacterium leprae*. Se encontró que todas las cuatro cepas de *N. caviae* estudiadas comparten determinantes del antígeno No. 21, con las micobacterias. Todas las otras cepas de nocardia fueron negativas en estas pruebas de inmunoprecipitación directa. Comparado con el antígeno No. 21 del *M. leprae*, el antígeno de *N. caviae* dió una reacción de identidad parcial, igual que todas las cepas de las otras micobacterias estudiadas previamente, i.e., con un espolón para el antígeno del *M. leprae*. Hubo una reacción de identidad completa entre *N. caviae* y *M. avium-intracellulare* y *M. smegmatis*, respectivamente, con la mezcla de sueros lepromatosos usada como fuente de anticuerpo. Los resultados sugieren que el antígeno No. 21 de *N. caviae* está más estrechamente relacionado con el antígeno correspondiente del género *Mycobacterium* que con el antígeno equivalente al No. 21 de las otras especies de nocardia probadas.

RÉSUMÉ

Dix-huit souches de nocardia représentant cinq espèces différentes ont été étudiées par des méthodes d'immunoélectrophorèse croisée contre un pool de serum lépromateux, dans le but de rechercher la présence de l'antigène n°21. Cet antigène mycobactérien montre une hétérogénéité antigénique d'espèce spécifique pour les déterminants antigéniques définis pour *Mycobacterium leprae*. On a observé que toutes les souches de *N. caviae*, c'est-à-dire les quatre souches, possédaient des déterminants de l'antigène n°21 communs avec les mycobactéries. Toutes les autres souches de nocardia étaient négatives pour ces épreuves d'immunoprécipitations directes. Lorsqu'on le compare avec l'antigène *M. leprae* n°21, on constate que l'antigène *N. caviae* donne une réaction d'identité partielle, dans la même direction que toutes les autres souches de mycobacteries étudiées jusqu'à présent, c'est à dire avec "spurring" de l'antigène *M. leprae*. On a constaté une réaction de complète identité entre *N. caviae* et *M. avium-intracellulare* et *M. smegmatis* respectivement, avec le pool de sérum lépromateux utilisé comme dose d'anticorps. Ces résultats suggèrent que l'antigène n°21 de *N. caviae* est plus proche de l'antigène correspondant du genre *Mycobactérium* que de l'antigène n°21 équivalent trouvé dans les autres espèces de nocardia qui ont été étudiées.

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