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Analyse qualitative et quantitative du transcriptome Vβ du TCR dans la Sclérose en Plaques : caractérisation des cellules portant les altérations et corrélations avec les paramètres cliniques et radiologiques de la maladie.

THESE DE DOCTORAT

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Abréviations

BHE :Barrière Hémato-Encéphalique

CMH : Complexe Majeur d'Histocompatibilité

CPA : Cellules Présentatrices d'Antigène

CTL : Lymphocytes T cytotoxiques

EAE : Encéphalomyélite Autoimmune Expérimentale

ICAM-1 : InterCelullar Adhesion Molecule-1

LCR : liquide céphalo-rachidien

MBP : Protéine Basique de Myéline

MOG : Glycoprotéine de Myéline Oligodendrocytaire

PLP : ProtéoLipide Protéine

SEP : Sclérose en Plaques

SNC : Système Nerveux Central

TCR : Récepteur des Cellules T

VLA4 : Very Late Antigen 4

Introduction

La Sclérose en plaques (SEP) est une maladie complexe supposée auto-immune et responsable d'un handicap chez l'homme jeune.

C'est le neuropathologiste Jean-Martin Charcot qui a fait les premières descriptions de la maladie en 1868 notant l'accumulation de cellules inflammatoires avec une distribution péri-vasculaire au sein de la substance blanche cérébrale et médullaire chez des patients ayant des épisodes intermittents de dysfonction neurologique (Charcot, 1868).

Plus récemment, en 1948, Elvin Kabat décrivait une augmentation de la sécrétion d'immunoglobulines avec une répartition oligoclonale dans le liquide céphalo-rachidien de patients corroborant la nature inflammatoire de la pathologie (Kabat, 1948). La mise au point d'un modèle animal d'inflammation du système nerveux central (SNC) par Thomas River en 1933, par immunisation de singes par injections répétées de cerveau et de moelle épinière de lapin, a conduit à l'hypothèse généralement acceptée que la SEP est une maladie auto-immune se développant en réponse à des auto-antigènes issus de la myéline du SNC chez des individus génétiquement prédisposés. En effet, de nombreuses études portant sur des jumeaux mono et dizygotes ont pu démontrer l'importance des gènes dans le développement de la maladie (Sadovnick et al., 1993). Il faut souligner d'autre part que l'absence de mise en évidence d'un agent infectieux au sein des lésions de la maladie renforce l'hypothèse d'une réponse auto-immune. Néanmoins, une infection virale primitive du SNC peut conduire à une véritable maladie auto-immune telle que dans l'EAE induite par le virus de Theiler chez la souris. L'étude de ces modèles animaux a conduit à la notion de niveau d'activation de cellules T autoréactives périphériques nécessaires à l'induction d'une maladie inflammatoire dans le SNC. Une autre information importante dérivant des travaux réalisés chez l'animal est la

notion « d'épitope spreading » ou dispersion d'épitope, décrite par Elie Sercarz (Lehmann et al., 1992) au début des années 90. En effet l'injection d'un épitope d'une protéine de myéline chez la souris entraîne une EAE et l'activation périphérique d'autres lymphocytes T dirigés contre d'autres épitopes de la même protéine de myéline. Le transfert adoptif de ces cellules vers une autre souris est ensuite capable de provoquer une EAE, indiquant qu'au moment de l'inflammation cérébrale il existe une libération de nouveaux antigènes (antigènes cryptiques)

De nombreuses données chez l'homme indiquent la présence de lymphocytes T autoréactifs dans le sang des malades autant que les contrôles sains. Il semble cependant que ce soit l'activation de ces cellules chez les malades qui soit l'événement crucial induisant la maladie auto-immune.

Etiopathogénie de la Sclérose en Plaques

Article 1

La Revue du Praticien

In press

Etiopathogénie de la sclérose en plaques.

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Résumé

La sclérose en plaques est une maladie inflammatoire de l'adulte jeune touchant le système nerveux central et responsable de lésions démyélinisantes au sein de la substance blanche. L'activation de cellules autoréactives CD4+ mais aussi CD8+ dirigées contre des antigènes de la myéline du système nerveux central est un phénomène clé à l'origine des lésions de SEP. Son étiologie est inconnue mais implique l'existence de traits génétiques particuliers et d'un déclenchement par des phénomènes environnementaux tels qu'une infection virale. Cependant, à ce jour, aucun virus ou autre élément environnemental n'a pu être impliqué directement dans la survenue de la maladie. Des anomalies des systèmes de régulation de l'immunité tels que les cellules régulatrices pourraient être en partie impliquées dans le déclenchement de la maladie. Toutefois, il apparaît, sur la base des découvertes récentes que la sclérose en plaques n'est pas simplement une maladie du système immunitaire mais implique aussi des facteurs neurobiologiques spécifiques qui devront être déterminés dans les études à venir.

Summary

Multiple sclerosis is an inflammatory disease of the central nervous system responsible for demyelinating lesions within white matter in young adults. The activation of autoreactive CD4 + but also CD8 + T cells directed against myelin antigens of the central nervous system is a key phenomenon at the origin of multiple sclerosis lesions. Its etiology is unknown but implies the existence of genetic traits and a triggering by environmental factors such as viral infections. However, no virus or other environmental factor has been directly involved in the triggering of the disease. Functionnal abnormalities on regulation systems of immunity, such as

regulatory T cells, could be partially involved in the triggering of the disease. However, it appears, on the basis of recent findings, that multiple sclerosis is not only a disease of the immune system but also implies specific neurobiological factors which must be determined in studies to come.

Pour la pratique

- La Sclérose en plaques est une maladie inflammatoire du système nerveux central dont l'étiologie reste indéterminée à ce jour.
- Des facteurs génétiques semblent impliqués dans la survenue de la maladie. Les études sur jumeaux monozygotes ont montré une concordance pour la maladie entre les jumeaux de l'ordre de 40 %.
- Les gènes impliqués ne sont pas connus mis à part le gène codant pour une molécule de classe II du complexe majeur d'histocompatibilité DRB1 1501, ce qui concorde avec les données connues sur la physiopathologie de la maladie.
- Des facteurs environnementaux, tels que des virus, pourraient ainsi être impliqués dans le déclenchement de la maladie sur ce terrain génétique prédisposé. Des virus latents neurotropes tels que HHV6 semblent particulièrement intéressants, mais les résultats des études menées à ce jour sur son implication sont discordantes.
- Le mécanisme déclencheur de la maladie reste mal connu. Des phénomènes de mimétismes moléculaire entre des agents viraux et des composants myéliniques pourraient provoquer l'activation de cellules T autoréactives dans le sang des patients. De plus, récemment des défauts de régulation du système immunitaire ont été mis en évidence chez les patients, ce qui est concordant avec cette hypothèse.
- Une autre hypothèse est une atteinte primitive des oligodendrocytes, les cellules myélinisantes du système nerveux central, avec une mise en jeu secondaire du système immunitaire. Cette hypothèse est appuyée par la découverte récente d'une apoptose oligodendrocytaire étendue au moment de la formation des lésions de SEP, sans infiltrat inflammatoire.

Ce qui est nouveau :

- Des anomalies portant sur certains sous groupes de lymphocytes T, les cellules régulatrices CD4+CD25+^{high}, ont été mises en évidence dans la sclérose en plaques indiquant de probables défauts de régulation des cellules autoréactives dans le sang des patients. Le défaut fonctionnel de ces cellules pourrait ainsi expliquer l'émergence de la maladie.
- Alors que l'hypothèse principale expliquant la lésion de SEP est celle d'une activation périphérique de cellules T autoréactives dirigées contre des antigènes de la myéline qui vont ensuite migrer vers le parenchyme cérébral, des travaux neuropathologiques récents ont montré une hétérogénéité des mécanismes lésionnels dans cette maladie. Ces travaux montrent l'implication de l'ensemble des acteurs du système immunitaire dans la SEP (microglie, macrophage, anticorps, complément, lymphocyte T CD8+ et CD4+). De plus, la mise en évidence d'une apoptose précoce des oligodendrocytes au moment de la formation des lésions soulève l'hypothèse de l'implication de facteurs toxiques encore inconnus dans la genèse de la maladie.

Introduction

La Sclérose en Plaques (SEP) est une affection inflammatoire touchant le système nerveux central (SNC), c'est à dire l'encéphale et la moelle épinière, débutant habituellement entre 20 et 40 ans. Il s'agit de la première cause non traumatique de handicap chez l'adulte jeune, ses conséquences socio-économiques sont donc particulièrement importantes. La prévalence de la maladie est d'environ 60 à 200/100 000 dans les zones de haute prévalence dont l'Europe du Nord. Deux grandes formes de SEP sont décrites. La forme rémittente, la plus fréquente puisqu'elle touche environ 80 % des patients, est caractérisée par la survenue de poussées de la maladie, plus ou moins résolutives et évolue dans la majorité des cas vers une forme secondairement progressive. La forme progressive d'emblée, qui touche environ 20 % des malades, est caractérisée par un début souvent insidieux et une aggravation progressive et continue du handicap neurologique.

Les progrès concernant la compréhension de la physiopathologie de la maladie ont été possible notamment grâce à la mise en place d'un modèle animal, l'encéphalomyélite auto-immune expérimentale (EAE) où un animal (rat, souris, marmouset,…) est immunisé en périphérie avec un peptide ou une protéine de la myéline du SNC. La protéine ou le peptide est alors traité par des cellules spécifiques, les cellules présentatrices d'antigène (CPA), qui le présentent à leur surface via leur molécule du CMH de classe II. Ce peptide, qu'on dit apprêté, est alors reconnu par des lymphocytes T CD4+ qui, activés, peuvent franchir la barrière hémato-encéphalique, reconnaître le peptide de l'hôte et exercer leur effet délétère aboutissant à une inflammation et à la destruction de la myéline. Cet effet destructeur est dû aux lymphocytes T CD4+ capables de produire des cytokines de type Th1 (IL2, Interféron γ). Ce modèle animal a ainsi permis de modéliser la lésion de SEP.

Toutefois , en ce qui concerne la maladie humaine, les données ne sont pas aussi claires (voir chapitres spécifiques par B. Trapp et K. Smith) et un certains nombre de questions restent en suspens.

L'étiologie de la SEP reste mal connue, mais la maladie semble se développer sur un terrain génétique prédisposé sous l'influence de facteurs environnementaux présidant au déclenchement et éventuellement à l'entretien de la maladie. La responsabilités de facteurs génétiques est communément admise et de nombreuses études ont ainsi pu mettre en évidence un lien entre la SEP et le gène codant pour la molécule du complexe majeur d'histocompatibilité (CMH) de classe II DRB1-1501 ou DR2 (Yaouanq et al., 1997). Cependant le poids de ce gène dans la responsabilité de la survenue de la maladie est limité (représentant entre 10 à 60% du risque génétique). De nombreux efforts ont été dépensés pour déterminer l'identité des facteurs génétiques prédisposant à la maladie sans que d'autres candidats n'émergent, vraisemblablement parce que la susceptibilité à la maladie est contrôlée par plusieurs gènes ou familles de gènes pour lesquels la responsabilité de chacun dans le déclenchement de la SEP est faible. Les liens entre génétique et SEP seront détaillés dans le chapitre spécifique qui lui est consacré par D. Brassat.

En ce qui concerne la participation de facteurs environnementaux dans l'étiopathogénie de la SEP, leur rôle a été suggéré à la suite d'études sur les migrations de population qui ont montré des différences d'incidence de la maladie en fonction de l'âge de la migration suggérant ainsi un rôle possible d'un facteur environnemental dans l'enfance (Alter et al., 1966; Gale and Martyn, 1995). D'autre part, l'apparition « d'épidémies » de SEP comme dans le cas des îles Féroe au Danemark ou dans les îles Shetland en Ecosse ont fortement suggéré l'implication d'un facteur transmissible environnemental (virus, bactérie, autre,…) comme jouant

un rôle majeur dans le déclenchement de la maladie (Kurtzke, 1995). Cependant, à ce jour, aucune étude n'a pu déterminer avec certitude quel facteur viral ou autre pourrait être responsable du déclenchement de la SEP. Nous détaillerons ces points dans le chapitre suivant.

Mise à part le rôle causal éventuel d'infections virales ou d'autre origine dans la SEP, le débat actuel concerne aussi le lieu et la façon dont s'initie la réaction inflammatoire touchant le système nerveux central, responsable de la lésion aiguë de démyélinisation caractérisant la maladie. En effet, alors que de nombreux arguments plaident pour une initiation périphérique de la maladie (mise en jeu des lymphocytes T CD4+, gène du CMH de classe II, modèle animal, réponse aux immunosuppresseurs,…), des travaux récents suggèrent que la lésion aiguë de SEP pourrait être due à une atteinte primitivement oligodendrocytaire dont le mécanisme reste pour l'instant obscur.

L'objectif de cette revue sera donc de faire le point et de décrire les plus récentes observations concernant l'initiation de la maladie.

Facteurs environnementaux et SEP

L'étude de jumeaux monozygotes dont l'un des deux est atteints de SEP a permis de mettre en évidence les différences liées aux gènes dans l'expression de la maladie. Ainsi la concordance de taux de la maladie est relativement faible puisqu'elle varie entre 20 et 30 % selon les différentes études réalisées (Sadovnick et al., 1993), suggérant ainsi l'importance des facteurs environnementaux dans le déclenchement et/ou l'entretien de la SEP. L'influence des facteurs environnementaux dans le déclenchement de la maladie est soutenu par différents arguments :

- Il existe un gradient nord-sud de répartition de la prévalence de la maladie, avec des prévalences élevées au nord et plus faibles au sud (Haahr et al., 1997).
- L'étude des populations migrantes, en particulier caucasienne, chez qui la prévalence de la SEP est élevée, montre que la prévalence de la maladie est deux fois inférieure dans le pays d'accueil par rapport au pays d'origine (Gale and Martyn, 1995).
- L'étude des migrants en fonction de l'âge de la migration montre que les personnes ayant migré d'une zone de forte prévalence vers une zone de faible prévalence avant l'âge de 15 ans acquièrent le risque du pays d'accueil au contraire des personnes ayant migré à l'âge adulte. Ces données suggèrent l'influence d'un facteur environnemental pendant l'enfance sur la prévalence de la maladie (Alter et al., 1966).

Cette variation de prévalence de la maladie reflète également le niveau économique des pays. La prévalence de la SEP en Asie est globalement faible mais est plus importante au Japon, le pays le plus développé de la région sur le plan économique. De plus, dans ces pays l'incidence de la maladie a augmenté ces dernières années

de façon concomitante au développement socio-économique, ce qui peut être en relation avec l'industrialisation, le mode de vie urbain, la pollution, les changements d'habitudes alimentaires, etc…C'est l'hypothèse « hygiène » expliquant la survenue de la SEP : le système immunitaire étant moins confronté ou confronté plus tardivement aux pathogènes, il aurait une propension à développer des maladies auto-immunes ou allergiques.

Les agents infectieux : déclencheurs de la SEP ?

Les agents infectieux, viraux ou bactériens, apparaissent donc comme des candidats logiques dans le déclenchement de la SEP. De très nombreuses études se sont penchés sur leur responsabilité sans que des éléments de certitude se dégagent nettement. Des études prospectives sur la relation entre les infections virales, en particulier de la sphère ORL, ont montré que les poussées de la maladie étaient souvent précédées d'une infection parfois asymptomatique (Buljevac et al., 2002). Cependant, l'absence de corrélation avec une activité IRM de la maladie et l'absence de groupe « contrôle » rend l'interprétation de ces études non univoque. De la même façon, les études épidémiologiques rendant compte de véritables « épidémies » de SEP (îles Féroe notamment) ne sont pas exemptes de critiques méthodologiques rendant leur interprétation difficile.

D'autres arguments plaident pour une relation étroite entre infection et SEP. Certains modèles animaux de SEP, en particulier chez la souris, peuvent être induit par un virus spécifique, le virus de Theiler. Par ailleurs certaines maladies démyélinisantes chez l'homme sont induites par des virus comme le JC virus dans le cadre des infections opportunistes liées au SIDA (leucoencéphalites multifocales progressives, LEMP), ou dans le cadre des panencéphalites sclérosantes subaiguës liées au virus de la rougeole. Enfin, l'étude de souris transgéniques pour un récepteur de cellule T

(TCR) dirigé contre un antigène de la myéline du SNC, la protéine basique de myéline, MBP, montre que ces souris développent spontanément une maladie autoimmune démyélinisante lorsqu'elles sont élevées dans un contexte non stérile contrairement aux mêmes souris élevés dans une animalerie confinée (Goverman et al., 1993).

De nombreux virus ont été associés avec le développement de la SEP, en particulier ceux responsables des maladies infantiles (varicelle, oreillons, rougeole,…) mais ont été par la suite innocentés. Cependant, plus récemment l'intérêt s'est porté sur des virus ubiquitaires responsables d'infection latente comme les herpès virus et en particulier le HHV6 ou les rétrovirus. En effet, les herpès virus sont neurotropes, ubiquitaires et responsables d'une infection latente avec des réactivations. HHV6 et l'EBV (Epstein-Barr Virus) en sont les deux candidats majeurs. HHV6 a été récemment lié à la SEP depuis qu'il a été détecté dans les oligodendrocytes ainsi que dans le LCR des patients de façon plus fréquente que chez les témoins, en particulier pour le sous type A, le plus neurotrope. Cependant, les résultats de ces études sont controversés et la plus grande incidence d'infection par HHV6 chez les patients SEP demande à être confirmée (Clark, 2004).

Il faut noter aussi que des études randomisées utilisant un traitement antiviral ont été réalisées dans la SEP avec des résultats peu probants mais néanmoins intéressants. En effet, dans une étude contre placebo menée chez 60 patients pendant 2 ans, l'aciclovir a permis une réduction de 30 % environ du nombre de poussés cliniques sans que cela soit statistiquement significatif (Lycke et al., 1996). En revanche, l'utilisation du valaciclovir a permis de mettre en évidence une réduction significative de l'activité IRM de la maladie chez les patients qui avaient au moins une lésion

active sur l'IRM à l'entrée dans l'étude (Bech et al., 2002). Il est à noter que les doses utilisées dans ces études étaient insuffisantes pour inhiber HHV6 .

Les hypothèses du mécanisme lésionnel

Une des questions majeures concernant la SEP est le lieu où s'initie la maladie. En effet, basé sur les principes immunologiques, les modèles animaux et les réponses thérapeutiques aux drogues immunosuppressives, l'hypothèse qui prévalait était celle d'une initiation périphérique de la maladie. Cependant, récemment, sur des constatations neuropathologiques, une autre hypothèse a émergé : celle de l'atteinte primitive du SNC avec une mise en jeu secondaire du système immunitaire.

L'hypothèse périphérique

- **La séquence des évènements**

L'hypothèse ayant longtemps prévalu sur l'initiation de la SEP repose sur l'existence de cellules auto-réactives dirigées contre des épitopes myéliniques dans le sang des patients. A la faveur d'une activation ces cellules sont alors capables de franchir la barrière hémato-encéphalique et de pénétrer le parenchyme cérébral. Sous l'impulsion d'une nouvelle présentation d'antigène à l'intérieur du SNC, ces cellules vont proliférer et provoquer une cascade inflammatoire entraînant des lésions du SNC auparavant normal. Cette hypothèse sur le mécanisme lésionnel est particulièrement étudiée au travers d'un modèle animal, l'EAE, où on immunise un rat, une souris voire un primate, contre un antigène de la myéline par des injections périphériques associées à un adjuvant pro-immunisant, ou par transfert direct de cellules auto-réactives contre des antigènes de la myéline.

Les études initiales sur les cellules auto-réactives se sont essentiellement focalisées sur les lymphocytes T CD4+, cellules qui permettaient par ailleurs de transférer la maladie d'un animal à l'autre par simple transfert cellulaire. Ces cellules paraissaient

d'autant plus intéressantes qu'un lien entre la SEP et les molécules du CMH de classe II avait été retrouvé, ces molécules étant responsables de la présentation d'antigène à ces lymphocytes T CD4+. De nombreux travaux ont ainsi montré une augmentation de la fréquence des cellules auto-réactives et/ou l'expression par ces cellules de certains marqueurs d'activation ou de mémoire chez les patients par comparaison aux témoins sains. Enfin l'étude de l'ensemble des peptides reconnus par ces lymphocytes T indiquaient que certain d'entre eux avaient des séquences proches de peptides viraux et auraient ainsi pu être « confondu » avec eux. C'est la théorie du mimétisme moléculaire que nous verrons plus loin.

Plus récemment l'intérêt s'est aussi porté sur d'autres composantes du système immunitaire comme étant possiblement impliqué dans la SEP. Les lymphocytes T CD8+ cytotoxiques (CTL) ont ainsi la capacité de détruire leur cible en larguant certaines molécules telles que les perforines et granzymes. Ces cellules sont beaucoup plus représentées dans lésions aiguës de SEP que les lymphocytes T CD4+. Elles portent elles aussi des marqueurs d'activation indiquant leur capacité à franchir la BHE (Battistini et al., 2003). L'étude des lymphocytes T CD8+ au sein des lésions de SEP indique qu'il y a une répartition clonale de ces cellules au contraire des lymphocytes T CD4+, suggérant la participation des lymphocytes T CD8+ dans une réponse antigénique restreinte. D'autre part ces mêmes clones sont retrouvés dans le sang des patients et le répertoire lymphocytaire T de ces patients porte la marque de ces altérations prédominant sur les lymphocytes T CD8+ (Babbe et al., 2000; Laplaud et al., 2004). Par ailleurs, la présentation d'antigènes au LT CD8+ se fait via les molécules du CMH de classe I, qui sont justement exprimées par les cellules neurales en cas d'inflammation contrairement aux molécules de classe II du CMH qui ne peuvent être induite que sur certains sous-types cellulaires. Ces

données suggèrent que les lymphocytes T CD8+ pourraient participer à la destruction non seulement oligodendrocytaire mais aussi axono-neuronale.

Les anticorps pourraient aussi participer à la démyélinisation puis qu'ils ont été mis en évidence dans les études neuropathologiques au sein des lésions de SEP. En revanche ils ne peuvent participer à l'initiation de la maladie, ces molécules de haut poids moléculaire ne pouvant franchir la barrière hémato-encéphalique. En se liant avec leur antigènes myéliniques des anticorps spécifiques peuvent alors conduire à une démyélinisation via la reconnaissance de leur fragment Fc par les cellules microgliales et/ou macrophagiques, comme cela a été démontré dans des modèles animaux.

- **Mécanismes lésionnels**

Les principaux mécanismes pouvant expliquer l'activation de cellules T périphériques autoréactives lors de processus infectieux restent hypothétiques. Deux principaux sont décrits : le mimétisme moléculaire, où les antigènes de l'agent infectieux provoquent une réaction croisée avec les antigène s de l'hôte, et la « bystander activation » qui est l'activation non-spécifique des cellules autoréactives lors d'un processus inflammatoire quelconque.

- <u>Mimétisme moléculaire</u>

La notion de mimétisme moléculaire implique la reconnaissance par les cellules T et/ou B de peptides ou de déterminants antigéniques partagés par les agents infectieux et les antigènes du soi. Lors de la sélection thymique, les lymphocytes d'affinité moyenne pour les peptides du soi se retrouvent dans la circulation périphérique. La réactivité croisée de ces cellules potentiellement auto-réactives avec des antigènes « étrangers » peut conduire, au cours d'une infection, à leur activation, permettant alors la traversée de la barrière hémato-encéphalique et une

migration dans le système nerveux central où la reconnaissance d'antigène du soi pourrait alors conduire au relargage de cytokines pro-inflammatoires, à des lésions tissulaires et à une maladie auto-immunes de type SEP. De nombreuses études se sont intéressées aux analogies entre les peptides du soi d'origine myéliniques et les peptides de différents virus et/ou bactéries. Si l'existence de peptides étrangers strictement identiques est un phénomène rare, de nombreux peptides viraux ou bactériens sont suffisamment proches de certains peptides du soi pour interagir avec le TCR. De plus des travaux récents ont montré qu'un peptide viral de séquence différente d'un peptide du soi pouvait pourtant lui ressembler suffisamment pour provoquer une activation T, car son aspect structural lors de la présentation par les molécules du MHC et sa répartition de charges électriques pouvaient être similaire (Lang et al., 2002). Ces nouveaux concepts font envisager l'existence de réactivités croisées comme un phénomène fréquent, et une dérégulation de ce phénomène pourrait donc être impliquée dans la genèse de la SEP.

- By-stander activation

Les mécanismes conduisant à l'activation de cellules auto-réactives potentiellement responsables de la SEP peuvent être dûs à des phénomènes d'activation non-spécifique liés à l'inflammation ou à la découverte d'antigènes cryptiques du soi, habituellement non présenté par les molécules du CMH, conduisant alors à l'activation de cellules T spécifiques. Dans le premier phénomène, l'inflammation au cours d'une infection conduit à la libération de certains facteurs tels que des cytokines qui pourraient participer à l'activation de cellules spécifiques. En revanche ces molécules ne pourraient pas engendrer la maladie auto-immune qui reste spécifique d'antigène. Ce type de phénomène pourrait exister dans la genèse de poussées de la maladie par exemple. Une autre possibilité est l'existence de facteurs

viraux ou bactériens, les superantigènes, qui ont la capacité d'activer des lymphocytes T partageant une même partie variable de leur TCR (famille Vβ). Si au sein de la famille Vβ activée existent des lymphocytes T autoréactifs, ils peuvent être à leur tour activés, passer la BHE et engendrer une atteinte tissulaire lors d'une reconnaissance antigénique spécifique. Ce phénomène a été décrit dans un modèle animal de SEP, mais n'a pas été démontré chez l'homme (Brocke et al., 1993).

Un autre mécanisme de « bystander activation » est celui décrit dans le modèle animal de SEP chez la souris impliquant l'infection par le virus de Theiler. Dans ce modèle, les lymphocytes T dirigés contre les cellules infectées pénètrent dans le parenchyme cérébral et détruisent leur cible, provoquant la destruction de tissu sain et le relargage d'antigène du soi. La présentation des antigènes du soi concomitante aux facteurs viraux adjuvants pourrait provoquer l'activation de cellules spécifiques et engendrer une maladie auto-immune. En effet le processing d'antigène par les cellules présentatrice est différent dans ce contexte inflammatoire aboutissant à la présentation d'antigènes habituellement non présentés (antigènes cryptiques). La réaction immunitaire va alors « glisser » depuis la reconnaissance des antigènes viraux vers la reconnaissance des auto-antigènes, c'est le phénomène du spreading.

- <u>Cellules régulatrices défectives</u>

Tous ces phénomènes décrits auparavant, en particulier l'existence de cellules autoréactives, peuvent exister chez l'homme sain sans engendrer de maladie auto-immune car il existe des phénomènes de régulation induisant une tolérance. Un des mécanismes clé de cette tolérance naturelle est l'existence de cellules régulatrices (lymphocytes T CD4+CD25+^{high}). Une des possibilité quant à l'émergence de maladies auto-immunes est donc un défaut dans les capacité régulatrices de ces cellules chez les patients. Dans un récent travail, l'équipe de David Hafler a ainsi pu

démontrer l'existence d'une diminution de l'activité de ces cellules chez des patients SEP comparé aux témoins (Viglietta et al., 2004). Depuis, d'autres équipes ont pu retrouver des anomalies des cellules régulatrices dans la SEP.

Hypothèse de l'atteinte primitive du SNC

Cette hypothèse implique que les évènements initiateurs de la maladie se déroulent dans le système nerveux central. Comme dans la maladie induite par le virus de Theiler chez la souris, une infection persistante dans le SNC pourrait provoquer un relargage d'antigène induisant une réponse (auto)-immune. Il est en fait peu probable qu'une infection directe des cellules neurales chez l'homme soit responsable de la maladie étant donné l'efficacité clinique et radiologique des traitements immunosuppresseurs ou des anticorps monoclonaux. Toutefois, comme expliqué précédemment, il est possible que sous l'impulsion d'une infection latente, lors des phases de réactivation et au moment de l'inflammation, la découverte d'antigènes cryptiques ou des phénomènes de mimétismes moléculaires entraîne une dispersion de la réponse immunitaire et amène à une maladie auto-immune.

Dans un travail récent, Barnett et Prineas ont observé des modifications de l'aspect des oligodendrocytes évocateur d'une apoptose indépendante des caspases et pouvant correspondre au phénomène initial lors de la création de la plaques aiguë de SEP (Barnett and Prineas, 2004). Dans cette hypothèse, l'atteinte est d'abord oligodendrocytaire avec une mise en jeu de l'immunité naturelle (via la microglie) et secondairement une réponse immunitaire adaptative mettant en jeu les lymphocytes T. La raison de l'apoptose oligodendrocytaire n'est pas déterminée par leur travail mais pourrait correspondre à ce qui est exposé plus haut : une atteinte toxique, virale, etc…L'existence d'une atteinte primitivement oligodendrocytaire avait déjà été

décrite par Lucchinetti et coll. dans un travail neuropathologique où l'étude de cas autopsiques et de biopsies a permis de détailler l'hétérogénéité de l'atteinte lésionnelle au sein de la substance blanche, remettant en cause par là même l'idée simple, voire simpliste, d'une maladie seulement dépendante des lymphocytes T CD4+. Dans ce travail, fondamental pour la compréhension des processus physiopathologique sous-tendant la maladie, quatre patterns différents d'atteinte de la substance blanche sont décrits au sein des lésions actives (Lucchinetti et al., 2000). Si dans tous ces types d'atteinte l'inflammation dépendante des lymphocytes T est présente, d'autres facteurs tels qu'une activation macrophagique ou microgliale, le dépôt d'immunoglobuline, une oligodendriogliopathie distale ou une dégénérescence oligodendrocytaire primitive peuvent aussi être présente rendant compte de l'hétérogénéité des mécanismes physiopathologiques responsables des lésions d'un patient à l'autre.

En conclusion, ces travaux récents remettent en cause l'idée d'un *primum movens* périphérique initiateur de la SEP et soulignent la complexité majeure des phénomènes physiopathologiques impliqués dans la maladie. Néanmoins, l'implication des lymphocytes T dans l'initiation et la création des lésions aiguës de SEP semble un phénomène central, tel que cela a pu être démontré par des thérapeutiques les ciblant spécifiquement.

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Immunologie de la Sclérose en Plaques.

Immunologie du Système Nerveux Central.

Le SNC est un organe dit *immunologiquement privilégié*. En effet, il existe différents phénomènes régulateurs de l'(auto)immunité au sein du SNC.

L'isolement anatomique du SNC.

Le SNC est considéré comme un organe à part sur le plan immunitaire. En effet on a longtemps considéré, notamment à la suite d'allogreffe de cellules dans le SNC n'entraînant pas de rejet, qu'il existe un « privilège » immunitaire et que ces phénomènes de tolérance « naturelle » étaint dû à différents facteurs anatomiques particuliers au SNC. Cette notion doit cependant être révisée à la lumière des différentes expérimentations animales et humaines d'allogreffes de tissu neural où des phénomènes de rejet ont été décrits (voir (Winkler et al., 2005) pour revue).

Le drainage lymphatique du SNC (Figure 1).

Au contraire des organes périphériques, le liquide intercellulaire issu des cellules cérébrales n'est pas drainé vers les ganglions lymphatiques par un système individualisé. Il n'existe pas de circuit lymphatique dans le SNC et donc *a priori* une absence de drainage des antigènes cérébraux vers les ganglions périphériques.

En fait cette notion a été battue en brèche par les travaux de différents auteurs mettant en évidence l'existence d'un drainage du liquide intercellulaire cérébral et donc des antigènes cérébraux, vers les ganglions (Cserr et al., 1992; Cserr and Knopf, 1992). Le passage, même de grosses molécules comme l'albumine, a pu être démontré et ce de façon importante puisque jusqu'à 40 % de l'albumine injectée dans le cerveau de différents animaux a été retrouvée dans les ganglions cervicaux profonds en quelques heures. Environ 50% du volume total du LCR va ainsi transiter

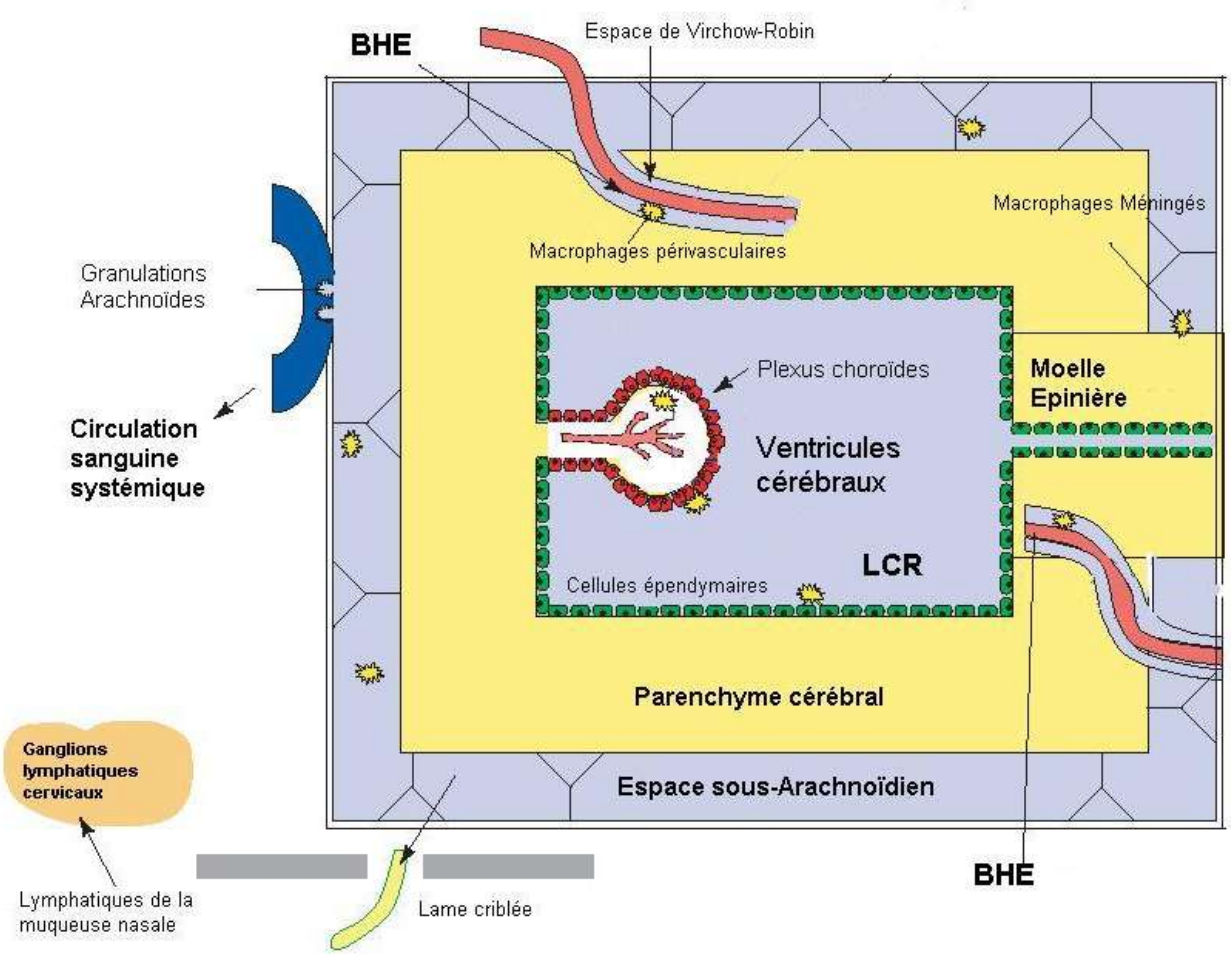

Figure 1 : voies afférentes et efférentes immunologiques du SNC, d'après Engelhardt et al, 2005. Les différents compartiments du SNC, plexus choroïdes, ventricules cérébraux, espaces sous-arachnoïdiens, parenchyme cérébral et médullaire sont montrés, avec leurs connections possibles avec le système immunitaire périphérique (lymphatiques de la muqueuse nasale). Les connections efférentes du système immunitaire vers le SNC prennent différentes formes incluant l'entrée de lymphocytes T circulant à travers la barrière hémato-encéphalique (BHE) vers le parenchyme cérébral ou médullaire ou à travers la barrière des plexus choroïdes vers les ventricules cérébraux. Les cellules présentatrices d'antigènes du SNC sont représentées en jaune et sont localisées au niveau des plexus choroïdes, des espaces sous-arachnoïdiens (macrophages méningés) et des espaces de Virchow-Robin (macrophages péri-vasculaires). Le liquide interstitiel du SNC se draine vers les espaces péri-vasculaires autour des artères à destinée cérébrale ainsi que le long de l'artère et du nerf olfactif (et des autres nerfs crâniens) vers la lame criblée. A partir de là il entre dans les canaux arachnoïdiens qui se drainent dans les lymphatiques de la muqueuse nasale, rejoignant ensuite les ganglions cervicaux.

via les ganglions cervicaux (Boulton et al., 1998a; Boulton et al., 1998b). De plus, l'injection intracérébrale de telles protéines induit une réponse immunologique au sein des ganglions, en particulier par la formation d'anticorps spécifiques, démontrant la fonctionnalité d'un réseau de drainage lymphatique même si celui-ci n'est pas conventionnel (voir Figure 1).

La Barrière Hémato-Encéphalique

Sur le plan anatomique, il existe une barrière entre le sang et le SNC, la barrière hémato-encéphalique (BHE) composée de cellules endothéliales spécialisées. Elles forment des capillaires non fenêtrés parce qu'elles sont reliées par des jonctions serrées (*zona ocludens*) qui limitent le trafic macromoléculaire entre le sang et le parenchyme cérébral. L'existence de cette barrière a été mise en évidence par les travaux de Ehrlich à la fin du 19ème siècle. L'injection intra-veineuse de colorant comme le Bleu Evans, de haut poids moléculaire, ne pénétrait pas dans le SNC alors qu'il marquait les autres organes, prouvant l'existence d'une barrière entre le sang et le cerveau. Cette barrière possède des caractéristiques spécifiques : elle est formée d'une seule couche de cellules endothéliale entourée d'une lame basale continue sur laquelle s'appuient les pieds astrocytaires jointifs issus des astrocytes périvasculaires voisins (réalisant la *glia limitans*). Les cellules endothéliales formant les capillaires cérébraux disposent de particularités : outre l'existence de jonctions serrées (voir Figure 2), on note la présence d'un transport vésiculaire important, une forte activité mitochondriale et l'absence de canaux transendothéliaux. Ces particularités rendent compte de l'absence de passage des molécules hydrosolubles et/ou des molécules de fort poids moléculaire depuis le sang vers le parenchyme cérébral, si ce n'est par un transport actif. D'autre part les cellules endothéliales n'expriment pas ou peu les

molécules du CMH de classe II ni les intégrines, rendant ainsi difficile le contact entre les cellules immunocompétentes et la BHE.

Régulation moléculaire de la tolérance naturelle du SNC

Il existe au sein du SNC un ensemble de facteurs solubles ou présent sur la membrane des cellules participant à la régulation immunitaire. Aucune des cellules du SNC, qu'elles soient gliales ou neuronales, n'expriment de façon constitutive les molécules du CMH de classe II, rendant impossible dans le cerveau sain la reconnaissance antigénique par les lymphocytes T.

De façon plus spécifique, les cellules microgliales, qui sont considérées comme de véritables macrophages cérébraux et qui à l'état activé ont vraisemblablement une activité de présentation d'antigène, expriment de façon peu importante les molécules de classe II du CMH à l'état quiescent aussi bien que les autres molécules immunologiques (molécules de costimulation, récepteur du fragment Fc, CD45, récepteurs du complément, des chimiokines,…) (Carson, 2002). L'absence de facteur de costimulation entraîne ainsi l'apoptose lymphocytaire lors de la présentation d'antigène. Des facteurs solubles comme TGFβ et IL-10 sécrétés par la microglie, participent à « l'immunodépression » naturelle du SNC en diminuant l'expression des molécules de classe II comme la prolifération des lymphocytes T et B

Il est à noter que les cellules du SNC expriment certains marqueurs comme Fas Ligand capable d'induire l'apoptose des lymphocytes T pénétrant dans le SNC et participent ainsi à la régulation de la réaction inflammatoire.

Caractérisation du passage des LT à travers la BHE (Figure 2).

En condition inflammatoire, les cellules endothéliales de la BHE sont capables d'exprimer les molécules de classe II du CMH ainsi que des molécules d'adhésion cellulaires comme VCAM (voir Figure 2). Les lymphocytes T activés sont alors capables de diapédèse à travers la BHE par adhésion grâce à leurs intégrines, en particulier les α4-intégrines comme VLA4, capable de se lier à VCAM. Lorsqu'il est activé, n'importe quel lymphocyte T exprimant VLA4 peut lier ses molécules d'adhésion à l'endothélium de la BHE et le franchir. Ce phénomène n'ést pas spécifique, et le blocage de l'interaction VLA4/VCAM prévient les poussée d'EAE dans le modèle chronique de la maladie (Yednock et al., 1992). L'étude neuropathologique des lésions aiguës de SEP permet de mettre en évidence la sur-expression de VLA4 sur les lymphocytes T présents autour des capillaires cérébraux, en parallèle avec les molécules du CMH de classe II et VCAM sur les cellules endothéliales. Chez l'homme, un anticorps monoclonal dirigé contre VLA4 a été réalisé et des études de phase II et phase III ont montré une efficacité très importante de ce traitement dans la prévention des poussées de la maladie avec une réduction de l'ordre de 90 % de l'apparition de nouvelles lésions sur l'IRM, et une atténuation de la rupture de la BHE (Miller et al., 2003).

Une fois la première étape du franchissement des cellules endothéliales réalisée les lymphocytes doivent traverser la matrice extra-cellulaire, principalement composée de collagène de type IV. Les protéinases de matrice (MMP pour Matrix Metalloproteases) jouent un rôle clé permettant aux lymphocytes T d'accéder à la substance blanche. Les MMP-2 et MMP-9 semblent avoir un rôle particulièrement important dans la formation des lésions de la SEP, et sont retrouvées autour des cellules endothéliales au niveau des lésions. Ces enzymes sont impliquées dans la

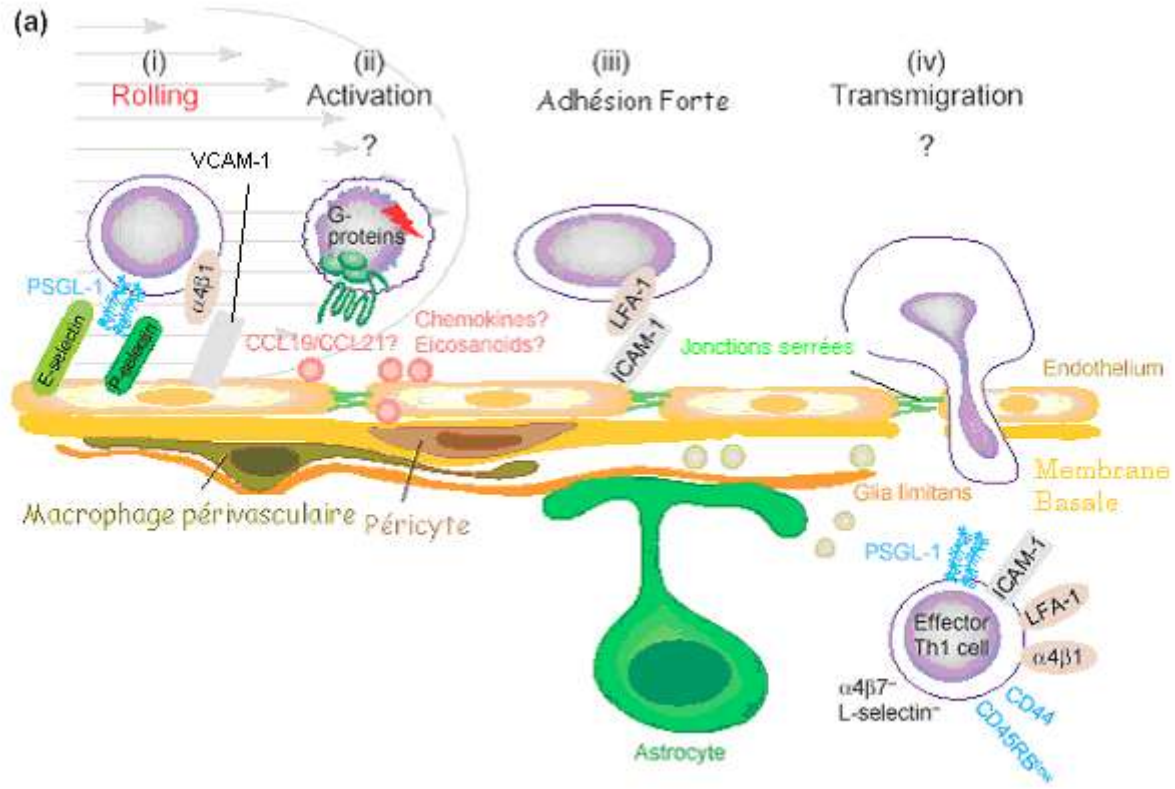

Figure 2 : Le franchissement de la BHE, d'après Engelhardt et al, 2005. La migration leucocytaire est un processus en plusieurs étapes. Un contact initial transitoire des leucocytes circulants avec l'endothélium vasculaire, généralement médié par l'interaction de molécules d'adhésion (sélectines avec leur ligand), ralentit le flux leucocytaire. Les lymphocytes peuvent alors " rouler " grâce à l'interaction des α4-intégrines avec leur ligand endothélial VCAM-1. Après ces contacts, les leucocytes roulent le long de la surface endothéliale avec une vélocité diminuée permettant une augmentation de leur sensibilité aux facteurs chimiotactiques (chimiokines) qui sont présents à la surface endothéliale. La liaison des chimiokines avec leur récepteur entraîne une activation lymphocytaire et l'expression de molécules d'adhésion de la famille des intégrines (LFA1), augmentant leur affinité et/ou leur avidité. Ces seules intégrines activées vont permettre une adhésions forte à la surface endothéliale via leur interaction avec les récepteurs situé sur les cellules endothéliales (ICAM-1). Ce processus conduit à la diapédèse leucocytaire à travers les cellules endothéliales ou entre elles. La diapédèse implique les intégrines et des molécules des jonctions serrées.

dégradation de la matrice extracellulaire et dans la protéolyse des composants myélinique. Elles ont une activité de convertase de TNFα, permettant le relargage de cette cytokine sous forme soluble à partir de la forme membranaire. Les MMP sont inhibées par des protéines spécifiques, les inhibiteurs tissulaires des MMP ou TIMP (pour Tissue Inhibitors of MetalloProteinases), permettant le blocage de la libération de TNFα et la régulation de l'expression des molécules d'adhésion. MMP-2 et MMP-9 sont retrouvées dans le LCR des patients SEP, ainsi qu'au niveau des lésions cérébrales, en particulier sur les cellules endothéliales, les péricytes, les macrophages péri-vasculaires et les astrocytes (Gijbels et al., 1992). Dans l'EAE la présence de MMP-9 est associée à la rupture de la membrane basale, et son rôle semble donc crucial dans la formation de la lésion de la SEP, en permettant par la suite aux cellules immunocompétentes issues de la circulation générale de disséminer dans le SNC.

Modélisation de la lésion aiguë de SEP

Lorsque les cellules sont parvenues dans la substance blanche la reconnaissance de l'antigène provoque la libération de facteurs cytokiniques en particulier pro-inflammatoire comme l'INFγ, le TNFα ou la lymphotoxine α. Ces molécules agissent sur la microglie et les macrophages périvasculaires provoquant leur activation et la libération d'oxyde nitrique (NO). Le NO semble être un acteur majeur de la réaction inflammatoire dans les maladies auto-immunes. Il s'agit d'un radical libre qui semble impliqué dans la mort des oligodendrocytes induite par la microglie (Merrill et al., 1993). La surexpression de l'enzyme iNOS a pu être mise en évidence au sein des lésions de SEP et est induite par le TNFα et l'INFγ dans les cellules microgliales, les astrocytes et les macrophages infiltrants. Ainsi l'effet combiné du NO, du

complément (car il y a rupture de la BHE et apports de facteurs humoraux), du TNFα et d'autres molécules inflammatoires provoque des lésions de la gaine de myéline et de l'oligodendrocyte. Les macrophages vont secondairement phagocyter ces larges parties de gaine de myéline abîmée.

L'activation microgliale et macrophagique provoque la libération de chimiokines, en particulier RANTES, IP-10 et IL-8, permettant le recrutement d'autres cellules comme les lymphocytes T CD8+, les monocytes, les lymphocytes B et les cellules mastocytaires à partir de la circulation générale, orchestrant ainsi la formation de la lésion inflammatoire. Les chimiokines sont des petites molécules impliquées dans le recrutement des cellules sur les sites d'inflammation et sont peu exprimées dans le cerveau sain. Elles ont aussi un rôle dans le développement cérébral normal (pour revue voir (Cartier et al., 2005)). Les études sur autopsies de cerveaux de patients atteints de SEP ont montré la sur-expression de différentes chimiokines (MCP-1, -2, -3, RANTES, IP-10, MIP-1α) sur les différentes cellules inflammatoires et dans la matrice extra-cellulaire. Les récepteurs étaient principalement retrouvés sur les macrophages et la microglie activée (McManus et al., 1998; Simpson et al., 2000; Van Der Voorn et al., 1999).

Le relargage de protéases par les cellules inflammatoires (mastocytes, lymphocytes T ou monocytes) induit alors la rupture de la BHE et la formation d'un œdème et, tel un véritable cercle vicieux, participe à l'entretien de la réaction inflammatoire. A ce stade très précoce, les lésions axonales, oligodendrocytaires et de la gaine de myéline sont déjà présentes. Au cours de ces étapes précédemment décrites, il semble que ce soient les lymphocytes T CD4+ qui initient et conduisent le processus alors que leur présence est secondaire dans la phase effectrice de la maladie (voir le chapitre Discussion). De nombreux processus peuvent conduire à la destruction de

la gaine de myéline et à la transection axonale, notamment l'implication des radicaux libres oxygéné, du TNFα, de la LTα, du dépôt de complément, les anticorps, la phagocytose par les macrophages, la lyse directe par les lymphocytes T cytotoxiques, la sécrétion de protéases ou l'apoptose oligodendrocytaire. De plus le glutamate, potentiellement mal dégradé ou produit en excès par les astrocytes au sein des lésions, peut entraîner une lésion oligodendrocytaire par excitotoxicité ainsi qu'un dysfonctionnement neuronal (Pitt et al., 2000).

L'ensemble des processus décrits ci-dessus est résumé dans la figure 3, tirée de (Sospedra and Martin, 2005).

Cependant ce schéma, qui reste simpliste, ne fait pas état des différences neuropathologiques retrouvées au sein des lésions de SEP. Ainsi la composition cellulaire et les molécules impliquées peuvent être différentes d'un patient à l'autre et réunies en 4 profils (Lucchinetti et al., 2000). Ceux-ci peuvent différer d'un patient à l'autre mais sont identiques chez le même patient dans différentes aires du SNC. Ces profils prennent en compte la présence et la quantité de cellules immunitaires, de complément et d'anticorps, ainsi que la perte myélinique et l'apoptose oligodendrocytaire.

Le profil 1 correspond à une prépondérance de lymphocytes T et de macrophages avec comme molécules effectrices candidates le TNFα, les radicaux libres et l'INFγ. Le profil 2 correspond à la prédominance d'anticorps et de complément. Des anticorps anti-MOG et anti-MBP sont retrouvés au sein des lésions. Le mécanisme de destruction de la myéline semble médié par l'immunité humorale. Dans le profil 3 les lésions sont caractérisées par une perte de myéline et une atteinte oligodendrocytaire. Un mécanisme vasculaire pourrait être suspecté. Dans le profil 4, il existe un atteinte dégénérative oligodendrocytaire. Ce profil est le moins fréquent et

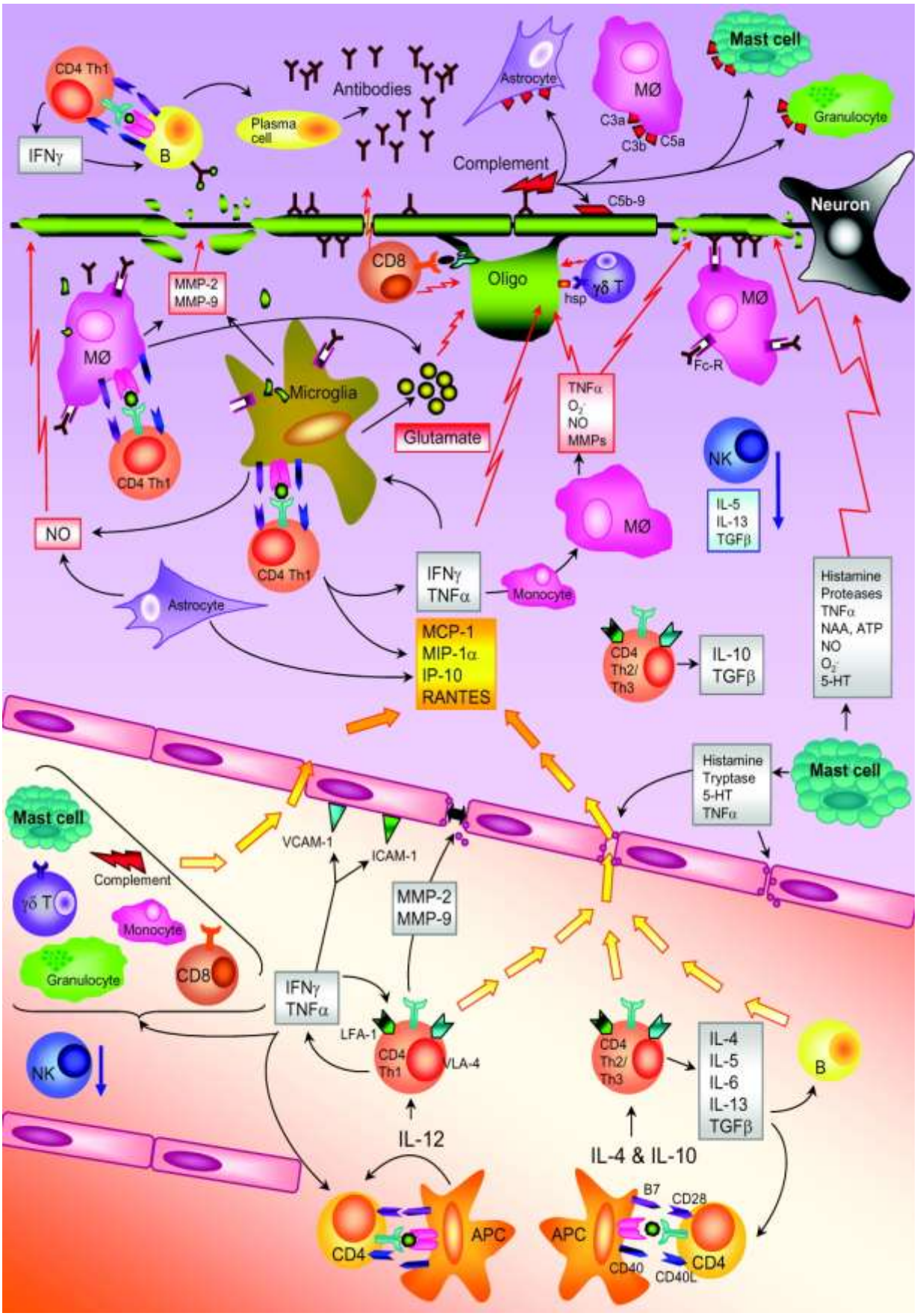

Figure 3 : Modélisation de lésion de SEP, tiré de Sospedra et al, 2005.

surtout présent dans les formes primitivement progressives de SEP.

Objectifs

Compte-tenu des éléments que nous connaissons de la physiopathologie de la maladie, une meilleure caractérisation des lymphocytes T circulants dans la SEP pourrait être utile et servir par exemple à évaluer la sévérité de la maladie, prévoir son évolution ou trouver de nouvelles pistes thérapeutiques.

Une façon de caractériser ces lymphocytes T possiblement impliqués dans la maladie est d'étudier leur répertoire Vβ à travers l'analyse de la région CDR3, qui la plus impliquée dans la reconnaissance antigénique. Nous avions à notre disposition au laboratoire une technologie d'analyse quantitative et qualitative du répertoire T que nous avons donc appliqués aux patients atteints de SEP (voir article 2), puis nous avons essayé de corréler les anomalies observées avec l'évolution clinique et radiologique des patients (voir article 3).

Article 2 : Blood T cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution.

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Blood T cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution.

Running title : TCRBV transcriptome alterations in multiple sclerosis

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Abstract

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system associated with T-cells autoreactive for myelin components. In this study we analyzed the T cell receptor (TCR) usage of the Vβ chain transcriptome in the blood of MS patients at various stages of the disease using a global and quantitative comparison of the complementary determining region 3 length distribution (CDR3-LD) of transcripts of the 26 Vβ genes. We investigated 35 patients; 12 with a high risk of MS, 10 with clinically definite MS, 13 with a relapsing-remitting worsening and active MS and 13 healthy individuals. Cells bearing the TCR transcripts with altered CDR3-LD were sorted and studied for CD4 or CD8 phenotype, cytokine transcript accumulation, and response to human Myelin Basic Protein (hMBP). We show that patients from all the groups have a significantly skewed blood T cell repertoire. Vβ transcriptome patterns were more altered in patients from the clinically definite MS group and the worsening and active MS group than in the high risk group. The T cells sorted from Vβ families with altered CDR3-LD concerned both CD4 and CD8 T cells with a more pronounced skewing in the CD8 compartment. These cells displayed a significantly increased level of IFNγ, IL2 and TNFα transcripts compared to their counterparts from the healthy individual group. Furthermore, using IFNγ Elispot assays, T cells from four out of seven altered Vβ families tested from MS patients responded to hMBP whereas no response was observed with human albumin or with altered Vβ families from healthy individuals. Our data support the concept of an early autoimmune component in the disease and emphasize the possible involvement of CD8 positive T cells in MS.

Word count for Abstract : 279

List of abreviations

AE C : 3-Amino-9-EthylCarbazol

CDMS : Clinically Definite Multiple Sclerosis

CDR3 : Complementary Determining Region 3

$CD4^{+ve}$: CD4 positive

$CD8^{+ve}$: CD8 positive

CNS : Central Nervous System

HPRT : Hypoxanthin-Phosphoryl-Ribosyl-Transferase

HRMS : High Risk of Multiple Sclerosis

HI : Healthy Individuals

LD : Length Distribution

MBP : Myelin Basic Protein

MS : Multiple Sclerosis

PBL : Peripheral Blood Lymphocytes

TCR : T Cell Receptor

WMS : Worsening Multiple Sclerosis

Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS) whose etiology remains unknown. Autoimmunity seems to play a key role in MS pathogenesis, as suggested by the presence of autoreactive T cells for myelin components in the peripheral blood and CNS of patients (Noseworthy et al., 2000). A better characterization of the peripheral blood T lymphocytes in MS would probably serve to evaluate the disease severity, to forecast its evolution and to adopt new therapeutic strategies. One way to characterize the peripheral lymphocytes possibly implicated in MS is to analyze the skewing of the complementary determining region 3 length distribution (CDR3-LD) of the T cell receptor β chain (TCRβ). Such an analysis was initially developed on the basis of a semi-quantitative assessment of CDR3-LD by RT-PCR technology (spectratype/Immunoscope® methods)(Pannetier et al., 1995). Several studies have addressed the question of CDR3-LD in unmanipulated T cells collected from the blood of MS patients and detected skewed β gene usage, a fact that supports the possibility of oligoclonal T cell expansions in the blood (Gran et al., 1998; Lozeron et al., 1998; Matsumoto et al., 2003; Muraro et al., 2002; Musette et al., 1996). In addition, some studies have focused on MBP-specific T lymphocytes, showing a preferential use of certain Vβ genes (Kotzin et al., 1991; Oksenberg et al., 1993; Wucherpfennig et al., 1990). However, these latter studies were performed following *in vitro* T cell clone stimulation with myelin peptides, a procedure that may select and promote the growth of low frequency committed T cells and therefore may not reflect the actual autoreactive T-cell pool size involved.

Recently, we developed an approach which integrates both the analysis of CDR3-LD alterations (Immunoscope®/spectratype) and quantitative real-time PCR-based measurements of Vβ/HPRT transcript ratios, for all the possible CDR3 lengths. The data are displayed as a global " T cell landscape " of the whole β chain transcriptome (Guillet et al., 2002; Guillet et al., 2001). In this study, we used this approach to investigate the Vβ CDR3-LD and more specifically to estimate the magnitude of the skewed T-cell repertoire at various stages of the disease, including at the appearance of the first clinical symptoms. Thirty-five patients suffering from different stages of MS (patients with a high risk of MS (HRMS), patients with clinically definite MS (CDMS) or patients with a relapsing-remitting worsening and active MS (WMS) and 13 healthy individuals (HI) were compared. Our data show that there is a detectable T-cell immune component in the blood of MS patients at all stages of the disease. The blood T cell CDR3-LD alterations concerned the CD4 and CD8 positive ($CD8^{+ve}$) cells but were prominent in the $CD8^{+ve}$ fraction, involving numerous β chain families in CDMS and WMS as compared to patients with HRMS. In addition, the T-cells from MS patients with a selected TCR accumulated more proinflammatory cytokine transcripts than their normal HI counterparts. Furthermore, using an IFNγ Elispot assay, we show that most of the T cells from altered Vβ families of the three groups of patients respond to human Myelin Basic Protein (hMBP) suggesting their possible role in the disease process.

Patients, Material and Methods

Patients

Thirty-five patients divided into two groups were studied (their clinical characteristics are summarized in table 1). *Group I* was composed of 22 patients at the onset of the disease. This group was divided into two subgroups. The *HRMS group* (n=12) was composed of patients presenting a first demyelinating event clinically well defined and confirmed by neurological or ophtalmological examination and the presence of at least 3 Barkhof's criteria on a spinal or cerebral MRI (Barkhof et al., 1997). Patients in this group were referred to as patients with a high risk of MS (2002). The *CDMS group* (n=10) was composed of patients undergoing a second or third relapse, reaching a clinically definite multiple sclerosis according to the Poser's criteria (Poser et al., 1983). Blood from group I patients was collected at the time of a relapse. None of the patients were under immunosuppressive or immunoregulatory drug treatment at the time of or before the study. All patients were interviewed to confirm the absence of infectious illness or other autoimmune diseases and all had a blood cell count within the normal range. *Group II* or the *WMS group* (n=13) was composed of patients with a clinically definite relapsing-remitting MS (according to the Poser's criteria) considered as requiring an immunosuppressive treatment with mitoxantrone (Edan et al., 1997). Patients from the WMS group were referred to as patients with a worsening and active MS. The criteria for mitoxantrone treatment were the loss of at least 1 point on the EDSS score during the previous 6 months and/or the occurrence of several relapses despite treatment compliance and the presence of at least one gadolinium-enhanced T1 lesion according to MRI. None of the patients suffered from other detectable autoimmune, inflammatory or infectious diseases. Results from laboratory tests performed at the time of blood sampling were within the normal

ranges. All WMS group patients had undergone an immunomodulatory treatment that had been stopped at least one month before testing. HLA-DR typing was performed for 32 patients. HLA class I typing was available for 14 patients.

Healthy Individuals. Blood from thirteen healthy individuals (HI) (Mean age: 31.8, 8 females, 5 males), who had been previously interviewed to rule out autoimmune or inflammatory disease, was taken for comparison. All MS patients and HI gave their informed consent to this study according to French legislation.

Blood harvesting, RNA extraction and cDNA synthesis.

One hundred ml of blood were collected by venopuncture. Peripheral Blood Lymphocytes (PBL) were recovered after a ficoll gradient (Eurobio, Les Ulis, France). After washing, 2.10^7 cells were frozen in Trizol® reagent (Invitrogen™, Life technologies, California) for RNA extraction according to the manufacturer's instructions. The rest of the cells were frozen at –80°C in AB serum containing 7.5 % DMSO. One day later, cells were transferred to a liquid nitrogen tank. The RNA concentration for each sample was determined by optical density measurement and a quality control on a 1% agarose gel was performed. Two μg of RNA were reverse transcribed using an Invitrogen cDNA synthesis kit (Boeringher Mannheim, Indianapolis, IN) and diluted to a final volume of 100 μl.

TCR repertoire analysis.

cDNA was amplified by PCR using a Cβ primer and one of the 26 Vβ specific primers. The amplifications were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA) as previously described (Gagne et al., 2000). Analysis of CDR3-LD was performed using Immunoscope® software (Brouard et al.,

1999; Douillard et al., 1996; Pannetier et al., 1995). The percentage of CDR3-LD alteration for each Vβ family and a global percentage of CDR3-LD alteration for each individual or each group was obtained as described in (Gorochov et al., 1998). Briefly, the percentage of alteration was defined as the difference between the frequency of each CDR3 length in the distribution profile of the Vβ family studied and the control distribution, calculated from the 13 age and sex-matched healthy individuals. The global CDR3-LD alteration is represented as a topview TcLandscape® (see below) enabling an easy appraisal of the "qualitative" measurement of the CDR3-LD bias. Only CDR3 lengths with an alteration above 25% were taken into account. The level of Vβ RNA was measured by real-time quantitative PCR and expressed as a ratio of a non or minimally regulated gene, the hypoxanthin-phosphoryl-ribosyl transferase gene, HPRT, in order to normalize the values, and the primers used were especially designed for quantitative PCR as previously described (Gagne et al., 2000). The data were displayed as a tridimentional TcLandscape® (Guillet et al., 2001; Sebille et al., 2001). Percentages of CDR3-LD alterations are represented as a color code, from deep blue (-30%) to dark red (+30%). The X-axis displays the 26 human Vβ families, the Y-axis gives the Vβ/HPRT ratios and the Z-axis gives the CDR3 lengths. The color code is the same for the tridimensionnal TcLandscape® and the corresponding topview.

Cytokine transcript quantification.

Cytokine transcript measurement was performed on RNA from sorted Vβ families. The cells were sorted using phyco-erythrin-coupled Vβ monoclonal antibodies (Immunotech, Marseille, France) on a FACSvantage (Becton-Dickinson, Mountain View, CA). The purity of sorted cells was above 95 %. RNA was extracted as

described above and cDNA obtained using a Boehringer SMART kit (Boehringer, IN) according to the manufacturer's recommendations. Briefly, RNA samples were mixed with cDNA synthesis primer and SMART II oligonucleotide, incubated at 72°C for 2 minutes and chilled on ice. First-strand buffer, DTT, dNTP and PowerScript Reverse Transcriptase were then added and the tubes incubated at 42°C for one hour, before being put on ice. A non-specific PCR was then performed, allowing a linear amplification. Pure water, Advantage 2 PCR buffer, dNTP, PCR primers and advantage polymerase Mix were added to the first strand cDNA obtained previously. The tubes were then placed in a Perkin-Elmer 9600 automate and thermal cycling performed using the following program : 1 min at 95°C, n cycles with 5 sec at 95°C, 5 sec at 65°C, 6 min at 68°C (the number of cycles was determined by the quantity of RNA as assessed by optical density measurement). Real-time quantitative PCR was subsequently performed using IL2, IFNγ, TNFα, IL10, IL13 and IL2-Rα (CD25) chain primers (IL2: 5'-AAACACAGCTACAACTGGAGCA-3' and 3'-GCTGATTAAGTCCCTGGGTCTT-5' ; IFNγ: 5'-TGTCCAACGCAAAGCAATACA-3' and 3'-TTCGCTTCCCTGTTTTAGCTG-5'; TNFα: 5'-TTAAGCAACAAGACCACCACT-3' and 3'-TCAAGGAAGTCTGGAAACATCT-5'; IL10: 5'-CTGCCTAACATGCTTCGAGATC-3' and 3'-AACCCTTAAAGTCCTCCAGCAA-5'; IL13: 5'-GGCAGCATGG TATGGAGCA-3' and 3'-TTCAGTTGAACCGTCCCTCG-5'; IL2-Rα: 5'-CAAGGGTCAGGAAGATGGATTC-3' and 3'-CCAGGACGAGTGGCTAGAGTTT-5') and normalized against HPRT transcript levels (Guillet et al., 2001).

CD4/CD8 T cell characterization.

$CD4^{+ve}$ or $CD8^{+ve}$ T cell selection was performed using MACS microbeads according to the manufacturer's recommendations (Miltenyi Biotec, Germany). The kit consists of an indirect magnetic labeling system composed of a hapten-monoclonal-antibody cocktail (anti-CD8/anti-CD4, CD16, CD56, CD11b, CD19 and CD36) and iron microbeads coupled with an anti-hapten antibody which enables the magnetic depletion of non-T cells. The magnetic bead-labeled cells are depleted by passing the cells through a MACS column in the magnetic field of an autoMACS. Purity was > 90%. The cells were mixed with Trizol® and the RNA extracted and retrotranscribed using the SMART procedure as explained above. The cDNA was then amplified and the CDR3-LD analysis performed as described above.

In order to assign a selected CDR3 length to a $CD4^{+ve}$ or $CD8^{+ve}$ cells population, the Immunoscope profiles of unselected PBL, $CD4^{+ve}$ and $CD8^{+ve}$ fractions were compared. The percentage corresponding to the frequency of a given selected CDR3 length in a Vβ family was calculated and compared in the PBL, $CD4^{+ve}$ and $CD8^{+ve}$ fractions. The skewing was assigned to the $CD8^{+ve}$ cells when the calculated percentage was higher for CD8 than for $CD4^{+ve}$ cells.

Elispot Assay protocol.

T cells from seven Vβ families with an altered CDR3 length were sorted from six patients (HRMS#4, Vβ17; HRMS#7, Vβ 3; HRMS#12, Vβ8 and Vβ14; CDMS#1, Vβ23; WMS#7, Vβ3; WMS#8, Vβ4), and three Vβ families with an altered CDR3 length were sorted from two Healthy Individuals (HI#9, Vβ21 and Vβ22; HI#10, Vβ2) and studied for human Myelin Basic Protein (MBP) reactivity. Additionnally, three other Vβ families with a gaussian-like CDR3-LD were sorted from three MS patients

(WMS#2, Vβ8, WMS#12, Vβ3 and HRMS#10, Vβ23) and also studied for MBP reactivity, in the same conditions. PBMC were thawed and washed in PBS. T cells with a Vβ family with an altered CDR3 length were sorted using the corresponding PE-labeled anti-Vβ antibody and anti-PE microbeads in an AutoMacs (Miltenyi Biotech, Germany). The purity of the sorted Vβ families was measured by flow cytometry and was above 75 %. The remaining fraction of the PBMC was irradiated for 315 sec at 15 Gray and was used as autologous antigen presenting cells (APC). First, human MBP (Sigma, France) at a concentration of 20 µg/ml, or human albumin (Sigma, France) at the same concentration was mixed with irradiated PBMC. Next, 4.10^5 PBMC were added to a 96-well anti-IFNγ coated Elispot plate (AID, Germany) and 4.10^5 of the T cells from sorted Vβ families were added to the plate. A control with irradiated PBMC alone at the same concentration was systematically performed. The cells were incubated for 24 h at 37 °C in 5% CO_2 and washed three times with washing buffer according to the manufacturer's instructions. A secondary biotinilated anti-IFNγ antibody was then added at 100 µl/well for 2.5 h. Plates were washed 3 times with buffer, and streptavidin-HRP was added for 2 hours at room temperature. Plates were washed in buffer and spot color was developed for a maximum of 1 h by adding AEC substrate diluted in acetate buffer containing H_2O_2. Plates were then washed with distilled water to stop the reaction. After drying, images of the wells were acquired using the AID Elispot software. All the experiments were run at least in duplicate or triplicate depending on the number of cells available. The results were expressed as means of the du(tri)-plicates.

Statistical analysis.

A Chi squared test, a Kruskal-Wallis test and a Dunn's multiple comparison test were performed between each group of MS patients (HRMS, CDMS and WMS) and HI for comparison of the Vβ/HPRT transcript ratios and the global CDR3-LD profiles. A Kruskal-Wallis test was performed on cytokine transcript values. A Mann-Whitney test was performed for the different Elispot assays. Differences were defined as statistically significant when $p < 0.05$.

Results

Healthy individuals display few Vβ families with significant CDR3-LD alterations with low Vβ/HPRT transcript ratios.

Fig 1 shows one representative example of the CDR3-LD profiles from the HI group. The TcLandscape pattern of every normal individual tested is available online (*www.nantes.inserm.fr/u437/sitesconnexes.html*). Only two of the 13 normal individuals expressed Vβ families with a significantly altered CDR3 length (>25%). All details concerning the alteration of Vβ families in the HI group are given in fig 5. Furthermore, the mean global percentage of CDR3-LD alteration in this group was 10.5 +/- 1.9 % (fig 6a). The mean Vβ/HPRT transcript ratio in the HI group was 2.3 +/- 0.9. In addition, 67 % of the Vβ families of healthy individuals had a Vβ/HPRT transcript ratio between 0 and 2 while 23 % of the Vβ families had a ratio between 2 and 5 and 10 % a ratio above 5 (fig 6c).

HRMS patients express a significantly higher mean CDR3-LD alteration than Healthy Individuals.

Fig 2 displays the CDR3-LD of the 12 HRMS patients. Four of the 12 patients displayed a CDR3 length with an alteration above 25 %. Details of the CDR3 length alterations are given in fig 5. Neither the number of patients with significantly altered V□(presence of a CDR3 length alteration >25%) nor the number of Vβ families with a significantly altered CDR3 length were different from the HI group. However, the mean percentage of CDR3-LD alterations (16.5 +/- 2.9%, fig 6a) was significantly higher than that of the HI group (10.58 +/- 1.9%, $p<0.01$).

The blood T cells from CDMS patients display more extensive CDR3-LD alterations than those from HRMS patients and Healthy Individuals.

Nine of the ten CDMS patients displayed a CDR3 length with an alteration above 25% (TcLandscapes and topwiews, fig. 3). Details of the Vβ families concerned are given in fig 5. The number of significant CDR3 length alterations in the CDMS group was significantly different from that of the HI and HRMS groups ($p<0.01$). The number of patients displaying at least one altered CDR3 length in the CDMS group was also significantly different from the HI and HRMS groups ($p<0.01$). In addition, the mean percentage of CDR3-LD alterations (17.8 +/- 1.6%, fig 6a) was significantly different from that observed in HI (10.58 +/- 1.98%, $p<0.001$) and in the HRMS group (16.5 +/- 2.9%, $p<0.03$).

The blood T cells from WMS patients display significantly more CDR3-LD alterations than those from Healthy Individual and HRMS patients.

Ten of the thirteen WMS patients displayed CDR3 lengths with alterations above 25% (TcLandscapes® and topwiews, fig. 4). Details concerning the Vβ families with significant CDR3 length alterations are summarized in fig 5. Patients with at least one significantly altered CDR3 length (alteration>25%) were significantly more numerous than in the HI and HRMS groups ($p<0.01$ and $p<0.05$ respectively). Furthermore, the number of alterations above 25 % in the WMS group was significantly different from the HI group ($p<0.05$). Additionally, the mean percentage of CDR3-LD alterations in WMS patients (15.5 +/- 1.5%, fig 6a) was significantly higher than that of the HI group (10.58 +/- 1.9%, $p<0.01$) but not that of HRMS patients (16.5 +/- 2.9%).

Vβ/HPRT transcript ratios.

Vβ/HPRT transcript ratio data are an indirect reflection of the pool size of T cell populations with different CDR3-LD. The mean Vβ/HPRT transcript ratios in the HRMS (0.94 +/- 0.5), CDMS (0.6 +/- 0.4) and WMS groups (0.7 +/- 0.3) were significantly lower from than those in the HI group (2.3 +/- 0.9, $p<0.001$, fig 6b). Furthermore, when Vβ/HPRT transcript ratios were compared according to a grading scale of 0-2, 2-5 and above 5, their distribution was significantly different as compared to the HI group (fig 6c, $p<0.01$).

Vβ transcript CDR3-LD alterations according to HLA class I and class II typing and MRI activity.

Distribution of CDR3-LD alterations according to HLA-DR typing was analyzed in 32 patients, 13 of them being DR2 positive. Ten of these 13 patients displayed significant alterations versus 12 of the 18 negative for DR2 (NS). No public skewed CDR3-LD was observed in HLA-DR2 patients. No correlation between Vβ family alterations and HLA class I typing was found. Correlation of CDR3-LD alterations with T1 gadolinium enhanced lesions was also studied. For the patients of group I, 14 exhibited at least one gadolinium enhanced lesion. Eight of them exhibited at least an altered CDR3 length above 25 % versus four out of eight with no gadolinium-enhanced lesion (NS).

T cells from MS patients with altered CDR3-LD Vβ accumulate proinflammatory cytokine transcripts.

In order to better understand the role played by the altered T cells identified by TcLandscape®, five Vβ families with highly altered CDR3-LD were sorted from three

different patients (one patient per group, HRMS#8: Vβ 3; CDMS#4: Vβ7.1, Vβ7.2 and Vβ17; WMS#1: Vβ17). These families were compared with sorted Vβ families with gaussian-like CDR3-LD (HI#10: Vβ 3 and Vβ 7; HI#11: Vβ 17; HI#12: Vβ 4 and Vβ 17; HI#13: Vβ 1and Vβ 16) and three Vβ families with altered CDR3-LD from HI (HI#9 and #12, Vβ 21, Vβ 22 and Vβ 13.1 respectively) for accumulation of different cytokine transcripts. The data are summarized in fig 7. The mean IFNγ and IL2 mRNA levels in sorted Vβ families with altered CDR3-LD from MS patients was significantly higher than for families with a gaussian-like pattern or altered CDR3-LD in the HI group ($p<0.01$ and $p<0.05$ respectively). TNFα mRNA was also significantly accumulated in the MS group, as compared to the HI group ($p<0.05$). IL10, IL13 and IL2 receptor α chain mRNA transcripts were not significantly different between the 3 groups. Despite the number of patients analyzed was low, these data suggest the presence of T cells with highly altered CDR3-LD able to produce proinflammatory cytokines in the blood of MS patients.

The alterations of CDR3-LD are more prominent in $CD8^{+ve}$ T cells.

Sorted $CD4^{+ve}$ and $CD8^{+ve}$ T cells from 9 patients (sixteen Vβ families) and 2 Healthy Individuals (6 Vβ families) were studied and their immunoscope profile compared to that of unselected PBL (fig. 8a and 8b). In some cases, the CD4 or CD8 nature of a selected CDR3 length was obvious (fig 8a, examples CDMS#1 and CDMS#9). In other cases (fig 8b, HRMS#12: Vβ 14; CDMS#4: Vβ 15, Vβ 17 and Vβ 21; WMS#7: Vβ 7; WMS#9 and WMS#10, HI#9: Vβ 6.4) the correspondence of the immunoscope profiles of total PBL with that of the fraction studied (CD4 or CD8) suggested that the selected CDR3 length in question belonged to this T cell subpopulation, which was corroborated by the comparison of the frequency of the prominent CDR3 length in the

three cellular fractions. Finally, in other V families (fig 8b, CDMS#4: Vβ 7; CDMS#7; CDMS#10; WMS#7: Vβ 4 and Vβ 15, HI#9: Vβ 21, Vβ 22 and Vβ 24, HI#7: Vβ8 and Vβ 24) no clear assignment to $CD4^{+ve}$ or $CD8^{+ve}$ cells was possible. Using this approach, 11 of the 16 Vβ families from MS patients and one of the 6 Vβ families from Healthy Individuals with an altered CDR3 length were found to have a more prominent skewing in the $CD8^{+ve}$ T cells suggesting a more pronounced bias in the $CD8^{+ve}$ repertoire in MS than in Healthy Individuals.

Sorted T cells from Vβ families with an altered CDR3-LD from MS patients produce IFNγ in the presence of human MBP.

IFNγ Elispot assays were performed with T cells from Vβ families with altered CDR3–LD from six MS patients (three from the HRMS group, one from the CDMS group and two from the WMS group) and two Healthy Individuals. In addition, because the analysis of Vβ families with gaussian-like CDR3-LD from patients already studied for their skewed Vβ family was technically impossible (due to an insufficient quantity of cells), three Vβ families with gaussian-like CDR3-LD were studied from three other patients. Only background Elispot reactivity (<10 spots) was detected when irradiated PBMC (used as APC) were tested (data not shown). Fig 9a shows the Elispot score obtained when hMBP was added to a culture of purified sorted T cells and irradiated PBMC used as APC (black boxes). For comparison, the same cultures were tested with human albumin (white boxes). In addition, the irradiated PBMC alone were also stimulated by hMBP (grey boxes). The figure shows that purified T cells from MS patients were highly reactive in response to hMBP ($p<0.01$ versus human albumin). Some reactivity was also observed in the irradiated PBMC fraction when hMBP was present in the culture. However, the sorted T cell response was much higher than

that of the PBMC alone in 4 out of 7 cases. Importantly, figure 9b shows that sorted T cells from the HI counterparts cultured in the same conditions with syngenic PBMC were not reactive to hMBP compared to altered Vβ families from MS patients (p<0.05). Furthermore, the number of IFNγ spots obtained with the gaussian Vβ families from the 3 additionnal patients was as low as for the altered Vβfamilies from Healthy Individuals (mean number of spots : 2.3 +/- 1), suggesting a specific response of the skewed Vβ families from MS patients.

Discussion

In this study, we analyzed the TCR Vβ chain transcriptome both qualitatively (alteration of CDR3-LD) and quantitatively (amount of transcripts concerned) in three groups of patients with relapsing-remitting MS at various stages of their disease compared to age-matched healthy individuals. Despite our study only concerned Vβ biases (analysis of Vα chain patternsmay provide further informations), significant T cell repertoire alterations, which were more prominent in $CD8^{+ve}$ T cells and mostly concerned Vβ families reactive to hMBP, can be detected in the blood of these patients from the onset of the disease (occurrence of first clinical symptoms [HRMS]). Furthermore, we show that sorted, circulating T cells from families with altered CDR3-LD are characterized by a transcriptional pattern with significant accumulations of IFNγ, IL2 and TNFα mRNA, without any further in vitro stimulation. Our data provide new information that supports the concept of early peripheral T cell activation in MS. Our results also suggest that MS patients could benefit from immunoregulatory treatment early in the course of their disease.

TCR biases in patients with MS have been reported by several groups in the past(Gran et al., 1998; Kotzin et al., 1991; Lozeron et al., 1998; Matsumoto et al., 2003; Muraro et al., 2002; Musette et al., 1996; Oksenberg et al., 1993). However, this study is the first to additionally analyze a cohort of patients identified as HRMS patients. Although healthy age-matched individuals also exhibited some level of CDR3-LD alterations (~10%), HRMS patients displayed highly significant global CDR3-LD alterations (~16 %). However, the Vβ/HPRT transcript ratios did not suggest blood accumulation of the T cell populations using the altered CDR3 Vβ transcript species. This apparent lack of peripheral accumulation may be related to a

continuous flux of these selected T cells toward other compartments including the CNS. Such a flux has recently been suggested by the effect of natalizumab, a monoclonal antibody inhibiting T cell homing into the CNS compartment through endothelial cells (Miller et al., 2003).

Significant global CDR3-LD alteration at a stage where the disease is in fact only suspected suggests that strategies aimed at inhibiting activated T cell transfer to the CNS or at regulating activation of selected T cells could be useful in the very early stage of the disease. One study has analyzed patients at the onset of a confirmed disease (CDMS) (Musette et al., 1996). However only a partial exploration of the T cell repertoire was performed with only a few Vβ families analyzed. This latter study of patients with confirmed disease (i.e. at a later stage than HRMS) was performed on Vβ 5 and 17 and also reported CDR3-LD alterations. Our data confirm and extend these initial observations by showing that most of the patients at the CDMS stage have both significantly more families with altered CDR3-LD and a more altered global transcriptome (allowing a statistical comparison) than patients at the HRMS stage (see fig 5 and 6a). The fact that 11 of the 26 Vβ families exhibited selected CDR3-LD at this stage of the disease suggests early epitope spreading. Spreading of T cell responses against different epitopes has been well characterized in experimental models of MS (Vanderlugt and Miller, 2002). In humans, Tuhoy *et al* have also described a serial analysis showing the decreasing frequency of clones directed against a single epitope during the progression of the disease from a monosymptomatic demyelinating syndrome (a group of patients similar to our HRMS group) to the stage of CDMS and the appearance of new clones directed against other epitopes of the same molecule or of other myelin proteins (Tuohy et al., 1999).

It is known that the CD8 T cell repertoire in healthy subjects is more altered than the CD4 repertoire (Gorochov et al., 1998). In this study, only six altered Vβ families from two HI were observed and only one alteration clearly belonged to the $CD8^{+ve}$ cell fraction. To our knowledge, the CD4/CD8 distribution of selected T cells in the blood of MS patients has been rarely investigated (Monteiro et al., 1996). The reason for a prominent bias in $CD8^{+ve}$ T cells in MS may be due to a response against viruses since it has been shown that the relapses of the disease are favorized by infections (Buljevac et al., 2002; Edwards et al., 1998). However, this explanation is not likely because the patients were not enroled in the study in case of a recent or ongoing clinical infection. Furthermore, the patients from the WMS group were not included at the moment of a relapse. Five patients of the CDMS group were able to be analyzed more closely for the phenotype of the T cells of Vβ families with altered CDR3-LD and six out of the nine Vβ families analyzed turned out to correspond in majority to $CD8^{+ve}$ cells (see fig 8a and 8b). The fact that the altered CDR3 length species represent a large majority of the T cells of these families (as assessed from the area under the curve of the immunoscope pattern of the amplified CDR3 segments) strongly suggests that these selected clones belong more to the CD8 than the CD4 phenotype. These observations are in agreement with the findings of Battistini et al. who have reported that circulating $CD8^{+ve}$ T cells from MS patients express adhesion molecules allowing them to cross the blood-brain barrier(Battistini et al., 2003) and may explain the significant $CD8^{+ve}$ T cell infiltrate seen in CNS lesions of MS (Booss et al., 1983; Gay et al., 1997). Moreover these data suggest that, even if HLA class II restricted $CD4^{+ve}$ cells play a role in disease susceptibility (Haines et al., 1998), spreading involving different class I restricted effectors occurs rapidly throughout the course of the disease, possibly by a phenomenon of cross-presentation (see

(Carbone et al., 1998) for review). In this respect, these data also suggest that besides class II tetramers (Reddy et al., 2003), class I tetramers may be a pertinent tool to test the blood T cells of MS patients. Interestingly, the only patient analyzed at the HRMS stage also displayed alterations of $CD8^{+ve}$ T cells (fig 8a and 8b). The concept that $CD8^{+ve}$ T cells may be important in MS has been suggested by the possibility of inducing autoreactive $CD8^{+ve}$ T cells for myelin peptides (Tsuchida et al., 1994). Furthermore, the role of CD8 T-cells has been recently highlighted by the findings that the majority of cells with clonal expansions and memory phenotype in the cerebro-spinal fluid and brain lesions of patients with MS were also $CD8^{+ve}$ T cells (Babbe et al., 2000; Jacobsen et al., 2002). These data and our own showing circulating $CD8^{+ve}$ CDR3-LD selected T cells in the blood of MS patients support the possibility that $CD8^{+ve}$ CTL could damage class I expressing brain cells including oligodendrocytes and neurons (Liblau et al., 2002; Medana et al., 2001; Neumann et al., 2002). Our observations also suggest a potential use of selected anti-Vβ antibodies in patients exhibiting expansions of circulating T cells with altered CDR3-LD as an alternative therapy.

This trend of $CD8^{+ve}$ selection was also found in two out of five Vβ families with altered CDR3-LD in the worsening MS group. However, these patients that had been defined as presenting an exacerbated disease (see materials and methods, all of them were included in a mitoxantrone regimen and complied with the usual criteria for worsening disease) exhibited fewer alterations of their Vβ transcriptome than CDMS patients, despite still being significantly different from age-matched healthy individuals. Furthermore, these patients did not have more Vβ̃/HPRT transcript ratios with altered CDR3-LD in their blood than normal individuals. Whether this lack of more severe global Vβ transcriptome alterations in this group of patients with active

disease is related to the fact that the blood sampling was not concomitant to disease exacerbation, that most of these patients (as opposed to those of other groups) had previously received immunologically oriented treatments or that an exacerbated flux of activated circulating T cells into the CNS occurred is unknown.

We were able to further analyze 3 patients (one from each of the clinical groups) for their transcriptional profile (real-time PCR) of the major Th1/Th2 related cytokines as well as TNFα. This study was performed immediately following cell sorting using anti-Vβ antibodies, without in vitro stimulation to avoid artifactual selection. Interestingly, despite the low number of Vβ families studied, the transcriptional pattern of T cells from altered families from MS patients significantly differed from those observed in families with either biased or gaussian CDR3-LD from normal individuals, reinforcing the idea that these expanding peripheral T cells may be involved in the disease process. Furthermore, IFNγ elispot assays were performed to test T cells from Vβ families with altered CDR3 lengths in each group of patients and HI. There was a striking difference in response to hMBP between T cells from Vβ families with altered CDR3-LD in MS patients and those from the controls. The T cells from more than half of such Vβ families from MS patients produced IFNγ when stimulated with hMBP whereas none of the Vβ families tested from Healthy Individuals did so. The reactivity of these cells to hMBP is likely due to the CD4 fraction of these cells since it is known that peripheral APC may not be optimal for crosspresentation. The lack of response of three Vβ families from MS patients could be explained by a reactivity to other myelin or non-myelin antigen(s) or a production of other cytokines than the one tested.

These observations fit with other studies which also reported IFNγ and/or TNFα mobilization in this disease (Huang et al., 1999; Pelfrey et al., 2000; Tejada-Simon et

al., 2001) despite the fact that in two of these three studies, the patterns were analyzed following in vitro stimulation with a putative MS antigen and that TNFα/IFNγ producing $CD8^{+ve}$ T cell clones directed against myelin peptides were found (Tsuchida et al., 1994).

Taken together, our data support the concept of circulating TCR selected T cells in MS and that $CD8^{+ve}$ T cells may be important in the pathophysiological processes of the disease. Our data also suggest that surveying the blood T cell abnormalities in MS may be helpful for drug response studies, particularly when global Vβ transcriptome analysis is used.

Acknowledgements

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Legends

Table 1 : Main clinical characteristics of the patients (y: years, m: months). NA : not available.

Figure 1 : TcLandscape® of blood T cells from one representative healthy individual. The data are displayed for each individual as a tridimentional TcLandscape® and as a Topview for easier assessment of global CDR3-LD alterations. The percentages of CDR3-LD alterations are represented as a color code. The X-axis displays the 26 human Vβ families, the Y-axis gives the Vβ/HPRT ratios and the Z-axis gives the 13 possible CDR3 lengths.

Figure 2 : TcLandscape® and Topview representation of blood T cells from patients with a high risk of MS (HRMS group). CDR3 length alterations above 25% appear in red.

Figure 3 : TcLandscape® and Topview representation of blood T cells from patients with a clinically definite MS (CDMS group). CDR3 length alterations above 25% appear in red.

Figure 4 : TcLandscape® and Topview representation of blood T cells from patients with a worsening MS (WMS group). CDR3 length alterations above 25% appear in red.

Fig 5 : CDR3 length alterations for all the patients and healthy individuals. CDR3 length alterations above 25% appear in black.

Fig 6 : a) Values of global CDR3-length distribution (LD) for each patient and HI. The mean value for each group is indicated by a bar. A kruskal-Wallis test and a Dunn's multiple comparison test was performed, $^{**}p<0.01$. **b)** Distribution of the mean Vβ/HPRT transcript ratios in the patient groups and HI (each dot represent the mean Vβ/HPRT transcript ration for each patient and HI). A Kruskal-Wallis test and a Dunn's multiple comparison test were performed to compare values. A bar indicates the mean value for each group. $^{**}p<0.01$. **c)** Distribution of the Vβ/HPRT transcript ratios between the groups according to a grading scale of 0-2, 2-5 and above 5. A Chi squared test was performed to compare distribution between the groups.

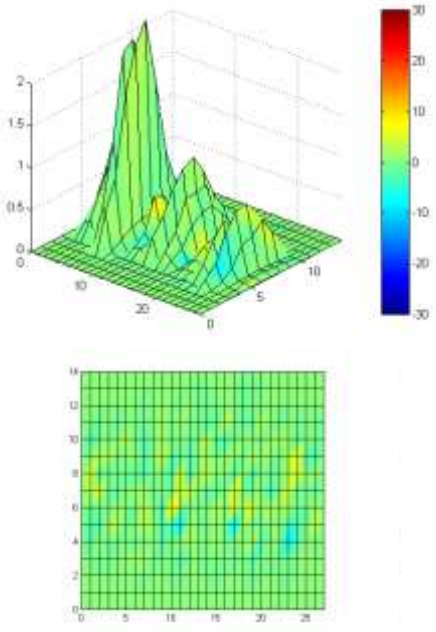
Figure 7 : Cytokine/HPRT transcript ratios from sorted T cells. Cytokine mRNA was studied for T cells sorted from families with CDR3 length alteration >25 % in MS patients as compared to T cells sorted from Vβ families with a gaussian-like pattern (HI-G) or an altered CDR3 length (HI-A) from healthy individuals. The median range is indicated for each group. Kruskal-Wallis test, $^{*}p<0.05$, $^{**}p<0.01$.

Figure 8 : CD4/CD8 phenotype of blood T cells from Vβ families with an altered CDR3 length. a) Examples from Vβ families with an altered CDR3 length. The phenotype was obtained by comparison of the Immunoscope profile of each population (CD4, CD8 and unselected PBL). **b)** Summary of the CD4/CD8 pattern of the Vβ families studied. CD8>CD4 means that the $CD8^{+ve}$ fraction of T cells is more represented in the selected CDR3 length than the $CD4^{+ve}$ fraction (see material and methods). The percentage indicated represents the frequency of the more prominent CDR3 length in the Vβ family studied. The comparison of the percentages for the

same CDR3 length in the different cell fractions (PBL, CD4 and CD8) indicates the fraction that is responsible for the skewing.

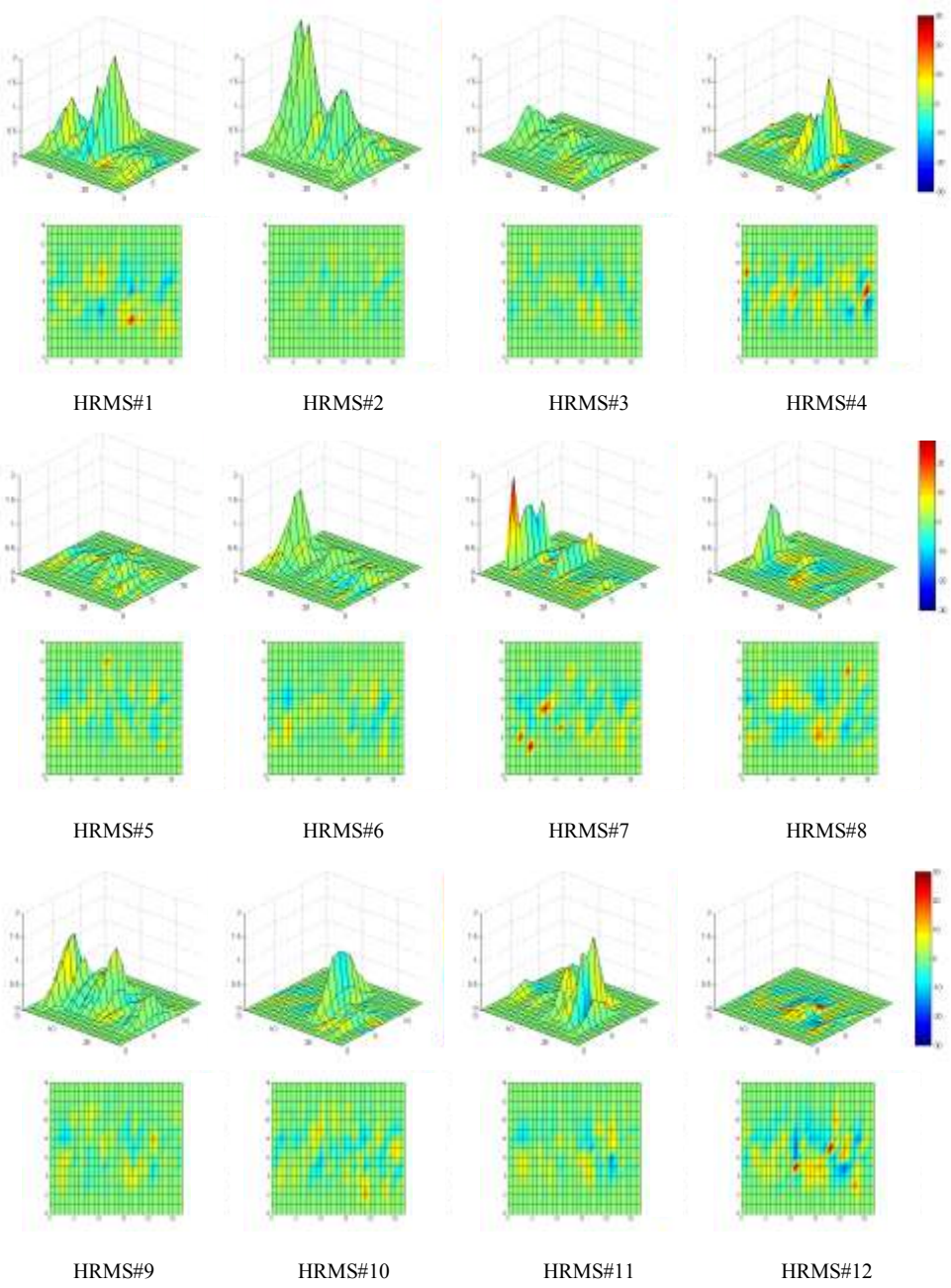
Figure 9 : IFNγ Elispot in T cells sorted from Vβ families with an altered CDR3 length. a) Elispot score of irradiated PBMC (APC) in MS patients (grey boxes), irradiated PBMC with T cells from sorted CDR3-LD altered Vβ families in the same MS patients (black boxes) in the presence of human MBP. White boxes represent the control score obtained with PBMC and sorted T cells from the same MS patients in the presence of human albumin. **b)** Comparison of reactivity for hMBP in irradiated PBMC with T cells sorted from CDR3-LD altered Vβ families (black squares) or healthy individuals (white squares). Mann-Whitney test, $p<0.01$.

	Age	Gender	Gadolinium^{+ve} lesions	HLA		
HRMS#1	21	F	0	DR2-DR4		
HRMS#2	42	F	1	A3-A11-B44-B51-DR2-DR5		
HRMS#3	29	F	9	DR1-DR6		
HRMS#4	41	M	0	DR1-DR7		
HRMS#5	30	F	1	NA		
HRMS#6	35	M	0	DR2-DR4		
HRMS#7	22	F	11	DR5-DR6		
HRMS#8	41	F	2	A2-A3-B27-B51-DR1-DR8		
HRMS#9	20	M	9	DR5-DR6		
HRMS#10	24	M	0	NA		
HRMS#11	31	M	0	DR4-DR7		
HRMS#12	19	F	9	DR6-DR8		
mean	*29.5*				**Disease duration**	
CDMS#1	30	M	2	A1-A31-B44-B40-DR2-DR4	1 y	
CDMS#2	46	F	3	NA	6 m	
CDMS#3	34	F	2	A2-A3-B7-B27-DR1-DR3	7 y	
CDMS#4	27	F	1	A2-A23-B15-B43-DR2	1 y	
CDMS#5	37	M	3	DR2-DR4	2 y	
CDMS#6	48	F	1	DR2	4 y	
CDMS#7	38	F	0	A1-A3-B8-B1402-DR3-DR6	NA	
CDMS#8	19	F	15	DR2-DR5	3 m	
CDMS#9	39	M	0	A3-B7-B8-DR2-DR3	1 y	
CDMS#10	38	M	0	DR4-DR5	1.5 y	
mean	*35.6*					**Immunosupp. drugs**
WMS#1	22	F	8	DR2-DR4	5 y	-
WMS#2	36	F	8	A2-A23-B13-B15-DR4-DR5	10 y	azathioprine
WMS#3	30	F	3	A1-A2-B8-B18-DR3-DR4	3 y	-
WMS#4	41	F	1	DR3	5 y	IFN □
WMS#5	32	F	1	A1-A2-B37-B51-DR5-DR6	3 y	-
WMS#6	38	F	1	DR2-DR7	13 y	IFN □
WMS#7	32	F	1	A3-A11-B37-B51-DR2-DR4	1 y	-
WMS#8	32	F	1	A1-A29-B8-B56-DR3-DR8	3 y	IFN □
WMS#9	27	M	1	DR3-DR6	2 y	IFN □
WMS#10	33	M	2	DR5-DR6	3 y	-
WMS#11	44	F	1	A2-A3-B7-B27-DR2-DR4	5 y	IFN □
WMS#12	46	F	2	A2-A28-B15-B37-DR4-DR6	3 y	IFN □
WMS#13	32	F	8	DR4-DR6	7 y	azathioprine
mean	*34.2*					



HI #4

Figure 1


HRMS#1
HRMS#2
HRMS#3
HRMS#4
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HRMS#9
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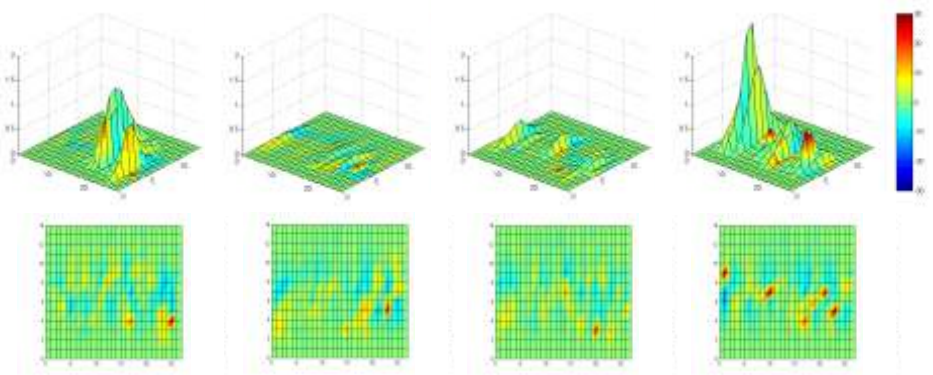
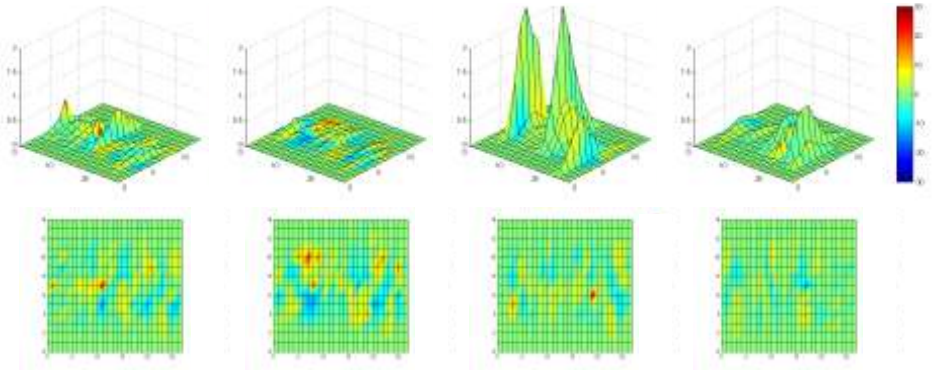
HRMS#11
HRMS#12

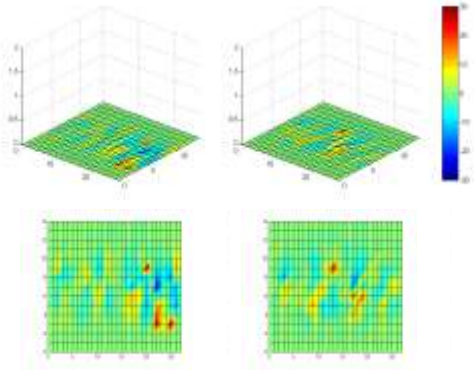
Figure 2



CDMS#1 CDMS#2 CDMS#3 CDMS#4

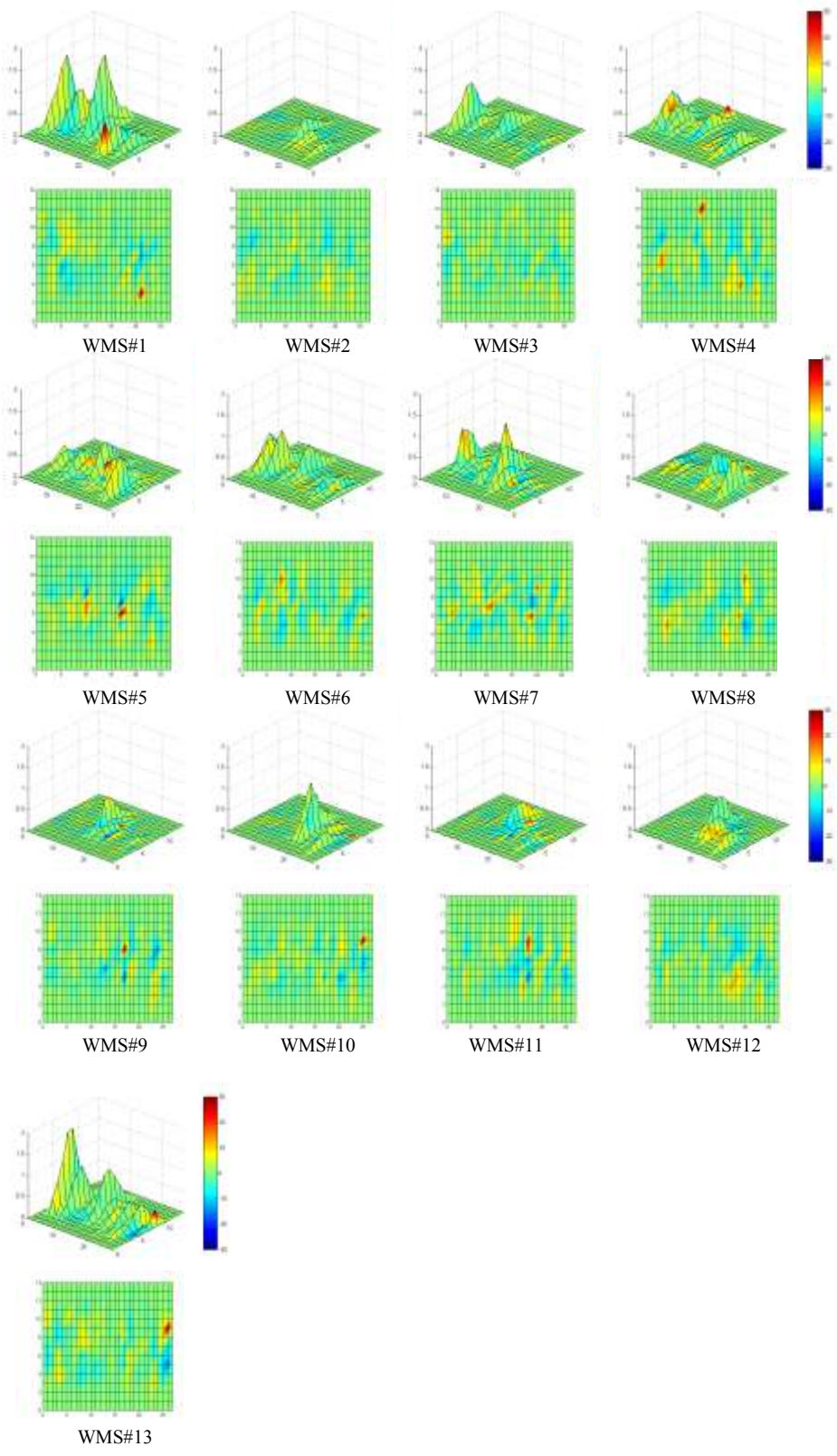


CDMS#5 CDMS#6 CDMS#7 CDMS#8



CDMS#9 CDMS#10

Figure 3


WMS#1
WMS#2
WMS#3
WMS#4
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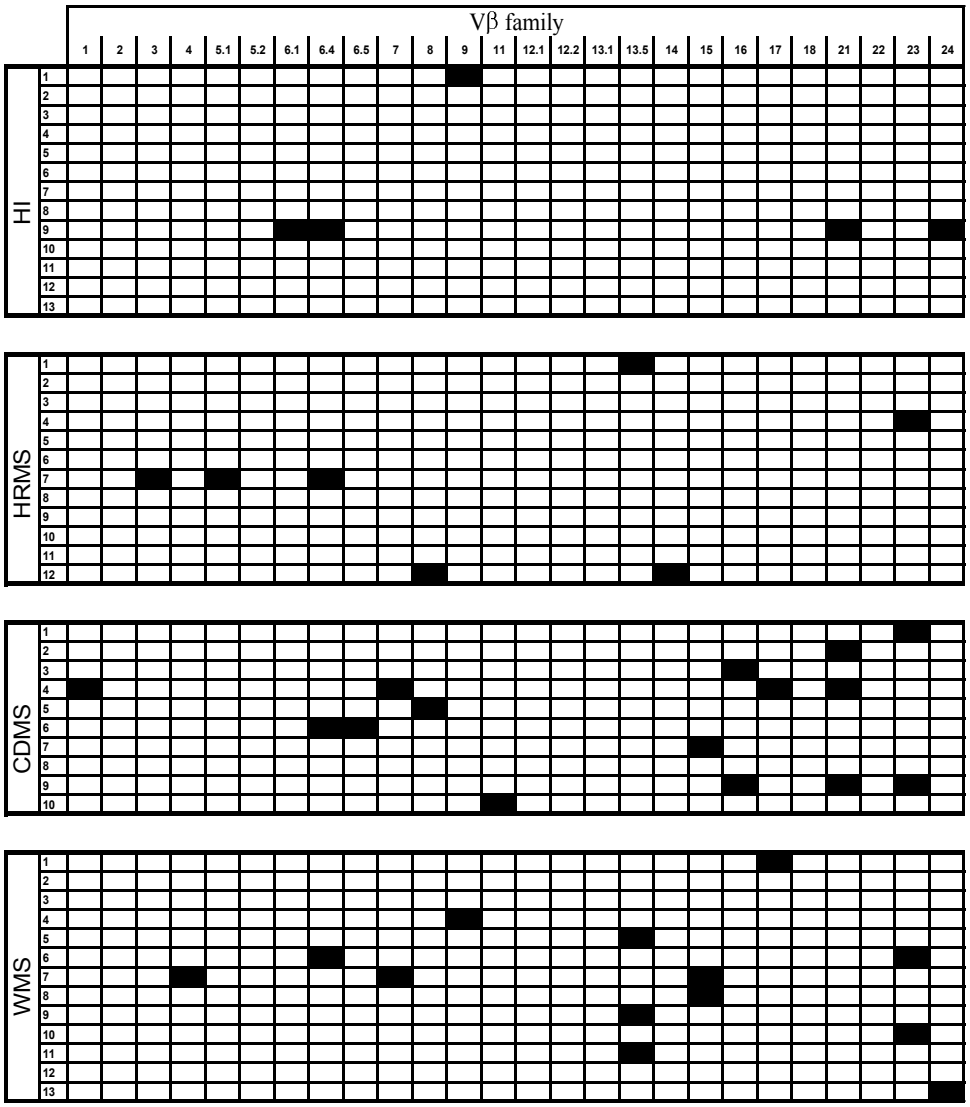
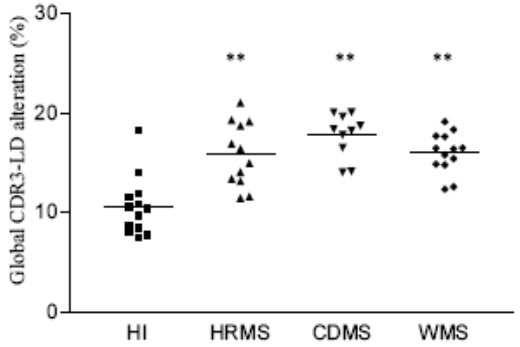
WMS#11
WMS#12
WMS#13

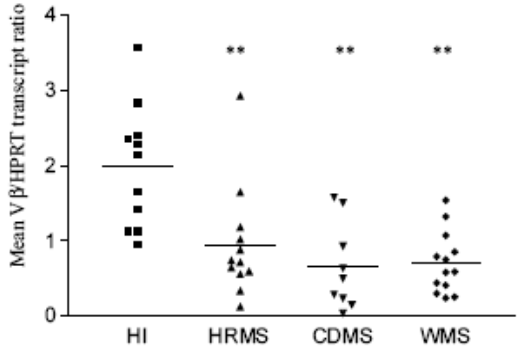
Figure 4


Vβ family
1 2 3 4 5.1 5.2 6.1 6.4 6.5 7 8 9 11 12.1 12.2 13.1 13.5 14 15 16 17 18 21 22 23 24
HI
1 2 3 4 5 6 7 8 9 10 11 12 13
HRMS
1 2 3 4 5 6 7 8 9 10 11 12
CDMS
1 2 3 4 5 6 7 8 9 10
WMS
1 2 3 4 5 6 7 8 9 10 11 12 13

a)



b)



c)

Vβ/HPRT ratio	[0;2[	[2;5[	>5	*p*
HI	67.13%	22.37%	10.48%	-
HRMS	84.93%	10.89%	4.16%	<0.01
CDMS	92.30%	5.38%	2.30%	<0.01
WMS	90.23%	8.87%	0.88%	<0.01

Figure 6

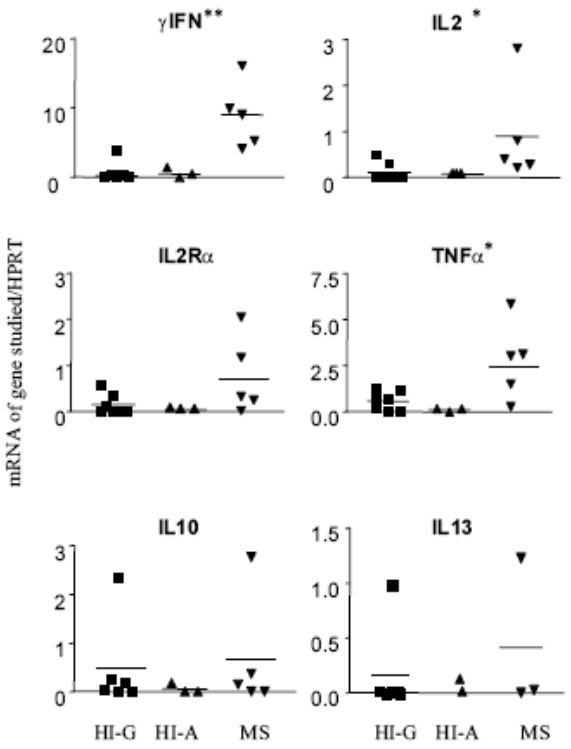
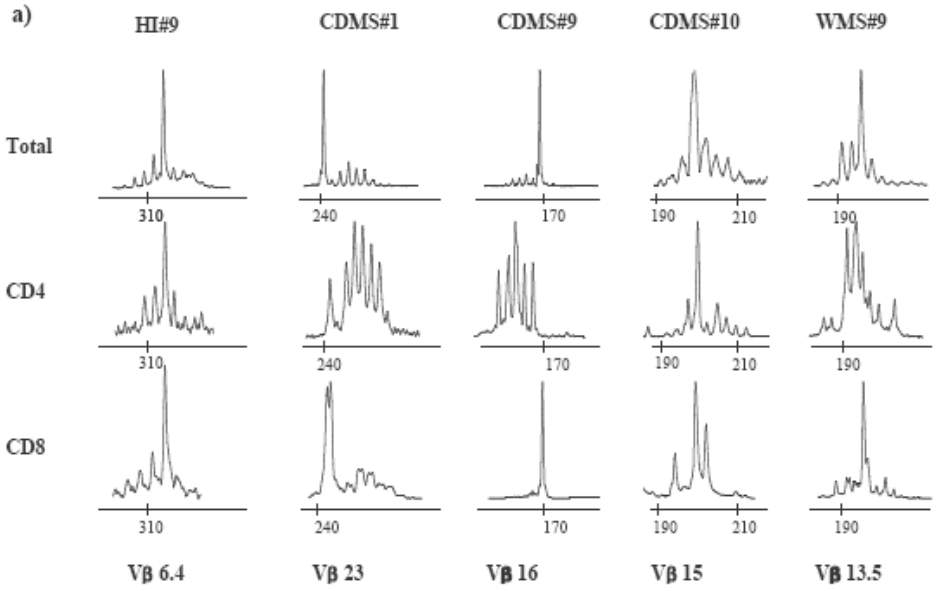

γIFN**
IL2 *
IL2Rα
TNFα*
IL10
IL13
mRNA of gene studied/HPRT
HI-G
HI-A
MS
HI-G
HI-A
MS

Figure 7

a)



b)

Patient	Altered Vβ studied	Percentage of the selected CDR3 in CD4+CD8	CD4	CD8	CD4/CD8
HI#7	Vβ 8	52%	**50%**	**49%**	**Both**
	Vβ 24	41%	**36%**	**38%**	**Both**
HI#9	Vβ 6.4	56%	41%	**57%**	CD8>CD4
	Vβ 21	31%	**30%**	**32%**	**Both**
	Vβ 22	42%	**43%**	**40%**	**Both**
	Vβ 24	36%	**33%**	35%	**Both**
HRMS#12	Vβ 8	100%	45%	**100%**	CD8>CD4
	Vβ 14	57%	17%	**60%**	CD8>CD4
CDMS#1	Vβ 23	53%	12%	**54%**	CD8>CD4
CDMS#4	Vβ 7	61%	**42%**	**38%**	**Both**
	Vβ 15	41%	28%	**43%**	CD8>CD4
	Vβ 17	54%	37%	**55%**	CD8>CD4
	Vβ 21	50%	32%	**45%**	CD8>CD4
CDMS#7	Vβ 15	49%	**50%**	**48%**	**Both**
CDMS#9	Vβ 16	75%	15%	**91%**	**CD8**
	Vβ 23	85%	0%	**60%**	**CD8**
CDMS#10	Vβ 15	41%	**29%**	**32%**	**Both**
WMS#7	Vβ 4	44%	**41%**	**45%**	**Both**
	Vβ 7	41%	16%	**33%**	CD8>CD4
	Vβ 15	40%	**25%**	**27%**	**Both**
WMS#9	Vβ 13.5	46%	**34%**	**57%**	CD8>CD4
WMS#10	Vβ 23	68%	0%	**100%**	**CD8**

Figure 8

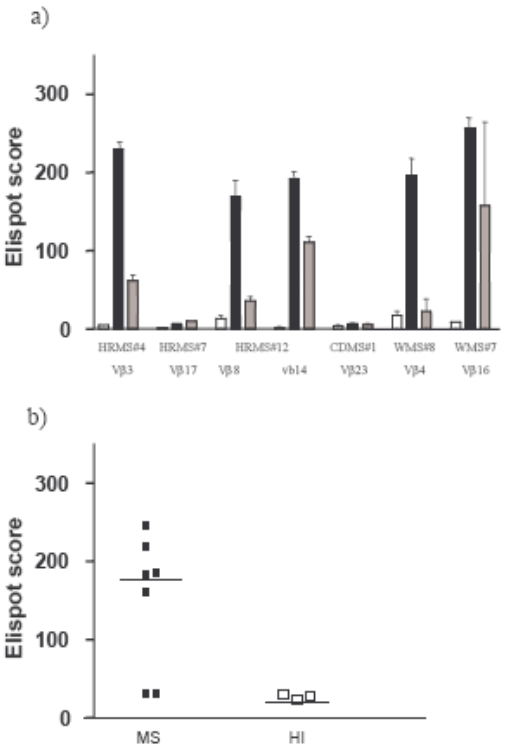

a)
Elispot score
300
200
100
0
HRMS#4
Vβ3
HRMS#7
Vβ17
HRMS#12
Vβ8
vb14
CDMS#1
Vβ23
WMS#8
Vβ4
WMS#7
Vβ16
b)
Elispot score
300
200
100
0
MS
HI

Figure 9

Article 3 : Serial blood T cell repertoire alterations in multiple sclerosis patients ; correlation with clinical and MRI parameters.

Journal of Neuroimmunology, En révision

Serial blood T cell repertoire alterations in multiple sclerosis patients ; correlation with clinical and MRI parameters.

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Key words : Multiple sclerosis, T cell repertoire, V beta genes, CDR3, TcLandscape, MRI

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List of abbreviations :

BBB : Blood Brain Barrier

CDR3 : Complementarity Determining Region 3

CNS : Central Nervous System

EDSS : Expanded Disability Status Scale

HPRT : Hypoxanthin-Phosphoryl-Ribosyl-Transferase

HI : Healthy Individuals

LD : Length Distribution

MBP : Myelin Basic Protein

MS : Multiple Sclerosis

PBMC : Peripheral Blood Mononuclear Cells

RR : Relapsing-Remitting

CP : Chronic-progressive

TCR : T Cell Receptor

Abstract

A significant skewing of the peripheral T cell repertoire has been shown in relapsing-remitting multiple sclerosis (MS). Most of the studies already performed in this field are cross-sectional and therefore, little is known of the T cell repertoire evolution over time in MS and the correlation of T cell repertoire variation with clinical and MRI parameters. This study was performed on serially harvested frozen PBMC from nine untreated MS patients (28 samples) and 14 healthy individuals. The blood T cell repertoire of each patient was analysed at the complementarity determining region 3 (CDR3) level and compared with a monthly MRI scan performed over a six month period with assessment of T2 lesion load and gadolinium enhancing lesions. A significant blood T cell repertoire skewing was observed in MS patients as compared with healthy controls (p<0.001) and the variation of the number of oligoclonal Vβ families with time correlated significantly with the variation of lesion activity as assessed by MRI (r=0.56, p<0.04). These findings suggest an association between the magnitude of TCRBV CDR3 length distribution alterations in the peripheral blood of MS patients and the disease process.

Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) disease associated with auto-immunity against myelin determinants (Noseworthy et al., 2000; Zamvil et al., 1985). A large body of evidence indicates that myelin reactive T cells are implicated at least in the early phase of the disease process, such cells being predominant in the active MS plaques (Booss et al., 1983). Recently, a humanized antibody directed against the T cell molecule VLA4 has shown its ability to reduce the formation of new gadolinium enhancing lesions in the CNS of MS patients, suggesting that activated autoreactive blood T cells cross the blood brain barrier (BBB) and contribute to MS lesions (Miller et al., 2003). Investigations into the selective T cell repertoire of MS patients have shown preferential usage of certain selected Vβ families, with different individual-specific (private) TCR repertoire restrictions (Gran et al., 1998; Laplaud et al., 2004; Lozeron et al., 1998; Matsumoto et al., 2003; Muraro et al., 2002; Musette et al., 1996). Analysis of the entire T cell repertoire has also shown a significant global alteration at the very onset of the disease (Laplaud et al., 2004). However, the precise location where these T cells undergo selection, whether it be in the periphery or in the CNS itself, is still controversial (see (Prat and Antel, 2005) for review) as is the issue of cell migration fluxes from the blood to the CNS (and vice versa) (de Vos et al., 2002; Melchior et al., 2005). T cell clones with similar TCRBV chain hypervariable regions, as assessed by the complementarity determining region 3 (CDR3) length or sequence, have been observed in the blood as well as in the CNS of MS patients (Babbe et al., 2000). We have also shown that most of

the T cells from the blood TCR V families presenting an altered CDR3 length distribution pattern are in fact CD8 cells (Laplaud et al., 2004). CD8+ cells expressing adhesion molecules such as P-selectin ligand have been shown to efficiently cross the BBB (Battistini et al., 2003). In addition, we have shown that a high frequency of sorted T cells belonging to Vβ families with strongly biased TCR usage are MBP-reactive (6000 per 10^6) when tested in vitro (Laplaud et al., 2004).

In this study, we collected serial blood samples from MS patients and longitudinally studied TCR repertoire skewing at the Vβ CDR3 length distribution (LD) level on a patient by patient basis. We found that TCR usage alterations and the association of altered Vβ families correlated with clinical parameters. We confirm and extend previous observations that MS patients exhibit altered blood CDR3-LD compared to normal individuals. We also show that relapsing-remitting MS patients present a significantly more altered TCR repertoire than chronic-progressive MS patients. Moreover, we observed a significant positive correlation between the appearance of new oligoclonal Vβ families and the variation of lesion activity over time, as assessed by MRI. If confirmed, these data suggest that blood T cell repertoire analysis could be used as an additional non invasive method for disease follow-up in MS.

Patients and study design.

Patients. Thirty MS patients were included in this longitudinal study between October 1994 and April 1995; 14 patients with Relapsing-Remitting (RR) MS and 16 patients with Chronic Progressive (CP) MS. All were followed at the MS Center of the Brigham and Women's Hospital in Boston, and represented the placebo arm of a randomized, double-blind, placebo-controlled, phase II trial of linomide in the treatment of MS. All gave their informed consent before participating in this study, which was approved by the local IRB. None of the patients were on treatment with interferon, steroids or other immunosuppressive drugs for at least 3 months prior to entering the study. The patients included 21 females (11 with RR MS, 10 with CP MS) and 9 males (3 with RR MS and 6 with CP MS), with an age range of 18 to 50 years. Their mean Expanded Disability Status Scale (EDSS) at baseline was 2.3 for RR MS patients and 5.4 for CP MS patients. All were clinically examined by a neurologist at baseline, 12, 24, 36 and 48 weeks.

Healthy controls. Blood from 14 healthy Individuals for whom PBMC were stored in liquid nitrogen tank since 1996, corresponding to a same time period of freezing than MS patients, were used. For three other healthy individuals, blood was drawn every month for three months, to determine the variations in CDR3-LD alterations (TcLandscape® Topview, see below) with time in a healthy population. All HI gave their informed consent to this study according to the local IRB.

Blood sampling and processing

PBMC preparation and freezing . Blood from MS patients was drawn at baseline and then every 4 weeks until week 48. Peripheral Blood Mononuclear Cells (PBMC) were subsequently isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation and re-suspended at 2.10^6 / ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM Hepes buffer, 50 units/ ml penicillin, and 50 mg/ml streptomycin (all from BioWhittaker, Walkersville, MD). After washing, the medium was discarded and 10.10^6 cells were re-suspended in one ml of Flash Freeze Buffer containing 10% DMSO and 90% heat inactivated fetal bovine serum before being frozen in liquid nitrogen. Frozen cells from healthy controls were obtained using the same process at a concentration of 10 10^6 per tube.

RNA extraction and cDNA synthesis. PBMC were thawed and washed in PBS. The cells were then resuspended in Trizol® reagent for RNA extraction according to the manufacturer's instructions. The concentration and quality of RNA for each sample was accurately determined using nanoRNA Chips (Agilent®, United Kingdom). According to these criteria, only 29 of the 65 frozen samples studied from the thirty MS patients, were of good enough quality and were retained for analysis (corresponding to nine MS patients; six RR-MS and three CP-MS patients), the remaining samples showing some RNA degradation, likely due to the long freezing time, were thus discarded. None of the samples from HI showed RNA degradation. Next, two µg of RNA

were reverse transcribed using an Invitrogen cDNA synthesis kit (Boeringher Mannheim, Indianapolis, IN) and diluted to a final volume of 100 µl.

TCR repertoire analysis. cDNA was amplified by PCR using a Cβ primer and one of the 26 Vβ specific primers. The amplifications were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA) as previously described (Gagne et al., 2000). Analysis of CDR3-LD was first performed using Immunoscope® software (Brouard et al., 1999; Douillard et al., 1996; Pannetier et al., 1995). The percentage of CDR3-LD alteration for each Vβ family and the global percentage of CDR3-LD alteration for each individual or each group was calculated as described (Gorochov et al., 1998). Briefly, the percentage of alteration was defined as the difference between the frequency of each CDR3 length in the distribution profile of the Vβ family studied and the control distribution, calculated from 13 healthy individuals. Global CDR3-LD alterations were represented as a "topview" TcLandscape® (see below) enabling an easy visual appraisal of the CDR3-LD biases. In this representation percentages of CDR3-LD alterations are represented as a color code, from deep blue (≤-30%) to dark red (≥+30%). The X-axis displays the 26 human Vβ families and the Y-axis gives the CDR3 lengths. A Vβ family is considered oligoclonal when its percentage of alteration is >25%, and appears as a red spot on the TcLandscape® Topview.

MR imaging and Image evaluation.

MRI scans were carried out for each patient at baseline, then every 4 weeks for 6 months. Imaging was performed on a General Electric Sigma 1.5-T unit (General Electric, Milwaukee, WI). Proton-density and T2-weighted images were obtained on the whole brain by contiguous 3 mm thick slices with an in-plane voxel size of 0.94 mm x 0.94 mm (24 cm field of view with a 256 x 192 acquisition matrix). Images were also obtained by applying a T1-weighted spin-echo pulse sequence after administration of an i.v. bolus of 10 ml of 0.5 M gadopentate dimeglumine (Gd-DTPA) (Magnevist, Berlex). Post contrast T1-weighted (Gd+) images in the axial plane resulted from a 600/19/1 (repetition time/echo time/excitations) spin-echo sequence. Slice thickness was four mm with one mm gap. Proton density- and T2-weighted images were analyzed using an automated computerized procedure allowing for a reproducible method of lesional volume assessment. Gd-enhanced T1-weighted images were evaluated by two radiologists for each patient data set. The total number of enhancing lesions and the number of new enhancing lesions at each time point were determined independently by each rater and the average number was used in the analysis.

The following 3 parameters were measured: total number of enhancing lesions, number of new enhancing lesions and total volume of T2 lesions.

Statistical procedures.

For all usual statistical tests the SPSS v 12.00 software was used and a $p<0.05$ was considered significant. A *t* test was used for comparison of global percentage of CDR3-LD alteration in each patient group versus controls. A non parametric kruskal-Wallis test was used for comparison of each Vβ family in the different patient and control groups. For the comparison of TcLandscape® Topview topologies, a multiple testing procedure was performed using SAS software (Westfall et al., 1999). The multiple testing procedure addresses the multiple testing problem when many simultaneous null hypotheses are performed, as is the case for TcLandscape® Topviews. This test takes into account the percentage of alteration of each Vβ family and the co-ordinate of each CDR3 length for all Vβ families.

The variation of gadolinium positive lesions with time was assessed by a dedicated score, G_i. This score depends on the appearance, the persistence and the loss of gadolinium positive lesions and, thus, is related to lesion activity. Special weighting was attributed for each of these 3 events. The evolution of a patient's status between t_i and t_{i+1} could be summarized as follows by the gadolinium positive lesions score, $G_{i,\,i+1}$:

$$G_{i,\,i+1} = a * (N_{i+1}) + p * (S_{i+1} - N_{i+1}) + d * (S_i + N_{i+1} - S_{i+1})$$

Where:

a is the weight of a lesion appearance;

p is the weight of a persistent lesion;

d is the weight of a lesion loss;

S_i is the total number of lesions at t_i;

S_{i+1} is the total number of lesions at t_{i+1};

and N_{i+1} is the number of new lesions at t_{i+1}

The basic way of scoring these 3 kinds of event leads to the score "*+1"* for the appearance or persistence of a lesion and "*-1"* for the loss of a lesion.

We then compared the evolution of the total volume of T2 lesions and the gadolinium positive lesion score. Evolution of the total volume of T2 lesions between t_i and t_{i+n} was calculated by the difference between the lesion volume at t_{i+n} and at t_i. A linear relationship (r^2 = 0.75) was found between the evolution of the lesion volume and the gadolinium positive lesion score. The correlation between these two variables was significant at $p<0.01$ (non-parametric Spearman ρ=0.884) and bringing confidence on the relevance of the clinical parameters tested.

The correlation between the number of oligoclonal V□ families (defined as a percentage of alteration >25%) or a variation in the number of oligoclonal V□ families with time, and either the T2 lesion load, the number of gadolinium positive lesions, the variation in gadolinium G_i scores or the variation in the T2 lesion load, was calculated using a non-parametric Spearman correlation test. Finally, we also analyzed the variations in the CDR3 length distributions of each Vβ family with time by quantifying the dissimilarities between two Immunoscope profiles as a percentage of alteration between two time points. The average "distance" between two samples is then equivalent to the mean of the percentage of alteration computed between each Vβ profile.

Results

CDR3 length distribution in normal individuals.

First, 14 Healthy Individuals (HI) whose PBMC had been frozen for a period of time similar to that of the MS samples (8 years) were used as controls to investigate the normal range of the level of CDR3-length distribution (CDR3-LD) alterations. The global percentage of CDR3-LD alteration in frozen samples was measured of 17% +/- 8%. Nevertheless, alterations above 25 % (corresponding to oligoclonal Vβ families) in PBMC, even following long-term freezing remained rare. Figure 1 shows the six Vβ families with oligoclonal patterns of CDR3-LD on the TcLandscape® Topview display. Collectively, only four of the 13 normal individuals tested displayed Vβ families with >25% CDR3-LD alteration. These results were summarized in figure 2.

MS patients exhibited higher global percentages of blood T cell CDR3-LD alterations than normal controls.

We then compared the level of CDR3-LD alterations in samples from MS and healthy individuals that had been frozen for the same length of time. Several Vβ families were not investigated in all the MS samples because the signal was too weak (white bands for corresponding Vβ families in figure 2). The global percentage of CDR3-LD alterations in MS patients and appropriate normal individual controls was significantly different with a percentage of alteration ranging from 15% to 31% in MS patients and from 14% to 19% in controls ($p<0.001$, *t* test).

CDR3-LD alterations in MS blood T cells preferentially affected certain Vβ families, as shown by the side by side comparison of each Vβ family from MS patients and normal individuals. Significant skewing involved several Vβ families : Vβ 25.1, 5.2, 7, 8, 12.1, 13.1 to 14, 16, 21, 23 and Vβ 24 (p<0.05, Kruskal-Wallis test). Using a multiple testing procedure (Westfall et al., 1999), this skewing was confirmed in the following Vβ families : Vβ 5.1, 5.2, 8, 12.1, 13.1, 14, 16, 21 and Vβ 23 (p<0.05, multiple testing). The comparison of the number of oligoclonal Vβ families, defined as a percentage of alteration >25% and shown as a red spot on the TcLandscape® Topview, revealed the presence of 24 oligoclonal Vβ families in the nine different MS patients, as compared to 6 oligoclonal Vβ families found among the 13 healthy Individuals. Taken collectively, these data confirm a significant skewing of blood T cell repertoire diversity in MS patients.

Serial analysis of TCR alterations in MS patients.

In MS patients the presence of altered Vβ families was roughly stable over time in a given patient but differed from one patient to another. During the follow-up, some “red spots” corresponding to Vβ family transcripts with strong CDR3-LD alterations appeared and/or disappeared, as shown in the example given in figure 3 (patient SR5) and summarized in figure 2. As shown in the example concerning SR5, the Vβ 2 family exhibited a strong Vβ CD3-LD alteration and this spot was roughly stable over time. Nevertheless, other Vβ

families exhibited strong CDR3-LD alterations appearing over the follow-up period and summarized in figure 2 : Vβ 1 at month (M) 3, Vβ 3 and Vβ 5.1 at M 6, Vβ 6.1 at M 1, M2 and M4, Vβ 6.4 at M 0, Vβ 12.1 at M 5, Vβ 14 at M 1, Vβ 18 at M 0 and M 6 and Vβ 24 at M 1. As shown in figure 2, with the exception of one patient (patient JB4) all patients displayed at least one oligoclonal Vβ family, as compared with only four among the 13 healthy individuals.

The level of TCR CDR3-LD alterations significantly differed according to the clinical status of MS patients.

CDR3-LD was then analysed in the different clinical groups of MS patients. The global percentage of CDR3-LD alterations was compared in relapsing remitting versus chronic progressive MS patients. The global percentage of CDR3-LD alteration was 22% (ranging from 15% to 31%) in RR-MS patients and 17% (ranging from 16% to 24%) in CP-MS patients ($p<0.02$, *t* test). Comparison of the TcLandscape® Topviews obtained from RR-MS patients with those from CP-MS patients revealed more numerous oligoclonal Vβ families in the group of RR-MS patients than in CP-MS patients (18 oligoclonal families in 6 RR-MS patients vs 3 oligoclonal families in 3 patients). The number of oligoclonal Vβ families is summarized in figure 2. The systematic comparison of the percentage of CDR3-LD alteration in each Vβ family did not reveal statistically significant differences for a specific Vβ family. Only trends were noted for Vβ 6.1, 6.5, 8, 13.1, 13.5 and Vβ 17. However, the difference

reached significance for Vβ 9 in RR-MS patients as compared with CP-MS patients (p<0.05, Kruskal-Wallis test).

Correlation of variations in CDR3-LD alterations over time with MRI parameters.

First, we compared the level of CDR3-LD alterations in patients with and without gadolinium enhancing lesions on their MRI and found no difference between the two patient groups: 20% +/- 11% and 20% +/- 12% respectively (NS). Nevertheless, considering the level of alteration ie the number of oligoclonal Vβ families (alteration above 25%) in each group of patients, a significant difference emerged. Sixty one (61) oligoclonal Vβ families were noted in patients with gadolinium positive lesions on the MRI compared to 31 Vβ families among patients without gadolinium positive lesions (p<0.05, Kruskal-Wallis test).

We then studied possible correlation between the number of oligoclonal Vβ families and the number of gadolinium enhancing lesions and the T2 lesion load. Despite the relatively low number of patients, a significant correlation was also observed between the variation in gadolinium enhancing lesions with time (as assessed with the gadolinium G_i score, see materials and methods) and the appearance of new oligoclonal Vβ families (r=0.56, p<0.04, figure 4a). In addition, we compared the "amount" of T cell repertoire diversity changes that occurred at each time interval (by comparing the variation in the percentage of alteration for each available Vβ family between two samples,

see materials and methods) with the evolution of gadolinium G_i scores for two patients (JL5 and SR5). As shown in figure 4b, for patient JL5, the level of TCR biases roughly paralleled the evolution of G_i. Altogether, these data suggest an association between the lesion activity seen on the MRI and new oligoclonal Vβ families in the blood of RR-MS patients.

Finally, the correlation between the T2 lesion load and the number of oligoclonal Vβ families also correlated significantly (r=0.42, $p<0.05$, figure 4c).

Discussion

Multiple sclerosis is thought to be an autoimmune T cell driven disease. In this study we serially analysed and monitored the TCR repertoire in the blood of MS patients with different disease types using an analysis of CDR3-LD alteration. In addition, for the first time we tested the hypothesis of possible correlations between TCR biases and clinical parameters in patients who had monthly MRI scans during the follow-up. The fact that all of the patients in this cohort belonged to the placebo arm of an interrupted therapeutic trial rendered the biological study of this cohort even more interesting since this enabled us to analyse patients in the absence of disease modifying therapies. Finally, the MRI data available for each patient at the time of each blood harvesting made it possible for us to perform correlations between the T cell repertoire biases and the different clinical parameters. Only samples giving high quality mRNA (as assessed by Agilent technology) were analysed.

Because little is known about the long-term effect of T cell freezing on TCR alterations and because the PBMC of MS patients had been frozen for several years, we also used as controls a set of blood samples from healthy individuals that had been prepared similarly as MS samples and frozen for a length of time similar to samples from MS patients. Using these appropriate controls, we confirm and extend the findings of our previous study of the TCR Vβ chain repertoire in fresh blood of MS patients (Laplaud et al., 2004). It is not likely in our group of patients that the skewing observed could be due to the age of the patients since it has been described oligoclonal expansions of CD8 T cells with age, because the patients were relatively young (see Effros et al,

for review). In addition we show that blood TCR biases principally affect patients with RR-MS rather than those with CP-MS, suggesting that immune driven inflammatory processes are less predominant in this latter type of MS disease where more degenerative processes are believed to operate. TCR immunoscope profiles have been rarely investigated in CP-MS patients. In a recent study by Sun et al, the TCR repertoire of four CP-MS patients was investigated at baseline and after autologous haematopoietic stem cell transplantation (Sun et al., 2004), and were also mostly gaussian.

The TCR skewing observed in our study involved several Vβ families, including Vβ 5.2 that has been extensively studied in other studies, and was confirmed by two different statistical tests (Multiple testing procedure and *t* test). Alteration of CDR3-LD in the Vβ 5.2 family has already been shown in MS patients, and this Vβ family has been reported to be frequently used by MBP-specific clones from HLA-DR2 MS patients (Kotzin et al., 1991). We also found other Vβ families with significant skewing such as Vβ 12 and Vβ 13 that were also reported to be used by MBP specific cells (Hong et al., 1999; Wucherpfennig et al., 1990). Finally, the Vβ 8, Vβ 21 and Vβ 23 families, which were significantly altered in the MS group, have also been reported to be biased by other authors (Matsumoto et al., 2003).

We first studied the stability of TCR alteration over time in three healthy individuals over a period of three months. The TCR profiles showed some Vβ families with altered CDR3-LD which are reproducibly found over time (data not shown, see supplementary file). Such private alterations have already

been reported in healthy individuals and might correspond to the T cell response occurring during different virus infections such as influenza, CMV or EBV viruses (Bitmansour et al., 2001; Maini et al., 2000; Prevost-Blondel et al., 1997). The presence of persisting peripheral blood TCR CDR3-LD alterations are likely linked to the “immunological history” of each normal individual. It is likely that these patterns are also superimposed in MS patients.

The stability of the TCR alterations over time in MS (for instance Vβ 5.2 and V□ 11) has been only investigated in a small number of patients (Matsumoto et al., 2003; Muraro et al., 2002). In the latter studies, the global percentage of CDR3-LD alteration was roughly stable over time in a given patient. However, although some Vβ family alteration patterns remained stable throughout the follow-up, we also observed time-related variations, suggesting, as shown by Muraro et al (Muraro et al., 2002), that some epitope spreading can also occur in the periphery.

The clinical significance of blood TCR skewing has not been investigated before in MS patients. In our previous study of TCR alterations, we showed that the T cells responsible for the skewing were mainly CD8 cells producing Th1 cytokines, and importantly, that some of these cells produced IFNγ when stimulated with human MBP (Laplaud et al., 2004). TCR skewing has also been correlated to clinical symptoms in other diseases, such as AIDS (Gorochov et al., 1998), melanoma (Degauque et al., 2004) or in renal allograft recipients (Brouard et al., 2005). In this study, we found a positive correlation between blood TCR biases and lesion activity. This correlation, which was

assessed by a G_i score taking into account the new gadolinium positive lesions, the persisting gadolinium positive lesions and the disappearance of gadolinium positive lesions, may be of potential relevance for the clinical management of MS. More importantly, a positive correlation was also detected between the variations in the TCR biases with time and the variations in gadolinium positive lesions in the same time period, suggesting that the level of TCR bias may be a dynamic marker of disease activity. However, even though we studied correlations between lesion activity and TCR biases by two different methods, our observations are based on a small cohort of patients, and should thus be interpreted with caution. Taken together, these data suggest a direct involvement of the T cells from oligoclonal Vβ families in the pathophysiological process. To our knowledge, this is the first study showing correlations between TCR alterations and clinical parameters such as clinical presentation (RR-MS, CP-MS), level of CNS lesion load or radiological activity as assessed by MRI in MS. The differences reported were statistically significant despite a relatively small number of patients. This work therefore provides new insight into the significance of the potential role of peripheral blood T cells in the physiopathology of MS and suggests that the exhaustive study of Vβ transcriptome alteration may be of some value in the management of MS. This now needs confirmation on a larger patient cohort.

Legends

Table 1: Clinical characteristics of the patients at the time of inclusion.

Figure 1: TcLandscape® Topviews of healthy Individuals (HI) for whom PBMC had been frozen for a similar time period as those from MS patients. The X axis displays the Vβ families from Vβ1 to Vβ 24. The Y axis displays the CDR3 length in amino-acid number for each Vβ family. The colour code represents the TCR skewing for each Vβ family from a deep blue (-30% of alteration) to a dark red (+30% of alteration). The circles indicate the oligoclonal Vβ families, as defined by an alteration >25 %.

Figure 2 : CDR3 length alterations for all of the multiple sclerosis patients and Healthy Individuals. A green square indicates a global gaussian distribution of the expressed Vβ family. A red square indicates an oligoclonal Vβ family (CDR3-LD alteration >25%). A surrounded red square indicates the appearance of a new oligoclonal Vβ family as compared to the preceding sample. A white square corresponds to missing data for the Vβ family.

Figure 3: A significant example of TcLandscape® Topviews from patient SR5 and their variations with time. Arrows indicate an example of persisting oligoclonal Vβ families throughout the follow-up period. The Immunoscope

profile of the persisting oligoclonal family is shown. Circles indicate examples of newly appearing oligoclonal Vβ families over the 6 month follow-up period .

Figure 4: Correlation between MRI parameters and oligoclonal Vβ families. a) Correlation between the appearance and disappearance of gadolinium enhancing lesions as assessed by G_i scores (see material and methods) and the number of new oligoclonal Vβ families (r=0.56, p<0.04). The X axis indicates the number of lesions assessed by MRI and the Y axis indicates the number of oligoclonal Vβ families. b) Correlation with time between Gadolinium G_i scores and oligoclonal Vβ families in patient JL5. The X axis indicates time (month) and the Y axis indicates Gi score and oligoclonality. The dotted line indicates the variation in G_i score with time for patient JL5. The black line indicates the variation of oligoclonality with time, as assessed by a hamming distance (see materials and methods) for patient JL5. A strong correlation exists between G_i score and oligoclonality for patient JL5. c) Correlations between the number of oligoclonal Vβ families and T2 lesion load (r=0.42, p<0.005). The X axis indicates the volume of T2 lesions expressed in cm^3 assessed by MRI and the Y axis indicates the number of oligoclonal Vβ families.

Supplementary File : Analysis of the stability over time of TcLandscape® Topviews from Healthy individuals. The global percentage of CDR3-LD alteration was roughly stable as well as the presence of the same oligoclonal

Vβ families for each individual (indicated by arrowheads) over a three month period.

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Patient	Gender	Disease Type	age (year)	disease duration	date of bleeding	lesion volume	Gado+ lesions	New Gado+ lesions	EDSS
TG4	M	RR	27		M0	3.1799	0	0	-
					M1	2.8055	0	0	-
					M2	3.3302	0	0	0
					M4	3.9973	0	0	-
					M6	4.0135	0	0	2,5
SR5	F	RR	45	3	M0	10.4098	1	-	3
					M1	10.9371	3	2	-
					M2	10.7552	1	0,5	-
					M3	11.3906	1,5	1	3,5
					M4	11.3036	1,5	1	-
					M5	10.8158	0	0	-
JL5	F	RR	31	4	M0	6.4178	4	-	-
					M1	5.6426	1	1	-
					M2	6.5733	5	5	-
					M3	7.5384	5	3	-
					M4	6.1277	2	2	-
					M6	6.2701	0	0	-
ET2	F	RR	51	6	M0	8.7275	3,5	0,5	-
EL3	F	RR	42	2	M0	6.2965	0	0	1,5
CM3	F	RR	37		M1	0.9255	0	0	2
					M6	2.2755	0	0	2
JO4	M	CP	45	8	M0	9.3973	2	0,5	-
					M1	9.3683	2	2	-
JB4	F	CP	38		M0	14.4466	0	0	6
SH5	F	CP	39	12	M0	5.0625	0	0	3
					M2	4.6644	0	0	-
					M5	4.8727	0	0	3

Table 1

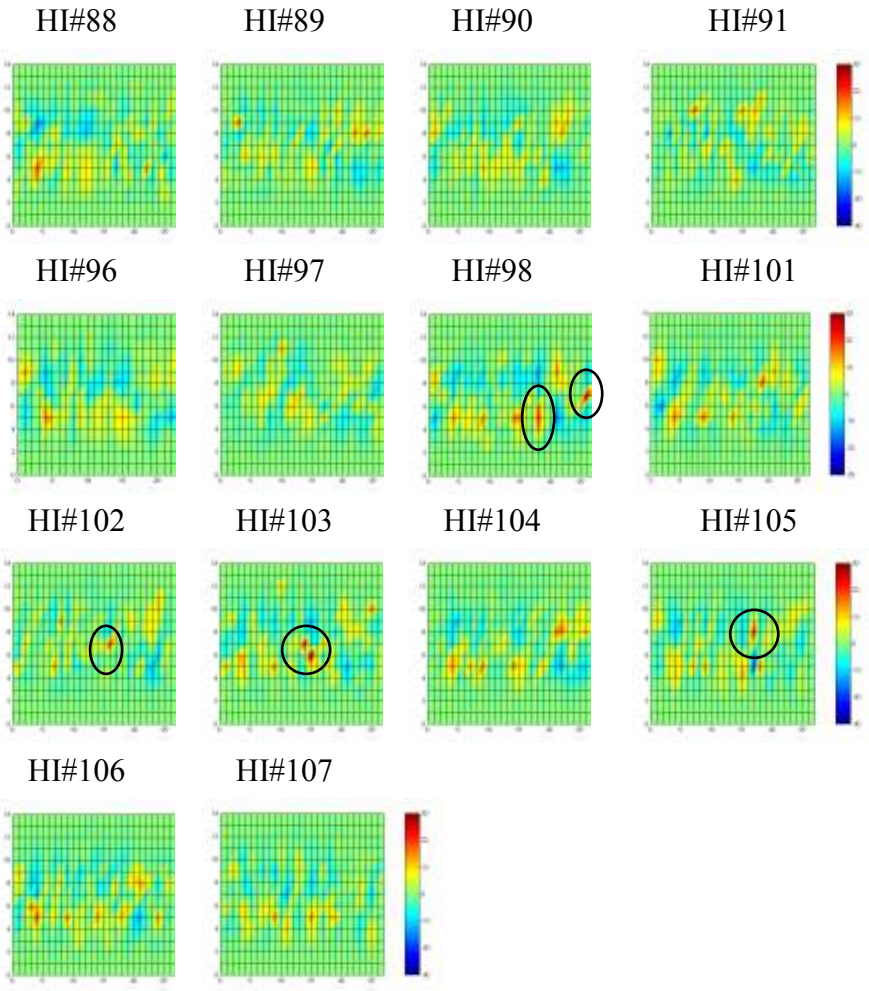
HI#88
HI#89
HI#90
HI#91
HI#96
HI#97
HI#98
HI#101
HI#102
HI#103
HI#104
HI#105
HI#106
HI#107

Figure 1

	Month / Vβ	1	2	3	4	5.1	5.2	6.1	6.4	6.5	7	8	9	11	12.1	12.2	13.1	13.5	14	15	16	17	18	21	22	23	24	Disease type	Gd + Lesions	New Gd+ lesion	T2 variation volume (cm3)
HI#88	-																											-	-	-	-
HI#89	-																											-	-	-	-
HI#90	-																											-	-	-	-
HI#91	-																											-	-	-	-
HI#96	-																											-	-	-	-
HI#97	-																											-	-	-	-
HI#98	-																											-	-	-	-
HI#101	-																											-	-	-	-
HI#102	-																											-	-	-	-
HI#103	-																											-	-	-	-
HI#104	-																											-	-	-	-
HI#105	-																											-	-	-	-
HI#106	-																											-	-	-	-
HI#107	-																											-	-	-	-
TG4	M0																											RR	0	0	-
	M1																												0	0	-0.3744
	M2																												0	0	0.5247
	M4																												0	0	0.6671
	M6																												0	0	0.016
SR5	M0																											RR	1	-	-
	M1																												3	2	0.5273
	M2																												1	0.5	-0.1819
	M3																												1.5	1	0.63534
	M4																												1.5	1	-0.0867
	M5																												0	0	-0.4878
	M6																												-	-	-
JL5	M0																											RR	4	-	
	M1																												1	1	-0.7752
	M2																												5	5	0.9307
	M3																												5	3	0.9651
	M4																												2	2	-1.4107
	M6																												0	0	0.1424
ET2	M0																											RR	3.5	0.5	-
EL3	M0																											RR	0	0	-
CM3	M1																											RR	0	0	-
	M6																												0	0	1.350
JO4	M0																											CP	2	0.5	-
	M1																												2	2	-0.029
JB4	M0																											CP	0	0	-
SH5	M0																											CP	0	0	-
	M2																												0	0	-0.3981
	M5																												0	0	0.2083

Figure 2

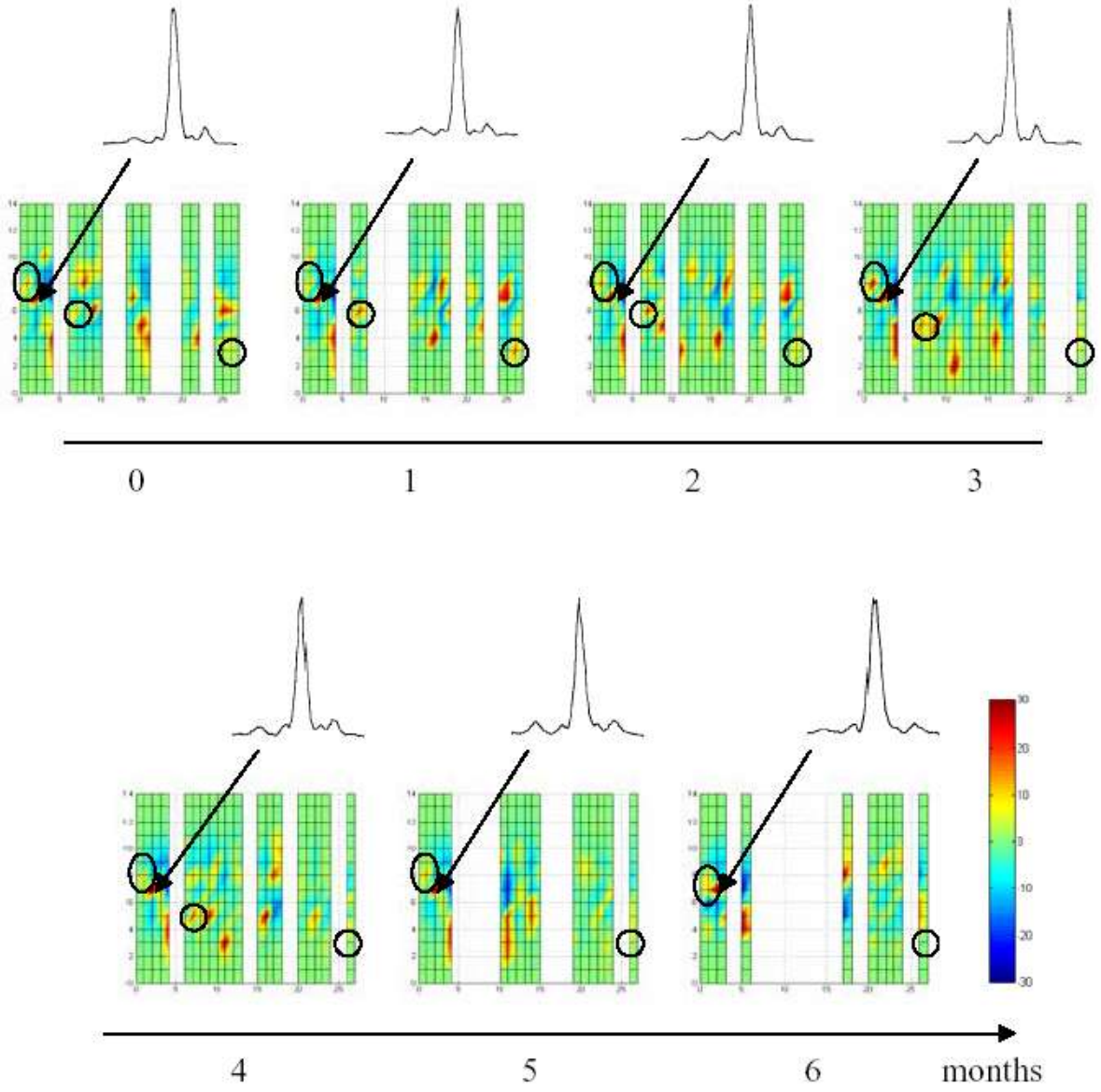

Figure 3

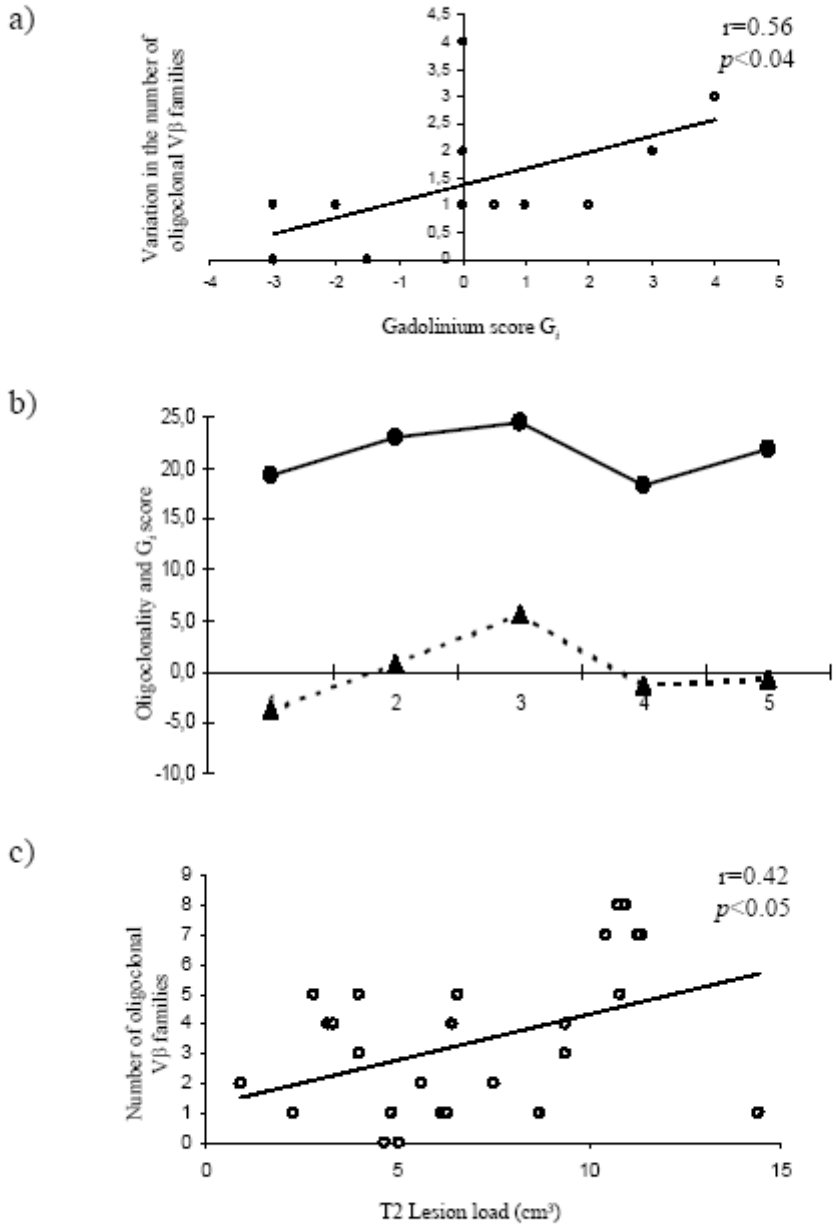
a)
r=0.56
p<0.04
Variation in the number of oligoclonal Vβ families
Gadolinium score G1
b)
Oligoclonality and G1 score
c)
r=0.42
p<0.05
Number of oligoclonal Vβ families
T2 Lesion load (cm³)

Figure 4

Discussion

Arguments cliniques et paracliniques de l'implication de Lymphocytes T CD8+ dans la SEP

Dans la SEP les lymphocytes T CD4+ ont longtemps été considéré comme les « responsables » de la maladie, ou en tous cas les cellules impliquées de façon prépondérante dans la physiopathologie de la SEP. Cette hypothèse était principalement basée sur les études de liaisons génétiques de la maladie et sur celle des modèles animaux.

Tout d'abord l'association de la maladie avec l'allèle du HLA DR2 codant pour les molécules de classe II du CMH a été mise en évidence, la présence de cet allèle conférant un risque relatif de la maladie de 4 (Olerup and Hillert, 1991). D'autre part des TCR spécifiques pour la MBP issus de lymphocytes T CD4+ ont été trouvés au sein des lésions de SEP dans le cerveau des patients (Oksenberg et al., 1993), alors que certains patients présentaient également un peptide immunodominant de la MBP complexé avec le HLA-DR2 et exprimé par les CPA au niveau des lésions (Krogsgaard et al., 2000).

De plus, l'étude du modèle animal de SEP, l'EAE, dans lequel des souris sont immunisées avec soit des extraits de moelle épinière, soit de la protéine MBP entière ou encore avec des épitopes encéphalitogènes de cette même protéine, met en évidence l'implication des lymphocytes T CD4+ dans la maladie. En effet le transfert de ces cellules d'un animal malade vers un animal naïf provoque une EAE, renforçant la notion que ces cellules jouent un rôle pratiquement exclusif dans la physiopathologie de la maladie (Zamvil et al., 1985).

Cependant ce concept a été sévèrement battu en brèche lorsque l'utilisation d'un anticorps monoclonal dirigé contre les CD4+ n'a montré aucun effet notamment dans la réduction du nombre de lésion prenant le gadolinium sur une IRM pratiquée chaque mois pendant 9 mois alors même qu'il existait une déplétion importante du

taux de CD4+ dans le sang des patients (van Oosten et al., 1997). Ce résultat pourrait signifier que les cellules T CD4+ ne sont pas impliquées de façons prépondérantes dans la SEP. Cependant, une autre interprétation est que les cellules les plus sensibles au traitement sont les cellules CD4+ naïves (CD45RA+/RO-) ne produisant pas d'INFγ (Rep et al., 1997).

Dans ce contexte, il faut noter que l'utilisation d'anticorps monoclonaux tels que l'anti-CD52 (Campath-1H) ou le natalizumab, dirigés contre le VLA-4, permettent non seulement une déplétion globale des populations lymphocytaires, mais diminuent encore de façon drastique le nombre de poussée de SEP et le nombre lésions actives sur l'IRM (Miller et al., 2003; Paolillo et al., 1999).

Ces observations plaident donc en faveur d'un rôle relativement modéré des lymphocytes T CD4+ dans la physiopathologie de la maladie, alors que les autres composantes du système immunitaire, en particulier les lymphocytes T CD8+, pourraient avoir un rôle prépondérant dans la formation des lésions démyélinisantes et axonales. Comme nous allons le voir, de nombreux arguments directs et indirects plaident effectivement en faveur du rôle important de ces cellules dans la maladie.

Arguments neuropathologiques

Comme expliqué précédemment, le SNC a longtemps été considéré comme un site immunologique privilégié du fait notamment de l'absence d'expression de molécules « immunogènes », en particulier les molécules de classe I du CMH (Joly et al., 1991) et donc protégé des lésions induites par les lymphocytes T (cf. Chapitre Immunologie du SNC). En fait toutes les cellules gliales et neuronales peuvent exprimer les molécules de classe I du CMH après stimulation appropriée in-vitro (Neumann et al., 2002) en particulier l'INFγ. De plus, les oligodendrocytes humains, in-vitro, peuvent

être lysés par les lymphocytes T CD8+ (Jurewicz et al., 1998). Dans le cerveau humain sain, une expression constitutive de molécule de classe I du CMH a été décrite sur les cellules endothéliales, les macrophages péri-vasculaires et la microglie (Hoftberger et al., 2004). L'étude du parenchyme cérébral des patients atteints de SEP met en évidence une sur-expression de ces molécules au niveau des lésions sur les cellules pré-citées. D'autre part, une étude récente utilisant des anticorps plus sensibles et plus spécifiques montrait que toutes les cellules du SNC peuvent exprimer les molécules de classe I du CMH en fonction du type de lésion. Ainsi la péri-plaque dans la substance blanche et les lésions inactives expriment de façon importante les molécules de classe I du MHC en particulier sur les cellules microgliales. En revanche, au sein des lésions actives tous les types cellulaires expriment ces molécules, les cellules microgliales, les astrocytes, les oligodendrocytes ainsi que les neurones (Hoftberger et al., 2004). Une des conclusions qui peuvent être tirées de ces travaux est que l'ensemble de ces cellules peut donc être la cible de l'attaque de lymphocytes T CD8+ cytotoxiques.

Le cas des neurones est particulier car on a longtemps cru que ces cellules étaient inertes sur le plan immunologique. L'équipe de Neumann et collaborateurs a ainsi montré que les neurones n'exprimaient les molécules de classe I du MHC que dans des conditions très stringentes : les neurones doivent avoir perdu leur capacité électrique et être stimulés par l'INFγ (Neumann et al., 1995). Plusieurs études in-vitro ont analysé la sensibilité des neurones aux lymphocytes T cytotoxiques (Rall et al., 1995). Il a été décrit notamment la susceptibilité à l'apoptose induite par l'interaction Fas/FasL et non aux perforines (Medana et al., 2000). Cependant, la plupart des études se sont concentrées sur la sensibilité des corps cellulaires neuronaux et non ou peu de leurs prolongements. En fait, les travaux de Neumann et collaborateurs

montrent que des lymphocytes T CD8+ cytotoxiques forment des complexes stables avec les neurites lorsque la reconnaissance de la combinaison peptide antigénique/Molécule de Classe I est correcte. La visualisation continue de l'interaction neurite/lymphocytes T DC8+ cytotoxique met en évidence l'apparition de transection des prolongements neuronaux (figure 4, (Medana et al., 2001).

D'autres part de nombreuses preuves histologiques font la part belle à la responsabilité des lymphocytes T CD8+ cytotoxiques dans la création de la lésion de SEP : alors que leur fréquence dans le sang périphérique de témoins est d'environ moitié inférieure à celle des lymphocytes T CD4°+, ils sont pourtant 3 à 10 fois plus nombreux que ces mêmes cellules au sein des lésion actives (Babbe et al., 2000; Booss et al., 1983). Les CD8+ sont observés de façon très proches des oligodendrocytes et des axones démyélinisés au sein des lésions de SEP, avec une polarisation de leur granules cytolytiques (Neumann et al., 2002). De plus, les lésions axonales observées au niveau des lésions sont bien corrélées avec le nombre de lymphocytes T CD8+ mais pas avec les cellules CD4+ (Bitsch et al., 2000).

Association des molécules de classe I du CMH avec la SEP

Les premières études d'association du HLA avec la maladie datent du début des années 70. Elles montraient une association avec l'antigène de classe I du MHC, HLA-A3, ou possiblement avec le HLA-B7 qui est en déséquilibre de liaison avec A3 (Jersild et al., 1972; Naito et al., 1972). Plus tard, l'association de la maladie avec l'haplotype HLA DR2, et en particulier avec le HLA DRB1-1501, codant pour les molécules de classe II du CMH a été clairement établi (Kenealy et al., 2003). Plus récemment et avec des méthodes améliorées et sur de plus grandes séries,

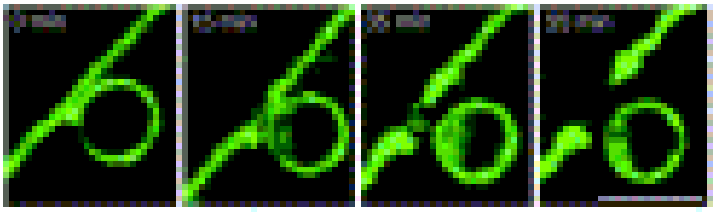

Figure 4 : Lymphocyte T CD8+ responsables d'une transection axonale dans un modèle in-vitro. Les Neurites de neurones de l'hippocampe sont mis en culture avec des lymphocytes T cytotoxiques. Les lésions neuritiques apparaissent en 15 à 30 minutes. Figure tirée de (Medana et al., 2001)

l'association avec les molécules de classe I a été clarifiée notamment avec le HLA A3 qui double le risque de SEP et ce, indépendamment du déséquilibre de liaison avec le HLA-DR2 (Fogdell-Hahn et al., 2000; Harbo et al., 2004). Dans ces travaux, la présence à la fois de HLA-A3 et DR2 confère un risque de développement de la SEP supérieur, de façon non additive puisque HLADR2 confère un risque génétique relatif (GRR) de 7 et A3 un GRR de 3 alors que la présence de ces deux allèles donne un GRR de 15 (Odds Ratio à 5,2). Ce travail montre que A3 module le risque conféré par HLADR2. De la même façon, la présence de HLA-A2 réduit le risque de développement de la SEP lorsque HLA-DR2 est présent avec un risque relatif passant de 3,5 à 1,5. L'ensemble de ces données est résumé dans le Tableau 1, tiré de (Friese and Fugger, 2005).

Arguments tirés des essais cliniques

D'autres arguments indirects plaident pour l'importance des lymphocytes T CD8+ cytotoxiques dans la physiopathologie de la SEP et en particulier les enseignements tirés de certains essais cliniques.

Alors que la déplétion sélective des lymphocytes T CD4+ semblait prometteuse dans des essais pilotes chez des patients souffrant de SEP (Lindsey et al., 1994a; Lindsey et al., 1994b) ou dans le modèle animal (Steinman, 1999), la réalisation d'un essai contrôlé, randomisé en double-aveugle, n'a pas apporté les confirmations attendues (van Oosten et al., 1997).Il s'agissait d'un essai de phase II contre placebo utilisant un anticorps monoclonal chimérique anti-CD4. Bien que le traitement permit une diminution significative et durable du nombre de cellules CD4+ dans le sang des patients traités, aucune différence statistique n'a été mie en évidence sur l'objectif principal qui était le nombre de lésions actives (c-a-d prenant le gadolinium) sur des

HLA Haplotype	Odds Ratio
HLA-A*0201	0.52-0.7
HLA-A*0301	1.9-2.1
HLA-DR2, HLA-DQB1*06	2.9-3.6
HLA-DR2, HLA-DQB1*06+HLA-A*0201	1.5
HLA-DR2, HLA-DQB1*06+HLA-A*0301	5.2-6.8
HLA-A*0201+HLA-A*0301	1.0

Tableau 1 : Associations du HLA et de la SEP, tiré de (Friese and Fugger, 2005)

IRM réalisées mensuellement pendant 9 mois. L'analyse *post-hoc* révèle sur des critères secondaires que l'activité lésionnelle semble corrélée au degré de déplétion en CD4, permettant de soutenir l'hypothèse qu'une déplétion plus marquée aurait permis de mettre en évidence une efficacité sur le critère primaire d'évaluation au cours de cette étude. On peut peut-être souligner le fait que si c'était le cas, d'autres études l'aurait sûrement corroboré depuis. Une des raisons soulignées expliquant l'absence d'efficacité de la molécule était que les lymphocytes T CD4+ les plus sensibles à l'anticorps étaient ceux qui n'étaient pas impliqués dans le processus physiopathologique de la maladie (lymphocytes T naïfs, non producteurs d'INFγ) (Rep et al., 1997).

En revanche d'autres thérapies ciblant de façon spécifique les lymphocytes ont montré leur efficacité dans la maladie : il s'agit du Campath-1H, un anticorps monoclonal dirigé contre le CD52 et le natalizumab, un anticorps monoclonal dirigé contre VLA4. Ces deux traitements qui déplètent autant les lymphocytes T CD8+ que les CD4+ ont clairement démontré leur efficacité sur des critères radiologiques et cliniques avec une diminution drastique du nombre de lésions actives et du nombre de nouvelles poussées de la maladie (Miller et al., 2003; Paolillo et al., 1999).

Compte-tenu de ces éléments, il paraît démontré que les lymphocytes T CD8+ ont un rôle majeur dans la lésion de SEP et que de nouvelles thérapies les ciblant spécifiquement pourraient être particulièrement intéressantes.

Conclusions – Perspectives

A travers ce travail nous avons mis en évidence l'existence de perturbations du répertoire T dans le sang de patients souffrant de SEP rémittente, quelque soit le stade de la maladie, et même de façon très précoce. Ces anomalies du répertoire qui sont par ailleurs aussi présentes chez des témoins sains, le sont de façon significativement plus fréquentes chez les malades. La présence de familles Vβ oligoclonales semble par ailleurs corrélée à l'activité de la maladie sur un plan radiologique, c'est à dire avec l'apparition de nouvelles lésions, suggérant que les cellules T responsables des anomalies du répertoire T pourraient être impliquées dans ces lésions.

La caractérisation de ces cellules montre qu'il s'agit essentiellement de lymphocytes T CD8+ capables pour un certain nombre d'entre elles de produire de l'INFγ en réponse à une stimulation avec de la MBP humaine, et que ces cellules accumulent des transcrits de cytokines pro-inflammatoires comme IL-2, TNFα ou IFNγ.

Comme nous l'avons vu précédemment (cf Discussion), de nombreux arguments plaident ainsi pour l'implication importante des lymphocyte T CD8+ dans la physiopathologie de la SEP, et ce travail apporte quelques arguments supplémentaires.

Pour autant, de nombreux progrès restent à faire dans la caractérisation des lymphocytes T CD8+ dans cette maladie et en particulier sur les antigènes du SNC et les épitopes reconnus par ces cellules. En effet peu d'études se sont focalisées sur les épitopes des molécules myéliniques ou d'autre origine reconnus par les CD8. La plupart des épitopes connus à ce jour ont été découverts par l'utilisation d'algorithmes mathématiques de prédiction mais n'ont pas été clairement identifiés au sein de lésion de SEP in-vivo. Finalement, moins d'une dizaine d'épitopes différent ont été actuellement décrits chez l'homme (cf Tableau 2) et principalement

Protéine	Type HLA
MBP	A2 : 87-95, 110-118
PLP	A2 : 80-88
	A3: 45-53
MAG	A2 : 287-295, 509-517, 556-564

Tableau 2 : Epitopes immunodominants de protéines de la myéline présentés par les molécules de classe I du CMH chez l'homme

avec le HLA-A2, alors qu'il s'agit d'un haplotype peu fréquent dans la maladie car protecteur. Par ailleurs, ces études n'ont pas ou peu été menées chez des témoins et la fréquence de la réponse CD8+ est donc inconnue chez l'homme sain.

Nous avons donc débuté un travail afin de décrire les différents épitopes issus de la MBP, la PLP et la MOG, reconnus par les lymphocytes T CD8+ issus de patients et témoins et capables d'induire une réponse INFγ positive. Nous espérons ainsi pouvoir décrire dans un avenir proche la fréquence de ces cellules dans la pathologie humaine et caractériser les CD8 reconnaissant ces épitopes myéliniques.

Annexes

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Blood T-cell receptor β chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution

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Summary

Multiple sclerosis is an inflammatory demyelinating disease of the CNS associated with T cells autoreactive for myelin components. In this study, we analysed the T-cell receptor (TCR) usage of the variable β (Vβ) chain transcriptome in the blood of multiple sclerosis patients at various stages of the disease using a global and quantitative comparison of the complementarity-determining region 3 length distribution (CDR3-LD) of transcripts of the 26 Vβ genes. We investigated 35 patients: 12 with a high risk of multiple sclerosis, 10 with clinically definite multiple sclerosis, 13 with a relapsing–remitting worsening and active multiple sclerosis and 13 healthy individuals. Cells bearing the TCR transcripts with altered CDR3-LD were sorted and studied for CD4 or CD8 phenotype, cytokine transcript accumulation and response to human myelin basic protein (MBP). We show that patients from all the groups have a significantly skewed blood T-cell repertoire. Vβ transcriptome patterns were more altered in patients from the clinically definite multiple sclerosis group and the worsening and active multiple sclerosis group than in the high risk group. The T cells sorted from Vβ families with altered CDR3-LD concerned both CD4 and CD8 T cells, with a more pronounced skewing in the CD8 compartment. These cells displayed a significantly increased level of interferon-γ, interleukin-2 and tumour necrosis factor-α transcripts compared with their counterparts from the healthy individual group. Furthermore, using interferon-γ enzyme-linked immunospot (ELISPOT) assays, T cells from four out of seven altered Vβ families tested from multiple sclerosis patients responded to human MBP, whereas no response was observed with human albumin or with altered Vβ families from healthy individuals. Our data support the concept of an early autoimmune component in the disease and emphasize the possible involvement of CD8-positive T cells in multiple sclerosis.

Keywords: CD4/CD8; CDR3; cytokines; multiple sclerosis; T cells; Vβ genes

Abbreviations: APC = antigen-presenting cell; CDMS = clinically definite multiple sclerosis; CDR3 = complementarity-determining region 3; CDR3-LD = complementarity-determining region 3 length distribution; ELISPOT = enzyme-linked immunospot; HI = healthy individuals; HLA = histocompatibility leukocyte antigen; HPRT = hypoxanthine phosphorylribosyl transferase; HRMS = high risk of multiple sclerosis; IFN-γ = interferon-γ; IL = interleukin; MBP = myelin basic protein; PBL = peripheral blood lymphocyte; PBMC = peripheral blood mononuclear cell; TCR = T-cell receptor; TNF-α = tumour necrosis factor-α; Vβ = variable β; WMS = worsening multiple sclerosis

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Introduction

Multiple sclerosis is a chronic inflammatory and demyelinating disease of the CNS whose aetiology remains unknown. Autoimmunity seems to play a key role in multiple sclerosis pathogenesis, as suggested by the presence of autoreactive T cells for myelin components in the peripheral blood and CNS of patients (Noseworthy *et al.*, 2000). A better characterization of the peripheral blood T lymphocytes in multiple sclerosis would probably serve to evaluate the disease severity, to forecast its evolution and to adopt new therapeutic strategies. One way to characterize the peripheral lymphocytes possibly implicated in multiple sclerosis is to analyse the skewing of the complementarity-determining region 3 length distribution (CDR3-LD) of the T-cell receptor (TCR) variable β chain (Vβ). Such an analysis was developed initially on the basis of a semi-quantitative assessment of CDR3-LD by reverse transcription–polymerase chain reaction (RT–PCR) technology (spectratype/Immunoscope® methods) (Pannetier *et al.*, 1995). Several studies have addressed the question of CDR3-LD in unmanipulated T cells collected from the blood of multiple sclerosis patients and detected skewed β gene usage, a fact that supports the possibility of oligoclonal T-cell expansions in the blood (Musette *et al.*, 1996; Gran *et al.*, 1998; Lozeron *et al.*, 1998; Muraro *et al.*, 2002; Matsumoto *et al.*, 2003). In addition, some studies have focused on myelin basic protein (MBP)-specific T lymphocytes, showing a preferential use of certain Vβ genes (Wucherpfennig *et al.*, 1990; Kotzin *et al.*, 1991; Oksenberg *et al.*, 1993). However, these latter studies were performed following *in vitro* T-cell clone stimulation with myelin peptides, a procedure that may select and promote the growth of low frequency committed T cells and therefore may not reflect the actual autoreactive T-cell pool size involved.

Recently, we developed an approach which integrates both the analysis of CDR3-LD alterations (Immunoscope®/spectratype) and quantitative real-time PCR-based measurements of Vβ/hypoxanthine phosphoribosyl transferase (HPRT) transcript ratios, for all the possible CDR3 lengths. The data are displayed as a global 'T-cell landscape' of the whole β chain transcriptome (Sebille *et al.*, 2001; Guillet *et al.*, 2002). In this study, we used this approach to investigate the Vβ CDR3-LD and, more specifically, to estimate the magnitude of the skewed T-cell repertoire at various stages of the disease, including at the appearance of the first clinical symptoms. Thirty-five patients suffering from different stages of multiple sclerosis [patients with a high risk of multiple sclerosis (HRMS), patients with clinically definite multiple sclerosis (CDMS) or patients with a relapsing–remitting worsening and active multiple sclerosis (WMS)] and 13 healthy individuals (HI) were compared. Our data show that there is a detectable T-cell immune component in the blood of multiple sclerosis patients at all stages of the disease. The blood T-cell CDR3-LD alterations concerned the CD4- and CD8-positive cells but were prominent in the $CD8^+$ fraction, involving numerous β chain families in CDMS and WMS compared with patients with HRMS. In addition, the T cells from multiple sclerosis patients with a selected TCR accumulated more proinflammatory cytokine transcripts than their normal HI counterparts. Furthermore, using an interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay, we show that most of the T cells from altered Vβ families of the three groups of patients respond to human MBP, suggesting their possible role in the disease process.

Material and methods

Patients

Thirty-five patients divided into two groups were studied (their clinical characteristics are summarized in Table 1). Group I was composed of 22 patients at the onset of the disease. This group was divided into two subgroups. The HRMS group (n = 12) was composed of patients presenting a first demyelinating event clinically well defined and confirmed by neurological or ophthalmological examination and the presence of at least three Barkhof's criteria on a spinal or cerebral MRI (Barkhof *et al.*, 1997). Patients in this group were referred to as HRMS patients (CHAMPS Study Group, 2002). The CDMS group (n = 10) was composed of patients undergoing a second or third relapse, reaching a clinically definite multiple sclerosis according to Poser's criteria (Poser *et al.*, 1983). Blood from group I patients was collected at the time of a relapse. None of the patients were under immunosuppressive or immunoregulatory drug treatment at the time of or before the study. All patients were interviewed to confirm the absence of infectious illness or other autoimmune diseases, and all had a blood cell count within the normal range. Group II or the WMS group (n = 13) was composed of patients with a clinically definite relapsing–remitting multiple sclerosis (according to Poser's criteria) considered as requiring an immunosuppressive treatment with mitoxantrone (Edan *et al.*, 1997). Patients from the WMS group were referred to as patients with a worsening and active multiple sclerosis. The criteria for mitoxantrone treatment were the loss of at least 1 point on the Expanded Disability Status Score during the previous 6 months and/or the occurrence of several relapses despite treatment compliance and the presence of at least one gadolinium-enhanced T1 lesion according to MRI. None of the patients suffered from other detectable autoimmune, inflammatory or infectious diseases. Results from laboratory tests performed at the time of blood sampling were within the normal ranges. All WMS group patients had undergone an immunomodulatory treatment that had been stopped at least 1 month before testing. Histocompatibility leukocyte antigen (HLA)-DR typing was performed for 32 patients. HLA class I typing was available for 14 patients.

HI group

Blood from 13 HI (mean age: 31.8, eight females, five males), who had been interviewed previously to rule out autoimmune or inflammatory disease, was taken for comparison. All multiple sclerosis patients and HI gave their informed consent for this study according to French legislation.

Table 1 *Main clinical characteristics of the patients*

	Age (years)	Gender	Gadolinium* lesions	HLA	Disease duration (years)	Immunosuppressive drugs
HRMS1	21	F	0	DR2–DR4		
HRMS2	42	F	1	A3–A11–B44–B51–DR2–DR5		
HRMS3	29	F	9	DR1–DR6		
HRMS4	41	M	0	DR1–DR7		
HRMS5	30	F	1	NA		
HRMS6	35	M	0	DR2–DR4		
HRMS7	22	F	11	DR5–DR6		
HRMS8	41	F	2	A2–A3–B27–B51–DR1–DR8		
HRMS9	20	M	9	DR5–DR6		
HRMS10	24	M	0	NA		
HRMS11	31	M	0	DR4–DR7		
HRMS12	19	F	9	DR6–DR8		
Mean	29.5					
CDMS1	30	M	2	A1–A31–B44–B40–DR2–DR4	1	
CDMS2	46	F	3	NA	6 months	
CDMS3	34	F	2	A2–A3–B7–B27–DR1–DR3	7	
CDMS4	27	F	1	A2–A23–B15–B43–DR2	1	
CDMS5	37	M	3	DR2–DR4	2	
CDMS6	48	F	1	DR2	4	
CDMS7	38	F	0	A1–A3–B8–B1402–DR3–DR6	NA	
CDMS8	19	F	15	DR2–DR5	3 months	
CDMS9	39	M	0	A3–B7–B8–DR2–DR3	1	
CDMS10	38	M	0	DR4–DR5	1.5	
Mean	35.6					
WMS1	22	F	8	DR2–DR4	5	–
WMS2	36	F	8	A2–A23–B13–B15–DR4–DR5	10	Azathioprine
WMS3	30	F	3	A1–A2–B8–B18–DR3–DR4	3	–
WMS4	41	F	1	DR3	5	IFN β
WMS5	32	F	1	A1–A2–B37–B51–DR5–DR6	3	–
WMS6	38	F	1	DR2–DR7	13	IFN β
WMS7	32	F	1	A3–A11–B37–B51–DR2–DR4	1	–
WMS8	32	F	1	A1–A29–B8–B56–DR3–DR8	3	IFN β
WMS9	27	M	1	DR3–DR6	2	IFN β
WMS10	33	M	2	DR5–DR6	3	–
WMS11	44	F	1	A2–A3–B7–B27–DR2–DR4	5	IFN β
WMS12	46	F	2	A2–A28–B15–B37–DR4–DR6	3	IFN β
WMS13	32	F	8	DR4–DR6	7	Azathioprine
Mean	34.2					

F = female; M = male; NA = not available.

Blood harvesting, RNA extraction and cDNA synthesis

A 100 ml aliquot of blood was collected by venopuncture. Peripheral blood lymphocytes (PBLs) were recovered after a Ficoll gradient (Eurobio, Les Ulis, France). After washing, 2×10^7 cells were frozen in Trizol® reagent (Invitrogen™, Life Technologies, CA) for RNA extraction according to the manufacturer's instructions. The rest of the cells were frozen at −80°C in AB serum containing 7.5% dimethylsulfoxide. One day later, cells were transferred to a liquid nitrogen tank. The RNA concentration for each sample was determined by optical density measurement, and a quality control on a 1% agarose gel was performed. A 2 µg aliquot of RNA was reverse transcribed using an Invitrogen cDNA synthesis kit (Boeringher Mannheim, Indianapolis, IN) and diluted to a final volume of 100 µl.

TCR repertoire analysis

cDNA was amplified by PCR using a Cβ primer and one of the 26 Vβ-specific primers. The amplifications were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, Foster City, CA) as previously described (Gagne *et al*., 2000). Analysis of CDR3-LD was performed using Immunoscope® software (Pannetier *et al*., 1995; Douillard *et al*., 1996; Brouard *et al*., 1999). The percentage of CDR3-LD alteration for each Vβ family and a global percentage of CDR3-LD alteration for each individual or each group was obtained as described in Gorochov *et al*. (1998). Briefly, the percentage of alteration was defined as the difference between the frequency of each CDR3 length in the distribution profile of the Vβ family studied and the control distribution, calculated from the 13 age- and sex-matched HI. The global CDR3-LD alteration is represented as a Topview TcLandscape® (see below) enabling an easy appraisal of

the 'qualitative' measurement of the CDR3-LD bias. Only CDR3 lengths with an alteration >25% were taken into account. The level of Vβ RNA was measured by real-time quantitative PCR and expressed as a ratio of a non- or minimally regulated gene, the HPRT gene, in order to normalize the values, and the primers used were especially designed for quantitative PCR as previously described (Gagne *et al*., 2000). The data were displayed as a three-dimensional TcLandscape® (Guillet *et al*., 2001; Sebille *et al*., 2001). Percentages of CDR3-LD alterations are represented as a colour code, from deep blue (–30%) to dark red (+30%). The *x*-axis displays the 26 human Vβ families, the *y*-axis gives the Vβ/HPRT ratios, and the *z*-axis gives the CDR3 lengths. The colour code is the same for the three-dimensional TcLandscape® and the corresponding Topview.

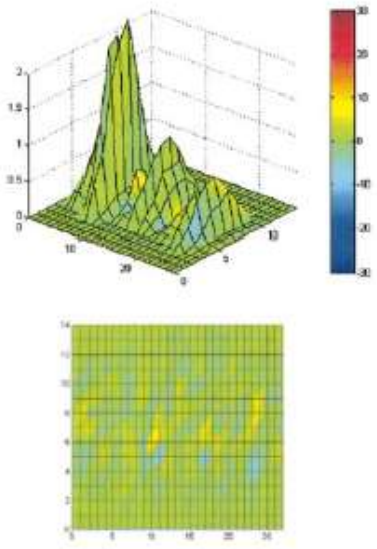
Cytokine transcript quantification

Cytokine transcript measurement was performed on RNA from sorted Vβ families. The cells were sorted using phycoerythrin-coupled Vβ monoclonal antibodies (Immunotech, Marseille, France) on a FACSvantage (Becton-Dickinson, Mountain View, CA). The purity of sorted cells was >95%. RNA was extracted as described above and cDNA was obtained using a Boehringer SMART kit (Boehringer, IN) according to the manufacturer's recommendations. Briefly, RNA samples were mixed with cDNA synthesis primer and SMART II oligonucleotide, incubated at 72°C for 2 min and chilled on ice. First-strand buffer, dithiothreitol, deoxynucleotide triphosphate (dNTP) and PowerScript Reverse Transcriptase were then added and the tubes incubated at 42°C for 1 h, before being put on ice. A non-specific PCR was then performed, allowing a linear amplification. Pure water, Advantage 2 PCR buffer, dNTP, PCR primers and Advantage Polymerase Mix were added to the first strand cDNA obtained previously. The tubes were then placed in a Perkin-Elmer 9600 automater, and thermal cycling was performed using the following program: 1 min at 95°C, *n* cycles with 5 s at 95°C, 5 s at 65°C, and 6 min at 68°C (the number of cycles was determined by the quantity of RNA as assessed by optical density measurement). Real-time quantitative PCR was performed subsequently using interleukin (IL)-2, IFN-γ, tumour necrosis factor-α (TNF-α), IL-10, IL-13 and IL-2-Rα (CD25) chain primers (IL-2, 5′-AAACACAGCTACAACTGGAGCA-3′ and 3′-GCTGATTAAGTCCCTGGGTCTT-5′; IFN-γ, 5′-TGTCCAACGCAAAGCAATACA-3′ and 3′-TTCGCTTCCCTGTTTTAGCTG-5′; TNF-α, 5′-TTAAGCAACAAGACCACCACT-3′ and 3′-TCAAGGAAGTCTGGAAACATCT-5′; IL-10, 5′-CTGCCTAACATGCTTCGAGATC-3′ and 3′-AACCCTTAAAGTCCTCCAGCAA-5′; IL-13, 5′-GGCAGCATGG TATGGAGCA-3′ and 3′-TTCAGTTGAACCGTCCCTCG-5′; IL-2-Rα, 5′-CAAGGGTCAGGAAGATGGATTC-3′ and 3′-CCAGGACGAGTGGCTAGAGTTT-5′) and normalized against HPRT transcript levels (Guillet *et al*., 2001).

CD4/CD8 T cell characterization

CD4+ or CD8+ T-cell selection was performed using MACS microbeads according to the manufacturer's recommendations (Miltenyi Biotec, Germany). The kit consists of an indirect magnetic labelling system composed of a hapten–monoclonal antibody cocktail (anti-CD8/anti-CD4, CD16, CD56, CD11b, CD19 and CD36) and iron microbeads coupled with an anti-hapten antibody which enables the magnetic depletion of non-T cells. The magnetic bead-labelled cells are depleted by passing the cells through a MACS column in the magnetic field of an autoMACS. Purity was >90%. The cells were mixed with Trizol® and the RNA extracted and retrotranscribed using the SMART procedure as explained above. The cDNA was then amplified and the CDR3-LD analysis performed as described above.

In order to assign a selected CDR3 length to a population of CD4+ or CD8+ cells, the Immunoscope profiles of unselected PBL, CD4+ and CD8+ fractions were compared. The percentage corresponding to the frequency of a given selected CDR3 length in a Vβ family was calculated and compared in the PBL, CD4+ and CD8+ fractions. The skewing was assigned to the CD8+ cells when the calculated percentage was higher for CD8 than for CD4+ cells.



HI #4

Fig. 1 TcLandscape® of blood T cells from one representative HI. The data are displayed for each individual as a three-dimensional TcLandscape® and as a Topview for easier assessment of global CDR3-LD alterations. The percentages of CDR3-LD alterations are represented as a colour code. The *x*-axis displays the 26 human Vβ families, the *y*-axis gives the Vβ/HPRT ratios, and the *z*-axis gives the 13 possible CDR3 lengths.

ELISPOT assay protocol

T cells from seven Vβ families with an altered CDR3 length were sorted from six patients (HRMS4, Vβ 17; HRMS7, Vβ 3; HRMS12, Vβ 8 and Vβ 14; CDMS1, Vβ 23; WMS7, Vβ 3; and WMS8, Vβ 4), and three Vβ families with an altered CDR3 length were sorted from two HI (HI9, Vβ 21 and Vβ 22; and HI10, Vβ 2) and studied for human MBP reactivity. Additionnally, three other Vβ families with a Gaussian-like CDR3-LD were sorted from three multiple sclerosis patients (WMS2, Vβ 8, WMS12, Vβ 3; and HRMS10, Vβ 23) and also studied for MBP reactivity, in the same conditions. Peripheral blood mononuclear cells (PBMCs) were thawed and washed in phosphate-buffered saline. T cells with a Vβ family with an altered CDR3 length were sorted using the corresponding phycoerythrin-

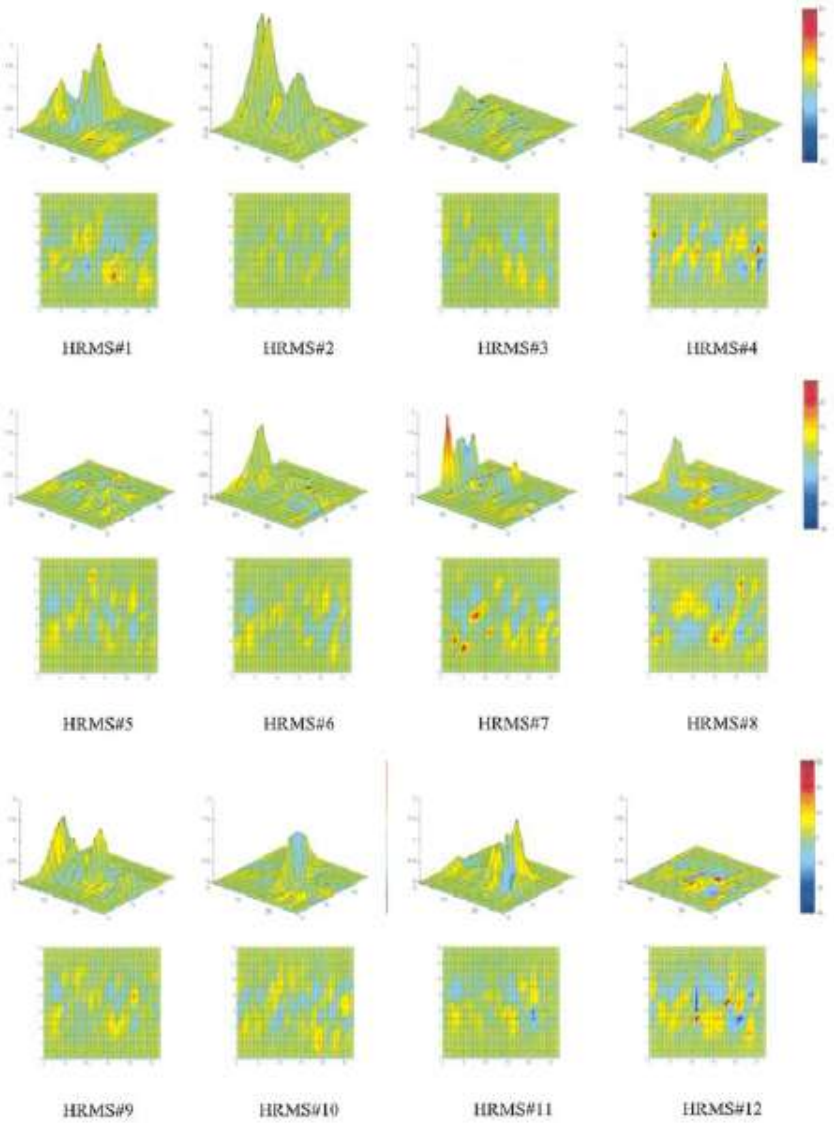


Fig. 2 TcLandscape® and Topview representation of blood T cells from the HRMS group. CDR3 length alterations >25% appear in red.

labelled anti-Vβ antibody and anti-phycoerythrin microbeads in an autoMACS (Miltenyi Biotech, Germany). The purity of the sorted Vβ families was measured by flow cytometry and was >75%. The remaining fraction of the PBMCs was irradiated for 315 s at 15 Gy and they were used as autologous antigen-presenting cells (APCs). First, human MBP (Sigma, France) at a concentration of 20 µg/ml, or human albumin (Sigma, France) at the same concentration was mixed with irradiated PBMCs. Next, 4×10^4 PBMCs were added to a 96-well anti-IFN-γ-coated ELISPOT plate (AID, Germany) and 4×10^4 of the T cells from sorted Vβ families were added to the plate. A control with irradiated PBMCs alone at the same concentration was performed systematically. The cells were incubated for 24 h at 37°C in 5% CO_2 and washed three times with washing buffer according to the manufacturer's instructions. A secondary biotinylated anti-IFN-γ antibody was then added at 100 µl/well for 2.5 h. Plates were washed three times with buffer, and streptavidin–horseradish peroxidase was added for 2 h at room temperature. Plates were washed in buffer and spot colour was developed for a maximum of 1 h by adding 3-amino-9-ethylcarbazol substrate diluted in acetate buffer containing H_2O_2. Plates were then washed with distilled water to stop the reaction. After drying, images of the wells were acquired using the AID ELISPOT software. All the

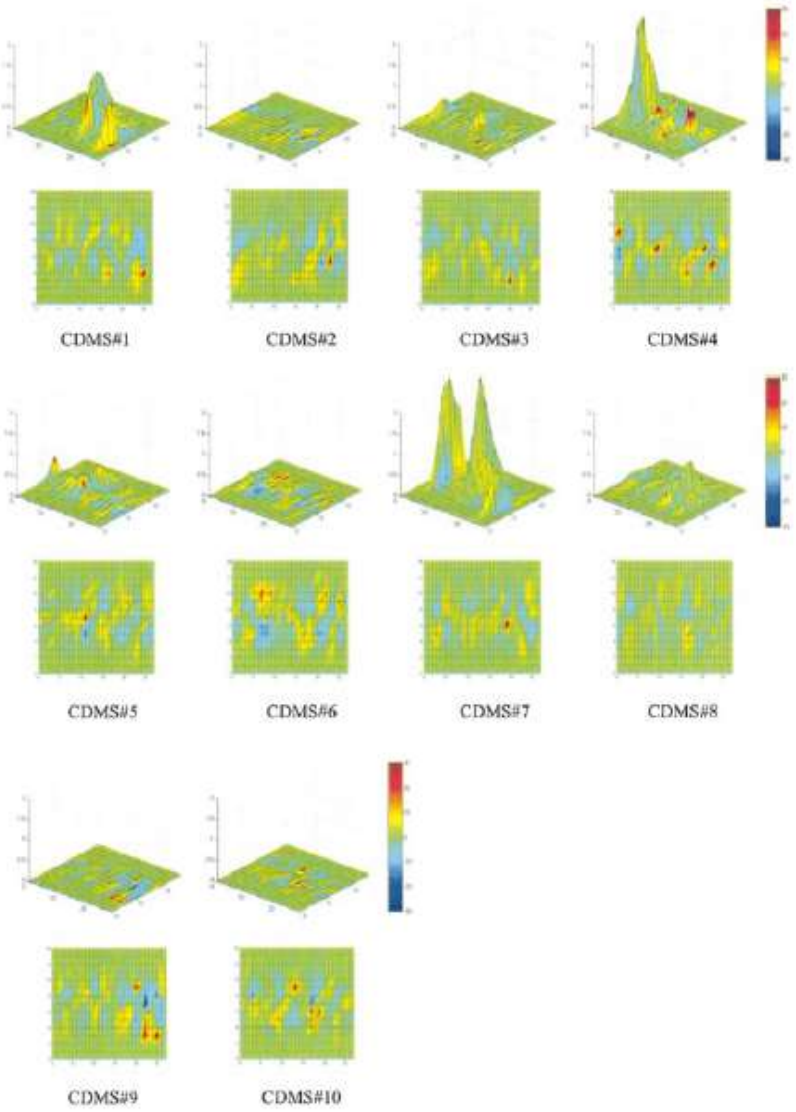


Fig. 3 TcLandscape® and Topview representation of blood T cells from the CDMS group. CDR3 length alterations >25% appear in red.

experiments were run at least in duplicate or triplicate depending on the number of cells available. The results were expressed as means of the du(tri)plicates.

Statistical analysis

A χ^2 test, a Kruskal–Wallis test and a Dunn's multiple comparison test were performed between each group of multiple sclerosis patients (HRMS, CDMS and WMS) and HI for comparison of the Vβ/HPRT transcript ratios and the global CDR3-LD profiles. A Kruskal–Wallis test was performed on cytokine transcript values. A Mann–Whitney test was performed for the different ELISPOT assays. Differences were defined as statistically significant when $P < 0.05$.

Results

HI display few Vβ families with significant CDR3-LD alterations with low Vβ/HPRT transcript ratios

Figure 1 shows one representative example of the CDR3-LD profiles from the HI group. The TcLandscape pattern of every normal individual tested is available online

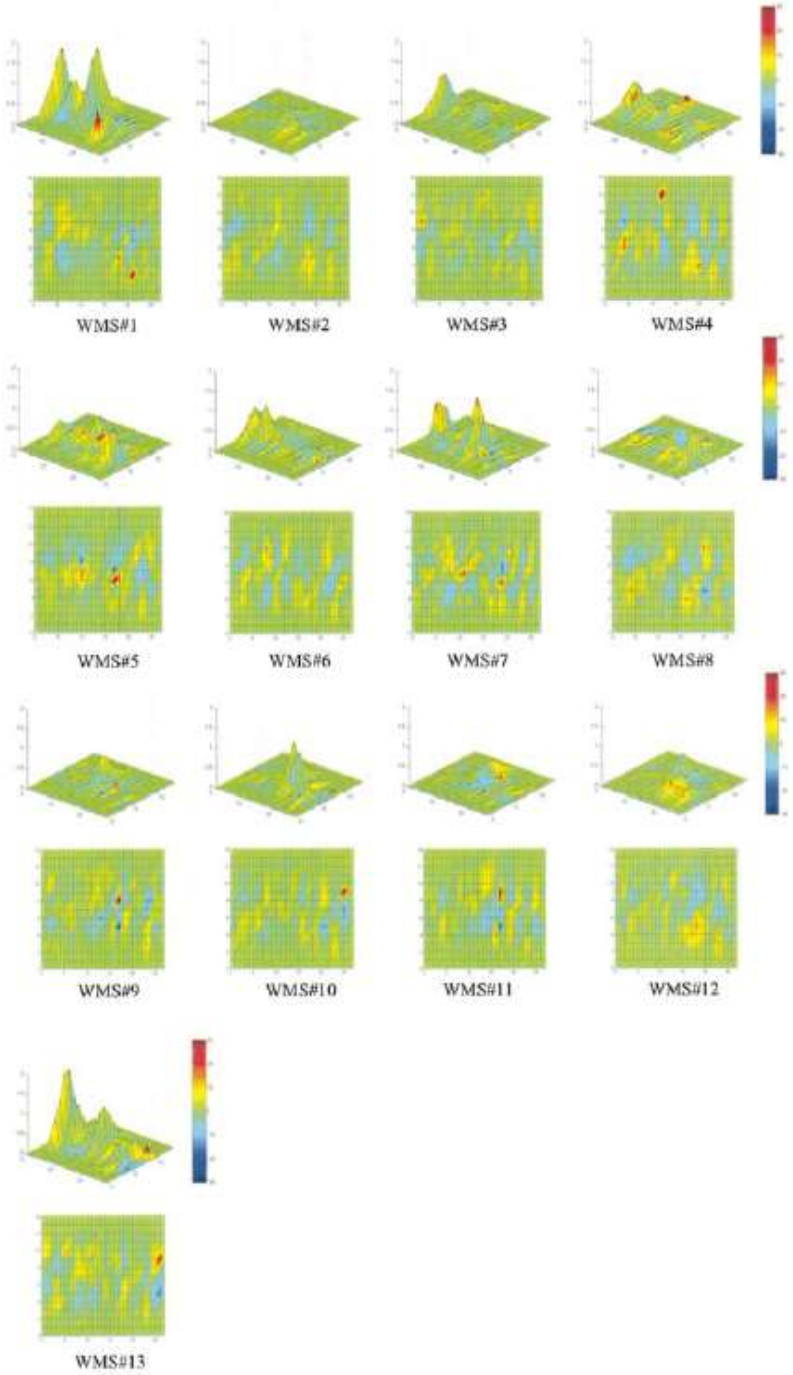


Fig. 4 TcLandscape® and Topview representation of blood T cells from the WMS group. CDR3 length alterations >25% appear in red.

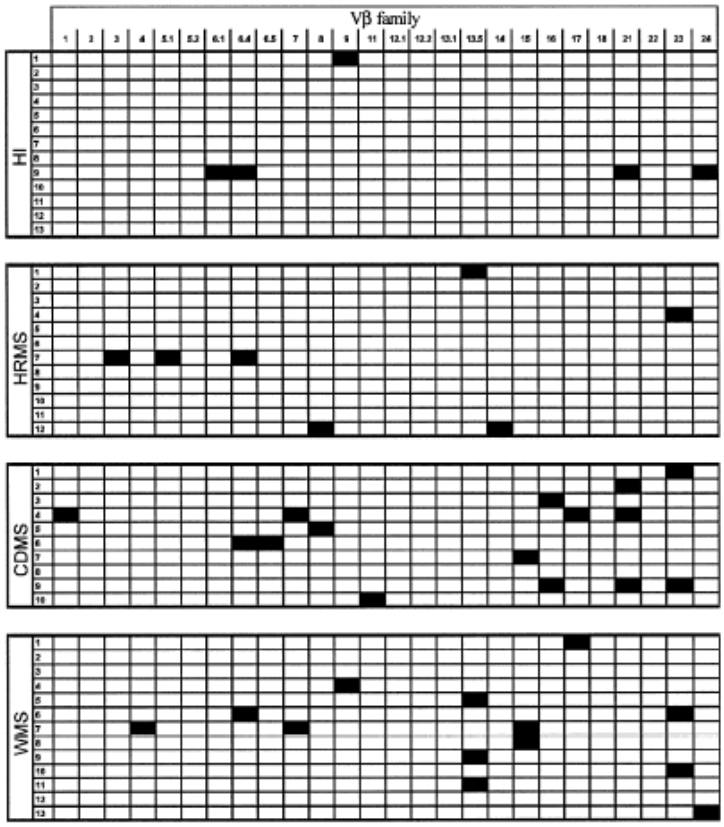


Fig. 5 CDR3 length alterations for all the patients and HI. CDR3 length alterations >25% appear in black.

(www.nantes.inserm.fr/u437/sitesconnexes.html). Only two of the 13 normal individuals expressed Vβ families with a significantly altered CDR3 length (>25%). All details concerning the alteration of Vβ families in the HI group are given in Fig. 5. Furthermore, the mean global percentage of CDR3-LD alteration in this group was 10.5 ± 1.9% (Fig. 6A). The mean Vβ/HPRT transcript ratio in the HI group was 2.3 ± 0.9. In addition, 67% of the Vβ families of HI had a Vβ/HPRT transcript ratio between 0 and 2, while 23% of the Vβ families had a ratio between 2 and 5, and 10% a ratio >5 (Fig. 6C).

HRMS patients express a significantly higher mean CDR3-LD alteration than HI

Figure 2 displays the CDR3-LD of the 12 HRMS patients. Four of the 12 patients displayed a CDR3 length with an alteration >25%. Details of the CDR3 length alterations are given in Fig. 5. Neither the number of patients with significantly altered Vβ (presence of a CDR3 length alteration >25%) nor the number of Vβ families with a significantly altered CDR3 length were different from the HI group. However, the mean percentage of CDR3-LD alterations (16.5 ± 2.9%, Fig. 6A) was significantly higher than that of the HI group (10.58 ± 1.9%, $P < 0.01$).

The blood T cells from CDMS patients display more extensive CDR3-LD alterations than those from HRMS patients and HI

Nine of the 10 CDMS patients displayed a CDR3 length with an alteration >25% (TcLandscapes® and Topviews, Fig. 3). Details of the Vβ families concerned are given in Fig. 5. The number of significant CDR3 length alterations in the CDMS group was significantly different from that of the HI and HRMS groups ($P < 0.01$). The number of patients displaying at least one altered CDR3 length in the CDMS group was also significantly different from the HI and HRMS groups ($P < 0.01$). In addition, the mean percentage of CDR3-LD

alterations (17.8 ± 1.6%, Fig. 6A) was significantly different from that observed in HI (10.58 ± 1.98%, $P < 0.001$) and in the HRMS group (16.5 ± 2.9%, $P < 0.03$).

The blood T cells from WMS patients display significantly more CDR3-LD alterations than those from HI and HRMS patients

Ten of the 13 WMS patients displayed CDR3 lengths with alterations >25% (TcLandscapes® and Topviews, Fig. 4). Details concerning the Vβ families with significant CDR3 length alterations are summarized in Fig. 5. Patients with at least one significantly altered CDR3 length (alteration >25%) were significantly more numerous than in the HI and HRMS groups ($P < 0.01$ and $P < 0.05$, respectively). Furthermore, the number of alterations >25% in the WMS group was significantly different from the HI group ($P < 0.05$). Additionally, the mean percentage of CDR3-LD alterations in WMS patients (15.5 ± 1.5%, Fig. 6A) was significantly higher than that of the HI group (10.58 ± 1.9%, $P < 0.01$) but not that of HRMS patients (16.5 ± 2.9%).

Vβ/HPRT transcript ratios

Vβ/HPRT transcript ratio data are an indirect reflection of the pool size of T-cell populations with different CDR3-LD. The mean Vβ/HPRT transcript ratios in the HRMS (0.94 ± 0.5), CDMS (0.6 ± 0.4) and WMS groups (0.7 ± 0.3) were significantly lower than those in the HI group (2.3 ± 0.9, $P < 0.001$, Fig. 6B). Furthermore, when Vβ/HPRT transcript ratios were compared according to a grading scale of 0–2, 2–5 and >5, their distribution was significantly different compared with the HI group (Fig. 6C, $P < 0.01$).

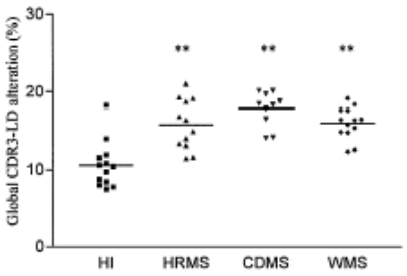
Vβ transcript CDR3-LD alterations according to HLA class I and class II typing and MRI activity

Distribution of CDR3-LD alterations according to HLA-DR typing was analysed in 32 patients, 13 of them being DR2 positive. Ten of these 13 patients displayed significant alterations versus 12 of the 18 negative for DR2 (NS). No public skewed CDR3-LD was observed in HLA-DR2 patients. No correlation between Vβ family alterations and HLA class I typing was found. Correlation of CDR3-LD alterations with T1 gadolinium-enhanced lesions was also studied. For the patients of group I, 14 exhibited at least one gadolinium-enhanced lesion. Eight of them exhibited at least an altered CDR3 length >25% versus four out of eight with no gadolinium-enhanced lesion (NS).

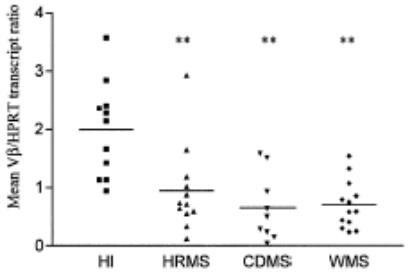
T cells from multiple sclerosis patients with altered CDR3-LD Vβ accumulate proinflammatory cytokine transcripts

In order to better understand the role played by the altered T cells identified by TcLandscape®, five Vβ families with highly altered CDR3-LD were sorted from three different patients (one patient per group: HRMS8, Vβ 3; CDMS4, Vβ7.1, Vβ7.2 and Vβ17; WMS1, Vβ17). These families were compared with sorted Vβ families with Gaussian-like CDR3-LD (HI10, Vβ 3 and Vβ 7; HI11, Vβ 17; HI12, Vβ 4 and Vβ 17; HI13, Vβ 1 and Vβ 16) and three Vβ families with altered CDR3-LD from HI (HI9 and HI12, Vβ 21, Vβ 22 and Vβ 13.1, respectively) for accumulation of different cytokine

(A)



(B)



(C)

Vβ/HPRT ratio	[0 ; 2[	[2 ; 5[	>5	p
HI	67.13%	22.37%	10.48%	-
HRMS	84.93%	10.89%	4.16%	<0.01
CDMS	92.30%	5.38%	2.30%	<0.01
WMS	90.23%	8.87%	0.88%	<0.01

Fig. 6 (A) Values of global CDR3-LD for each patient and HI. The mean value for each group is indicated by a bar. A Kruskal–Wallis test and a Dunn's multiple comparison test were performed, $^{**}P < 0.01$. (B) Distribution of the mean Vβ/HPRT transcript ratios in the patient groups and HI (each dot represents the mean Vβ/HPRT transcript ratio for each patient and HI). A Kruskal–Wallis test and a Dunn's multiple comparison test were performed to compare values. A bar indicates the mean value for each group. $^{**}P < 0.01$. (C) Distribution of the Vβ/HPRT transcript ratios between the groups according to a grading scale of 0–2, 2–5 and >5. A χ^2 test was performed to compare the distribution between the groups.

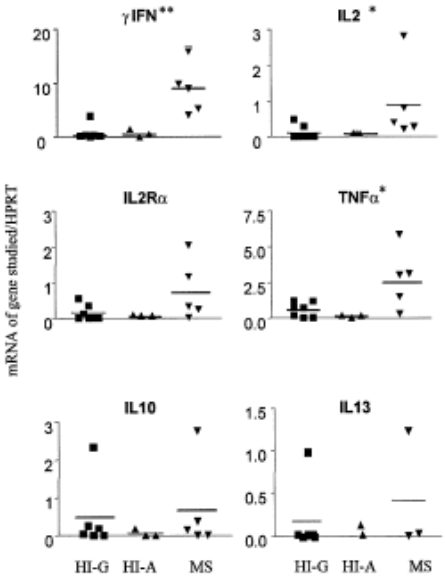


Fig. 7 Cytokine/HPRT transcript ratios from sorted T cells. Cytokine mRNA was studied for T cells sorted from families with CDR3 length alteration >25% in multiple sclerosis patients compared with T cells sorted from Vβ families with a Gaussian-like pattern (HI-G) or an altered CDR3 length (HI-A) from healthy individuals. The median range is indicated for each group. Kruskal–Wallis test, $*P < 0.05$, $**P < 0.01$.

transcripts. The data are summarized in Fig. 7. The mean IFN-γ and IL-2 mRNA levels in sorted Vβ families with altered CDR3-LD from multiple sclerosis patients was significantly higher than for families with a Gaussian-like pattern or altered CDR3-LD in the HI group ($P < 0.01$ and $P < 0.05$, respectively). TNF-α mRNA was also significantly accumulated in the multiple sclerosis group, compared with the HI group ($P < 0.05$). IL-10, IL-13 and IL-2-Rα chain mRNA transcripts were not significantly different between the three groups. Despite the fact that the number of patients analysed was low, these data suggest the presence of T cells with highly altered CDR3-LD able to produce proinflammatory cytokines in the blood of multiple sclerosis patients.

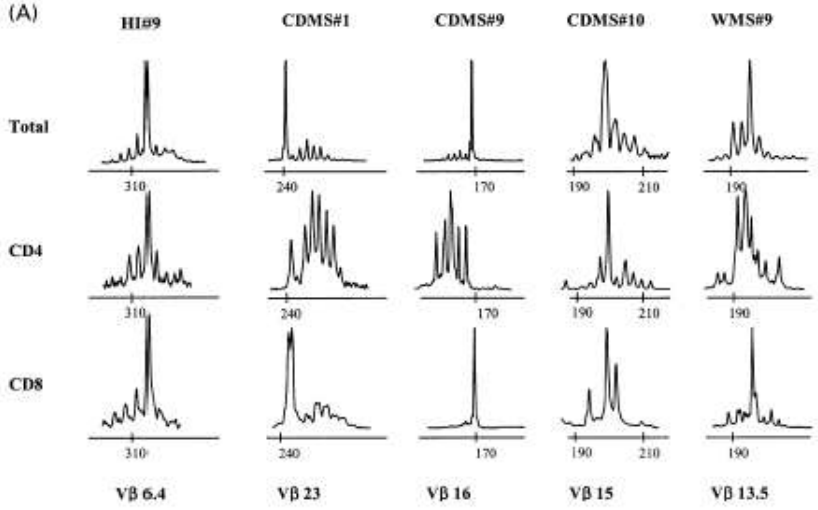
The alterations of CDR3-LD are more prominent in CD8⁺ T cells

Sorted CD4⁺ and CD8⁺ T cells from nine patients (16 Vβ families) and two HI (six Vβ families) were studied and their immunoscope profile compared with that of unselected PBLs (Fig. 8A and B). In some cases, the CD4 or CD8 nature of a selected CDR3 length was obvious (Fig. 8A, examples CDMS1 and CDMS9). In other cases (Fig. 8B: HRMS12, Vβ 14; CDMS4, Vβ 15, Vβ 17 and Vβ 21; WMS7, Vβ 7; WMS9, WMS10 and HI9, Vβ 6.4), the correspondence of the immunoscope profiles of total PBLs with that of the fraction studied (CD4 or CD8) suggested that the selected CDR3 length in question belonged to this T-cell subpopulation, which was corroborated by the comparison of the frequency of the prominent CDR3 length in the three cellular fractions. Finally, in other Vβ families (Fig 8B: CDMS4, Vβ 7; CDMS7; CDMS10; WMS7, Vβ 4 and Vβ 15; HI9, Vβ 21, Vβ 22 and Vβ 24; HI7, Vβ8 and Vβ 24), no clear assignment to CD4⁺ or CD8⁺ cells was possible. Using this approach, 11 of the 16 Vβ families from multiple sclerosis patients and one of the six Vβ families from HI with an altered CDR3 length were found to have a more prominent skewing in the CD8⁺ T cells, suggesting a more pronounced bias in the CD8⁺ repertoire in multiple sclerosis than in HI.

Sorted T cells from Vβ families with an altered CDR3-LD from multiple sclerosis patients produce IFN-γ in the presence of human MBP

IFN-γ ELISPOT assays were performed with T cells from Vβ families with altered CDR3–LD from six multiple sclerosis patients (three from the HRMS group, one from the CDMS group and two from the WMS group) and two HI. In addition, because the analysis of Vβ families with Gaussian-like CDR3-LD from patients already studied for their skewed Vβ family was technically impossible (due to an insufficient quantity of cells), three Vβ families with Gaussian-like CDR3-LD were studied from three other patients. Only background ELISPOT reactivity (<10 spots) was detected when irradiated PBMCs (used as APCs) were tested (data not shown). Figure 9A shows the ELISPOT score obtained when human MBP was added to a culture of purified sorted T cells and irradiated PBMCs used as APCs (black boxes). For comparison, the same cultures were tested with human albumin (white boxes). In addition, the irradiated PBMCs alone were also stimulated by human MBP (grey boxes). The figure shows that purified T cells from multiple sclerosis patients were highly reactive in response to human MBP ($P < 0.01$ versus human albumin). Some reactivity was also observed in the irradiated PBMC fraction when human MBP was present in the culture. However, the sorted T-cell response was much higher than that of the PBMCs alone in four out of seven cases. Importantly, Fig. 9B shows that sorted T cells from the HI counterparts cultured in the same conditions with syngenic PBMCs were not reactive to human MBP compared with altered Vβ families from multiple sclerosis patients ($P < 0.05$). Furthermore, the number of IFN-γ spots obtained with the Gaussian Vβ families from the three additional patients was as low as for the altered Vβ families from HI (mean number of spots: 2.3 ± 1), suggesting a specific response of the skewed Vβ families from multiple sclerosis patients.

(A)



(B)

Patient	Altered Vβ studied	Percentage of the selected CDR3 in CD4+CD8	CD4	CD8	CD4/CD8
HI#7	Vβ 8	52	**50**	**49**	**Both**
	Vβ 24	41	**36**	**38**	**Both**
HI#9	Vβ 6.4	56	41	**57**	**CD8>CD4**
	Vβ 21	31	**30**	**32**	**Both**
	Vβ 22	42	**43**	**40**	**Both**
	Vβ 24	36	**33**	**35**	**Both**
HRMS#12	Vβ 8	100	45	**100**	**CD8>CD4**
	Vβ 14	57	17	**60**	**CD8>CD4**
CDMS#1	Vβ 23	53	12	**54**	**CD8>CD4**
CDMS#4	Vβ 7	61	**42**	**38**	**Both**
	Vβ 15	41	28	**43**	**CD8>CD4**
	Vβ 17	54	37	**55**	**CD8>CD4**
	Vβ 21	50	32	**45**	**CD8>CD4**
CDMS#7	Vβ 15	49	**50**	**48**	**Both**
CDMS#9	Vβ 16	75	15	**91**	**CD8**
	Vβ 23	85	0	**60**	**CD8**
CDMS#10	Vβ 15	41	**29**	**32**	**Both**
WMS#7	Vβ 4	44	**41**	**45**	**Both**
	Vβ 7	41	16	**33**	**CD8>CD4**
	Vβ 15	40	**25**	**27**	**Both**
WMS#9	Vβ 13.5	46	**34**	**57**	**CD8>CD4**
WMS#10	Vβ 23	68	0	**100**	**CD8**

Fig. 8 CD4/CD8 phenotype of blood T cells from Vβ families with an altered CDR3 length. (A) Examples from Vβ families with an altered CDR3 length. The phenotype was obtained by comparison of the immunoscope profile of each population (CD4, CD8 and unselected PBLs). (B) Summary of the CD4/CD8 pattern of the Vβ families studied. CD8>CD4 means that the $CD8^+$ fraction of T cells is more represented in the selected CDR3 length than the $CD4^+$ fraction (see Material and methods). The percentage indicated represents the frequency of the more prominent CDR3 length in the Vβ family studied. The comparison of the percentages for the same CDR3 length in the different cell fractions (PBL, CD4 and CD8) indicates the fraction that is responsible for the skewing.

Discussion

In this study, we analysed the TCR Vβ chain transcriptome both qualitatively (alteration of CDR3-LD) and quantitatively (amount of transcripts concerned) in three groups of patients with relapsing–remitting multiple sclerosis at various stages of their disease compared with age-matched HI. Despite the fact that our study only concerned Vβ biases (analysis of Vα chain patterns may provide further information), significant T-cell repertoire alterations, which were more prominent in $CD8^+$ T cells and mostly concerned Vβ families reactive to

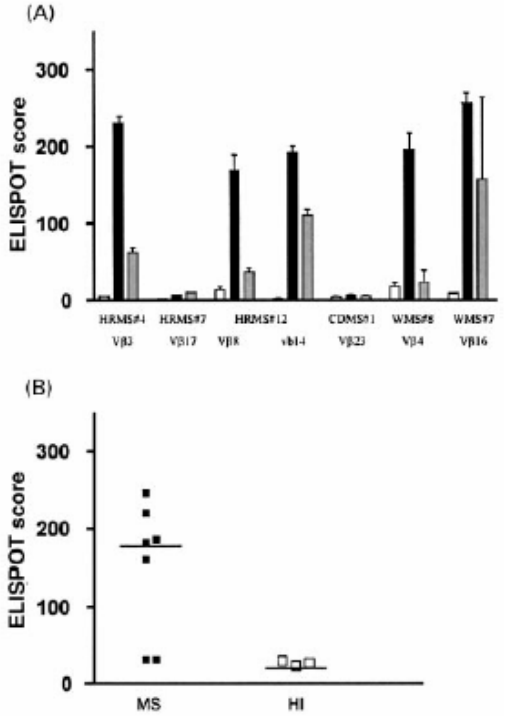


Fig. 9 IFN-γ ELISPOT in T cells sorted from Vβ families with an altered CDR3 length. (**A**) ELISPOT score of irradiated PBMCs (APCs) in multiple sclerosis patients (grey boxes) and irradiated PBMCs with T cells from sorted CDR3-LD-altered Vβ families in the same multiple sclerosis patients (black boxes) in the presence of human MBP. White boxes represent the control score obtained with PBMCs and sorted T cells from the same multiple sclerosis patients in the presence of human albumin. (**B**) Comparison of reactivity for human MBP in irradiated PBMCs with T cells sorted from CDR3-LD-altered Vβ families (black squares) or HI (white squares). Mann–Whitney test, $P < 0.01$.

human MBP, can be detected in the blood of these patients from the onset of the disease [occurrence of first clinical symptoms (HRMS)]. Furthermore, we show that sorted, circulating T cells from families with altered CDR3-LD are characterized by a transcriptional pattern with significant accumulations of IFN-γ, IL-2 and TNF-α mRNA, without any further *in vitro* stimulation. Our data provide new information that supports the concept of early peripheral T-cell activation in multiple sclerosis. Our results also suggest that multiple sclerosis patients could benefit from immunoregulatory treatment early in the course of their disease.

TCR biases in patients with multiple sclerosis have been reported by several groups in the past (Kotzin *et al*., 1991; Oksenberg *et al*., 1993; Musette *et al*., 1996; Gran *et al*., 1998; Lozeron *et al*., 1998; Muraro *et al*., 2002; Matsumoto *et al*., 2003). However, this study is the first to analyse additionally a cohort of patients identified as HRMS patients. Although healthy age-matched individuals also exhibited some level of CDR3-LD alterations (~10%), HRMS patients displayed highly significant global CDR3-LD alterations (~16%). However, the Vβ/HPRT transcript ratios did not suggest blood accumulation of the T-cell populations using the altered CDR3 Vβ transcript species. This apparent lack of peripheral accumulation may be related to a continuous flux of these selected T cells toward other compartments including the CNS. Such a flux has been suggested recently by the effect of natalizumab, a monoclonal antibody inhibiting T-cell homing into the CNS compartment through endothelial cells (Miller *et al*., 2003).

Significant global CDR3-LD alteration at a stage where the disease is in fact only suspected suggests that strategies aimed at inhibiting activated T-cell transfer to the CNS or at regulating activation of selected T cells could be useful in the very early stage of the disease. One study has analysed patients at the onset of a confirmed disease (CDMS) (Musette *et al*., 1996). However, only a partial exploration of the T-cell repertoire was performed, with only a few Vβ families analysed. This latter study of patients with confirmed disease (i.e. at a later stage than HRMS) was performed on Vβ 5 and Vβ 17 and also reported CDR3-LD alterations. Our data confirm and extend these initial observations by showing that most of the patients at the CDMS stage have both significantly more families with altered CDR3-LD and a more altered global transcriptome (allowing a statistical comparison) than patients at the HRMS stage (see Figs 5 and 6A). The fact that 11 of the 26 Vβ families exhibited selected CDR3-LD at this stage of the disease suggests early epitope spreading. Spreading of T-cell responses against different epitopes has been well characterized in experimental models of multiple sclerosis (Vanderlugt and Miller, 2002). In humans, Tuohy *et al*. (1999) have also described a serial analysis showing the decreasing frequency of clones directed against a single epitope during the progression of the disease from a monosymptomatic demyelinating syndrome (a group of patients similar to our HRMS group) to the stage of CDMS and the appearance of new clones directed against other epitopes of the same molecule or of other myelin proteins.

It is known that the CD8 T-cell repertoire in healthy subjects is more altered than the CD4 repertoire (Gorochov *et al*., 1998). In this study, only six altered Vβ families from two HI were observed and only one alteration clearly belonged to the $CD8^+$ cell fraction. To our knowledge, the CD4/CD8 distribution of selected T cells in the blood of multiple sclerosis patients has rarely been investigated (Monteiro *et al*., 1996). The reason for a prominent bias in $CD8^+$ T cells in multiple sclerosis may be due to a response against viruses since it has been shown that the relapses of the disease are favoured by infections (Edwards *et al*., 1998; Buljevac *et al*., 2002). However, this explanation is not likely because patients with a recent or ongoing clinical infection were not enrolled in the study. Furthermore, the patients from the WMS group were not included at the moment of a relapse. Five patients of the CDMS group were able to be analysed

more closely for the phenotype of the T cells of Vβ families with altered CDR3-LD, and six out of the nine Vβ families analysed turned out to correspond in the majority to CD8+ cells (see Fig. 8A and B). The fact that the altered CDR3 length species represent a large majority of the T cells of these families (as assessed from the area under the curve of the immunoscope pattern of the amplified CDR3 segments) strongly suggests that these selected clones belong more to the CD8 than the CD4 phenotype. These observations are in agreement with the findings of Battistini *et al.* (2003) who have reported that circulating CD8+ T cells from multiple sclerosis patients express adhesion molecules allowing them to cross the blood–brain barrier and may explain the significant CD8+ T-cell infiltrate seen in CNS lesions of multiple sclerosis (Booss *et al.*, 1983; Gay *et al.*, 1997). Moreover, these data suggest that, even if HLA class II-restricted CD4+ cells play a role in disease susceptibility (Haines *et al.*, 1998), spreading involving different class I restricted effectors occurs rapidly throughout the course of the disease, possibly by a phenomenon of cross-presentation (for a review see Carbone *et al.*, 1998). In this respect, these data also suggest that besides class II tetramers (Reddy *et al.*, 2003), class I tetramers may be a pertinent tool to test the blood T cells of multiple sclerosis patients. Interestingly, the only patient analysed at the HRMS stage also displayed alterations of CD8+ T cells (Fig. 8A and B). The concept that CD8+ T cells may be important in multiple sclerosis has been suggested by the possibility of inducing autoreactive CD8+ T cells for myelin peptides (Tsuchida *et al.*, 1994). Furthermore, the role of CD8 T cells has been highlighted recently by the findings that the majority of cells with clonal expansions and memory phenotype in the CSF and brain lesions of patients with multiple sclerosis were also CD8+ T cells (Babbe *et al.*, 2000; Jacobsen *et al.*, 2002). These data and our own showing circulating CD8+ CDR3-LD selected T cells in the blood of multiple sclerosis patients support the possibility that CD8+ cytotoxic T lymphocytes could damage class I-expressing brain cells including oligodendrocytes and neurons (Medana *et al.*, 2001; Liblau *et al.*, 2002; Neumann *et al.*, 2002). Our observations also suggest a potential use of selected anti-Vβ antibodies in patients exhibiting expansions of circulating T cells with altered CDR3-LD as an alternative therapy.

This trend of CD8+ selection was also found in two out of five Vβ families with altered CDR3-LD in the WMS group. However, these patients, who had been defined as presenting an exacerbated disease (see Material and methods; all of them were included in a mitoxantrone regimen and complied with the usual criteria for worsening disease), exhibited fewer alterations of their Vβ transcriptome than CDMS patients, despite still being significantly different from age-matched HI. Furthermore, these patients did not have more Vβ/HPRT transcript ratios with altered CDR3-LD in their blood than normal individuals. Whether this lack of more severe global Vβ transcriptome alterations in this group of patients with active disease is related to the fact that the blood sampling was not concomitant with disease exacerbation, that most of these patients (as opposed to those of other groups) had previously received immunologically oriented treatments or that an exacerbated flux of activated circulating T cells into the CNS occurred is unknown.

We were able to analyse three patients further (one from each of the clinical groups) for their transcriptional profile (real-time PCR) of the major Th1/Th2-related cytokines as well as TNF-α. This study was performed immediately following cell sorting using anti-Vβ antibodies, without *in vitro* stimulation to avoid artefactual selection. Interestingly, despite the low number of Vβ families studied, the transcriptional pattern of T cells from altered families from multiple sclerosis patients differed significantly from those observed in families with either biased or Gaussian CDR3-LD from normal individuals, reinforcing the idea that these expanding peripheral T cells may be involved in the disease process. Furthermore, IFN-γ ELISPOT assays were performed to test T cells from Vβ families with altered CDR3 lengths in each group of patients and HI. There was a striking difference in response to human MBP between T cells from Vβ families with altered CDR3-LD in multiple sclerosis patients and those from the controls. The T cells from more than half of such Vβ families from multiple sclerosis patients produced IFN-γ when stimulated with MBP, whereas none of the Vβ families tested from HI did so. The reactivity of these cells to human MBP is probably due to the CD4 fraction of these cells, since it is known that peripheral APCs may not be optimal for cross-presentation. The lack of response of three Vβ families from multiple sclerosis patients could be explained by a reactivity to other myelin or non-myelin antigen(s) or a production of cytokines other than the one tested.

These observations fit with other studies which also reported IFN-γ and/or TNF-α mobilization in this disease (Huang *et al.*, 1999; Pelfrey *et al.*, 2000; Tejada-Simon *et al.*, 2001) despite the fact that in two of these three studies, the patterns were analysed following *in vitro* stimulation with a putative multiple sclerosis antigen and that TNF-α/IFN-γ-producing CD8+ T-cell clones directed against myelin peptides were found (Tsuchida *et al.*, 1994).

Taken together, our data support the concept of circulating TCR-selected T cells in multiple sclerosis and that CD8+ T cells may be important in the pathophysiological processes of the disease. Our data also suggest that surveying the blood T-cell abnormalities in multiple sclerosis may be helpful for drug response studies, particularly when global Vβ transcriptome analysis is used.

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Article 4 : Increase in multiple sclerosis relapse rate following in-vitro fertilization.

Neurology, sous presse.

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Introduction

The immune and hormonal systems are known to be closely linked. In several animal models of immune diseases as well as in multiple sclerosis (MS), sex hormones have been shown to modulate clinical course (Confavreux et al., 1998; Voskuhl and Palaszynski, 2001). Some women followed for MS may suffer from infertility resulting in treatment for in vitro fertilization (IVF). In this regard, the type of drugs used, which modify hormonal status, might affect disease activity in MS patients.

Methods

By matching MS and IVF databases from the University Hospital of Nantes, France, we found four MS patients who had undergone six IVF cycles over the past ten years. All patients met the McDonald criteria for MS (McDonald et al., 2001).

Two additional patients who underwent four IVF cycles were also reported by their referring neurologist. One of them had a clinically isolated syndrome after IVF with the presence of all the Barkhof criteria on MRI. This patient developed a clinically definite MS over the course of the one year follow-up period.

Once the list of patients had been obtained, their clinical file was retrospectively studied to compare the number of MS relapses in a three-month period following IVF to a three-month period preceding IVF and to two other three-month periods one year before and one year after IVF. A patient was considered as having a relapse when it was specifically noted in the patient's file and that the neurological exam results were different to those previously reported.

The non parametric Wilcoxon and Friedman tests were used to compare the number of relapses in the different three-month periods. The results were considered significant when $p < 0.05$.

Results

All patients had a relapsing-remitting form of MS with an age at disease onset ranging from 19 to 29 years. For treated patients, disease modifying therapies were stopped at least one year before IVF. Two patients underwent three IVFs while the others had only one. The treatments used for IVF consisted of either LHRH agonists (in six cases) or LHRH antagonists (in four cases), both associated with recombinant-FSH. Two patients suffered a spontaneous miscarriage during the first trimester after IVF.

None of the patients suffered a relapse during the three months preceding IVF while the annualized relapse-rate (ARR) for the following three months was 2.4 +/- 2.8 (Wilcoxon test for paired samples, $p<0.04$).

A significant difference was also observed in the ARR after IVF (2.4 +/- 2.8) as compared to the ARR one year before IVF (0.5+/- 1.2) and one year after IVF (0.4 +/- 1.2, $p<0.05$, Friedman test, figure 1). The difference was still significant when the two additional patients were removed from the analysis.

The influence of the type of treatment used was also analyzed. All of the patients treated with LHRH agonists (four patients) suffered at least one MS relapse in the three months following IVF. Similarly, LHRH agonists were used six times and a relapse was noted in five cases while LHRH antagonists were used four times and no relapses were noted.

Discussion

The present study suggests a significant increase in the relapse rate after IVF, concerning only patients treated with LHRH agonists. To exclude a bias due to a regression to the mean because no relapse was noted in the three months preceding IVF, we also compared the number of relapses in the three-month period following IVF to two control periods of three months. Another possible bias could be due to a better medical surveillance in the months following IVF, thereby artificially increasing the opportunity of noting a relapse. However, because there was no reason to believe that such treatments could induce a relapse, no increased neurological surveillance was performed during the post-IVF period.

Several studies have shown that LHRH agonists can have a direct effect on T cells both in-vitro and in-vivo (Batticane et al., 1991; Jacobson et al., 1994) and that functional LHRH receptors on T cells can trigger the expression of adhesion molecules that can be relevant in MS physiopathology (Chen et al., 2002). Furthermore, in a murine model of lupus erythematosus, LHRH agonists worsened the disease while LHRH antagonists improved it, independent of an estrogenic effect (Jacobson et al., 1994).

The patients in our study were also treated with other drugs able to modulate sex hormones, such as rec-FSH and Clomifen citrate. Although the effects of FSH on the immune system has been rarely investigated, it does not seem to have an impact on T cell proliferation (Biffoni et al., 1998). The two patients treated with Clomifen citrate in association with LHRH antagonists did not suffer any relapses after treatment. Taken together, these results suggest that LHRH agonists should be used with caution in relapsing-remitting MS patients.

Further studies are now underway to validate these results on a larger scale by including all cases reported in France.

Figure 1 : Comparison of the annualized relapse rate (ARR) during the different three-month periods. The white column indicates the three-month period before in vitro fertilization (IVF), the ARR is 0. The black column indicates the three-month period following IVF, the ARR is 2.48. The hatched column indicates the first control period, ARR is 0.48. The remaining column (black squares) indicates the second control period, ARR is 0.44. * $p<0.04$, Wilcoxon test, and ** $p<0.05$, Friedman test.

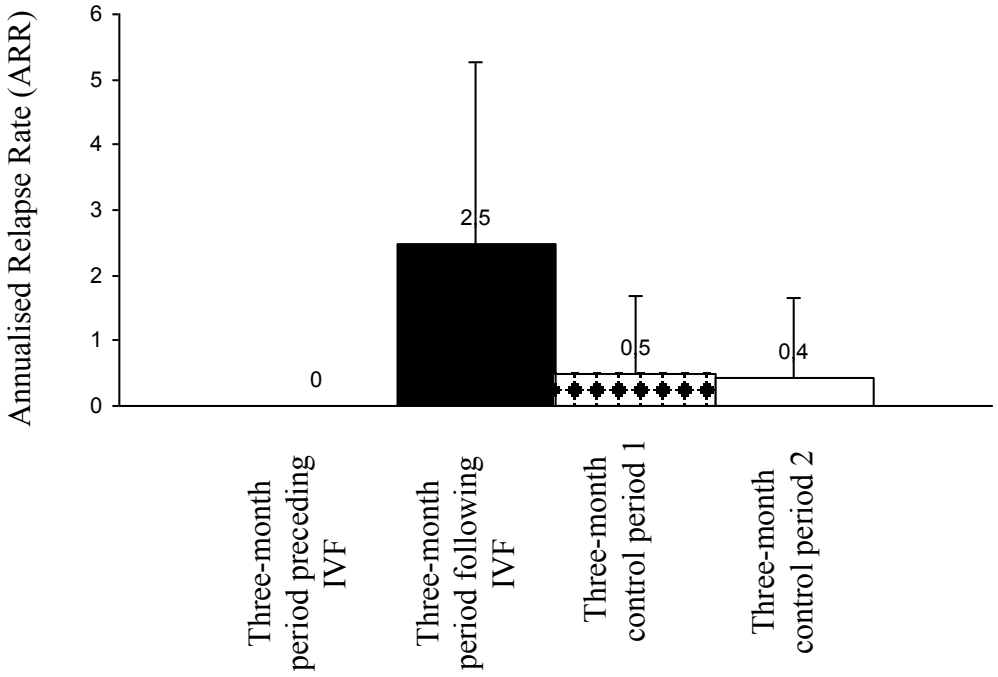
Annualised Relapse Rate (ARR)
6
5
4
3
2
1
0
0
2.5
0.5
0.4
Three-month period preceding IVF
Three-month period following IVF
Three-month control period 1
Three-month control period 2

Figure 1

Article 5 : Ectopic expression of the TrkA receptor in adult dopaminergic mesencephalic neurons promotes retrograde axonal NGF transport and NGF-dependent neuroprotection

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Ectopic expression of the TrkA receptor in adult dopaminergic mesencephalic neurons promotes retrograde axonal NGF transport and NGF-dependent neuroprotection

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Abstract

A recombinant adeno-associated virus (rAAV) was used to investigate the impact of an ectopic expression of the NGF high-affinity receptor in adult neurons. The rat TrkA cDNA cloned in a pCMX vector was first tagged with a human c-Myc sequence. The resulting vector was shown to encode a functional receptor which promoted the expression of TrkA immunoreactivity upon transfection of 293 fibroblasts or nnr5 cells, a TrkA-defective variant of PC12 cells. These cells also accumulate TrkA transcripts upon transfection and extended neurites in the presence of NGF. Therefore, the $TrkA_{myc}$ cassette was inserted into the pSSV9 plasmid. The new vectors shared properties similar to pCMX $TrkA_{myc}$ in 293 and nnr5 cells and enabled the preparation of rAAV $TrkA_{myc}$ viruses. Unilateral injection of this rAAV into the substantia nigra (SN) resulted in a protracted expression of TrkA (or c-Myc) immunoreactivity in numerous cell bodies, including tyrosine-hydroxylase (TH)-positive dopaminergic neurons. The presence of TrkA receptors in corresponding striatal dopaminergic endings was demonstrated by the advent of a striato-nigral retrograde axonal transport of ^{125}I-NGF. Likewise, ectopic expression of TrkA in neurons of the parafascicular thalamic nucleus promoted a striatofuge transport of NGF toward this structure. To investigate whether ectopic expression of TrkA in SN neurons may confer neuroprotection, lesions were induced by 6-hydroxydopamine in striata located ipsilateral to the virus injection site. NGF or vehicle were next delivered dorsally to the virus-treated SN for 2 weeks, before sacrifice and processing of brains for TH-immunohistochemistry. NGF treatment, in contrast to treatment with vehicle, significantly enhanced the number of dopaminergic neurons counted in the lesioned SN. These data suggest that ectopic TrkA can mediate the trophic actions of NGF and influence neuronal plasticity in vivo.

Keywords: Alzheimer's disease; Parkinson's disease; Nerve growth factor; Gene therapy; Neurotrophin; Neurotrophic factor; Cell death

Introduction

Nerve growth factor (NGF) is a neurotrophic factor belonging to the neurotrophin family. In the central nervous system (CNS), NGF exerts trophic effects on the cholinergic neurons of the basal forebrain and striatum (Mobley et al., 1985; Williams et al., 1986), as well as acting on some noncholinergic neuronal populations located in more caudal positions (Holtzman et al., 1995). As a mirror image, target areas of NGF-responsive neurons are the major sources of NGF in the brain. This is the case for the hippocampus and cortex and, to a lesser extent, the striatum, which provides NGF to cholinergic neurons in the forebrain (Ayer-LeLievre et al., 1988; Korsching et al., 1985; Rennert and Heinrich, 1986; Senut et al., 1990). Astrocytes constitute another

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source of NGF, which is less influenced by regional clues (Houlgatte et al., 1989; Spranger et al., 1990). The neurotrophic effects of NGF are mediated by a high-affinity receptor, a *trans*-membrane tyrosine-kinase referred to as TrkA (Kaplan et al., 1991; Klein et al., 1991; Meakin et al., 1992), which is expressed by the NGF-responsive neurons of the CNS (Holtzman et al., 1992, 1995). Although NGF may also interact with a low-affinity coreceptor, $p75^{NTR}$, ligation of TrkA is sufficient in driving complex downstream phosphorylation cascades which mediate its major trophic actions (reviewed in Chao and Hempstead, 1995; Kaplan and Miller, 2000). Moreover, TrkA is associated with cytoskeletal components (MacDonald et al., 1999; Yano et al., 2001), and, in the brain, is subjected to retrograde axonal transport, irrespective of the presence of the low-affinity coreceptor (Curtis et al., 1995; DiStefano et al., 1992; Holtzman et al., 1995; Seiler and Schwab, 1984; Venero et al., 1995). However, in the adult brain, endogenous production of NGF may be insufficient in ensuring the survival of NGF-responsive neurons following exposure to drastic traumatic conditions. In contrast, survival may be obtained by increasing the local availability of NGF. Such rescue experiments were initially based on a direct intracerebral infusion of NGF (Altar et al., 1992; Hefti, 1986; Stromberg et al., 1990), or implantation of biodegradable NGF-loaded microspheres (Menei et al., 2000). Other strategies have involved the transplantation of cells or injection of viral vectors that had been genetically modified to produce NGF (Klein et al., 2000; Martinez-Serrano et al., 1995; Rosenberg et al., 1988; Stromberg et al., 1990). Similarly, vector-derived production of a factor such as glial cell line-derived neurotrophic factor (GDNF) has been reported to protect injured dopaminergic mesencephalic neurons (reviewed in Bjorklund et al., 2000).

The potential benefit of a reciprocal strategy, based on an ectopic expression of TrkA, has been much less documented. Expression of TrkA in neurons which naturally fail to synthesize this high-affinity receptor should confer a certain degree of responsiveness to NGF. This assumption is supported by in vitro studies involving cultures of peripheral embryonic neurons, or pheochromocytoma PC12 variant cells lacking TrkA. In both cases, ectopic expression of TrkA enhanced and/or triggered NGF-dependent biological effects (Allsopp et al., 1993, 1994; Loeb et al., 1991).

In the present study, we investigated the possibility of genetically modifying adult brain neurons in vivo, in order to trigger a sustained synthesis of TrkA in cells which do not normally express this receptor. For this purpose, we made use of a recombinant adeno-associated virus (rAAV), since this type of vector is known to efficiently transduce certain CNS neurons, without inducing any significant inflammatory reaction within the host brain (Lo et al., 1999; Mandel et al., 1997). Thus, a rAAV was constructed, in which the rat TrkA cDNA was placed under the control of a CMV early promoter. The ability of this recombinant vector to drive a protracted production of TrkA was investigated using substantia nigra (SN) dopaminergic neurons as target cells. These neurons play a pivotal role in the control of movement and the pathophysiology of Parkinson's disease (Hirsch et al., 1997; Smith and Kieval, 2000). They project their axons into the striatum, and they constitute a typical population which fails both to express TrkA and to respond to NGF (Holtzman et al., 1995; Hyman et al., 1994; Knusel et al., 1991; Schwab et al., 1979). The data presented here indicate that the rAAV is able to drive a prolonged expression of TrkA in SN dopaminergic neurons. This ectopic expression triggers retrograde axonal transport of NGF from striatal terminals and confers a neuroprotection of lesioned dopaminergic neurons upon delivery of exogenous NGF.

Materials and methods

Plasmid construction

Rat TrkA cDNA was subcloned into the plasmid vector pCMX as described previously (Meakin et al., 1997). For the addition of a tag, a sequence encoding an antigenic peptide fragment of the human Myc protein (EQKLISEEDL), as well as a GP stretch to help the tag hang away from the receptor, was subcloned between the signal peptide and the mature end of rat TrkA between amino acids Ala [34] and Ser [35]. This was achieved by overlap polymerase chain reaction (PCR) mutagenesis using wild-type TrkA primers as the outside anchors. The final PCR product (approx 620 bp) was cloned into the TrkA *Apa*1 and *Pvu*II sites. The inserted PCR fragment contained an internal and unique *Eco*RV site which was used to confirm the presence of the tag on the receptors. The identity of the PCR-generated TrkA construct was confirmed by DNA sequencing.

The resulting $TrkA_{myc}$ cDNA was then subcloned from pCMX $TrkA_{myc}$ into the unique *Xho*1 site in pSSV9 (Du et al., 1996) to produce pSSV9 $TrkA_{myc}$. Plasmids were propagated in SURE 2 supercompetent cells (Stratagene, La Jolla, CA), extracted, and then purified using the Endofree Plasmid Maxi Kit (Qiagen, USA). Some control experiments involved pSSV9 LacZ which drives the expression of *Escherichia coli* β-galactosidase (Du et al., 1996; Salvetti et al., 1998).

AAV production

Type II rAAV was produced by the Gene Vector Production Network (GVPN, University Hospital of Nantes, France) using the procedure described in detail by Salvetti et al. (1998). Briefly rAAV $TrkA_{myc}$ was produced by transfection of 293 cells with pSSV9 $TrkA_{myc}$ and a plasmid vector pDG containing the *rep* and *cap* genes (Grimm et al., 1998). Cells were infected 6 h after transfection with a wild-type adenovirus. After 48 h, cells were harvested and lysed. The rAAV was purified on an iodixarol gradient,

through a heparin column and then was dialyzed as described by Zolotukhin et al. (1999). Two different methods were used to measure the rAAV titer. Dot blot analysis, which is based on the quantification of viral DNA, gave a range of 10^{11} to 10^{12} particles/ml. Modified Replicant Center Assay (RCA) gave a value of 10^{10} to 10^{11} infectious particles/ml, depending on the preparation. A stock of rAAV LacZ control virus was prepared from plasmid pSSV9 LacZ under conditions similar to those described above (kind gift of Dr. A. Salvetti).

Cell culture and transfection

Rat pheochromocytoma PC12 and nnr5 cells (a gift from Dr. M. Magazin, Sanofi, Labège, France) were cultured as described by Loeb et al. (1991) in culture dishes coated with rat tail collagen (Jacques Boy, Lyon, France) or 50 mg/ml poly-L-lysine (from Sigma-Aldrich, Saint-Quentin-Fallavier, France). Human kidney E293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich). Plasmid transfection was achieved with the non-liposomal formulation, FuGene 6 Transfection Reagent (Boehringer-Mannheim, Germany) using 2 μg of plasmid for 80% cell confluence in 35-mm tissue culture dishes in the presence of FCS. Human recombinant βNGF (Sigma-Aldrich) was used at a final concentration of 50 ng/ml to assess the function of the $TrkA_{myc}$ protein. nnr5 cells were considered as positive when neuritic length exceeded twice the cell diameter (Loeb et al., 1991).

Immunocytochemistry

After 24 or 48 h, transfected E293 and nnr5 cells were fixed with 4% paraformaldehyde for 20 min, treated for 5 min with 0.3% H_2O_2 in phosphate-buffered saline, and then with normal goat serum. Cells were exposed overnight at 4°C to rabbit polyclonal anti-TrkA antibody (1:5000; kindly provided by Dr. L. Reichardt, Howard Hughes Medical Institute, San Francisco, CA) or mouse anti-c-Myc 9E10 antibody (1:500; Biomol, Plymouth Meeting, PA). Cells were subsequently incubated for 1 h with a biotinylated anti-rabbit or anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) and then treated with the ABC kit Vectastain (Vector, Burlingame, CA), using diaminobenzidine or very intense purple, and counterstained with hematoxylin/lithium carbonate. Positive and negative cells were counted in 10 different microscopic fields. A total of at least 2500 cells was monitored for each experimental condition.

RNase protection assays

RNase protection assays for the detection of *TrkA* RNA were performed as previously described (Naveilhan et al., 1996). Briefly, 15 μg of RNA isolated from cell cultures by the lithium chloride-urea procedure was hybridized with cRNA probes labeled with [^{32}P]CTP (Amersham-Pharmacia-Biotech, Saclay, France) using the RPAII kit (Ambion, Austin, TX). PC12 and nnr5 cells served as positive and negative controls, respectively. The rat TrkA antisense cRNA probe was prepared as previously described. In order to control and standardize RNA loading, an antisense probe detecting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts was added to each sample. Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing conditions. Gels were fixed, dried, and exposed to a Molecular Dynamics (Palo Alto, CA) Phospho Imager screen. Gels were exposed for 1 day or 6 h (for cells transfected with the TrkA-expressing vector) to X-ray films at −70°C with intensifying screens.

Animals and unilateral stereotaxic injection of AAV $TrkA_{myc}$

All procedures involving animals were in compliance with our Institutional guidelines. Adult female outbred Sprague-Dawley rats (Laboratoires Janvier, Le Genest-Saint-Isle, France) were weighted (280–300 g) and anesthetized with 0.3 ml of a mixture of 2% Rompun and 50 mg/ml ketamine administered intraperitoneally. The injection coordinates for the right SN pars compacta were: bregma −5.3 mm antero-posterior (AP), −2.4 mm medial-lateral (ML), −7.5 mm dorsal-ventral (DV); incisor bar at −3.3 mm. Viral stocks (1.5×10^{10} pi/ml) were injected with a 10-μl Hamilton syringe mounted on a automated microinjector (Phymep, Paris, France). The pump delivered 5 μl over a 5-min period, and the needle remained in place for 5 additional min. A similar procedure was used for the rAAV LacZ control virus.

Immunohistochemistry and confocal image analysis

Anesthetized animals were perfused with 100 ml of 0.9% NaCl, followed by 300 ml of cold 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. Brains were removed and cryoprotected in two successive solutions of 15 and 30% sucrose/phosphate-buffered saline at 4°C and stored at −80°C. Free floating coronal sections (40 μm) of midbrain were exposed for 24 h at 4°C to several antibodies: rabbit anti-TrkA antibody (1:5000), mouse anti-c-Myc 9E10 antibody (1:500), and rabbit anti-TH antibody (1:500; Pel Freez, Rogers, AR). Sections were incubated for 1 h with an appropriate secondary biotinylated antibody and revealed with the ABC kit and diaminobenzidine, as described above. Controls included sections where the primary antibody was omitted. The possible induction of the low-affinity neurotrophin receptor $p75^{NTR}$ in response to rAAV $TrkA_{myc}$ administration was monitored by immunohistochemistry using the MC192 monoclonal antibody (Boehringer-Mannheim). When the rAAV LacZ control virus was used, expression of β-galactosidase was detected with an antibody from ICN Pharmaceuticals (Orsay,

France). To further unravel the identity of TrkA-positive cell bodies observed following injection of the rAAV $TrkA_{myc}$ virus, selected sections were double-labeled using a sheep FITC-conjugated anti-TH antibody (Jackson ImmunoResearch) and a biotinylated anti-rabbit antibody, visualized using a streptavidin-Texas red complex. Slides were analyzed with a Leica confocal microscope using TCS NT software.

Retrograde transport of ^{125}I-NGF

The rAAV $TrkA_{myc}$ was administered to the right SN (n = 2) or parafascicular thalamic nucleus (n = 1). Animals were anesthetized after 24 weeks, and 5 μl (1 μCi) of ^{125}I-NGF (specific activity, 1366 Ci/mmol; Amersham-Pharmacia-Biotech) was injected at two different sites in each striatum. This corresponded to an input of 20 ng of NGF per striatum. Stereotaxic coordinates were bregma +1.0 mm AP, ±2.8 mm ML, −5.2 mm DV; and +0.5 mm AP, ±2.5 mm ML, −5.2 mm DV, incisor bar at −3.3 mm, with a Hamilton syringe, at 1 μl/min. Animals were anesthetized and perfused 20 h later and their brains cryoprotected as described above. For autoradiography, coronal cryostat sections (30 μm) performed at the level of the midbrain were mounted onto gelatin-treated slides and were firstly placed in contact with Kodak films (Paris, France) for 2 months. After development, which revealed the presence of radiolabeled material on at least 24 successive sections (740 μm) or more, part of the slides were dehydrated in successive baths of alcohol and dipped in Kodak emulsion NTB-2. Development was performed 4 months later, and preparations were counterstained with cresyl violet. Other slides were used to monitor the presence of TrkA immunoreactivity. However, TrkA immunohistochemistry turned out to be negative, even in brain areas such as the septum which naturally contains neurons expressing the NGF receptor. This was probably due to damage of the tissue sections as a result of their 2-month storage period at room temperature for autoradiography. Eventually, immunoreactivity was recovered by first treating the sections for 40 min at 95°C in 10 μM citrate buffer, pH 6.0, before they were processed as described above.

Assessment of neuroprotection

Eight weeks following unilateral injection of rAAV in the right SN, performed as described above, 7 animals received two ipsilateral intrastriatal injections (Sauer and Oertel, 1994) of 5 μl of 6-hydroxydopamine (6-OHDA; free base from Sigma-Aldrich) at 2 mg/ml, dissolved in 1 mg/ml ascorbic acid-0.9 % NaCl, pH 4.3. The coordinates were: bregma +0.5 mm AP, −2.5 mm ML, −5.0 mm DV; and −0.5 mm AP, −4.2 mm ML, −5.0 mm DV, incisor bar at 0. Four days later, a stainless-steel cannula was sealed to the skull of each animal, with its tip at the level of the dorsal side of the ipsilateral SN (−5.3 mm AP, −2.4 mm ML, −7.0 mm DV, incisor bar at −3.3 mm). The cannula were connected to Alzet 2002 mini osmotic pumps (Alza Corp, Palo-Alto, CA), and 4 animals (group *a*) received a continuous delivery of 0.7 μg/day of human recombinant NGF (Sigma-Aldrich) in 124 mM NaCl, 24 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 1.4 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM Na_2SO_4, and 6 mM glucose at pH 7.4 (Tuszynski, 2000). The flow rate was 0.5 μl/h for 14 days. The second group of three animals (group *b*) received pumps delivering vehicle alone. Three more animals were included in the study, as an additional control group. Unlike the 2 former groups, these animals had no prior administration of rAAV, but they were lesioned with 6-OHDA and received pumps delivering NGF (n = 2) or vehicle (n = 1). The results obtained under both conditions were so similar that the three animals were pooled and will be referred to as group *c*. All animals were sacrificed at the end of the treatment. After perfusion, brains were processed for TH immunohistochemistry as described above. Free floating coronal sections (40 μm) were prepared and those showing the medial terminal nucleus of the optic track served as references and were assumed to correspond to (or to be close to) position AP −5.3 mm to bregma, according to the atlas of Paxinos and Watson (Paxinos and Watson, 1982; Sauer and Oertel, 1994). TH-immunoreactive neurons were counted in this, as well as neighboring sections at 240 μm intervals, thus allocated to coordinates AP −4.82, −5.06, −5.30, and −5.54 mm to bregma. These calculated values were used by convenience, knowing that actual positions are probably less accurate. Sections stained for TH immunoreactivity were viewed on a computer screen and contralateral, untreated SN were delineated to exclude the ventral tegmental area (German and Manaye, 1993). The same area of interest was delineated on the lesioned side. For each rostrocaudal position, the mean percentage of TH-positive cell bodies remaining in the lesioned SN was established, ascribing a value of 100% to the counts found in the nonlesioned contralateral side. The mean percentages are presented +/− SEM. To obtain an overview of the data, the average of the values found in the 4 successive sections of each experimental group was calculated. The differences were analyzed using one-factor analysis of variance (ANOVA).

Results

Expression of $TrkA_{myc}$ in cell cultures

The rat TrkA cDNA cloned into plasmid pCMX was tagged with a human c-Myc sequence, and the resulting pCMX $TrkA_{myc}$ plasmid was used to transfect human kidney E293 cells. Staining 24 h later with an anti-TrkA antibody showed that about 15–20 % of the cells expressed TrkA immunoreactivity (Figs. 1A and B). A similar pattern was observed with an anti-c-Myc antibody (not shown). In order to test the functionality of the tagged receptor, nnr5

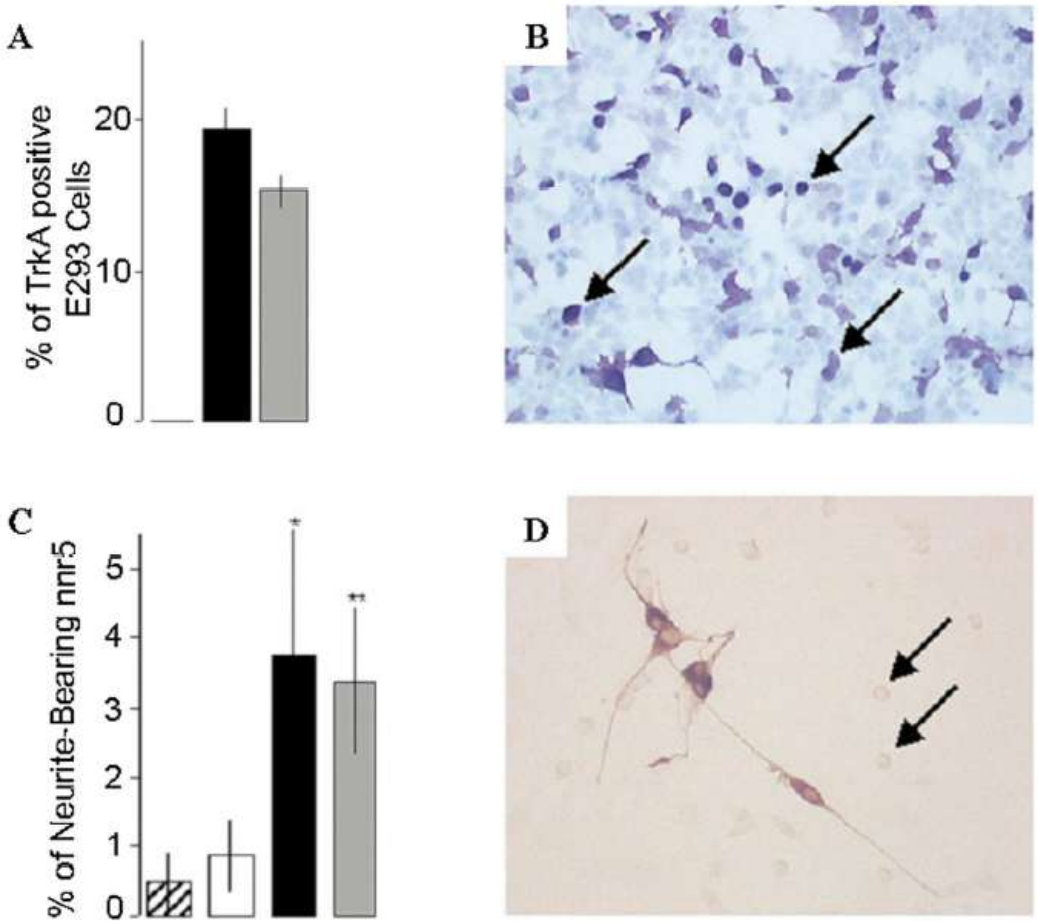


Fig. 1. Expression of TrkA in E293 and nnr5 cells. (A) E293 cells were transfected with plasmids pSSV9 LacZ (—), pCMX TrkA$_{myc}$ (black bars), and pSSV9 TrkA$_{myc}$ (gray bars). The histogram summarizes the percentage of TrkA-immunoreactive cells after 24 h, +/− standard deviation. Cells exposed to the nonrelevant plasmid pSSV9 remained negative while about 15–20% of cells transfected with either of the TrkA-expressing plasmids displayed TrkA-immunoreactivity. (B) E293 fibroblasts 24h after transfection with the pCMX TrkA$_{myc}$ plasmid. TrkA-immunoreactive cells are depicted in dark blue (arrows) and are scattered among a majority of negative cells, visualized by a counterstaining with hematoxylin (light blue) (X200). (C) Neurite extension by nnr5 cells. Cells were either untreated (hatched bar) or were transfected with plasmids pSSV9 LacZ (open bar), pCMX TrkA$_{myc}$ (black bar), or pSSV9 TrkA$_{myc}$ (gray bar). Cells were incubated with 50 ng/ml NGF for 48 h. Transfection with TrkA$_{myc}$ expression vectors significantly increased the fraction of neurite-bearing cells. Data are presented as percentage +/− standard deviation (*pCMX TrkA$_{myc}$ vs pSSV9 LacZ: $P < 0.005$; ** pSSV9 TrkA$_{myc}$ vs pSSV9 LacZ: $P < 0.0005$; Student's t test). (D) TrkA-immunoreactive nnr5 cells form neurites in the presence of NGF. nnr5 cells transfected with pCMX TrkA$_{myc}$ were exposed for 48 h to 50 ng/ml NGF and were subsequently processed for TrkA immunocytochemistry. Immunoreactive neurite-bearing cells are found among a majority of round, TrkA-negative cells (arrows) (× 300).

variant cells of the PC12 strain were transfected with the same plasmid. In this case, the fraction of TrkA-immunoreactive cells was much lower than in the case of E293 cells. However, most TrkA-immunoreactive cells extended neurites when exposed for 48 h to 50 ng/ml of NGF. Furthermore, the fraction of neurite-bearing cells was significantly elevated compared to untransfected cells, or cells transfected with a control plasmid (Figs. 1C and D). An RNase protection assay confirmed that transfection of nnr5 cells with the pCMX TrkA$_{myc}$ plasmid restored a high expression of *TrkA* mRNA in these cells (Fig. 2). In view of these results, the TrkA$_{myc}$ cassette was subcloned into the pSSV9 plasmid. Transfection of E293 cells with the resulting plasmid pSSV9 TrkA$_{myc}$ resulted in the appearance of TrkA

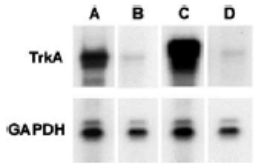


Fig. 2. RNase protection assays of *trkA* transcripts (top panel) and *GAPDH* transcripts (lower panel). A, detection of *trkA* and *GAPDH* transcripts in PC12 cells; B, in nnr5 cells; C, in nnr5 cells transfected with pCMX $TrkA_{myc}$, and D, with an irrelevant plasmid, pSSV9 LacZ (exposition time was 24 h, and 6 h for *trkA* transcripts in B).

immunoreactivity in approximately 15% of the cells, as in the case of the pCMX $TrkA_{myc}$ plasmid. Likewise, TrkA-positive nnr5 cells responded to NGF by an outgrowth of neurites (Figs. 1A and C).

Long-lasting expression of TrkA in neurons

The rAAV $TrkA_{myc}$ was prepared from the pSSV9 $TrkA_{myc}$ plasmid and injected into the right SN. Animals were sacrificed after 6 ($n = 2$), 11 ($n = 3$), 20 ($n = 4$), 24 ($n = 2$), and 33 ($n = 2$) weeks, and their brains processed for immunohistochemistry. TrkA-immunoreactive cell bodies were observed at all time points and in all animals, around the virus injection site. In the example presented in Fig. 3, the brain was analyzed 6 weeks after unilateral viral administration. TH staining did not reveal any obvious anatomical differences between the contralateral (Fig. 3A) and ipsilateral sides (Fig. 3B). TrkA-immunoreactive somata were found at the level of TH-positive neurons (Figs. 3B and D) in the ipsilateral side only (Figs. 3A and C). The anti-c-Myc antibody gave a similar staining pattern (Figs. 3E and F). Immunohistochemistry performed with an anti-$p75^{NTR}$ antibody remained negative, irrespective of a prior administration of the rAAV $TrkA_{myc}$ to the SN (data not shown). To further characterize the specificity of the TrkA-expressing rAAV, controls were performed with a rAAV LacZ vector. The virus was injected into the right SN, under conditions similar to those described for the rAAV $TrkA_{myc}$. After 8 weeks, the rAAV LacZ led to the expression of β-galactosidase in cell bodies located around the injection site, as detected by immunohistochemistry. In contrast, it failed to induce any TrkA immunoreactivity (data not shown).

Colocalization of TrkA and TH immunoreactivity by confocal microscopy confirmed that dopaminergic neurons permitted a long-term expression of the TrkA transgene (Fig. 4). Many immunoreactive neurites were observed, around cell bodies located in the pars compacta. In contrast, analyses performed on the striatum were not conclusive, because unlike the substantia nigra, this structure contains TrkA-immunoreactive interneurons. No significant differences in the staining intensity between either the ipsilateral or the contralateral sides could be distinguished. In parasagittal sections, the anti-TrkA antibody revealed scarce ascending axons between the substantia nigra and the striatum (not shown), possibly corresponding to projections of dopaminergic neurons. However, this result was not supported by staining performed with the anti-c-Myc antibody, possibly because of a high background staining. Likewise, no differences could be detected between either the ipsilateral or the contralateral sides of the striatum, when analyzed on coronal sections with the same antibody.

Striatofuge transport of TGF

Retrograde axonal transport was used to determine whether ectopically expressed TrkA was positioned along the striatal axonal endings of dopaminergic neurons. Two animals whose substantia nigra was exposed 24 weeks earlier to a unilateral stereotaxic administration of the rAAV $TrkA_{myc}$ virus received two bilateral, intrastriatal injections of ^{125}I-NGF. Brains were collected 20 h later and coronal sections, performed at the level of the midbrain, were subjected to autoradiography. Labeled neuronal cell bodies were detected in the virus-treated substantia nigra (Figs. 5B and E), whereas the contralateral side was negative (Fig. 5A). Control experiments performed by immunohistochemistry on adjacent sections confirmed the existence of TrkA-immunoreactivity at the level of radiolabeled cell bodies (not shown). One animal displayed radioactive somata scattered in the parafascicular thalamic nucleus. It was verified that in this animal, TrkA-immunoreactive cells were present in this structure, ipsilaterally to the virus injection site (Figs. 5C, D, and F).

Neuroprotection of SN dopaminergic neurons

The rAAV $TrkA_{myc}$ vector was injected into the right SN and 8 weeks later, the dopaminergic system was lesioned by two intrastriatal injections of 6-OHDA. Four days later, NGF ($n = 4$; group *a*) or vehicle ($n = 3$; group *b*) was delivered dorsal to the virus injection site for a period of 14 days. The third group (group *c*) included animals which were not exposed to the rAAV, but which received the lesion and were treated for 14 days with either NGF ($n = 2$) or vehicle ($n = 1$). These three animals were pooled, because the type of treatment had no significant influence on the results. All brains were collected at the end of the treatments and were processed for TH immunohistochemistry. For each experimental group, percentages of TH-immunoreactive neurons present ipsilateral to the lesion (% of contralateral) were established in 4 successive coronal sections, positioned as described in materials and methods. Scores found for a similar rostrocaudal position were averaged and values +/− SEM are shown in Fig. 6A. The results indicate that in comparison to the non-lesioned contralateral side, the intrastriatal lesion consistently de-

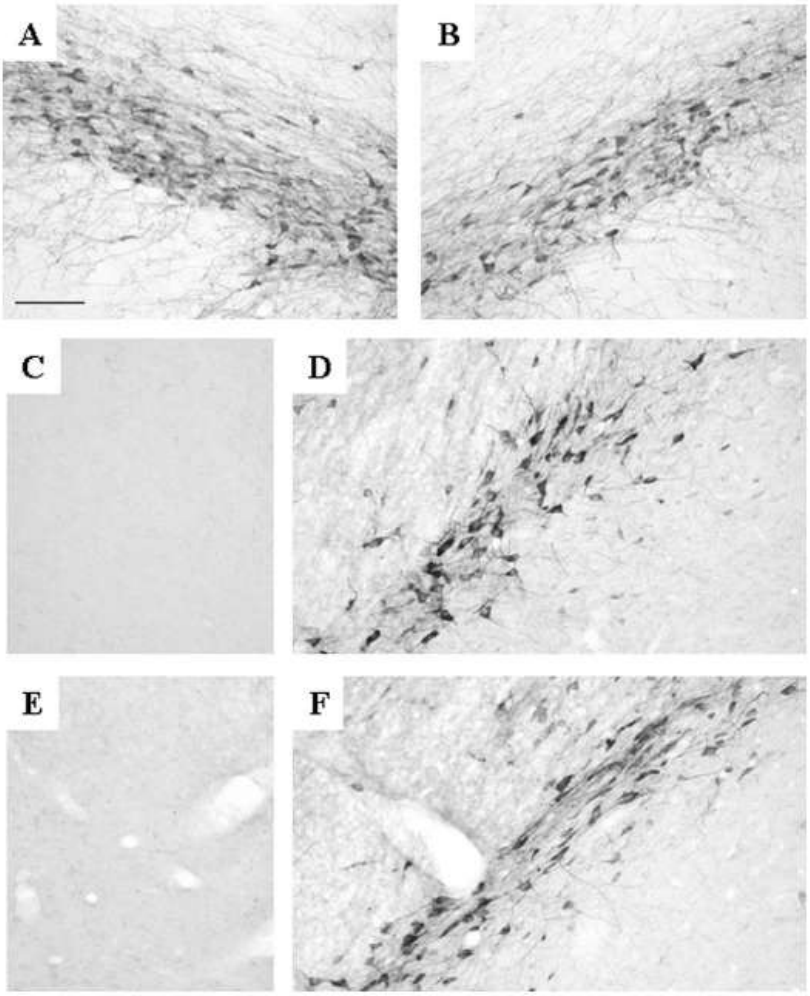
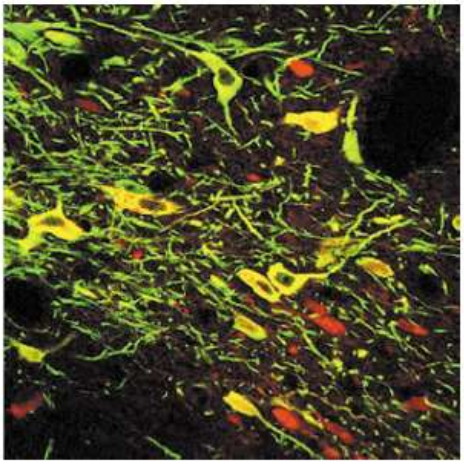


Fig. 3. Detection of TrkA or c-Myc immunoreactivity 6 weeks following unilateral stereotaxic injection of rAAV $TrkA_{myc}$. The virus was administered to the right SN. (A and B) TH staining identifies dopaminergic neurons in the SN located both contralateral (A) and ipsilateral (B) to the injection site. (C and D) TrkA immunohistochemistry evidences cell bodies ipsilaterally (D), but not contralaterally (C) to the virus injection site. (E and F) c-Myc immunohistochemistry, which stains cell bodies in the ipsilateral SN only (F), and is absent in the contralateral side (E), as in the case of TrkA-expressing cells. Scale bar: 100 μm.

creased the number of TH-positive neurons in the ipsilateral SN. The numbers of dopaminergic cell bodies were minimal in the 3 animals of control group *c* (lesion plus supply of either NGF or vehicle, without any prior rAAV administration), as well as in group *b* (SN exposed to the virus and subsequently lesioned, with a supply of vehicle only). In striking contrast, TH-positive somata were more numerous in group *a* (SN exposed to the vector and subsequently lesioned, with a delivery of NGF). This was observed in 3 successive sections, which were the closest to the tip of the device delivering the trophic factor (AP: −4.82, −5.06, and −5.30 mm to bregma). In order to obtain an overview of these results, mean scores corresponding to the 4 sections were pooled and the average was calculated (Fig. 6B). One-factor analysis of variance (ANOVA) indicated a significant neuroprotective effect of NGF treatment in brains that had been first exposed to the TrkA-expressing vector ($P < 0.001$, group *a* vs *b*; $P < 0.003$, group *a* vs *c*).



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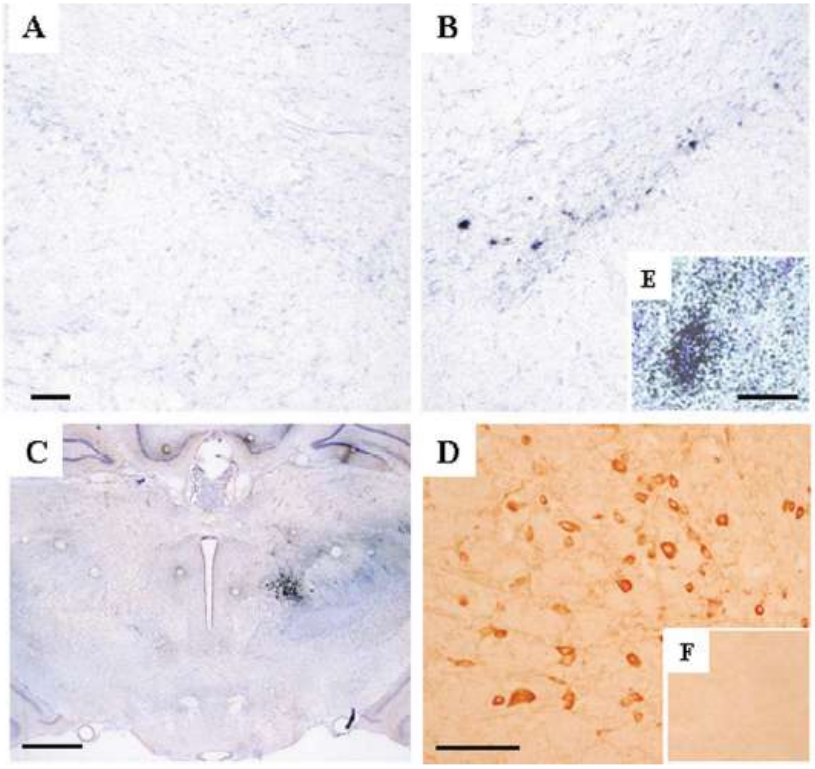

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Fig. 4. Expression of TrkA in TH-positive neurons. Confocal microscopic observation of a SN 6 weeks after injection of rAAV $TrkA_{myc}$. The anti-TH antibody was labeled with FITC (green) and the anti-TrkA antibody with Texas red. Superposition of both images shows that most TH-positive neurons present in the field express TrkA immunoreactivity, as shown by their yellow color.

Fig. 5. Striatofuge transport of ^{125}I-NGF. Animals received a unilateral injection of AAV TrkAmyc. After 24 weeks, a bilateral injection of radiolabeled NGF was performed in both striata. After 20 h, animals were sacrificed and midbrain slices subjected to autoradiography, as described under materials and methods. After development, preparations were counterstained with cresyl violet. No radiolabeled cells could be detected in the SN located contralaterally to the virus injection site (A). In contrast, labeled cells were present in the ipsilateral SN (B and E). In one animal, labeled somata were found in the parafascicular thalamic nucleus located in the virus-treated hemisphere (C). This coincided with the presence of TrkA immunoreactivity in the same structure (D). Such immunoreactive cell bodies are lacking in the contralateral side (F). Scale bars: A, B, D, 110 μm; C, 1 mm; E, 20 μm.

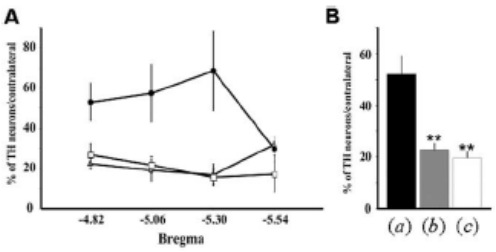

Fig. 6. Neuroprotection associated with rAAV $TrkA_{myc}$ administration. (A) The virus was administered to the right SN, in animals which were subjected 8 weeks later to two ipsilateral, intrastriatal insults with 6-OHDA. This was followed by a 14 day supply of NGF (group *a*, ●; $n = 4$) or vehicle (group *b*, △; $n = 3$), at the level of the virus injection site. Three other animals did not receive any virus, but were subjected to the unilateral intrastriatal 6-OHDA lesions and received NGF ($n = 2$) or vehicle ($n = 1$). These 3 additional control animals were pooled to form one group (group *c*, □). Brains were processed for TH immunhistochemistry. Cell bodies were counted in 4 successive sections, whose coordinates were assessed as described under materials and methods. The graph depicts the average percentages of TH-immunoreactive somata counted ipsilaterally in each section, and for each group (% of values found contralaterally) +/− SEM. (B) Overview of results. Data presented in A, which corresponded to 4 successive midbrain sections, were pooled and their average was calculated. Survival in group *a* (black bar) is significantly higher than in groups *b* (gray bar) and *c* (open bar) (**$P < 0.001$, group *a* vs *b*; $P < 0.003$, group *a* vs *c*; ANOVA).

Discussion

Our results show that tropism of the type 2 AAV vector used in this study enables an efficient transduction of SN dopaminergic neurons, in which the cytomegalovirus (CMV) promoter of the construct remained functional for at least 33 weeks. Such a long-term neuronal expression confirms data obtained with other recombinant AAV and is in contrast with the poor efficiency of transduction of striatal rostral neurons (Davidson et al., 2000; Klein et al., 2000; Mandel et al., 1997; Tenenbaum et al., 2000).

While doublelabeling experiments unambiguously showed that TrkA immunoreactivity was present in TH-positive somata and dendrites, axonal localization of the receptor could not be assessed using immunohistochemical techniques alone, despite the Myc-tag. In contrast, mesencephalic neurons exposed several weeks earlier to the rAAV were able to translocate NGF from the striatum to cell bodies in the SN. As pointed out above, this process of retrograde axonal transport relies on the expression of TrkA. In agreement with data of Schwab et al. (1979), it was not observed in the contralateral SN, which was not treated with the TrkA-expressing virus. Although the number of animals included in this study was limited ($n = 2$), these results indicate that ectopically synthesized $TrkA_{myc}$ receptors are positioned along the striatal axonal efferences of the nigral dopaminergic neurons. Data also support the concept that $TrkA_{myc}$ molecules are functional, at least with respect to their interaction with the anterograde and retrograde trafficking machineries. It is worth noting that this transport was observed in the absence of any detectable expression of $p75^{NTR}$ immunoreactivity in the SN, a fact which supports the possibility that the low-affinity neurotrophin receptor is not required for NGF axonal transport in certain CNS neurons (DiStefano et al., 1992; Holtzman et al., 1995; Mufson et al., 1994; Venero et al., 1995). This does not exclude that conditions leading to a coexpression of the $p75^{NTR}$ receptor in DA neurons might modify qualitative or quantitative parameters characterizing NGF axonal flux (Kramer et al., 1999).

Mesencephalic dopaminergic neurons respond to other neurotrophins (Hyman et al., 1994; Knusel et al., 1991), in particular, they express TrkB (Numan and Seroogy, 1999) and promote retrograde axonal transport of its ligand, brain-derived neurotrophic factor (BDNF) (Mufson et al., 1994). Therefore, it is possible that the transducing machinery of the TrkB receptor may contribute to the promotion of retrograde axonal transport of NGF liganded to TrkA. The ectopic expression of TrkA in the parafascicular thalamic nucleus also resulted in the retrograde axonal transport of radiolabeled NGF injected into the striatum whereas no such transport was seen in the contralateral side. This result is explained by the existence of a projection of parafascicular neurons toward the striatum, which has been described using other techniques of axonal retrograde tracing (Beckstead, 1984; Berendse and Groenwegen, 1990). Neurons present in the same structure were reported to transport BDNF but not NGF in a retrograde manner, as do SN dopaminergic neurons (Mufson et al., 1994, 1996a). Hence, data do not make it possible to determine whether an ectopic expression of TrkA can promote a retrograde transport of NGF in neurons that are lacking any other Trk-related receptor and its corresponding transducing apparatus. The TrkA-expressing rAAV vector may serve to unravel this question.

A hallmark of NGF and other neurotrophins is the promotion of neuronal survival. In the case of NGF, the signaling role of retrograde axonal transport remains a matter of controversy (MacInnis and Camperot, 2002; Riccio et al., 1997; Tsui and Ginty, 1999; Watson et al., 1999). The Trk receptor family transduces signals via several intracellular pathways (Chao and Hempstead, 1995; Kaplan and Miller, 2000) which are redundant to some extent (Stephens et al., 1994). However, responses may differ, according to the cell type or development stage. Furthermore, several arguments indicate that these receptors are not equivalent (Bradbury et al., 1998; McAllister et al., 1997; Yamada et al., 2002) and that under certain conditions, NGF is more effective than other neurotrophins in promoting neuronal survival (Frim et al., 1993; Meakin et al., 1997). In order to investigate whether ectopically expressed TrkA could exert a positive action on the survival of SN dopaminergic neurons, we used the model described by Sauer and Ortel (1994) of intrastriatal insult with 6-OHDA. This lesion is followed by a striatofuge, progressive degeneration of SN dopaminergic neurons. We verified that the infusion of NGF alone to the

injured SN had no effect on the number of residual dopaminergic neurons. This is in agreement with previous work demonstrating the lack of trophic action of NGF on SN dopaminergic neurons (Hyman et al., 1994; Knusel et al., 1991; Schwab et al., 1979). Likewise, the expression of ectopic TrkA alone in SN neurons was insufficient to promote dopaminergic cell survival (or in slowing down the cell death process), since the number of dopaminergic neurons remaining in the lesioned SN was not influenced by a previous administration of rAAV $TrkA_{myc}$. This suggests that endogenous production of NGF (Altar et al., 1992; Korsching et al., 1985) is not sufficient to protect TrkA-expressing SN dopaminergic neurons. In contrast, exogenous supplementation of NGF significantly enhanced the number of TH-immunoreactive neurons in SN that were initially exposed to the TrkA-expressing vector. Vehicle delivery was inefficient under the same experimental conditions. It seems, therefore, that NGF transduces a survival signal in adult dopaminergic SN neurons via ectopic TrkA. As stated above, this effect took place in the absence of any observable expression of the low-affinity $p75^{NTR}$. However, further studies will be required to determine to what extent this survival effect mobilizes transducing elements of TrkB or other related neurotrophin receptors.

Taken together, our data indicate that transduction of TrkA by a viral vector provides a convenient way to modify the plasticity of adult CNS neurons in vivo. This could have interesting applications in repair strategies, for instance in conjunction with agonists of TrkA (Maliartchouk et al., 2000) or compounds able to enhance NGF synthesis in the brain or spinal cord (Saporito et al., 1993; Wion et al., 1991). Moreover, several reports have shown that in Alzheimer disease, loss of cholinergic neurons of the basal forebrain nuclei is preceded by a decreased expression of the TrkA receptor (Boissiere et al., 1997; Mufson et al., 1996b; Salehi et al., 1996). Recombinant vectors driving the ectopic expression of this receptor might provide new strategies to maintain or restore the responsiveness of these neurons to NGF, and could contribute to enhancing the therapeutic potential of this factor.

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Titre : Analyse qualitative et quantitative du transcriptome Vβ du TCR dans la Sclérose en Plaques. Caractérisation des cellules portant les altérations et corrélations avec les paramètres cliniques et radiologiques de la maladie.

La SEP est une maladie inflammatoire et démyélinisante du SNC associée à des lymphocytes T autoréactifs contre des composants myéliniques. On pense que ces lymphocytes T autoréactifs, portant des TCR particuliers, pourraient se développer de façon clonale au cours de la maladie. Il pourrait donc être intéressant d'étudier le répertoire T dans le sang des patients afin d'identifier ces cellules autoréactives.
Nous avons donc étudié la chaîne β du TCR à la fois sur un plan qualitatif et quantitatif dans différentes séries de patients et à différents moments, ainsi que chez des témoins sains.
Les résultat suggèrent que le répertoire T des lymphocytes circulants est dévié à tous les stades de la maladie et de façon plus prononcée chez les patients ayant une maladie cliniquement définie ou une forme agressive que chez les patients ayant un syndrome cliniquement isolé. Le répertoire T est aussi plus biaisé dans les formes rémittentes que dans les formes progressives. Les déviations observées impliquent différentes familles Vβ, sans altération publique, même chez les patients ayant un même type HLA-DR. Le suivi des patients pendant plusieurs mois montrait une stabilité relative des altérations observées, mais aussi l'apparition de nouvelles familles oligoclonales au cours du temps. Une corrélation positive a été mise en évidence entre les paramètres IRM (charge lésionnelle en T2 et activité lésionnelle) et l'apparition de nouvelles familles oligoclonales. Les lymphocytes T isolés appartenant à ces familles Vβ oligoclonales appartenait à des populations essentiellement CD8+. Ces cellules accumulaient de façon importante des transcrits de cytokines pro-inflammatoires (IFNγ, TNFα et IL-2) comparativement aux témoins sains. De plus, l'utilisation d'Elispot IFNγ montrait que ces familles Vβ oligoclonales produisaient de l'INFγ en présence de MBP humaine, une protéine de la myéline, comparativement aux familles oligoclonales issues de témoins sains. Ces données suggèrent la présence d'une réponse auto-immune précoce dans la maladie, et argumente sur le rôle important des lymphocytes T CD8+ dans la SEP.

Titre : Qualitative and quantitative analysis of TCRBV transcriptome in multiple sclerosis patients. Characterization of the T cells responsible for the skewing and correlation with clinical and MRI parameters.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system associated with myelin-reactive T cells. It has been postulated that these autoreactive T cells, bearing particular types of TCRs, are clonally expanded during the course of MS. Thus, the study of TCR repertoire in the blood of MS patients could allow to identify the autoreactive T cells.
We analyzed the TCR Vβ chain, both qualitatively and quantitatively, in different series of MS patients and at different time points, and in healthy subjects.
The results suggested that the circulating T cell repertoire was biased at all the stages of MS, and more pronounced in clinically definite MS and in active MS than in patients with a clinically isolated syndrome. The T cell repertoire was also more biased in relapsing remitting than in chronic progressive MS. The skewing of TCR usage involved different Vβ families without public alterations, even in patients with identical HLA-DR typing. The follow-up of the patients for several months showed a relative stability of the alterations observed, but also the appearance of new oligoclonal Vβ families A positive correlation was found between MRI parameters (T2 lesion burden and lesional activity) and appearance of oligoclonal Vβ families. The T cells sorted from oligoclonal Vβ families concerned both CD4 and CD8 positive populations with a more pronounced skewing in the CD8 compartment. These cells displayed a significantly increased level of IFNγ, IL2 and TNFα transcripts compared to their counterparts in healthy subjects. Furthermore, using IFNγ Elispot assays, T cells from oligoclonal Vβ families tested from MS patients responded to hMBP whereas no response was observed with human albumin or with altered Vβ families from healthy individuals. Our data support the concept of an early autoimmune component in the disease and emphasize the possible involvement of CD8 positive T cells in MS.

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