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CHARACTERIZATION AND FOLLOW-UP OF THE IgG ANTIBODY RESPONSE AGAINST *BORRELIA BURGDORFERI* USING WESTERN BLOT IN A SEROPOSITIVE (ELISA) POPULATION FROM AN ENDEMIC AREA

by

LISE GERN, SUZANNE LEUBA-GARCIA AND ELLEN FROSSARD

WITH 4 FIGURES

INTRODUCTION

Since the identification of *Borrelia burgdorferi* as the causative agent of Lyme borreliosis in the United States and Europe (BURGDORFER *et al.* 1982), the number of clinical and pathological reports of this disease continues to increase. The criteria for diagnosis of Lyme borreliosis are not clearly defined and are complicated by the fact that culture or direct visualization of spirochetes are often unsuccessful. Hence serological testing has been the only practical laboratory aid in diagnosis.

In a prospective study done in 1986 (GERN *et al.* 1989), we studied a rural population from the Swiss Plateau (Aarberg), an endemic area, showing no signs of Lyme borreliosis but presenting a high percentage (26.6%) of persons with IgG antibodies (ELISA) against *B. burgdorferi*. These results prompted us to test this population using Western blot and to continue this study by following the seropositive persons. This paper describes the serological and clinical follow-up of some of these individuals using ELISA and Western blot techniques.

MATERIALS AND METHODS

Studied populations. A rural population frequently exposed to tick bites ($n = 491$) from an endemic area was examined on hospital admission in 1986 (GERN *et al.* 1989). The only condition for inclusion in the study was that active Lyme borreliosis was not suspected and was not diagnosed during hospitalization. Of the patients examined, 131 had IgG antibodies against *B. burgdorferi* detected by ELISA (GERN *et al.* 1989). To obtain a better indication on the frequency of the different proteins of *B. burgdorferi* reacting with the sera from asymptomatic seropositive individuals, 61 sera

collected in 1986 were tested by Western blot. Thirty four of these individuals were reexamined in 1989 using ELISA and Western blot. In 1989, each person was asked about possible manifestations of Lyme borreliosis during the last 3 years.

A control population (blood donors; $n = 84$) from a non endemic area, where *I. ricinus* ticks are absent, was investigated by Western blot and ELISA.

Enzyme Linked Immuno Sorbent Assay. The ELISA sonicate antigen (strain B31) was prepared as described by Russell *et al.* (1984). The ELISA was done as previously described (FAHRER *et al.* 1991). Briefly, microtitre plates (Dynatech, USA) were coated overnight at 4°C with 50 µl of sonicate antigen diluted in 0.1 M carbonate buffer (pH 9.6). The optimal coating concentration was 6.5 µg of protein/ml. All serum samples were tested in duplicate at a dilution of 1/200. Antibody-antigen reactions were visualized with peroxidase conjugated goat anti-human IgG (GaHu/IgG (Fc)/PO Nordic) and ortho-phenyldiamine as substrate. In each assay, a negative serum and a strongly positive one (diluted 6 times from 1:300 to 1:9600) were included. The curve derived from the results with the positive serum was used as reference. The optical density (OD) of each serum sample was compared with the positive control curve and the result was expressed as the logarithm of the dilution (log dil) of the positive serum corresponding to this OD. The positive cut-off level for IgG antibodies was defined as a log dil of <3.74 which corresponded to 2 standard deviations below the mean log dil of 51 sera of people living at high altitude, in a non endemic area. At this cut-off level, the specificity of the test is 96% if we exclude other spirochetes like leptospires and treponemes, and the sensitivity of the serologic test varied from 26% to 100% depending on clinical manifestations in cases of definite Lyme borreliosis (GERN 1991; FAHRER *et al.* 1991).

Immunoblot. *B. burgdorferi* strain B31 was used for antigen preparation. BSK II medium containing 10^8 organisms was centrifuged at $17{,}000 \times g$ for 30 min and washed 3 times in PBS with 5 mM $MgCl_2 . 6H_2O$. The final pellet was resuspended (1:2) in sample buffer containing Tris HCl 0,5M, 12.5% Glycerol, 4% sodium dodecyl sulfate, 10% mercaptoethanol and 0.1% bromophenol blue. A 12,5% polyacrylamide gel (12 cm plate, 0.80 mm gel thickness; 30.0:0,8 acrylamide:bisacrylamide) was used to separate 12 µg of spirochetal protein per lane. A current of 20 mA was applied for 30 min. (concentration gel) followed by 30 mA for 1 hour 30 (separation gel). Proteins were electrophoretically transferred from the gel to nitrocellulose paper (NC-0.2 µm) using a current of 0.8 mA/cm^3 of gel for 1 hour. After transfer, the paper was cut into strips and the blots were blocked by immersion in tris-buffered saline (TBS; pH 7.5) (Tris-HCl 1M, NaCl 5M) with 5% fatfree milk (TSM) for 2 hours 30 and then were washed three times with TBS (pH 7.5) at room temperature.

The blocked strips were incubated individually with patient sera diluted 1:200 in TSM (pH 7.5) and left overnight at room temperature with gentle agitation. After three washings, strips were incubated in horseradish peroxidase conjugated goat anti-human IgG (Nordic; diluted 1:1000 in TSM).

Bands were visualized with 4-chloro-1-naphtol (Fluka) for 3-5 min. Finally, all strips were washed with distilled water. All visible bands were counted, irrespective of their intensity. All immunoblots were read by one of the authors (SLG). Low molecular weight standards of 14.4-97.4 kDa (Biod-Rad) were used for size estimation of the bands.

The specificity of the Western blot test was assessed using sera from 3 patients with leptospirosis, 13 with syphilis, 2 with pneumonia, 5 with bacterial intestinal diseases and 5 patients with Tick Borne Encephalitis. The cross-reactive antigens were the 41 kDa and 66 kDa proteins which appeared respectively in 22/28 (11/13 syphilis) and 11/28 sera. No proteins with a molecular weight <41 kDa reacted in the Western blot of these individuals.

The 34 sera from 1986 and 1989 were tested simultaneously in the same immunoblot and the same ELISA assay.

Statistical analysis. Statistical analysis of paired data (1986-1989) were performed using Student's T-test.

RESULTS

Control population. All individuals ($n = 84$) of the control population were seronegative when tested by ELISA. When tested by Western blot, 7 sera showed no bands, 15 demonstrated traces of reaction with the 41 kDa flagellar protein and 62 had weak reactions with mainly 2, sometimes 3 bands of 41, 66 and 97 kDa.

Frequency of responses to antigenic components of *B. burgdorferi* in the asymptomatic population. The frequency of responses to antigenic components of *B. burgdorferi* of the 61 tested sera are shown in Fig. 1. These sera reacted with as many as 37 distinct proteins. The most frequent reactions were observed with proteins 41, 97, 66, 80/81, 32, 31 and 22 kDa (listed in order of frequency). Four sera did not react with the 41 kDa protein but two of them reacted with the highly specific proteins OspA and pC and one reacted with the 39 kDa and OspA. The sera reacted with a mean of 7.1 bands (3.2 bands <41 kDa and 3.9 bands >41 kDa). A minimum of 2 bands was observed in the immunoblot pattern of 60/61 individuals (98%) and of 5 bands in 45/61 (74%). Of the sera tested 35/61 (57%) reacted with a band corresponding to a 41 kDa protein and at least one band corresponding to low proteins of 18, 21.5 or 23 kDa.

Follow-up 1986-1989. ELISA. Serum antibody titers of the 34 studied individuals are shown in Fig. 2. All had positive IgG titers in 1986, and 26 were positive in 1989. The eight negative individuals showed a log dil value above the 3.74 cut-off value, but only four of them demonstrated a significant decrease in antibody titer (FAHRER *et al.* 1991). In contrast, a significant increase in titer was observed for three individuals.

Immunoblot. The evolution, between 1986 and 1989, of the number of bands observed in the Western blot of the 34 subjects is presented in Fig. 3

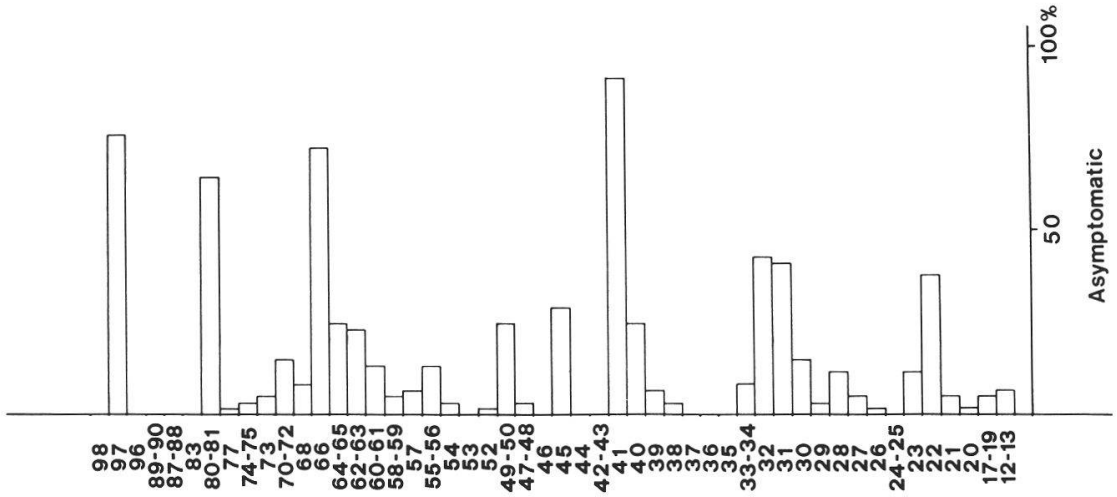

Fig. 1. Frequency of antigenic proteins of *Borrelia burgdorferi* (B31) detected in Western Blot of sera from 61 asymptomatic persons (indicated in %). The abscissa represents the molecular weights (kDa) of the proteins detected in the Western Blot and the ordinate the percentage of Western Blot in which the proteins reacted.

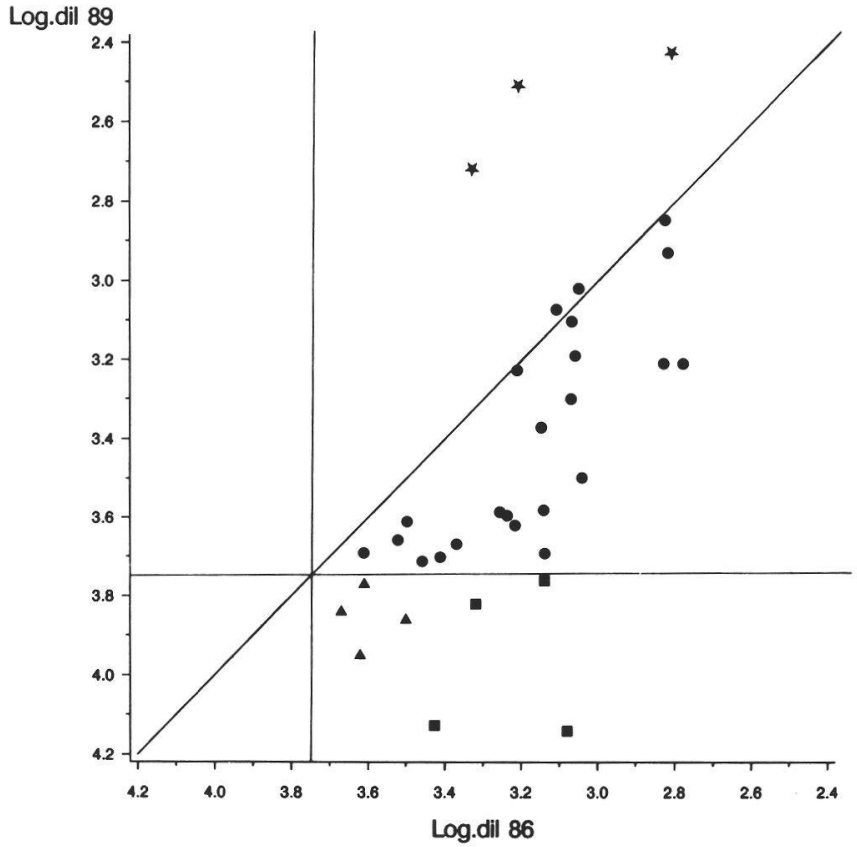

Fig. 2. Plot of IgG ELISA tests for anti-*Borrelia burgdorferi* antibodies in serum samples from 34 asymptomatic individuals, obtained in 1986 and 1989. Cut-off level for a positive ELISA is log. dilution <3.74.

■: Sera positive in 1986 and negative in 1989, presenting an increase in log.dil of at least 0.40

▲: Sera positive in 1986 and negative in 1989, presenting an increase in log.dil of less than 0.40 (not relevant)

★: Sera presenting a relevant antibody concentration increase.

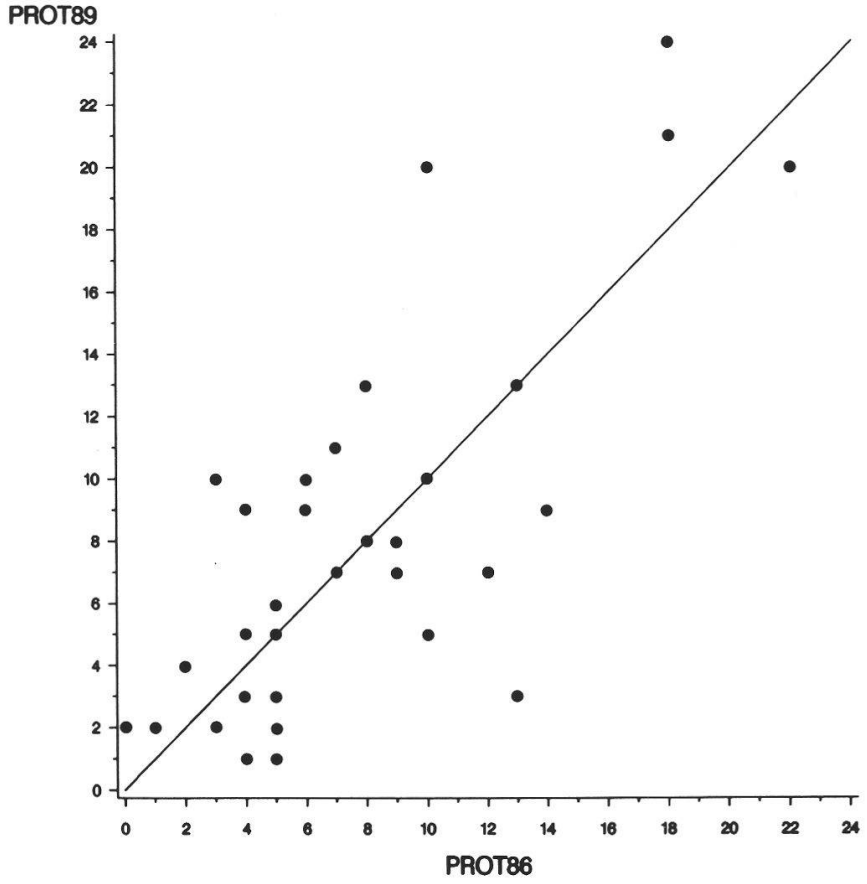

Fig. 3. Plot of the number of *Borrelia burgdorferi* proteins detected by Western Blot in the sera of 34 asymptomatic persons in 1986 and 1989. Note that 2 sera presented no reaction in 1986 and a reaction against 2 proteins in 1989; and 2 sera presented a reaction against 3 proteins in 1986 and against 2 in 1989.

and representative immunoblots are shown in Figure 4. IgG bound to as many as 22 distinct proteins in 1986 and 24 in 1989. The sera reacted with a mean number of 7.37 bands in 1986 (3.48 < 41 kDa; 3.88 > 41 kDa) and 7.57 bands in 1989 (3.17 < 41 kDa and 4.4 > 41 kDa). No statistical difference was detected in either the number of reacting proteins during the follow-up ($p = 0.768$) or in the number of reacting proteins having molecular weight <41 kDa ($p = 0.411$) or >41 kDa ($p = 0.188$).

Nevertheless, the number of reacting proteins decreased in two cases from 13 and 10 proteins to 3 and 5, respectively, and increased in two other cases from 3 to 10 and from 4 to 9 proteins.

The most commonly recognized polypeptide was the 41 kDa flagellin antigen but many samples reacted with other proteins, frequently those with molecular weight of 97, 66, 80/81, 31 (OspA) and 22 kDa (presented in order of frequency). Counting all bands, a minimum of 2 bands was recognized by 91% of the sera in 1986 and in 1989, 5 bands by 69% in 1986 and 63% in 1989 and 15 bands by 9% in 1986 and 11% in 1989.

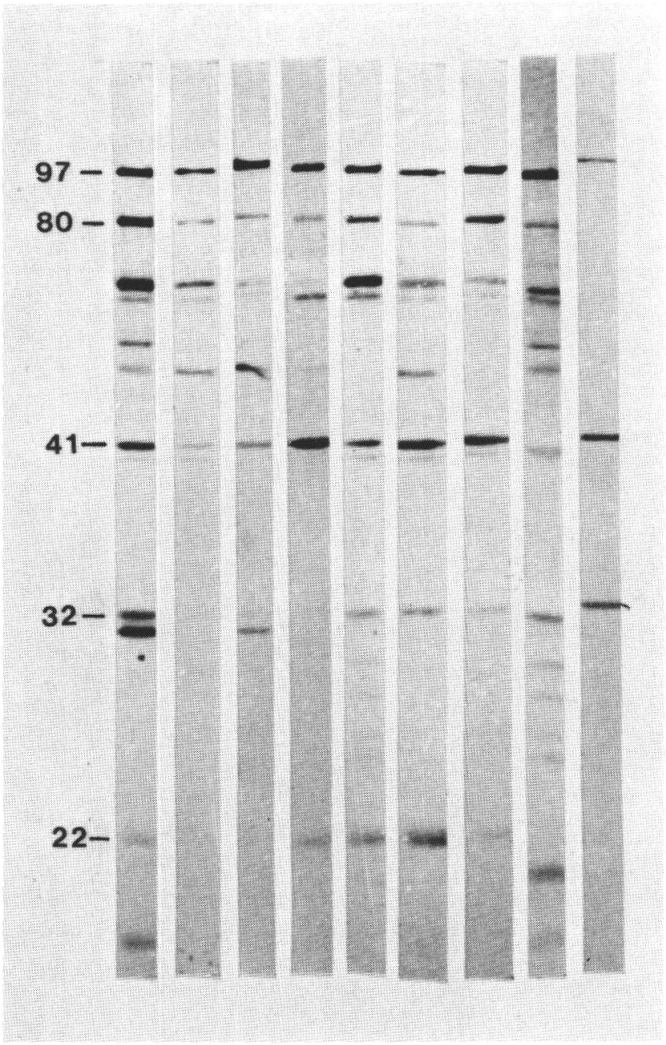

Fig. 4. Representative immunoblots (*Borrelia burgdorferi*, strain B31) of 9 asymptomatic individuals. Molecular weights (kDa) of some proteins are indicated on the left.

None of the 34 individuals available for the follow-up reported any clinical manifestations compatible with Lyme borreliosis during the 1986-1989 period.

DISCUSSION

The association of *B. burgdorferi* with Lyme borreliosis was first revealed by serology (BURGDORFER *et al.* 1982). Since that time, this disease has been diagnosed in laboratories by detection of specific antibodies to the spirochete, mainly by immunofluorescence or ELISA. Western blot has previously been evaluated for serodiagnosis and found to be more sensitive and specific than ELISA (GRODZICKI and STEERE, 1988; KARLSSON *et al.* 1989) and therefore has been proposed as a confirmation test of specific

antibodies (GRODZICKI and STEERE, 1988, ZÖLLER *et al.* 1991). The definition of positivity of Western blot has been discussed in many papers (GRODZICKI and STEERE, 1988, FISTER *et al.* 1989, KARLSSON *et al.* 1989, KARLSSON 1990, MAGNARELLI *et al.* 1989, NADAL *et al.* 1989, OLSSON *et al.* 1991, ZÖLLER *et al.* 1991). Recommendations were given to consider a Western blot positive if at least 2 to 5 bands were detected (KARLSSON *et al.* 1989, OLSSON *et al.* 1991, ZÖLLER *et al.* 1991). KARLSSON *et al.* (1989) considered an immunoblot as positive if containing a band to a 41 kDa and at least one band corresponding to low molecular weight proteins of 18, 21.5 or 23 kDa and ZÖLLER *et al.* (1991) observed the best specificity and sensitivity if at least 5 bands were present and a specificity of 97% if reactions with the 94 kDa, 73 kDa, 30 kDa and 21 kDa were observed.

In this study, the more frequent bands appearing in immunoblots were 41, 97, 66, 80/81, 32, 31 and 22 kDa. The flagellar 41 kDa band was the most frequent band observed. Reaction against this protein has been reported as being cross reactive with other spirochetes and bacteria, and also as being the first one appearing in *B. burgdorferi* infection (GASSMANN *et al.* 1989, LUFT *et al.* 1989). This cross reactivity was also observed when we assessed the specificity of our test. Proteins from 66 to 73 kDa were found to possess homologies to a heat shock protein by Luft *et al.* (results presented at the VI International Conference on Lyme Borreliosis in 1990, in Stockholm) and this could explain its high frequency and weak specificity. In this study, four groups of antigens within the range of 90 kDa, 80, 30 and 20 were frequent, they probably correspond to the proteins described by Zöller *et al.* (1991) as presenting the best specificity.

Interestingly, reactions against polypeptides of 30 kDa range (OspA) were frequent in the sera examined. The difference observed concerning the frequency of OspA in the immunoblot pattern of our samples with the results obtained by other European researchers (ZÖLLER *et al.* 1991) could be due to the distribution of the different antigenic strains in this area (HU *et al.* in prep) or to the quantity of antigenic stimulation (e.g. numerous tick bites) among the studied population.

The present study shows that in the 1989 follow-up, of 34 seropositive but asymptomatic individuals identified in 1986 (GERN *et al.* 1989), none reported clinical manifestation of Lyme borreliosis. Four individuals became seronegative whereas three individuals presented an increase in antibody titre. A significant difference was noticed in the number of proteins recognized by Western blot during this follow-up, in four cases only. This phenomenon, that qualitative immunoblot band patterns do not change, even after successful treatment of Lyme borreliosis, has already been described (ZÖLLER *et al.* 1989). The observed difference between both tests (ELISA and Western blot) is probably due to the different sensitivities of the tests, the difference in antigen preparation, and the fact that Western blot was not quantitative.

Actually, some studies, in an endemic area or in populations at risk, indicate that most infections with *B. burgdorferi* remain inapparent and lead to a high prevalence of specific antibodies (FAHRER *et al.* 1991, GERN *et al.* 1989, GERN *et al.* 1991, SATZ *et al.* 1988). This situation considerably

increases the difficulty of making a diagnosis of active disease using serological tests. We applied the Western blot testing to the asymptomatic population in this study, because this technique allows the visualization of a specific antibody response directed against the different polypeptides of the spirochete. As shown here, Western blot (IgG) gives no further definite information on the activity of the disease if compared with the diagnostic criteria described in other studies concerning patients with active disease (unpublished data; GRODZICKI and STEERE, 1988; FISTER *et al.* 1989; KARLSSON *et al.* 1989; KARLSSON 1990; MAGNARELLI *et al.* 1989; NADAL *et al.* 1989; OLSSON *et al.* 1991; ZÖLLER *et al.* 1991). Hence Western blot (IgG) is of little help in diagnosing Lyme borreliosis, in populations at risk and in endemic areas where seropositivity against *B. burgdorferi* is frequent. An IgG seropositivity detected by ELISA and/or by immunoblot, in this context, is mainly a sign of previous exposure to infectious tick bites. An immunoblot analysis of two successive serum samples might help to determine if a patient has an active disease or not. Detection of specific IgM may be another method of diagnosing active Lyme borreliosis, especially if IgM-capture ELISA is used (BERARDI *et al.* 1988, HANSEN *et al.* 1991).

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Summary

In 1986, we investigated the immune response to *Borrelia burgdorferi* of seropositive (ELISA) asymptomatic individuals ($n = 61$) from an endemic area using Western blot. The sera reacted with as many as 37 distinct proteins of the spirochete. A minimum of 2 proteins was observed in the Western blot of sera of 60/61 individuals (98%) and of 5 in 45/61 (74%). The most frequent proteins in the immunoblots were the 41, 97, 66, 80/81, 32, 31 and 22 kDa (listed in order of frequency).

The evolution of positive serological responses to *Borrelia burgdorferi*, detected by ELISA and Western blot, was followed over 3 years in 34/61 of these asymptomatic persons. During the follow-up, 8 of 34 persons had a decrease in IgG titers (ELISA) and the other 26 did not. In contrast, a significant increase in titer was observed for three individuals. The number of proteins in IgG Western blot increased in two cases (3 to 10; 4 to 9) and decreased in two other cases (13 to 3 and 10 to 5). It is concluded that Western blot is difficult to use for routine diagnosis of active disease in endemic areas or in populations at risk.

Résumé

Nous avons caractérisé et suivi la réponse immune contre *Borrelia burgdorferi*, l'agent de la borréliose de Lyme, chez une population asymptomatique séropositive provenant d'une région endémique (Aarberg, Suisse) pendant une période de 3 ans (1986-1989). En 1986, les sera testés en Western Blot ($n = 61$) ont réagi avec 37 protéines différentes. Une réaction avec au minimum 2 protéines a pu être observée chez 60/61 individus (98%) et avec 5 protéines chez 45/61 (74%). Les bandes antigéniques apparaissant le plus fréquemment présentaient des poids moléculaires de 41, 97, 66, 80/81, 32, 31 et 22kDa (présentées dans l'ordre de leur fréquence d'apparition).

En 1989, 34/61 personnes ayant déjà participé à l'étude en 1986 ont pu être retestées. Une diminution du titre d'anticorps IgG (ELISA) a été observée chez 8 personnes et une augmentation du titre chez 3. Le nombre de protéines ayant réagi en Western blot (IgG) a augmenté dans 2 cas (3 à 10 protéines et de 4 à 9) et diminué dans 2 autres cas (de 13 à 3 protéines et de 10 à 5). Ces résultats montrent que l'utilisation et l'interprétation du Western blot, pour diagnostiquer une borréliose de Lyme en zone endémique, peuvent poser quelques problèmes.

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