Detection of IgA anti-PGL-I specific antigen to *Mycobacterium leprae* in mangabey monkeys inoculated with *M. leprae*

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Summary Using sera from 4 pairs of mangabey monkeys inoculated with titrated doses of *Mycobacterium leprae* we demonstrated that IgA antibodies against *M. leprae* specific PGL-I antigen were present in 75% of inoculated monkeys' sera. High IgA antibody was detected in 50% (3/6) of infected animals and all three developed lepromatous leprosy (LL). Antibody titers correlated with PGL-I antigen in serum. The highest IgA peak appeared late and corresponded to the beginning of treatment, and in two of them appeared shortly after or corresponded with neurological damage. Low IgA response was found in the other 3 monkeys (50%—3/6), two of which developed indeterminate leprosy (I) and the other one LL. Low IgA levels appeared late after IgG and IgM, and shortly after neurologic signs. Both I monkeys were negative for PGL-I in serum. The remaining 2 monkeys (25%—2/8) did not show an IgA response; one of them developed LL but the disease regressed to I. IgM seemed to correspond to the appearance of PGL-I in serum. The other animal did not develop clinical symptoms of leprosy, and PGL-I in serum was negative.

Although there was no clear relation between the development of anti-PGL-I IgA and experimental leprosy, the finding of a high IgA response in some animals suggests that further studies are needed to evaluate the role of antigen-specific IgA in the disease process.

Introduction

Infectious diseases have had a big impact on human life. Among those diseases leprosy has presented and continues to present a significant problem in developing countries.

Leprosy is a chronic disease caused by *Mycobacterium leprae*, which has tropism to peripheral nerves, skin and mucosa where bacilli are found in large numbers in lepromatous leprosy (LL) patients. Infection with micro-organisms through skin and mucosa generally elicits specific antibody predominantly of the IgA class. However, the IgA antibody response in leprosy patients has so far escaped attention.

Since phenolic glycolipid-I (PGL-I) species-specific antigen to *M. leprae* was discovered,[1] several studies have attempted to evaluate the humoral response of the host.[2,3] However, despite the tropism of *M. leprae* to skin and mucosa we do not know if PGL-I elicits IgA class antibodies and what significance they might have for disease development. The lack of knowledge about the development of immune response in leprosy patients is due to the long incubation period between infection and clinical disease, so by the time the diagnosis is made the individual has already been ill for a long period of time. That makes it impossible to study the immunological response immediately after primary infection with *M. leprae*.

The sooty mangabey monkey (*Cercocebus torquatus atys*) has been reported to be a good model to study the immune response to leprosy, because it has a course of infection similar to humans.[3]

The aim of this paper is to evaluate the immune response of IgA class antibodies to species-specific PGL-I antigen by ELISA in the sera of 8 mangabey monkeys inoculated with *M. leprae* suspension and to correlate it with anti-PGL-I IgG and IgM titers, PGL-I in serum, and clinical course of the disease.

Material and methods

The animals' conditions, methods of *Mycobacterium leprae* inoculation, the clinical evaluation before and after inoculation, and IgG and IgM results have been described previously.[3] Briefly, four pairs of mangabey monkeys were inoculated with *M. leprae* suspension by combined intravenous and intradermal routes. Monkeys number D171 and D172 received 4.8×10^{10} acid-fast bacilli (AFB), D173 and D174 were inoculated with 4.8×10^9 AFB, D175 and D176 received 4.8×10^8 AFB, D177 and D178 received 4.8×10^7 AFB. Two hundred and four serum samples were obtained over a 95-month period. There were two samples from each monkey 3 months before *M. leprae* inoculation and an average of 25 samples from each animal over 90 months after inoculation.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR IGA

The disaccharide moiety of PGL-I coupled to bovine serum albumin (D-BSA, provided by WHO) was used to measure IgA antibody levels. The detection of antibody IgA anti-PGL-I by ELISA serological test was performed as briefly described.[4] Flat bottom, polystyrene microplates (Nunc) were coated with $50\,\mu$l/well of D-BSA and BSA, respectively, diluted 100 ng/ml in 0·05 M sodium carbonate buffer pH 9·6 for 2 h at 37°C.

The plate was blocked with 100 μl/well of 1% bovine albumin in phosphate-buffered saline containing 0·1% v/v Tween 20 (BSA-PBST). After incubation for 1 h at 37°C, 50 μl of test monkey serum diluted 1 : 10 in 1% BSA-PBST was added and incubated as

before. Then 50 μl/well of mouse monoclonal peroxidase labelled anti-human IgA antibody (Nordic) was added, diluted 1:300 in BSA-PBST. After incubation and washing 50 μl/well of substrate solution containing 18 mM 2-2' azino (3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS; Boehringer) and 2·9 mM H_2O_2 in citrate-phosphate buffer pH 5·0 were added. After incubation for 1 h at room temperature in the dark the reaction was stopped by the addition of 50 μl/well of 0·32% (w/v) NaF. Extinction (ΔE) was read with a double wavelength micro ELISA autoreader MR 580 (Dynatech) at 405 nm (versus 490 nm) against a blank made up of substrate and stop solution. Known positive and negative control reference sera were included on each tray. Blank antigen wells were assayed in parallel to ensure that positive readings were, in fact, due to specific interactions between antibodies and the antigen. Each serum was tested twice and in duplicate. A serum was considered positive when the OD exceeded by two standard deviations (SD) the mean of results obtained before monkeys' inoculation with *M. leprae* (cutoff = 0·17).

ELISA DETERMINATION OF IGG AND IGM ANTI PGL-I

The assays were performed as previously reported,[3,5] but with some modifications, described in detail below, to ensure maximum accuracy and reproducibility. Natural PGL-I was used as antigen and was provided by Dr Brennan (Fort Collins, CO, USA).

Briefly, 96-well plates were coated with antigen (Ag), washed and blocked with BSA, washed again and reacted with a previously determined optimal dilution of monkey serum. After incubation and washing, the plates were coated with peroxidase-labelled anti-human IgG or IgM Fc fragment gamma or μ-chain-specific antibody diluted according to prior titrations, incubated, washed, reacted with o-phenylenediamine plus H_2O_2, acidified and OD's were determined at 490 nm on an ELISA reader.[3] Final OD's represent the difference in absorbance between wells containing Ag minus wells lacking Ag. Each reagent in the ELISA was carefully titrated in a checkboard manner to determine dilutions that would give final OD values between 0·1 and 0·5 OD whenever possible to utilize the OD range most sensitive to small changes in OD. This way longitudinal changes also would be accurately reflected. All sera were assayed together at one time in given experiments to permit accurate relative comparisons. The same batch of peroxidase antibody was used throughout. All experiments were done at least twice. OD values obtained with these precautions were reproducible in a given sample from one assay to another to within ±0·05. The mean + 2 SD, taken as the cutoff points, respectively, were 0·043 and 0·073 for IgG and IgM.

DETECTION OF PGL-I ANTIGEN IN SERA

The PGL-I antigen assay was performed as described previously.[6] Briefly, for serum lipid extraction, 100 μl of serum was added to filter paper discs (0·5" in diameter) and dried completely. Lipids were then extracted using 2–3 ml of chloroform:methanol (2:1) solution and dried under N_2. Serum lipids were dissolved in chloroform and applied to fluorosil packed in a Pasteur pipette, 60–100 mesch (Sigma Chemical Co. St Louis, MO., USA) and eluted with chloroform, followed by 5% methanol in chloroform. The lipid fraction eluted with 5% methanol was saved and dried under N_2 and

examined for the presence of PGL-I by a dot-ELISA method as previously reported.[6] The lipid fraction was dissolved in 100 μl of hexane and a 5-μl portion was applied to a tuffryn (polysulphone) membrane (HT-200) (Gelman Sciences Inc., Ann Arbor, MI, USA), followed by anti-PGL-I antibody. A high titer of rabbit anti-PGL-I antibody (a gift from Dr P. J. Brennan) was used for the primary antibody and peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical Inc., Malvern, PA, USA) was used as the secondary antibody. For colour development, 4-chloro-1-naphthol (Biorad Laboratories, Richmond, CA, USA) was used and the results were read visually.

Results

We demonstrated that the majority of monkeys developed IgA antibody against PGL-I. In six animals (6/8, 75%) increasing IgA levels were detected after *M. leprae* inoculation (D171, D173, D174, D176, D177, D178).

Three monkeys (D174, D176 and D177—3/6, 50%) showed high anti-PGL-I IgA levels ($\Delta E > 0.5$) (Figure 1). All three monkeys developed lepromatous leprosy (LL) disease. Mangabey D174 developed LL by 10 months post-inoculation (pi) but regressed at 14 months. However, a relapse occurred at 35 months pi and persisted, requiring chemotherapy at 59 months (Figure 1(b)). Neurologic deformities appeared at 50 months and became worse after initiation of chemotherapy with a combination of rifampicin and clofazimine.[3] Early (within 3 months pi) moderate IgG and low IgM levels were seen which coincided with the appearance of the PGL-I concentration in serum (Figure 1(a) and (b)). Low anti-PGL-I IgA level appeared later after inoculation and corresponded with clinical reactivation at 36 months pi, and a second higher peak coincided with the beginning of chemotherapy and after neurologic deformities.

Monkey D176 showed a much more rapid clinical evolution of disease than any of the other animals, LL clinical symptoms were noted at 4 months with progressive evolution, thereafter requiring treatment at 27 months with rifampicin, and the complete remission of the disease was observed at 35 months.[3] No neurological signs have been noted. A low IgA peak appeared at 10 months pi and a second higher peak was seen at 24 months pi. On the other hand, D177 showed a slow development of clinical leprosy (LLs) by 26 months pi. The treatment with rifampicin was started at 42 months pi. Nerve enlargement was detected at 47 months pi and persisted after chemotherapy.[15] IgA appeared shortly after IgG and IgM and the highest peak of IgA appeared shortly (at 47 months pi) after treatment and corresponded to the beginning of the neurologic damage.

In these three monkeys early (within 3 months pi) anti-PGL-I IgG antibody titers were observed: moderate in D174, high in D176 and low in D177. IgM level in the same period was lower. The initial IgG and IgM were followed by a second or third peak that corresponded to periods of progression of the disease and higher serum concentration of PGL-I antigen in serum (Figure 1(a) and (b)). PGL-I in serum, as expected, decreased quickly after treatment. Anti-PGL-I IgG and IgM decreased slowly after treatment, but anti-PGL-I IgA decreased slower in D174 and D177 (Figure 1(a)).

In the other three monkeys (3/6, 50%) a low IgA response was found (D171, D173 and D178) (Figure 2). D171 developed severe clinical symptoms of LL at 5 months pi requiring treatment with rifampicin at 38 months pi.[3] Neurologic deformities appeared at 75 months pi and persevered beyond 90 months pi. A low anti-PGL-I IgA antibody
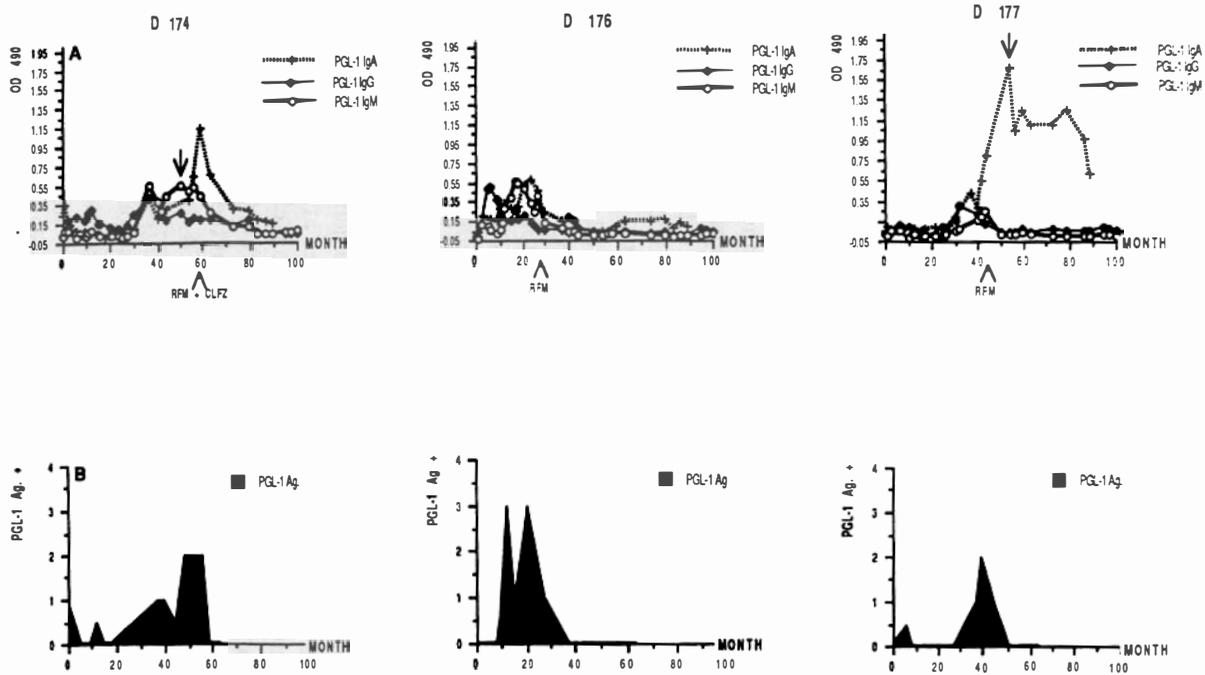
Figure 1. (A) Antibody responses (IgG, IgM and IgA) to PGL-I measured by ELISA in monkeys' sera. Mangabeys D174, D176 and D177 showed high anti-PGL-I IgA levels. ↓—development of neurologic signs. RFM—Rifampicin, CLFZ—clofazimine. (B) Detection of PGL-I antigen in monkeys' serum.

Detection of IgA anti-PGL-I specific antigen to M. leprae in mangabey monkeys
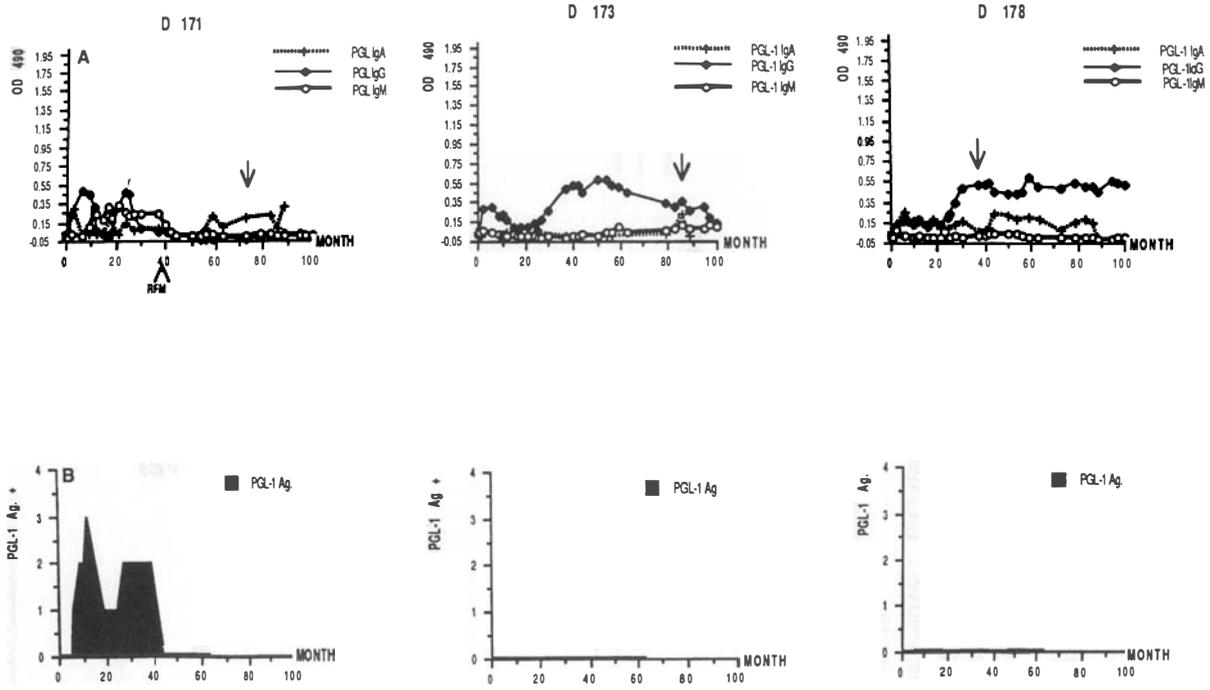
Figure 2. (A) Antibody responses (IgG, IgM and IgA) to PGL-I measured by ELISA in monkeys' sera. Monkeys D171, D173 and D178 showed low anti-PGL-I IgA levels. ↓—development of neurologic signs. RFM—Rifampicin. (B) Detection of PGL-I antigen in monkeys' serum.

titer was found late (at 60 months pi) after the appearance of IgG and IgM as well as after highest serum peak of PGL-I, and treatment. A second low IgA antibody peak, but higher than the first one, was detected at 90 months pi. Early (within 3 months pi), moderate IgG antibodies and very low IgM antibodies titers were found. IgG antibodies persisted and IgM antibodies rose corresponding with PGL-I concentration in serum and to the periods of clinical progression of disease. IgG and IgM antibodies and PGL-I antigen in serum decreased rapidly after chemotherapy, while IgA was still being elicited.

The animal D173 developed indeterminate leprosy (I) at 5 months pi but by 14 months the disease regressed.[3] Neurological signs appeared at 85 months pi, followed by progression to LL. Chemotherapy was given at 105 months pi.[15] Low IgA antibody titers appeared late after IgG and IgM and shortly (at 86 months pi) after aggravation of disease. Initial moderate IgG and very low IgM antibody levels were followed by a second high peak and low, respectively. IgG decreased slowly after 60 months pi. PGL-I antigen was not detected in serum.

D178 developed I leprosy by the 35th month pi. The indeterminate lesion of leprosy regressed spontaneously, however the nerve enlargement persisted.[3] A low early IgA peak appeared at the same time as IgG in the absence of disease, a second IgG peak
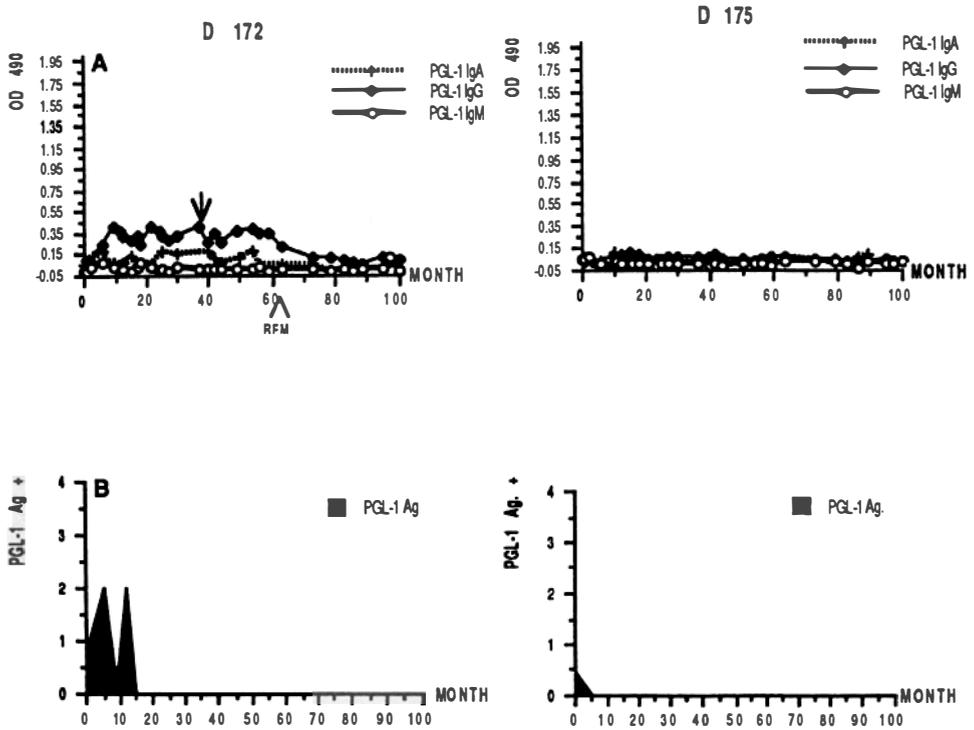
Figure 3. (A) Antibody responses (IgG, IgM and IgA) to PGL-I measured by ELISA in monkeys' sera. Monkeys D172 and D175 did not elicit IgA antibodies. ↓, development of neurologic signs; RFM, rifampicin. (B) Detection of PGL-I antigen in monkeys' serum.

appeared correlated with the clinical symptoms. Another slight increase of IgA was seen late (at 44 months pi) in the disease. IgM as well as PGL-I in serum were negative.

The remaining two monkeys (D172 and D175–2/8, 25%) did not show an IgA response (Figure 3). Monkey D172 developed LL at 9 months pi but the disease spontaneously regressed to I.[3] Relapse and regress of the disease happened several times during the follow-up (Figure 3(b)). It developed neurological signs at 39 months pi which persisted; chemotherapy was given at 65 months pi. IgG and IgM seemed to correspond to the appearance of PGL-I in serum and the development of the disease. PGL-I antigen disappeared much more rapidly in D172 than in any of the other monkeys. Mangabey D175 did not develop clinical symptoms of leprosy, mounted an early predominantly low IgG response and PGL-I was not detected in serum (Figure 3(b)).

Discussion

We report the detection of IgA to species-specific PGL-I antigen to *M. leprae* in serum of mangabey monkeys inoculated with *M. leprae* suspension over the entire course of the infection (over 90 months).

In this study the majority of animals presented increased levels of IgA after *M. leprae* inoculation (75%). An interesting finding was the presence of high levels of IgA after the appearance of clinical symptoms while the rise of IgG and IgM levels preceded the symptoms (D171, D173, D174, D176 and D177). It is already known that systemic immunization elicits preferentially IgM and IgG antibodies but a prolonged exposure to the antigens may induce high levels of serum IgA.[7]

It is commonly believed that human LL patients produce predominantly IgM anti-PGL-I, but our data show that in some monkeys anti-PGL-I IgG class is also elicited in significant quantities (4/7, 57%—D171, D172, D173, D178). One reason may be a difference between humans and monkeys, it may also be due to the inoculation routes or to the fact that our study looked at earlier time periods post-infection compared to most human leprosy studies.

It is not known whether IgG anti-PGL-I is elicited early in the disease, but IgM has been reported to be present before[8] and after disease symptoms. Our data show that IgG and IgM anti-PGL-I can be produced in significant levels early after the inoculation of monkeys with *M. leprae*. Gormus *et al*.[3,5,9] suggest that IgG antibody may correspond to resistance to clinical leprosy (D171, D172, D173 and D178) and that IgM antibody corresponds to susceptibility to LL forms of disease (D174, D176 and D177).

Five monkeys showed clinical evolution to lepromatous leprosy. In three of them (D174, D176 and D177) disease progression corresponded to the appearance of PGL-I in serum and high IgM anti-PGL-I, although the highest IgA peak came late after a serum PGL-I peak. In animals D171 (LL) and D172 (LL → I) only IgG and IgM corresponded to serum PGL-I antigen; IgA in D171 appeared late and was negative in D172. The monkeys D173 and D178 (both I) showed IgG and/or IgM and/or IgA in the absence of serum PGL-I antigen. These results suggest that mechanisms other than antigen load may be involved with IgA yield. Little is known about the production of IgA in leprosy but our data do not support the hypothesis that IgA would be useful for the detection of early leprosy infection,[10] since a significant anti-PGL-I IgA response in

the monkeys appeared later than IgG and IgM and most of the time corresponded to the beginning of treatment (D174, D176 and D177) or to the period shortly thereafter (D171). D172 had a different clinical evolution in comparison with the other monkeys. It seemed to be leprosy resistant (LL → I) and would probably not have developed clinical disease if it had received a low *M. leprae* dose.

It is established that a humoral response is dependent on the cooperation of B and T cells. On the other hand, the occurrence of lepromatous leprosy is due to a deficient cellular response to *M. leprae*, an obligate intracellular parasite. A high IgA response in animals with lepromatous leprosy, especially in those under treatment, then, seems to be a controversial finding. The existence of IgA titers may be explained by recent studies where it has been reported that the cytokine TGF-β (transforming growth factor beta) enhances the IgA production by LPS stimulated murine B cells even in the absence of T cells.[11] Bullock[12] links the high serum IgA concentration in LL patients under therapy to the persistence of killed *M. leprae* in the tissues but we believe that other mechanisms may also be activated. The association of TGF-β with immunosuppression was previously shown and increased production of this cytokine was reported in patients with the acquired immune deficiency syndrome (AIDS).[13] This suggests that TGF-β may play an important role in the spread of infection and/or disease progression.[13] This hypothesis may explain why a high IgA peak appears coincidently with the beginning of or shortly after treatment. Monkeys D171, D174, D176 and D177 received chemotherapy because the disease became severe. Perhaps high IgA peaks were elicited at this time by TGF-β action and not because of the chemotherapy effect. On the other hand, maybe an IgA response would be elicited to remove the circulating antigenic substances, incidentally this is the only function of IgA that is substantiated.

Another result of particular interest is that anti-PGL-I IgA antibodies were found shortly after or coincident with neurologic damage (D171, D173, D174, D177 and D178). Animals D174 and D177 produced IgA in excess of 1·0 OD. These monkeys seem to be more susceptible than the other animals. In D174 and D177, in which the clinical nerve symptoms persisted even after successful chemotherapy, the IgA antibodies remained at a high level for long periods of time (Figure 1). Mangabey monkeys D171, D173 and D178 seemed less susceptible to leprosy infection and produced low anti-PGL-I IgA antibody levels. Although D171 developed extensive disease in the beginning, it responded very well to chemotherapy and, as animal D173, developed neurologic signs later after regression of the disease. Mangabey D178 showed symptoms of leprosy nerve enlargement with indeterminate lesions of leprosy in the skin regressing spontaneously. Antibody responses to human nerve antigens have been reported in these monkeys, but it is not clear if these antibodies or anti-PGL-I IgA play some role in the pathogenesis of the disease.[15] If IgA antibodies were elevated only in the context of a wider humoral response against PGL-I of either IgG and IgM (as in D176), doubts may be cast on the possible significance of this immunoglobulin, but in some monkeys high IgA antibody levels were detected even when IgG or/and IgM were low or decreasing (D171, D173, D174 and D177). On the other hand, anti-PGL-I IgG and IgM have been elicited early pi and both immunoglobulins or one of them arose following the progression of the disease, so maybe the damage to peripheral nerves was originally caused by these two antibodies, and in this case the late increase of IgA may be involved in the amplification of the neurological damage. Or alternatively, anti PGL-I IgA may play a protective role from attack by humoral or cellular factors.

Several fluctuations in the antibody response and in the clinical evolution of disease between the monkeys studied were noted, which is perhaps influenced by intrinsic factors of the host, such as his cell-mediated immunity, environmental factors and the strain of *M. leprae.*

The interpretation of our data may not be simple. Leprosy seems to elicit a particular immune response. During the course of HIV induced-immunosuppression, tuberculosis, among other opportunistic infections, tends to appear. However, in a recent report no evidence of an association between the incidence of leprosy and HIV infection was found.[14] The authors suggest that leprosy may have a particular immune response mechanism. In our study the immunological events have been orchestrated since the monkeys were experimentally inoculated with *M. leprae.* Many cells and cytokines have played a role in the activation and suppression of the immune response and during this process the disease developed. The availability of an experimental model would be helpful in designing a longitudinal study to evaluate not only if IgA antibodies play a role in leprosy but also to study the interactions between lymphocyte subsets and cytokines produced and the humoral response in leprosy.

Acknowledgments

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