Interferon-γ induced increases in intracellular calcium in T lymphocytes from patients with multiple sclerosis precede clinical exacerbations and detection of active lesions on MRI

G Martino, M Filippi, V Martinelli, E Brambilla, C Gobbi, G Comi, L M E Grimaldi

Abstract

**Background**—Interferon (IFN)-γ exerts a multiplicity of actions potentially relevant for the pathogenesis of multiple sclerosis, including the expression of a transplasmalemma calcium (Ca2+) influx leading to an intracellular Ca2+ ([Ca2+]i) increase able to lower T lymphocyte threshold of excitability. It has been previously shown in a cross sectional cumulative study that this influx is associated with clinical and MRI evidence of disease activity.

**Methods**—To evaluate the temporal relation between disease activity and the IFN-γ activated Ca2+ influx in individual patients, a fluorimetric analysis was performed on peripheral blood lymphocytes from eight patients with relapsing-remitting multiple sclerosis every 15 days for one year. Results—Fluctuations of the influx were correlated with clinical events and monthly enhanced brain MRI. The influx was detected in a mean of 10.4 (range 7-17) times per patient during our analysis. In 61% of the occasions, influx induced [Ca2+], increases were recorded in each patient in more than two consecutive measurements, determining sustained [Ca2+], increases lasting for a mean of 31.5 days. Peak [Ca2+], increases preceded clinical attacks (P=0.04) or maximal detection of brain MRI enhancing lesions (P=0.05) by a mean of 30.8 and 34.2 days respectively. Spectral analysis of time series further indicated that the fluctuation frequency of [Ca2+], increases due to the influx over time were superimposable on the appearance of new MRI lesions in all patients and confirmed that in two thirds of the patients these [Ca2+], increases occurred significantly before (P<0.005) or concurred with new lesion appearance. Finally, the overall presence of the influx throughout the follow up period correlated (P=0.03) with the patients' exacerbation rates.

**Conclusions**—Intracellular events leading to T lymphocyte activation in multiple sclerosis occur in the peripheral blood before CNS specific events become evident and are, in part, sustained by cytokine induced Ca2+ mediated phenomena.

**Keywords**: multiple sclerosis; interferon-γ; lymphocytes; calcium; MRI

A major part in the pathogenic mechanisms leading to CNS demyelination in multiple sclerosis is played by activated T lymphocytes. Their activity is regulated by a complex network of cytokines among which interferon (IFN)-γ is considered essential.

We have recently shown that T lymphocyte activation in multiple sclerosis is partially dependent on a novel transmembrane IFN-γ activated calcium (Ca2+) influx which is mainly expressed on CD4+ cells. The influx is associated with phases of disease activity and facilitates T cell proliferation in response to activator stimuli.

To evaluate the temporal relation between this Ca2+ influx and clinical and MRI evidence of disease activity in multiple sclerosis, we correlated the presence of the influx with clinical exacerbations and gadolinium enhancing brain MRI lesions in eight patients with relapsing-remitting multiple sclerosis followed up every two weeks for one year.

**Materials and methods**

**PATIENT SELECTION**

Peripheral blood lymphocytes were obtained every 15 days for one year from eight patients (six women, two men; mean age 25, range 21-37 years) affected by clinically definite multiple sclerosis (mean disease duration 6.3, range 2-10 years) according to the criteria of Poser et al. The clinical course of the disease was relapsing-remitting in all patients. Patients were clinically evaluated using the expanded disability status scale (EDSS; mean score at entry 2.5). Patients were considered to be in an active phase of the disease when experiencing an episode of symptoms, documented on examination, indicative of a neurological abnormality attributable to acute demyelination in one or more regions of the CNS; the symptoms had to last more than 24 hours and be separated from a previous attack by at least one month. Patients experiencing a new attack of the disease were always treated with a two to three week course of steroids.
DETECTION OF THE IFN-γ ACTIVATED Ca2+ INFLUX BY FLUORIMETRIC ANALYSIS

To evaluate the presence of the IFN-γ activated Ca2+ influx, macrophage depleted human peripheral blood lymphocytes were analysed by fluorimetry every 15 days as recommended by Grynkiewicz et al.1 but modified according to the Ca2+ free Ca2+ reintroduction protocol.10 This protocol allows the characterisation of the increases of intracellular Ca2+ ([Ca2+]i) concentrations, measured by conventional fluorimetry, as due to Ca2+ release from intracellular stores or to Ca2+ influxes from outside the cell. Briefly, cell samples were supplemented with excess ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA; 3 mM) to chelate extracellular Ca2+ before fluorimetric analysis. After one minute 1 pg/ml IFN-γ (specific activity 1x107 U/mg; Genzyme, Cambridge, MA, USA), which was found to be the optimal dose to stimulate the IFN-γ activated Ca2+ influx in peripheral blood lymphocytes in preliminary dose-response experiments,1 was added. Then Ca2+ (3 mM) was reintroduced into the medium after four additional minutes and the ensuing [Ca2+]i peak, indicating Ca2+ influx from outside the cell due to the IFN-γ activated influx, was then measured.

The increase in [Ca2+]i response to IFN-γ occurring in peripheral blood lymphocytes from patients with multiple sclerosis was expressed as the percentage of [Ca2+]i increase over basal level. To calculate the percentage increase of [Ca2+]i over basal concentration due to the IFN-γ activated Ca2+ influx we previously subtracted from the IFN-γ induced [Ca2+]i, increase the percentage of [Ca2+]i increase due to the small cytosolic [Ca2+], changes occurring in non-stimulated cells as a consequence of medium Ca2+ chelation and reintroduction.11 However, due to the high frequency of spontaneous [Ca2+] i oscillations occurring in non-stimulated lymphocytes10 and to the necessity of standardising our assay to perform longitudinal studies on large cohorts of patients, we introduced more stringent criteria. We now perform three fluorimetric measurements, measured by conventional fluorimetry every 15 days as recommended by Grynkiewicz et al.9 but modified according to the Ca2+ free Ca2+ reintroduction protocol.10

The increase due to the small cytosolic [Ca2+], increase in non-stimulated cells was considered as a consequence of medium Ca2+ chelation and reintroduction. However, due to the high frequency of spontaneous [Ca2+]i oscillations occurring in non-stimulated lymphocytes and to the necessity of standardising our assay to perform longitudinal studies on large cohorts of patients, we introduced more stringent criteria. We now perform three fluorimetric analyses per patient on different cell preparations obtained from the same blood sample. Each analysis consists of two recordings of variations in [Ca2+], (performed in parallel on two different aliquots of the same cell preparation): the first one in the absence of any stimulus, and the second one in the presence of 1 pg/ml IFN-γ. At the end of both recordings, the [%Ca2+] increase after Ca2+ reintroduction in the absence of any stimulus (already subtracted by the small cytosolic [Ca2+], changes occurring as a consequence of the protocol used; see above) is subtracted from the [%Ca2+] increase recorded in response to IFN-γ. A patient is considered influx positive only when the difference of [Ca2+] increase is higher than 1% in at least two of three experiments. The final value is the mean of the values calculated in the three fluorimetric analyses. With these criteria we recorded an intra-assay variability <20% (a 1% [Ca2+] increase over basal concentration due to the IFN-γ influx corresponds to 1% (SD 0.05)).

MRI STUDIES

Brain MRI was performed monthly in all patients with a 1.5 Tesla machine (Siemens SP63, Erlangen, Germany). Five mm contiguous axial T1 weighted (SE 600/17; field of view=230 mm, matrix size=198x256; two excitations) slices throughout the whole brain were obtained five to seven minutes after the injection of gadolinium-diethylenetriamine penta-acetic acid (0.1 mmol/kg intravenously over one to two minutes). New or persisting enhancing lesions were counted in each scan by one of us (MF), unaware of the clinical status and fluorimetric results. An arbitrary scoring system was used to estimate the size of the lesions: 1 point was given for each lesion with a diameter 1-5 mm, 2 points for 6-10 mm, and 3 points for lesions>10 mm in size. The total score represented the lesional burden of each patient.

MEASUREMENTS OF TUMOUR NECROSIS FACTOR-α (TNF-α) mRNA

Total RNA was isolated from peripheral blood lymphocytes using guanidine-isothiocyanate solution and purified by ultracentrifugation on a caesium chloride gradient. A T primed first strand kit was used for the reverse transcription of total RNA into cDNA (Ready-to-go kit, Pharmacia, Upsala, Sweden). Polymerase chain reaction amplification of cDNA sequence specific for TNFα was performed using 1 µM cytokine primers, 200 µM of each dNTP, 50 mM KCl, 10 mM Tris HCl (pH 8.3), and 1.5 mM MgCl2. After a hot start at 95°C for five minutes, 1 U Taq polymerase was added and 25 polymerase chain reaction cycles were carried out. Each cycle consisted of one minute of amplification at 95°C, one minute of primer annealing at 60°C, and one minute of product dissociation at 72°C. The number of amplification cycles was selected based on preliminary experiments on cDNA from the U937 cell line. The number of cycles was varied from 10 to 40, in 5 cycle increments, and we found that 25 cycles were in the exponential part of the amplification curve. Amplified polymerase chain reaction products were visualised in ethidium bromide stained 1% agarose gels run in TAE buffer, blotted on to nylon membranes, and hybridised with specific 3P labelled probes (Ready Prime kit, Amersham, Buckinghamshire, UK) representing the amplified region. Probes were obtained by elution of RT polymerase chain reaction products from preliminary experiments on cDNA from the U937 cell line representing a specific sequence of TNFα. Autoradiograms of the probed TNFα polymerase chain reaction products were quantified by densitometry (Computing Densitometer, Molecular Dynamics; Image Quant Software, version 3.3). The raw data was normalised against the intensity of the β actin band. For comparisons between subject, the normalised intensities were further corrected using the normalised intensities of the U937 cell derived positive controls. All cDNA samples from each patient were run in the same polymerase chain reaction round. To minimise experimental errors, all cDNA samples from a given patient were amplified simultaneously.
and a positive control, U937 cell cDNA, was included in every polymerase chain reaction experiment.

The following primers were used: TNFα (product 490 bp), antisense 5'-GAC CTC TCT CTA ATC AGC CC-C3', sense 5'-CAG ACC CGT CCA GAT GAA AC-C3', β-actin (product: 658 bp); antisense 5'-CTA GAA GCA TTT GCG GTG GAC GAT G3', sense 5'-AGG GGG TCA CCC ACA CTG TGC-3'.

STATISTICAL ANALYSIS
As the presence of the IFN-γ activated Ca²⁺ influx as well as number of gadolinium enhancing MRI lesions tended to vary during the study, we plotted all [Ca²⁺], increases due to the influx and variations in MRI lesion number over time and obtained a temporal distribution of the two variables for each patient. IFN-γ induced maximal [Ca²⁺] increases of 23.7 (95% confidence interval 7.9 - 12.8) per patient. The influx determined a mean of maximal [Ca²⁺], increases of 23.7 (95% CI 17.7 - 29.7)% over basal concentrations.

We also evaluated whether the clinical activity of the disease was influenced by the presence of the influx by regression analysis: the area under the curves generated by the [Ca²⁺], fluctuations due to the influx (indicating how many influx positive cells for how long) was compared with the number of each patient's exacerbations during the follow up period. Area under the curves was calculated with the following formula:

\[
\text{area} = \frac{1}{2} \sum \left( T_{n-1} - T_n \right) \left( Y_{n-1} - Y_n \right)
\]

where T represents time of influx detection and Y the percentage increase of [Ca²⁺] over basal concentration due to the influx.

Results
FLUORIMETRIC ANALYSIS
Eighty five of the 183 (46%) fluorimetric evaluations in T lymphocytes from the eight patients with relapsing-remitting multiple sclerosis showed the presence of the IFN-γ activated Ca²⁺ influx. Influx positive lymphocytes were found a mean of 10.4 times (95% confidence interval (95% CI) 7.9 - 12.8) per patient. The influx determined a mean of maximal [Ca²⁺], increases of 23.7 (95% CI 17.7 - 29.7)% over basal concentrations.

In 61% of occasions the influx was detected for more than two consecutive fortnightly measurements. Consecutive detections were interpreted as persistence of the influx determining a sustained [Ca²⁺], increase. These increases lasted for a mean of 31.5 (95% CI 23.3 - 39.5) days.

The influx had already disappeared at the beginning of the steroid treatment for eight clinical attacks. In two attacks the influx continued to increase during steroid administration. In the remaining seven attacks, the influx was already decreasing at the beginning of steroid treatment (fig 1 A).

The persistence of the influx and its tendency to fluctuate determined different curves of [Ca²⁺], in each patient which were plotted over time. Maximal [Ca²⁺], increases within sustained curves (mean 4.5 peaks per patient, range 3-6; fig 1A) were considered for statistical analysis as markers of maximal T lymphocyte activation and were compared with clinical attack occurrence.

The eight patients with relapsing-remitting multiple sclerosis studied experienced 17 clinical attacks during the study (one patient four attacks; two patients three attacks; three patients two attacks; one patient one attack; one patient no attacks) (mean annual exacerbation rate 2.1). The influx was detectable at the beginning of 10 (59%) clinical attacks but had already subsided by the beginning of the remaining seven attacks. [Ca²⁺], peaks never coincided with the beginning of a clinical attack but were found within 45 days before the onset of clinical symptoms in 15 of the 17 (88%) clinical attacks. The increased frequency of the [Ca²⁺], peaks (mean 4.5 peaks/year/patient) made it difficult to interpret the temporal relation between T lymphocyte specific events and clinical attacks. However, as the time elapsed between a [Ca²⁺], peak and the next clinical attack was significantly shorter (P=0.05) (mean 30.8; 95% CI 11.6 - 49.9) days than that between a clinical attack and the ensuing [Ca²⁺], peak (mean 50.5; 95% CI 30.2 - 70.5 days) (fig 2 A), we favour a non-random
association between [Ca\(^{2+}\)]\(_i\) peaks and clinical exacerbations.

The area under the curves generated by the [Ca\(^{2+}\)]\(_i\) fluctuations due to the influx significantly correlated (r=0.72; P=0.03) with the patients' exacerbation rates during the follow up period further suggesting that the clinical activity of the disease was influenced by the presence of the influx.

**BRAIN MRI LESIONS AND IFN-\(\gamma\) INDUCED [Ca\(^{2+}\)]\(_i\) VARIATIONS**

During the study we performed 80 gadolinium enhanced brain MRI scans. Four patients had 12 monthly scans, two patients had 11 monthly scans, and two patients had five monthly scans. A total of 462 enhancing lesions were found in 66 scans (343 lesions were new and 119 were persisting from at least the previous scan); no enhancing lesions were detected in the remaining scans. Lesion scores varied between patients from 0 to 51 (mean 7.3 (SD 9.2)).

We first plotted the total number of active lesions per scan over time and generated for each patient curves reflecting disease activity as detected by MRI (fig 1 C). As the total score as well as the number of new enhancing lesions for each scan generated curves which were superimposable to those generated using the total number of enhancing lesions in all patients (total number of lesions v score: r=0.98, P<0.001; total number of lesions v new lesions: r=0.93, P<0.001), we adopted the maximal number of enhancing lesions (MRI peaks) as a marker of CNS inflammation. A total of 20 MRI peaks were recorded (mean

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**Figure 1** Time course of (A) IFN-\(\gamma\) activated Ca\(^{2+}\) influx, (B) TNF\(\alpha\) mRNA concentrations, and (C) number of brain MRI enhancing lesions in one representative patient with relapsing-remitting multiple sclerosis. The influx is expressed as % of [Ca\(^{2+}\)]\(_i\) increases over basal values in response to IFN-\(\gamma\) stimulation, TNF\(\alpha\) mRNA is expressed as arbitrary units (ratio between densitometric values of TNF\(\alpha\) and \(\beta\)-actin mRNA concentrations) and MRI lesions are represented by the number of gadolinium enhancing lesions disclosed by brain MRI. The black box at the bottom of the lower panel represents days of steroid treatment. The arrow and the dashed line indicate onset of clinical attacks. NA=not available.
After P = 0.05

IFN-γ activated Ca2+ influx and disease activity in multiple sclerosis

We now report on the temporal relation between the detection of the influx and clinical or MRI evidence of disease activity in eight patients with relapsing-remitting multiple sclerosis followed up every 15 days for one year. We found that the IFN-γ activated Ca2+ influx fluctuates over time in peripheral blood lymphocytes from patients with multiple sclerosis, becoming undetectable within 30 days of its appearance. As we noted in previous studies, the influx is not detectable in 40% of cases by the time of the clinical attack, having probably exhausted its contribution to the pathogenesis of multiple sclerosis in earlier stages (during T lymphocyte activation). However, we found that the fluctuations of the influx significantly heralds clinical attacks and the detection of enhancing lesions. The peak activity of the influx as well as its highest frequency (reflecting T cell activation in the peripheral circulation) falls about 25-30 days before clinical attacks and maximal MRI evidence of CNS inflammation and fosters almost 90% of all clinical attacks. The clinical relevance of the influx is also supported by the significant correlation between the detection of IFN-γ activated Ca2+ influx positive T lymphocytes over time and the annual exacerbation rates of patients with multiple sclerosis.

Why does it take four to five weeks for peripherally activated CNS specific clones to leave their peripheral location and reach the CNS? Although full blown immune responses require only a few days or weeks to take place, studies employing the chronic experimental allergic encephalomyelitis model in rodents and non-human primates have shown that widely ranging periods of time (from one week to six months) can separate immunisation from...
Figure 3  Left side panels represent spectra of the two time series (dashed line=numbers of gadolinium enhancing brain MRI lesions; continuous line=IFN-γ activated Ca²⁺ influx measured as % [Ca²⁺]i lymphocytic increase after IFN-γ stimulation) in each of the seven patients with multiple sclerosis studied (patients A-G). The spectra of the two variables are concordant in each patient. Right side panels show phase spectrum v frequencies. Regressions between the two variables are indicated. Negative slopes indicate that the [Ca²⁺]i increase series precedes the brain MRI gadolinium enhancing lesion series. Each row (left and right sided panels) describes results in one of the patients with multiple sclerosis.
appearance of active disease or two consecutive spontaneously occurring attacks. 

This finding can be in part explained by genetic predisposition (for example, HLA background) as well as environmental triggering factors (for example, viral infections). Our finding of a lag time of 10 to 50 days occurring between peak activity of the IFN-γ activated Ca²⁺ influx and MRI or clinical signs of disease activity in patients with multiple sclerosis is in line with experimental evidence. These findings, along with results of our spectral analysis, explain the apparent paradox we previously reported of a T cell phenomenon relevant for immune activation in multiple sclerosis but not detected in 20 to 30% of patients with multiple sclerosis at the time of clinical or MRI active phases of the disease. 

The activity of the influx actually signals one of the earliest immunological events leading to demyelination in patients with multiple sclerosis, as suggested by spectral analysis, which showed that the IFN-γ influx activity (frequency) precedes or concurs with phases of disease activity (MRI active lesions) in almost two thirds of the patients.

In a preliminary study involving four patients with relapsing-remitting multiple sclerosis tested at the beginning and at the end of a clinical attack, we reported that steroid treatment seemed to foster the disappearance of the influx. From the present more thorough longitudinal study, it seems that the response to steroids is not stereotyped. The influx either disappeared before, appeared after, or was active during the steroid treatment. Although this variability questions a specific role of steroids in influencing the influx kinetics and overall disease activity in multiple sclerosis, we cannot at present exclude that part of the delay from the beginning of the clinical attack to the appearance of the next [Ca²⁺] peak is due to the steroid treatment.

Increases in TNFα concentration have been recently shown to precede periods of disease activity in multiple sclerosis about one month before clinical exacerbations. We found in one patient with multiple sclerosis that a similar temporal occurrence differs between fluctuations of [Ca²⁺], due to the IFN-γ activated influx and variations of TNFα mRNA concentrations. Although preliminary, this finding, along with our recent finding of a synergistic effect of TNFα/interleukin (IL)-6/IL-2 with the IFN-γ activated influx in promoting non-antigen specific T cell activation in multiple sclerosis via two different but cooperating intracellular Ca²⁺-mediated pathways, (Martino et al, unpublished data) further supports the proposed synergistic effect between proinflammatory cytokines, such as TNFα and IFN-γ, in orchestrating immune mediated processes relevant for the pathogenesis of multiple sclerosis.

In conclusion, we found that the IFN-γ activated Ca²⁺ influx is a dynamic feature of T lymphocytes from patients with relapsing-remitting multiple sclerosis and usually operates before or during clinical or MRI evidence of disease activity. Moreover, as its early detection is associated with preclinical phases of disease activity, it might represent an immunological surrogate marker of disease activity in patients with multiple sclerosis.
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