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Immune Responses to Bovine Neural Antigens in Leprosy Patients. I. Absence of Antibodies to an Isolated Myelin Protein[1]

Robert N. Mshana, Morten Harboe, Gerald L. Stoner, R. A. C. Hughes, M. Kadlubowski, and Ayele Belehu[2]

Leprosy is the most common cause of peripheral neuropathy in the world ([8]). The pathogenesis of this neuropathy is still poorly understood, making a rational approach to its management difficult. *Mycobacterium leprae*, the causative organism, seems to have a special predilection for peripheral nerves and especially so for the Schwann cells of unmyelinated fibers ([7, 29, 30]). The reasons for this are not clear. Since *M. leprae* is remarkably non-toxic ([16]), it is thought that most of the tissue damage, including neural damage, seen in leprosy patients is due to the hosts' immune responses to *M. leprae* antigens ([3, 15, 16, 26]).

Ultrastructural studies of early leprosy neuropathy have shown that there are obvious and significant changes, including Schwann cell proliferation, thickening of perineurial basement membranes, endothelial cell proliferation, and segmental demyelination ([6, 7, 29, 30]). These alterations have been observed to occur in the absence of morphologically demonstrable *M. leprae* or infiltrating inflammatory cells, raising the possibility of either autoimmune humoral factors or biologically active substances, e.g., enzymes, secreted by cells at a distance and affecting nerves. Since demyelination is prominent in leprosy neuropathy, at least in early stages, one would expect such factors to be directed towards or to affect myelin components. Autoantibodies can frequently be detected in lepromatous leprosy (LL) patients although they do not seem to have any pathogenic significance ([24]). Wright, Hirst and Waters ([35]), using an immunofluorescent technique, were able to demonstrate anti-axonal antibodies in lepromatous leprosy patients and a few healthy control subjects composed of individuals from both leprosy endemic and non-endemic areas. This antibody was also found in patients with Guillain-Barré syndrome. The anti-axonal antibodies were not absorbed by BCG, indicating that the antibodies were not due to crossreactions between axons and mycobacterial antigens. In view of these findings, it was concluded that the presence of the antibody was not directly related to nerve damage but was rather an epiphenomenon following nerve injury. In no sera were they able to demonstrate anti-myelin antibodies.

Recently, refined radioimmunoassay (RIA) has been introduced to detect antibodies to P_2, the neuritogenic protein in peripheral nerve myelin, in animals with experimental allergic neuritis ([21, 28]). We have used this technique to detect antibodies to bovine peripheral nerve myelin basic protein, P_2, in patients with leprosy neuropathy. Both human and bovine P_2, when injected into susceptible animals, induce experimental allergic neuritis. This, together with the fact that these proteins show immunological crossreactivity as well being very similar biochemically, was the basis for using the easily available bovine material.

MATERIALS AND METHODS

Bovine sciatic nerve myelin basic proteins. Freshly dissected bovine sciatic nerves were collected in ice-cold isotonic saline

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[2] R. N. Mshana, M.D., Research Scientist, Armauer Hansen Research Institute (AHRI), P.O. Box 1005, Addis Ababa, Ethiopia. M. Harboe, M.D., Ph.D., Professor of Immunology, University of Oslo, Institute for Experimental Medical Research, Ullevaal Hospital, Oslo, Norway. G. L. Stoner, Ph.D., Research Scientist, National Institutes of Health, Bethesda, Maryland 20205, U.S.A. R. A. C. Hughes, M.D., F.R.C.P., Consultant Neurologist, M. Kadlubowski, Ph.D., Department of Medicine, Guys' Hospital Medical School, London, England. A. Belehu, Director, Armauer Hansen Research Institute (AHRI), P.O. Box 1005, Addis Ababa, Ethiopia.

containing a protease inhibitor, Trasylol®, at a concentration of 800 KIU/ml (Trasylol®, Bayer, Lever-Kusen, Germany) and cleaned of fat. Myelin basic proteins were then isolated according to the method of Uyemura, *et al.* ([33]). After separation, the proteins were stored at −70°C until used. The presence of P_2 was confirmed by double diffusion in gel using rabbit anti-P_2. Injection of this protein emulsified with Freund's complete adjuvant led to experimental allergic neuritis, clinically and histologically.

Human and animal sera. Sera were obtained from patients attending the All Africa Leprosy Rehabilitation and Training Centre (ALERT), Addis Ababa, Ethiopia. Leprosy was diagnosed clinically, and whenever possible a skin biopsy was taken for confirmation and classification of the disease. Each patient had at least one enlarged peripheral nerve, and signs and symptoms of acute neuritis (painful nerves) were particularly looked for. Functional nerve deficit was judged by the cotton wool and pinprick tests (for sensory loss) and voluntary muscle testing for motor functions. Control subjects were of two groups. One was composed of clinically normal individuals working at ALERT or at the Armauer Hansen Research Institute (AHRI). These individuals had had several years of direct contact with leprosy patients. The other group was composed of healthy individuals working at Guys' Hospital, London. These individuals had had no known contact with leprosy.

Rabbit anti-bovine myeline protein P_2 was prepared by repeated subcutaneous injections of P_2 in Freund's incomplete adjuvant. Immunoglobulins were isolated according to the method of Harboe and Ingild ([17]).

Radioimmunoassay (RIA). The protein concentration of the P_2 was determined according to Lowry, *et al.* ([24]). RIA was carried out as described previously ([21]). Briefly, 50 μl serum was incubated overnight at 4°C with approximately 2 ng ^{125}Iodine labelled P_2 making a 500 μl total volume. P_2 was radioiodinated with ^{125}I according to the method of Sarvas, *et al.* ([28]). Reacted complexes were isolated by the addition of 10% polyethylene glycol (PEG) and centrifugation. The radioactivity in the precipitates was then read in a radiocounter. The serum dilution used for the radioimmunoassay was 1:10 in phosphate buffered saline. Only 44 serum samples including the European controls were done at the Guys' Hospital, London. The remaining radioimmunoassays were done at AHRI using exactly the same technique and read using an LKB, 1216 Rackbeta 11 Counter (LKB, Finland). Absorption of sera with BCG was done as described previously ([18]).

Statistical analysis. Student's *t* test was used for statistical analysis.

RESULTS

Seventy-three sera were analyzed for anti-bovine P_2 activity *in vitro*; 23 of these were from patients classified as having borderline tuberculoid (BT) leprosy, 22 as having either borderline lepromatous (BL) or lepromatous leprosy (LL). Eleven sera were from patients showing evidence of reversal reaction with tender nerves. The remaining 17 sera were from clinically normal individuals—10 being from a randomly selected European population; the other 7 were from individuals who had had several years of direct contact with leprosy patients.

Rabbits immunized with P_2 developed a good anti-P_2 antibody response (Fig. 1). The antibody titer reached a peak at three weeks after immunization. This serum was then used as a known positive control for all human serum studies.

The anti-P_2 antibody activity and its distribution in leprosy patients and healthy controls is seen in Figure 2. It can be seen that most patients, irrespective of the disease classification, had anti-P_2 activity within two standard deviations of the normal controls. It was found that the Ethiopian controls had a slightly higher activity than the European controls who had had no known exposure to leprosy. The difference, however, is not statistically significant. There were no correlations between the number of enlarged peripheral nerves, duration of disease, degree of nerve function deficit, or presence of acute neuritis and the anti-P_2 titer.

Immunoglobulin concentrations, determined by single radial immunodiffusion, were found to be high both in leprosy patients and in normal Ethiopian controls. The controls from Ethiopia had slightly higher

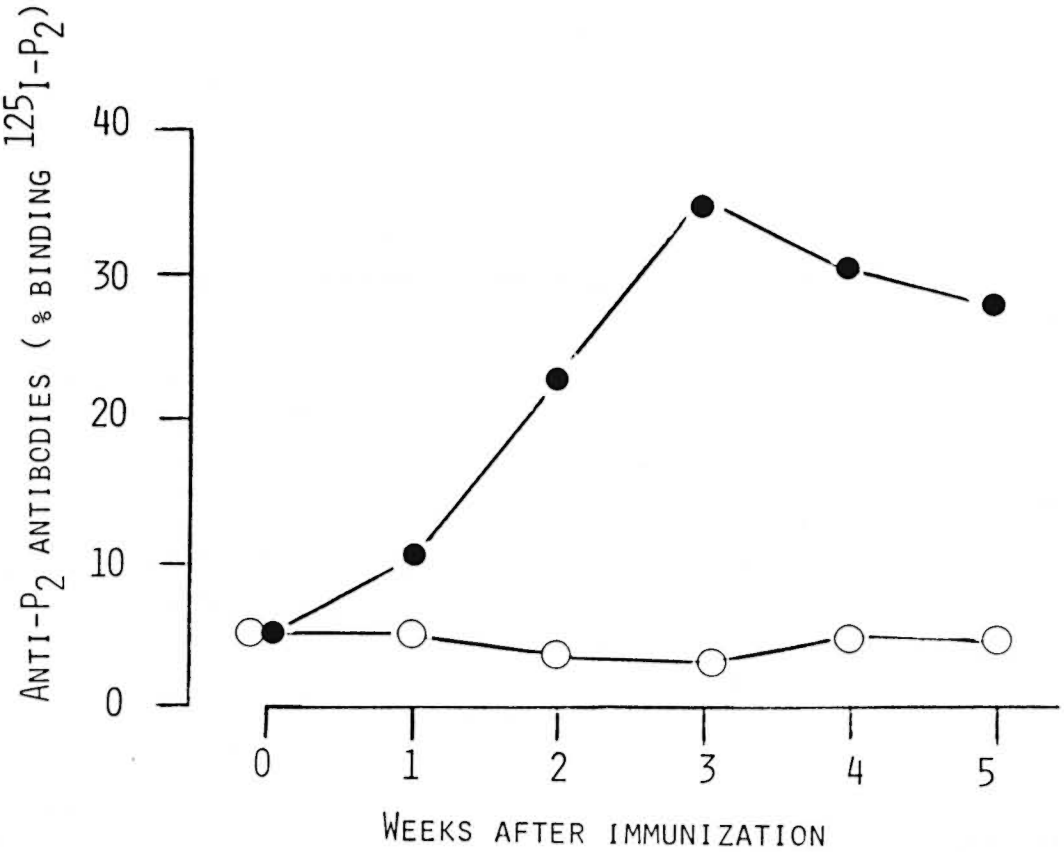

FIG. 1. Anti-bovine myelin protein, P_2, in a rabbit immunized with P_2 (●) and a normal rabbit (○). The assay was done with a serum dilution of 1:10.

values than those expected from healthy European subjects (The Table).

By absorbing leprosy sera with BCG sonicate we were not able to show a reduction in anti-P_2 activity, indicating that the anti-P_2 activity we detected was not due to cross-reacting determinants between mycobacteria and P_2.

DISCUSSION

The role of antibodies in the pathogenesis of peripheral neuropathy is not well understood. These antibodies could be involved in antibody dependent, cell-mediated demyelination ([32]) or cause demyelination by a complement dependent mechanism. Unactivated macrophages by themselves lead

THE TABLE. *Concentrations of different immunoglobulins in healthy controls and leprosy patients from Ethiopia.*

Group (N)	IgA (g/l)	IgG (g/l)	IgM (g/l)
Healthy controls (7)	1.6 ± 0.8[a]	16.5 ± 3.8	1.2 ± 0.5
Leprosy patients[b]			
BT (23)	1.7 ± 0.6	16.8 ± 4.5	1.3 ± 0.4
BL/LL (22)	1.9 ± 0.7	17.0 ± 3.5	1.6 ± 0.6
BT/RR (11)	1.8 ± 0.9	17.6 ± 3.8	1.3 ± 0.2

[a] Concentrations given as mean ±2 S.D.

[b] BT = borderline tuberculoid, BL/LL = borderline lepromatous or lepromatous leprosy, BT/RR = borderline tuberculoid complicated with reversal reaction.

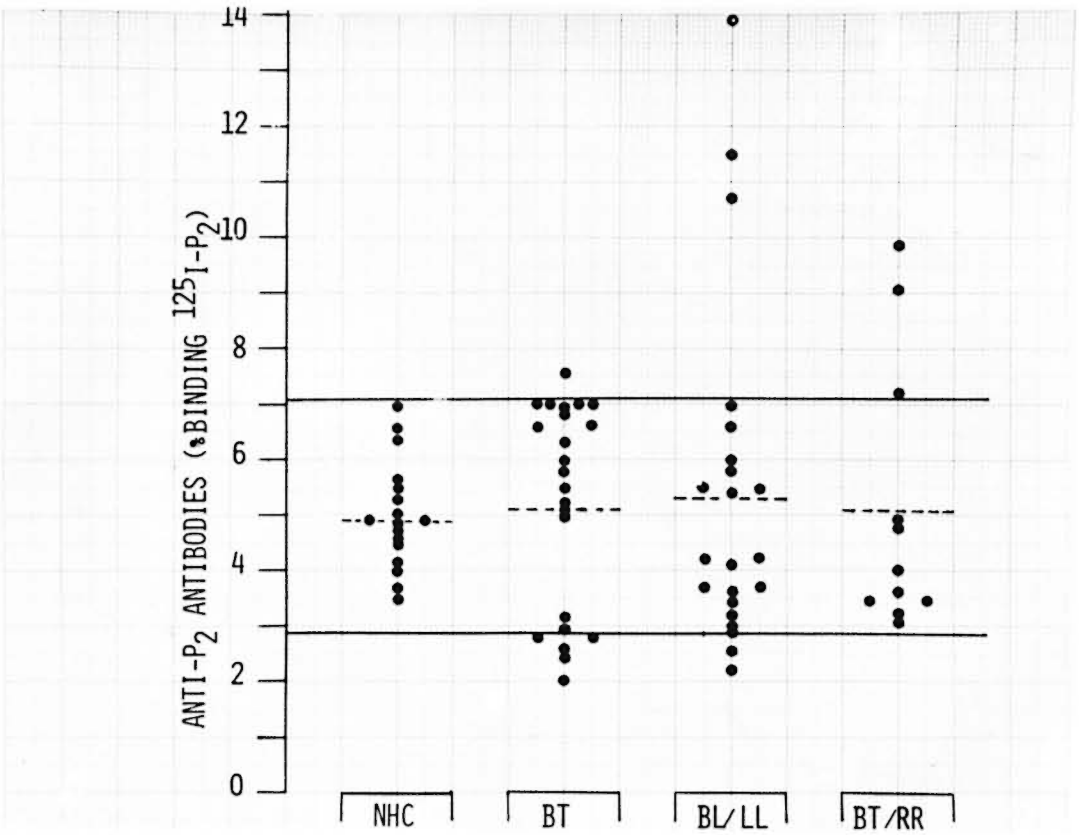

FIG. 2. Anti-bovine myelin protein, P_2, in healthy controls and patients with leprosy. The assay was done at a serum dilution of 1:10. Broken horizontal lines indicate the mean of each group, while the solid lines show ±2 S.D. of the normal values. NHC = Normal healthy control, BT = borderline tuberculoid leprosy, BL/LL = borderline lepromatous or lepromatous leprosy, and BT/RR = borderline tuberculoid with reversal reaction.

to very little demyelination but, in the presence of even small amounts of antibody, areas of primary focal demyelination are easily produced ([32]). Furthermore, antibody dependent lymphocyte cytotoxicity against myelin proteins has been thought to be involved in the pathogenesis of central nervous system neuropathies ([13, 14]). In the peripheral neuropathies the situation is less clear. Recently, however, Saida, *et al.* have been able to produce demyelination by intraneural injections of experimental allergic neuritis serum ([27]).

A blood-nerve barrier is present in healthy nerves ([6]). Both in experimental and human leprosy, however, a breach of this barrier has been reported ([4, 5]). In such a situation then, a high titer of anti-P_2 antibodies might, perhaps, contribute to the pathogenesis of neuropathy either directly or in collaboration with cells. The finding that early nerve damage in leprosy is not associated with the presence of *M. leprae* or inflammatory cells *in situ* would tend to support the former, while in established disease the later might be important even when *M. leprae* or its antigens are no longer detectable. Activated macrophages are capable of releasing factors that can lead to peripheral nerve damage. Phagocytosis of materials which are not easily digestible is known to stimulate activation of macrophages. These cells even at a distance from a nerve, therefore, when stimulated by injesting *M. leprae,* could release factors that would induce damage without their infiltrating the nerve per se. This could, perhaps, explain the early nerve changes, e.g., demyelination, without either *M. leprae* or inflammatory cells *in situ.* In established neuropathy, however, it is evident that more nerve damage is seen in association with inflammatory cells. Antibodies

could be involved at this stage in collaboration with lymphocytes or macrophages ([13, 14, 32]) or the cells could produce damage alone ([34]).

Our results do not show any significant elevations of antibodies to bovine P_2 myelin protein in leprosy patients. Bovine P_2 and human P_2 show both biochemical and immunological similarities. The anti-bovine P_2 antibodies detected in a few cases were of very low titers, the activity being detected at a dilution of 1:10. This, therefore, means that our data has to be viewed with caution. It is quite possible that the "antibody" activity we detected was actually due to non-specific binding of proteins to P_2. Myelin proteins have been shown to bind quite non-specifically to many serum proteins, glass and plastic tubes ([2, 22, 25]). In this regard it should be noted that high levels of immunoglobulins are commonly found in the sera of lepromatous patients. The slightly increased activity in the BT patients with reversal reaction could be on a similar basis, since an increase in non-specific immunoglobulins has been reported to occur during reversal reaction ([1]).

The controls from Ethiopia had higher anti-P_2 activities than those from London. This could be due to higher immunoglobulin concentrations in the sera of the former group which, in turn, could be due to many causes, especially repeated infections. That anti-mycobacteria antibodies are not responsible for this activity was shown by absorption experiments where no reduction of anti-bovine P_2 antibody activity was seen after absorption of the sera with BCG sonicate.

Edgington and Delissio ([12]) were able to show antibodies to myelin in 88% of normal individuals, although again the titers were low. They ascribed this finding to the normal process of growth. Recently Hughes, *et al.* ([20]) found lymphocyte transformation to myelin basic protein in normal individuals and thought that this protein might also be mitogenic.

M. leprae has strong adjuvant activity ([31]). In leprosy, therefore, where there is a prominent nerve involvement one would expect autoimmune reactions to peripheral nerve components to be generated. We have been unable to find antibodies to bovine myelin basic protein, P_2, in most patients. In those patients where P_2 binding activity was present in the serum, there was no correlation between the antibody titer and any clinical parameter. The possibility that anti-human myelin basic protein P_2 can be found in human beings with leprosy cannot be ruled out by our study since we used a bovine protein. It is quite possible that the determinant(s) for antibody responses to P_2 molecule may be recognized differently by different species ([28]). From experimental animals, however, the titer of anti-P_2 has not been shown to correlate with the clinical or histological changes in experimental allergic neuritis. It is also possible that the system we used, namely detecting anti-P_2 antibodies, might not be relevant in studying peripheral neuropathies. Search for antibodies to other peripheral nerve components might prove more useful. In this regard it is to be noted that the susceptibility of the various myelin proteins to plasminogen-dependent neutral proteinases varies from one to the other ([9]).

From our observations, circulating anti-P_2 antibodies probably are not involved in leprosy neuropathy. However, the role of locally secreted antibodies by lymphocytes remains unknown in leprosy neuropathy. These antibodies could lead to a localized nerve damage.

Although the basis of nerve damage seen in leprosy is thought to be the hosts' own immunological response, the exact mechanisms are not fully understood. A clear understanding of the mechanisms could lead to possibilities of minimizing or controlling the nerve damage seen in leprosy.

Recent results indicate that there may be differences in the mechanisms involved the pathogenesis of pure leprous sensory neuropathy and mixed or motor neuropathy ([10, 11, 19]). Both are, however, thought to be due to cell-mediated immune responses. In pure sensory neuropathy, where the bacilli are extremely few and difficult to find, Crawford, *et al.* have proposed that autoimmunity to non-myelin components of sensory nerves might be the underlying mechanism ([10, 11]). In the mixed or motor type of neuropathy, cell-mediated responses to *M. leprae* antigens ([3, 15]) may be involved just as is seen in experimental animals ([34]). Further studies on the interaction between neural antigens and *M. leprae* antigens, and the hosts' immune responses in the patho-

genesis of leprous neuropathy are underway.

SUMMARY

Recent electron microscopic demonstration that peripheral nerve demyelination can occur in leprosy patients even in the absence of both morphologically definable *Mycobacterium leprae* or inflammatory cells suggested that recognizable *M. leprae* or locally infiltrating cells need not be present to initiate leprosy neuropathy. The continued presence of *M. leprae* antigens or autoimmune humoral factors may thus be important.

We used bovine myelin protein P_2 to detect antibodies to this myelin protein in leprosy patients due to the scarcity of the human equivalent. Furthermore, both proteins show immunological crossreactivity. Sera from some of the patients were divided into two parts. One part was sent to Guys' Hospital, London, and the remaining portion studied at AHRI, Addis Ababa. For detecting anti-bovine P_2 antibodies, the sera were diluted 1:10, reacted with ^{125}I-labelled bovine P_2, the complexes precipitated by polyethylene glycol, and the radioactivity counted. Results from both laboratories were very similar. Seven out of 56 leprosy patients had anti-P_2 antibodies which were above two standard deviations from the normal control group. The increased anti-P_2 antibodies bore no correlation with any clinical parameter in terms of disease duration, number of enlarged or tender nerves, or severity of motor or sensory neural function loss. This activity is probably due to high immunoglobulin concentration in these patients and particularly so in the lepromatous group. Increased immunoglobulin levels could also explain the slight increase in anti-P_2 activity in Ethiopian (mean activity 5.11%) when compared to European controls (mean activity 4.85%). There were no significant differences in the mean values of anti-P_2 antibody activities of any of the groups studied.

It is surprising that in leprosy, a disease characterized by prominent nerve damage and the presence of mycobacteria which are strong adjuvants, no antibodies to a peripheral nerve myelin protein, P_2, were seen. Antibodies to other peripheral nerve components, however, should be searched for and their role in the pathogenesis of leprosy neuropathy studied.

RESUMEN

La reciente demostración por microscopía electrónica de que la desmielinización de nervios periféricos puede ocurrir en los pacientes con lepra aún en ausencia aparente de *Mycobacterium leprae* y/o de células inflamatorias, sugiere que estos elementos no necesitan estar presentes para iniciar la neuropatía leprosa. La continua presencia de los antígenos del *M. leprae* o de factores humorales autoinmunes puede ser una causa importante del daño neural.

En este estudio, debido a la carencia de material de orígen humano, se usó la proteína P_2 de la mielina bovina para buscar la presencia de anticuerpos contra esta proteína de la mielina en los pacientes con lepra. Ambas proteínas, la humana y la bovina, presentan reactividad cruzada. Los sueros de algunos de los pacientes se dividieron en dos partes; una parte se mandó al Guys' Hospital, en Londres, y la otra parte se estudió en el AHRI de Addis Ababa. Para la detección de anticuerpos anti P_2-bovina, los sueros se diluyeron 1:10, se hicieron reaccionar con P_2-bovina marcada con I^{125}, los complejos se precipitaron con polietilén glicol y en el precipitado se midió la radioactividad. Los resultados de ambos laboratorios fueron muy similares. Siete de 56 pacientes con lepra tuvieron anticuerpos anti-P_2 a niveles que estuvieron por arriba de 2 desviaciones estándar de la media encontrada en el grupo control. Sin embargo, los valores incrementados de anticuerpos anti-P_2 no tuvieron correlación alguna con parámetros tales como duración de la enfermedad, número de nervios agrandados o infartados, o severidad de la pérdida de la función motora o sensitiva. Probablemente la elevada reactividad sérica se debe a la alta concentración de inmunoglobulinas en estos pacientes y particularmente en el grupo lepromatoso. Los niveles incrementados de immunoglobulinas también podrían explicar el ligero incremento en la actividad anti-P_2 en etiopes (actividad media 5.11%) cuando se comparan con controles europeos (actividad media 4.85%). No hubieron diferencias significativas en los valores medios de las actividades de anticuerpo anti-P_2 entre los diferentes grupos estudiados.

Es sorprendente que en la lepra, una enfermedad caracterizada por un prominente daño a nervios y por la presencia de micobacterias que son potentes adyuvantes, no se encuentren anticuerpos contra una proteína de la mielina de los nervios periféricos, P_2. Es necesario, sin embargo, investigar la presencia de anticuerpos contra otros componentes de los nervios periféricos y estudiar su papel en la patogénesis de la neuropatía leprosa.

RÉSUMÉ

Des études récentes au microscope électronique ont montré que la demyélinisation des nerfs périphériques peut survenir chez des malades atteints de lèpre, même en l'absence de *Mycobacterium leprae* identifiables

morphologiquement, ou de cellules inflammatoires, ceci suggère qu'il n'est pas nécessaire que *M. leprae*, sous une forme reconnaissable, ou que des cellules d'infiltration locale, soient présents pour que s'amorce une névropathie lépreuse. La présence persistante d'antigènes de *M. leprae* ou de facteurs humoraux autoimmuns peut dès lors revêtir un rôle important.

Les auteurs ont utilisé la protéine myélinique P_2 de boeuf afin de détecter des anticorps à cette protéine de la myéline chez des malades de la lèpre, et ceci par suite de la rareté de l'équivalent de cette protéine chez l'homme. En tout état de cause, les deux protéines témoignent d'une réactivité croisée sur la plan immunologique. Des échantillons de sérum provenant de quelques-uns des malades ont été répartis en deux parties. Une partie a été envoyée au Guys' Hospital, à Londres, et la portion restante a été étudiée à AHRI, à Addis-Abéba. Dans le but de déceler des anticorps P_2 antibovins, les échantillons ont été dilués à 1:10, mis en contact avec la protéine bovine P_2 marquée à l'I^{125}, des complexes ont été précipités par le polyéthylène glycol, et la radioactivité a été mesurée. Les résultats obtenus dans l'un et l'autre de ces laboratoires étaient très semblables. Sur 56 malades de la lèpre, sept possédaient des anticorps anti-P_2, dont les taux se situaient à plus deux déviations standards de la normale établie dans le groupe témoin. L'augmentation des anticorps anti-P_2 ne présentait aucune corrélation avec un quelconque paramètre clinique, tel que la durée de la maladie, le nombre de nerfs épaissis ou sensibles au toucher, ou la gravité de la perte nerveuse motrice ou sensorielle. Cette activité était vraisemblablement due à une concentration élévée d'immunoglobulines chez ces malades, et particulièrement chez ceux du groupe lépromateux. Une élévation des taux d'immunoglobulines pourrait expliquer la légère augmentation de l'activité anti-P_2 chez les éthiopiens (activité moyenne 5.11%), comparée à celle observée chez les témoins européens (activité moyenne 4.85%). On n'a pas observé de différences significatives dans les valeurs moyennes de l'activité des anticorps anti-P_2 dans aucun des groupes étudiés.

Il est surprenant que dans la lèpre, une maladie qui est caractérisée par une atteinte nerveuse très importante et par la présence de mycobactéries qui sont connues pour leur pouvoir adjuvant marqué, aucun anticorps à la protéine myélinique P_2 des nerfs périphériques n'a été observée. Il faudrait cependant rechercher des anticorps à d'autres composés du nerf périphérique, et étudier leur rôle dans la pathogénèse des troubles nerveux dans la lèpre.

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