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The outer surface proteins of Lyme disease borrelia spirochetes stimulate T cells to secrete interferon-gamma (IFN-γ): diagnostic and pathogenic implications

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SUMMARY

Late stages of borrelia Lyme disease infections may be difficult to diagnose because of unspecific symptoms and unreliable laboratory tests, being too unspecific or insensitive. The T cell immune response was thus evaluated in these patients by using a sensitive ELISPOT T cell assay that detects the secretion of IFN-γ, i.e. a T helper 1 (Th1) response on the single-cell level. Three subcellular fractions of the Lyme borreliosis strain *Borrelia afzelii* were used for antigenic stimulation. The outer surface protein (Osp) fraction elicited the strongest response, discriminating between borrelia infections ($n = 15$) compared with other neurological diseases ($n = 10$) and normal controls ($n = 12$) ($P = 0{\cdot}0001$). The more heterogeneous sonicated borrelia fraction also elicited a strong response, however, also in some of the controls. The flagellin fraction did not have a similar T cell-stimulating effect. When looking at subgroups of borrelia infections, central nervous system (CNS) infections ($n = 7$) revealed a lower T cell response in blood ($P = 0{\cdot}0128$) compared with other borrelia manifestations ($n = 8$). Cerebrospinal fluid (CSF) lymphocytes were available from three patients with CNS borreliosis, and all showed a compartmentalization with higher responses to the Osp fraction in CSF compared with blood, also in the two patients without any intrathecal-specific antibody synthesis. The ELISPOT method is feasible for detecting a specific IFN-γ T cell response in borrelia infections. This Th1 response may well be of pathogenic relevance.

Keywords cerebrospinal fluid T cells interferon-gamma ELISPOT borrelia

INTRODUCTION

The demonstration of Lyme borreliosis is difficult because small numbers of spirochetes are found in pathologic lesions and body fluids. Cultivation of *Borrelia afzelii* from clinical specimens is difficult, time consuming, and requires considerable experience.

Antibody determination, especially of IgG, is a widely used method in the diagnosis of borreliosis. However, it is of limited use during an early stage of the infection because antibody production is not detected until after 8 weeks [1]. Dattwyler *et al.* [2] reported that borrelia-specific antibody response was lacking in patients with clinical signs of borreliosis. The antibodies may, on the other hand, persist for many years [3–5], making the diagnostic value later in life questionable. Furthermore, the occurrence of serum antibodies increases with age and time spent in the highly endemic areas [6–7]. These findings show that other methods should be useful, in combination with antibody testing, to diagnose and monitor borrelia infections.

Attempts have been made to study cellular reactivity against borrelia. The T cell response during Lyme borreliosis is pronounced in some patients, both against crude antigens, the flagellin and outer surface protein (Osp) proteins [8–11]. However, the false-positive rate is high [12], which may partly be due to the use of a crude mixture of borrelia antigens which may contain unspecific mitogens. In addition, the T cell assays used so far demand the proliferation of T lymphocytes, and may therefore not be sensitive enough.

There is obviously a need for more sensitive and specific diagnostic techniques, especially when the chronic type of infection is suspected. Furthermore, the pathogenic mechanisms which are at hand in the chronic disease are mainly unknown. By using cytokine secretion as a measure of T cell activation, it is possible to obtain qualitative information on

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the immunological response. IFN-γ is secreted by activated T cells belonging to a subgroup of T helper cells called Th1 cells [13,14]. In this study we present the usage of an enzyme-linked immunospot (ELISPOT) assay that enumerates single cells secreting IFN-γ in response to different *B. afzelii* proteins. A high number of IFN-γ-secreting cells was considered as indicative of T cell activation against the *B. afzelii* antigen.

PATIENTS AND METHODS

Patients and controls

A survey of clinical characteristics of patients and controls is given in Table 1. The seven patients with central nervous system (CNS) borreliosis are described in detail (Table 2). A non-borrelia reference group consisted of 10 patients with other neurological diseases (OND). Two patients with aseptic meningitis (AM) had typical clinical symptoms and pleocytosis in the cerebrospinal fluid (CSF) but no positive borrelia serology. Five patients had multiple sclerosis (MS), as diagnosed according to Rose *et al.* [15]. The remaining three patients had borrelia seronegative facial palsy, sudden deafness and paresthesia, respectively, all with normal CSF findings. The control group consisted of 12 healthy individuals who were all staff at the hospital. They were seronegative for IgM and IgG borrelia antibodies.

Preparation of cells

Heparinized peripheral blood was separated on lymphoprep (Nycomed Pharma AS, Oslo, Norway) according to Boyum [16], followed by washing twice with balanced salt solution (BSS) pH 7·2. The mononuclear cells were resuspended in tissue culture medium (TCM) consisting of Iscove's modification of Dulbecco's medium (Gibco BRL, Paisley, UK) supplemented with (given as final concentrations in the medium): L-glutamine (Flow Labs, Irvine, UK) 292 mg/*l*; sodium bicarbonate 3·024 g/*l*; penicillin 50 U/ml and streptomycin 50 μg/ml (Flow); 100 × non-essential amino acids 10 ml/*l* (Flow) and 5% heat-inactivated fetal calf serum (FCS; Flow).

CSF was centrifuged at 4°C for 10 min at 200 ***g***. The supernatant was removed and the cells were washed once with TCM. The cells were then resuspended in TCM, counted by phase contrast microscopy and diluted to a lymphocyte concentration of 1×10^6/ml, $0{\cdot}5 \times 10^6$/ml or $0{\cdot}25 \times 10^6$/ml, depending on the number of cells available. Peripheral blood lymphocytes (PBL) were always cultured in triplicate at a lymphocyte concentration of 1×10^6/ml, and in addition at the same concentration as CSF lymphocytes (CSF-L) (if the CSF-L concentration was one of the lower). CSF-L were added in triplicate, duplicate, or in a few cases single wells, dependent on the amount of cells available.

Antigens

Borrelia antigen fractions were prepared as previously described. In summary, 1·5 *l* of *B. afzelii* strain/ACA I culture (10^{11} cells in late log phase) was harvested, washed and, after addition of octyl β-D-glukopyranoside (OGP; Calbiochem, Novabiochem, San Diego, CA) to a final concentration of 2%, incubated at 37°C for 60 min. After centrifugation, the supernatant was incubated at 56°C for 30 min and the resulting flocculent precipitate was separated from the soluble material by a second centrifugation at 48 000 ***g*** for 30 min at 37°C. This supernatant was dialysed against glass-distilled water at 4°C. The precipitate formed in the dialysis bag was recovered by centrifugation at 48 000 ***g*** for 30 min at 25°C and then sonicated. This fraction was denoted borrelia fraction (BF).

An Osp-enriched fraction, fraction OF, was obtained from the precipitate of the OGP supernatant. After centrifugation at 48 000 ***g*** for 30 min, the precipitate was solubilized with

Table 1. Characteristics of patients with borrelia infection, patients with other neurological diseases (OND) and normal controls

				Number of patients with					
	n	Males/ females	Mean age (range), years	Positive serum serology IgM and/or IgG	Tick bite	EM	Neurological manifestations	Pleocytosis in cerebrospinal fluid	Intrathecal IgM and/or IgG antibody production
Patients with borrelia infection	15	7/8	57 (4–74)	14	12	9	12	7/14	4/14
Early stage									
EM	4	0/4	50 (31–71)	4	3	4	2	0/3	0/3
Late stage									
CNS	7	5/2	63 (4–64)	6	5	4	7	7	4
PNS	3	2/1	30 (30–74)	3	3	1	3	0	0
Arthralgia	1	0/1	43	1	1	0	0	0	0
Patients with OND	10	7/3	45 (21–62)	0	1	0	10	4/8	0/8
Aseptic meningitis	2	1/1	21, 56	0	0	0	2	2	0
Multiple sclerosis	5	3/2	43 (25–62)	0	0	0	5	2/3	0/3
Others	3	3/0	57 (41–62)	0	1	0	3	0	0
Normal controls	12	6/6	45 (39–60)	0	0	0	0	ND	ND

EM, Erythema migrans; PNS, peripheral nervous system.

PNS borrelia patients had facial palsy ($n = 1$), trigeminusneuralgia ($n = 1$) and radiculitis ($n = 1$). Others represent patients with facial palsy ($n = 1$), sudden deafness ($n = 1$) and paresthesia ($n = 1$).

ND, not done.

Table 2. Characteristics of patients with central nervous system borrelia infection

Patient number	Age (years)	Sex	Neurological symptoms	Duration of neurological symptoms (days)	Serum serology IgG (+/−)	Serum serology IgM (+/−)	CSF IgG borrelia antibody index (+/−)	CSF IgM borrelia antibody index (+/−)	IFN-γ-secreting cells/100 000 PBL fraction OF	IFN-γ-secreting cells/100 000 PBL fraction FF	IFN-γ-secreting cells/50 000 CSF-L fraction OF	IFN-γ-secreting cells/50 000 CSF-L fraction FF
1	63	M	EM, radiculitis	14	+	−	−	−	10*	21*	16	13
2	63	M	EM, headache	4	−	+	−	−	9*	22*	10	14
3	46	M	Facial palsy, headache	90	+	−	+	−	0*	5*	35	95
4	4	F	Fever, fatigue, headache, neck stiffness	7	+	+	−	−	38	38	ND	ND
5	40	M	EM, numbness	16	−	−	−	−	40	35	ND	ND
6	64	M	Rhizopathia, abdominal pain	36	+	−	+	−	11	5	ND	ND
7	64	F	Rhizopathia, headache	51	+	+	+	−	13	12	ND	ND

* Number of IFN-γ-secreting cells/50 000 PBL.
EM, Erythema migrans; fraction OF, Osp fraction; fraction FF, flagellin fraction.

sarkosyl (1% sodium lauryl sarcosinate in 10 mM Tris pH 7·4, 150 mM NaCl, 10 mM EDTA and 0·05% sodium azide (TSEA) and the resulting solution was centrifuged. The obtained supernatant was dialysed against methanol in glass-distilled water at 20°C and collected.

The flagellin fraction, fraction FF, was recovered from the pellet after the OGP treatment. This pellet was washed, centrifuged, and solubilized in sarkosyl, and thereafter centrifuged at 48 000 *g* for 30 min at 25°C. The resulting pellet was resuspended in 2% SDS in TSEA, incubated at 65°C for 30 min, and then centrifuged at 48 000 *g* for 30 min at 25°C. The resulting supernatant was dialysed against 25% methanol before lyophilization.

To confirm the presence or absence of the OspA, OspB and the flagellin in the various protein fractions, the respective murine MoAbs (H5332, 84C and H9724), obtained from Dr Alan G. Barbour and Dr Denee D. Thomas (University of Texas Health Science Centre, San Antonio, TX), were assayed against whole cell preparations and the fractions by immunoblot analysis [17–19].

Photohaemagglutinin A (PHA; Boehringer Mannheim, Mannheim, Germany), at a final concentration of 20 μg/ml, and purified protein-derived tuberculin (PPD; SBL, Stockholm, Sweden), at a final concentration of 5 μg/ml, were used as positive controls.

Stimulation of lymphocytes and enumeration of IFN-γ-secreting cells

The ELISPOT was performed essentially according to Czerkinsky *et al.* [20]. Sterile nitrocellulose-bottomed 96-well microtitre plates (Multiscreen HA; Millipore, Bedford, MA) were coated with 100 μl/well mouse anti-human IFN-γ 7B 61 MoAb (Mabtech, Stockholm, Sweden) in sterile PBS pH 7·4, at a concentration of 15 μg/ml, overnight at 4°C. The plates were then emptied by suction using a multiscreen vacuum manifold (Millipore) and washed eight times with 100 μl/well of sterile PBS. Unspecific binding sites on the nitrocellulose were blocked by incubation with TCM (including 5% FCS) for 30 min at 37°C. After emptying, aliquots of 100 μl cell suspension were applied together with 100 μl TCM with the three different antigens (fraction, OF, FF and BF). The antigens were tested in various concentrations, and for all three borrelia fractions optimal numbers of spots were found with a final concentration of 10 μg/ml, which was subsequently used. Lymphocyte cultures without antigen (non-stimulated) were run on the same microtitre plates to determine the number of cells spontaneously secreting IFN-γ.

The cells were then cultured undisturbed at 37°C in a humified atmosphere with 5% CO_2. After 48 h the plates were emptied and washed twice with PBS and twice with PBS containing 0·05% Tween 20 (PBS–T). Thereafter 100 μl of biotinylated anti-human IFN-γ 1-D1K MoAb (Mabtech) diluted to 1 μg/ml in PBS–T with 1% bovine serum albumin (BSA; Sigma, St Louis, MO) were added and incubated for 2 h at room temperature. After four washings with PBS–T, a final incubation for 1 h at room temperature was done with 100 μl/well of streptavidin-alkaline phosphatase (Mabtech) diluted 1 : 1000 in PBS–T with 1% BSA. Unbound reagent was removed by washing four times with PBS and pH was raised by 5 min incubation with Tris buffer 0·05 M pH 8·0. The spots were developed using an 'AP conjugate substrate kit' (BioRad, Hercules, CA). Spots were seen after 5–10 min and the reaction was allowed to proceed for another 5 min before the wells were rinsed with copious amounts of tap water, emptied, and dried overnight at room temperature.

The spots were counted in a dissection microscope. The mean value of the triplicates was calculated for each antigen and lymphocyte concentration. The value of the non-stimulated cells (the 'spontaneous spots') was subtracted from the values of the stimulated cells.

As a control some wells on each plate were incubated with TCM and antigens without cells, but otherwise they were treated as the other wells. No spots were found in any of these wells.

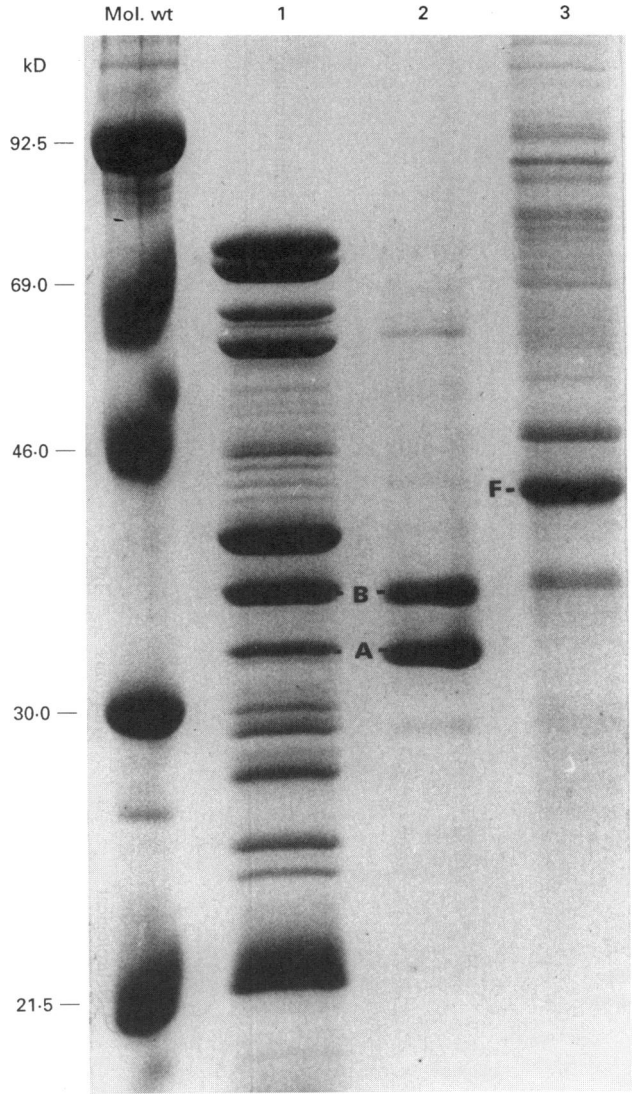

Fig. 1. Coomassie brilliant blue-stained proteins of fractionated *Borrelia afzelii* strain ACA I. Lane 1, BF, a subcellular fraction of 12 outer membrane proteins; lane 2, outer surface protein (Osp)-enriched fraction; lane 3, FF, flagellin-enriched fraction. The locations of the OspA (A) and OspB (B) and flagellin (F) are indicated by respective letter. The sizes of the molecular weight standards in kD are shown on the left.

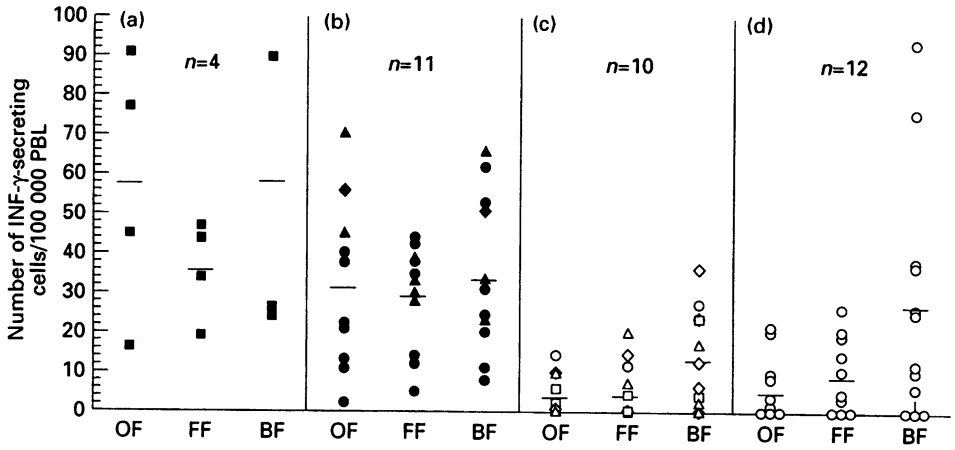

Fig. 2. Number of IFN-γ-secreting cells/100 000 peripheral blood lymphocytes (PBL) when stimulated with outer surface protein (Osp) antigen (OF), flagellin antigen (FF) and borrelia antigen (BF) in patients with (a) erythema migrans (EM) (■); (b) borrelia infections, except EM, with symptoms from other organs, i.e. central nervous system (●) and peripheral nervous system (▲), and arthralgia (◆); (c) aseptic meningitis (AM) (□), multiple sclerosis (MS) (◇) and others (△); (d) normal control subjects (○). Means are shown as horizontal lines.

Statistical analysis

Mann–Whitney *U*-test was used when comparing the results from PBL stimulations in different patient groups.

RESULTS

All three fractions of *B. afzelii* antigens shown in Fig. 1 elicited a PBL T cell response in patients with erythema migrans (EM) (Fig. 2a) and patients with other borrelia infections (Fig. 2b), as measured by number of IFN-γ-secreting cells after antigenic stimulation. The strongest response was obtained by the Osp fraction (OF) and borrelia outer membrane fraction (BF), compared with the FF. Patients with OND (Fig. 2c) in general showed a low T cell response, as did normal controls (Fig. 2d). However, the crude BF elicited a strong response in some patients with other neurological diseases and normal controls. When comparing the patients with borrelia infections with normal control subjects and with patients with OND, the OF discriminated best ($P < 0{\cdot}0001$) (Fig. 3). The BF and the FF did not significantly discriminate between borrelia patients and controls. Because of the small number of patients, it was not possible to perform statistical analyses on all patient subgroups.

When looking at the distribution of T cell responses within the group of borrelia patients, it is obvious that in PBL the Osp antigen (OF) induced rather weak responses in patients with CNS borreliosis (Fig. 3). The number of IFN-γ-secreting PBL was significantly lower ($P = 0{\cdot}0128$) in patients with CNS borreliosis compared with those with borreliosis with symptoms from the peripheral nervous system (PNS) or joints, i.e. radiculitis and arthralgia.

CSF-L were available from three patients with CNS borreliosis. The number of IFN-γ-secreting cells in the CSF after stimulation with OF was higher compared with peripheral blood in all three patients (Fig. 4, patient NB1–3). In contrast, this was not the case for FF and BF. In AM and MS the number of IFN-γ-secreting cells after stimulation was lower in CSF compared with the corresponding blood. In addition, the number of IFN-γ-secreting cells in CSF was lower in patients with AM and MS compared with patients with neuroborreliosis.

DISCUSSION

In this study we show that a sensitive ELISPOT T cell assay combined with the usage of selected *B. afzelii* antigens improves the possibility of studying T cell responses in borrelia infections.

The ELISPOT method detects activated T cells at a single-cell level. The commonly used proliferative assay measures T cell activity by radioactive labelled thymidine incorporation. However, when applying the proliferative assay, the number of T cells that respond in the form of mitosis may be low. Furthermore, the proliferative phase may be suppressed by secreted cytokines, e.g. IFN-γ [21]. The ELISPOT method, on the other hand, is able to measure low numbers of T cells secreting a cytokine in response to antigenic stimulation, i.e. activated T cells are detected even if they do not go into the proliferative phase. A correlation between the ELISPOT method measuring the proportion of T cells secreting a specific cytokine and the amount of cytokine secreted in supernatants of cultivated cells has been found, although the ELISPOT method proved more sensitive [22]. In the presently used technique IFN-γ secretion was used as an indication of antigenic activation. Another advantage with the ELISPOT method is that it can be applied to other cytokines as well. The ELISPOT method has recently proved successful for studies of T cell immunoreactivity in, for example, MS [23] and animal models of autoimmune diseases, e.g. NOD mice [24].

The choice of antigen for stimulation of T cells is important. The presently used subcellular fractions of *B. afzelii* is an attempt to use more defined outer surface protein preparations. Two of the three protein fractions used consist of different major outer surface proteins (OF and BF), and the third fraction consists mainly of the highly expressed flagellin

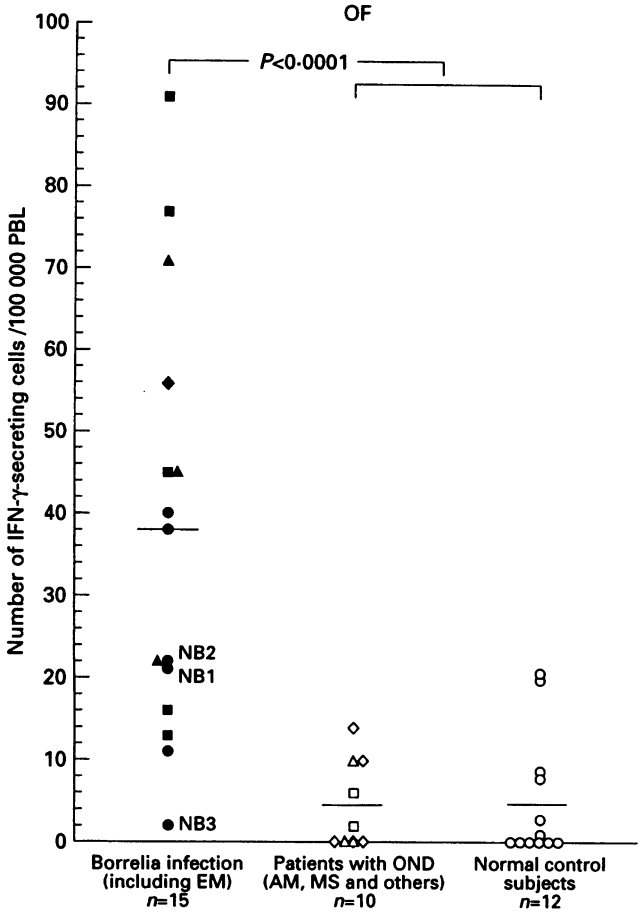

Fig. 3. Number of IFN-γ-secreting cells/100 000 peripheral blood lymphocytes (PBL) when stimulated with outer surface protein (Osp) antigen (OF), in patients with erythema migrans (EM) (■), borrelia infections except EM with symptoms from other organs, i.e. central nervous system (CNS; ●) and peripheral nervous system (▲) and arthralgia (◆) ($n = 15$), aseptic meningitis (AM) (□), multiple sclerosis (MS) (◇) and others (△) ($n = 10$), normal control subjects (○) ($n = 12$). Means are shown as horizontal lines. Note that patients with CNS symptoms (neuroborreliosis, NB 1–3 in figure) have low or moderate responses in PBL.

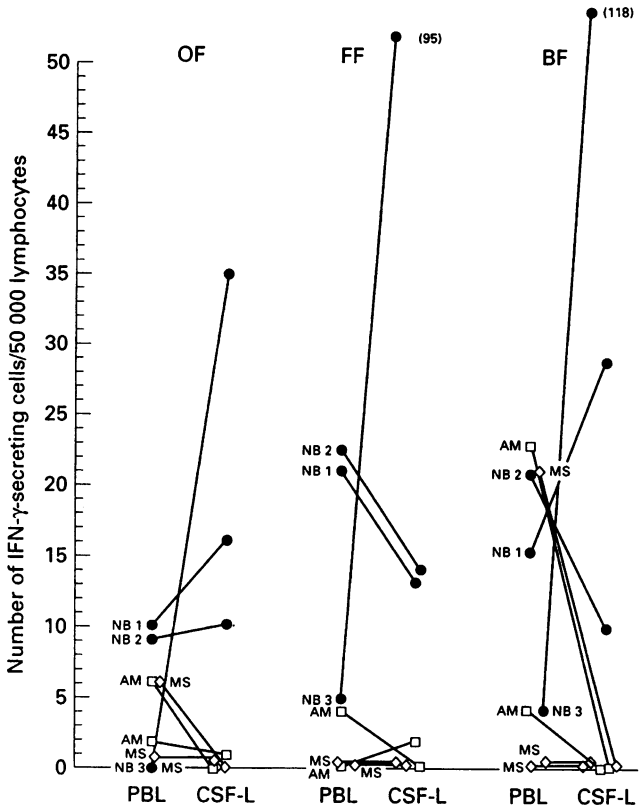

Fig. 4. Number of IFN-γ-secreting cells/50 000 lymphocytes from peripheral blood (PBL) and cerebrospinal fluid (CSF-L) when stimulated with outer surface protein (Osp) antigen (OF), flagellin antigen (FF) and borrelia antigen (BF) in patients with central nervous system borreliosis (●) (NB1–3), aseptic meningitis (□) (AM $n = 2$) and multiple sclerosis (◇) (MS $n = 3$).

protein, which is localized in the periplasmic space of the spirochete.

Interestingly, OF was the best discriminating antigen preparation when comparing the patient groups. This fraction, which contains primarily the outer membranes OspA 32 kD and OspB 36 kD of *B. afzelii* strain ACA I, is probably the most specific and sensitive antigen, considering the results of the PBL stimulation tests. The OspA and OspB proteins in the fraction OF are lipoproteins, which could partly explain the T cell response as there are several examples of lipoproteins that induce a vigorous T cell response [25,26].

Despite the small number of patients examined in this study, it seems that there was no substantial T cell reactivity in peripheral blood in patients with CNS neuroborreliosis compared with patients with other forms of borrelia infection. Interestingly, in three patients with CNS borrelia infection CSF was studied, and all showed high numbers of IFN-γ-secreting CSF cells upon stimulation with the OF antigen, even when the number of IFN-γ-secreting cells in peripheral blood was low. This points out the importance of studying the effector organ, i.e. the CNS, particularly in clinical suspected borreliosis when there is difficulty in establishing the diagnosis by conventional means. In fact, two of these three patients studied had no detectable antibodies in CSF or any evidence of specific intrathecal antibody production. Therefore, the present method seems to be promising for diagnosing a suspected neuroborreliosis when free circulating antibodies are undetectable. However, before we can draw any certain conclusion, more patients must be examined. In addition, one major restriction when using the ELISPOT method is that a sufficient number of cells must be available in the CSF, i.e. a cell count of > 3–5×10^6 mononuclear cells/l is desirable. It may, however, be possible to develop further the ELISPOT method, in order to use fewer cells.

CSF-L in AM and MS patients showed no reaction against any specific *B. afzelii* antigen, although MS patients are known to have markedly elevated numbers of IFN-γ-secreting T cells in response to different myelin antigens [27]. This fact further strengthens the notion of a specific reaction to *B. afzelii* in the ELISPOT assay presented here.

The presently used antigens clearly show that a purified antigen from the *B. afzelii* outer membrane was the most relevant. A further improvement would be to study other T cell epitopes including purified peptide antigens and fragments

of antigens. The ELISPOT method may prove useful in this respect, and it would be feasible to map the fine specificity of the T cell response.

In this study we have analysed IFN-γ secretion, usually attributed to the Th1 response. Th1 secretion of IFN-γ may have pathogenic implications on the development of nervous system involvement and neurological symptoms. IFN-γ promotes inflammation in the nervous system, e.g. by activation of macrophages [28], up-regulation [29] and induction [30,31] of MHC class II expression, and recruitment of T cells into the CNS [32]. The potential *in vivo* effect of IFN-γ on the CNS was demonstrated in MS, which is an immune-mediated CNS disease, where IFN-γ in a therapeutical trial caused deterioration of the clinical outcome [33]. Preliminary data from two patients with neuroborreliosis show that when a Th1 response is evident, a simultaneous suppression of Th2 cells, as determined by the production of IL-4, is present. Further studies of the Th2 response, i.e. the secretion of IL-4 or other cytokines potentially suppressing cellular immune reactivity, may be important tools to increase our understanding of prognostic and pathogenic factors in CNS borreliosis.

In conclusion, the presently used ELISPOT assay showed that a protein fraction of Lyme disease borrelia, including the OspA and OspB proteins, induces a substantial T cell response in patients with neuroborreliosis. The activated T cells were enriched in the CNS, i.e. the target organ, confirming the compartmentalization of the immune reaction in CNS neuroborreliosis. The ELISPOT method may prove useful to determine the fine specificity of T cell reactivity against borrelia antigens, and to elucidate the cytokine profile in relation to clinical outcome. Finally, T cell reactivity may become an important diagnostic tool, not least in neuroborreliosis.

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