

## Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations

Sylvaine GALIÈGUE<sup>1</sup>, Sophie MARY<sup>1</sup>, Jean MARCHAND<sup>1</sup>, Danielle DUSSOSSOY<sup>1</sup>, Dominique CARRIÈRE<sup>1</sup>, Pierre CARAYON<sup>1</sup>, Monsif BOUABOULA<sup>1</sup>, David SHIRE<sup>2</sup>, Gérard LE FUR<sup>3</sup> and Pierre CASELLAS<sup>1</sup>

<sup>1</sup> Sanofi Recherche, Immunopharmacology Department, Montpellier, France

<sup>2</sup> Sanofi Recherche, Labège, France

<sup>3</sup> Sanofi Recherche, Paris, France

(Received 28 April 1995) – EJB 95 0679/1

Two proteins with seven transmembrane-spanning domains typical of guanosine-nucleotide-binding-protein-coupled receptors have been identified as cannabinoid receptors; the central cannabinoid receptor, CB1, and the peripheral cannabinoid receptor, CB2, initially described in rat brain and spleen, respectively. Here, we report the distribution patterns for both CB1 and CB2 transcripts in human immune cells and in several human tissues, as analysed using a highly sensitive and quantitative PCR-based method. CB1 was mainly expressed in the central nervous system and, to a lower extent, in several peripheral tissues such as adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, thymus and tonsils. In contrast, the CB2 gene, which is not expressed in the brain, was particularly abundant in immune tissues, with an expression level 10–100-fold higher than that of CB1. Although CB2 mRNA was also detected in some other peripheral tissues, its level remained very low. In spleen and tonsils, the CB2 mRNA content was equivalent to that of CB1 mRNA in the central nervous system. Among the main human blood cell subpopulations, the distribution pattern of the CB2 mRNA displayed important variations. The rank order of CB2 mRNA levels in these cells was B-cells > natural killer cells ≫ monocytes > polymorphonuclear neutrophil cells > T8 cells > T4 cells. The same rank order was also established in human cell lines belonging to the myeloid, monocytic and lymphoid lineages. The prevailing expression of the CB2 gene in immune tissues was confirmed by Northern-blot analysis. In addition, the expression of the CB2 protein was demonstrated by an immunohistological analysis performed on tonsil sections using specific anti-(human CB2) IgG; this experiment showed that CB2 expression was restricted to B-lymphocyte-enriched areas of the mantle of secondary lymphoid follicles. These results suggest that (a) CB1 and CB2 can be considered as tissue-selective antigens of the central nervous system and immune system, respectively, and (b) cannabinoids may exert specific receptor-mediated actions on the immune system through the CB2 receptor.

**Keywords:** cannabinoid; cannabinoid receptors (CB1; CB2); human immune system; B cells; natural killer cells.

$\Delta$ -9-Tetrahydrocannabinol, the major active component of cannabis, as well as other cannabinoids, are known to exert a wide range of physiological effects such as drowsiness, alterations in cognition and memory, analgesia, orexigenic effects, anti-emetic effects, a decrease in intra-ocular pressure, anti-inflammatory effects and immunosuppression [1]. Many studies have been conducted to decipher the cannabinoid system. First attributed to non-specific cell membrane disruption, the major cannabinoid effects are now thought to be mediated through specific cannabinoid receptors. A guanosine-nucleotide-binding-protein-coupled receptor of 472 amino-acid residues, CB1, was initially characterized in rat brain [2] and further cloned both in rat [3] and human [4]. As the expression of the corresponding

mRNA and protein has been confined to the brain, CB1 was designated as the central cannabinoid receptor. However, using a sensitive and quantitative technique based on reverse transcription associated with the polymerase chain reaction (RT-PCR), we and others have described CB1 transcripts in peripheral tissues, although at a much lower abundance than in brain [5, 6]. The activation of this cannabinoid receptor inhibits adenylyl cyclase activity [7] as well as voltage-dependant N-type calcium channel activity in neurons [8]. In the astrocytoma cell line U373 MG, the CB1 receptor is coupled to the induction of the immediate-early gene *Krox-24* [9]. Recent pharmacological studies have provided new and powerful tools for investigating the function of the CB1 receptor. First, an endogenous ligand for the cannabinoid receptor, anandamide, (ethanolamide of arachidonic acid) was purified from porcine brain [10]. Second, we have described a potent and selective antagonist (SR 141716A) for the brain cannabinoid receptor [11].

Recently, the cloning of a second cannabinoid receptor subtype has been reported. This receptor (CB2), defined as peripheral receptor type, has been described in the macrophage population of the spleen [12]. It shares 44% identity with the CB1

*Correspondence to* P. Casellas, Sanofi Recherche, Immunopharmacology Department, 371 rue du Professeur Joseph Blayac, F-34184 Montpellier, France

**Abbreviations.** CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; CHO, Chinese Hamster ovary cells; PBL, peripheral blood leukocytes; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear neutrophils; RT-PCR, reverse transcription associated with PCR; NK cells, natural killer cells;  $\beta$ 2m,  $\beta$ 2 microglobulin.

amino-acid sequence and 68% similarity if the transmembrane regions only are considered. The distribution of this receptor has not been defined precisely in other tissues.

In addition to their psychoactive effects, cannabinoids are known to alter immune functions; they inhibit proliferative responses of T lymphocytes [13, 14], cytotoxic T-cell activity [15], antibody synthesis, microbiocidal activity of macrophages [16] and reduce  $\gamma$ -interferon production [17]. Thus, cannabinoid-derived compounds devoid of mood-altering effects may be potential therapeutic agents, provided that their cellular mechanism of action is clearly elucidated.

Kaminski and coworkers [18] showed on murine spleen cells that cannabinoids exhibit highly specific binding and inhibit the adenylyl cyclase-cAMP second-messenger system. They also showed that suppression of the humoral immune response by cannabinoids is at least partially mediated by inhibition of adenylyl cyclase through a pertussis-toxin-sensitive guanosine-nucleotide-binding-protein-i-subunit-coupled cannabinoid receptor [19]. This strongly suggested that immunomodulation by cannabinoid compounds is mediated by cannabinoid receptors present on immunocytes.

To date, no specific agonist that can discriminate between cannabinoid receptor subtypes has been published. Pharmacological studies have indeed been performed using cannabinoid agonists that are equipotent in their binding to CB1 or CB2. For this reason, the identification of the receptor subtype responsible for immunomodulation has, so far, not been achieved.

We have previously described CB1 transcripts in human spleen, tonsils and peripheral blood leukocytes (PBL) [5]. In contrast, CB2 was identified in spleen macrophages [12]. However, nothing is known about the expression of both CB1 and CB2. Our approach for resolving this consists of a comparative analysis of the distribution patterns of both cannabinoid receptors among various immune tissues. Here, we present the precise and detailed CB1 and CB2 mRNA distribution profiles in human immune tissues, in purified immune cell subpopulations, as well as in monocytic and leukemia cell lines. For this purpose, we used a quantitative and sensitive RT-PCR method. We further enlarged the analysis to include most human tissues. The CB2 protein expression was also examined by immunohistological analysis using specific anti-CB2 serum.

## MATERIALS AND METHODS

**Biological samples.** Human peripheral blood was obtained from healthy volunteer donors after obtaining their informed consent. Mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN) were isolated from peripheral blood by density gradient centrifugation using a ready-made solution provided by J. Bio (Les Ulis, France). Purification of leukocyte cell subpopulations [T4 cells, T8 cells, natural killer (NK) cells, B cells and monocytes] was performed as previously described [20]. B cells were purified from surgical specimens of human tonsils. The purities of the different cell subpopulations were determined by a three-colour immunofluorescence flow-cytometry analysis as already described [21].

The human monocytic cell line U937, the human promyelocytic cell line HL60, the human T leukemia cell line Molt-4, the human B lymphoblastoid cell line DAUDI and the human astrocytoma cell line U373 MG were purchased from ATCC. Cell lines were maintained at 37°C in humidified 5% CO<sub>2</sub>, 95% air in RPMI 1640 medium (Gibco) supplemented with 25 mM Hepes, 2.5 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum. Chinese hamster ovary cells (CHO), stably transfected with CB1

cDNA [9] or CB2 cDNA (results not shown) were grown as monolayers in minimum essential medium supplemented with 10% dialysed fetal calf serum, 2 mM glutamine, 40 µg/ml L-proline, 60 µg/ml Tylocine (Gibco BRL, Life Technologies), 1 mM sodium pyruvate and 5 µg/ml gentamycin. Wild-type CHO cells were grown in the same medium excepted that it contained 10% fetal calf serum. Wild-type and CHO cell lines transfected with CB1 cDNA or CB2 cDNA are referred to as CHO-wt, CHO-CB1 and CHO-CB2, respectively.

**RNA preparation, RT-PCR analysis and Northern-blot analysis.** Total cellular RNA was isolated from cells by the guanidinium isothiocyanate/cesium chloride method [22]. The integrity of RNAs was assessed by analysing 18S and 28S rRNA contents on a denaturing 1% agarose gel. RNA was treated with RNase-free DNase as described [5].

5 µg RNA was reverse-transcribed using the Ready-To-Go™ T-Primed First-Strand kit, according to manufacturer's recommendations (Pharmacia). Synthesis of cDNA was monitored by determining incorporation of [ $\alpha$ -<sup>32</sup>P]dCTP and cDNA was adjusted to 1 ng/µl and stored at -80°C in a Tris/EDTA buffer (5 mM Tris/HCl, pH 7.6, 0.5 mM EDTA) until use. Quantification of CB1 and CB2 transcripts in tissues from non-hematopoietic lineage were performed on total brain, cerebellum, cerebral cortex, pituitary gland, thyroid, retina, adrenal gland, placenta, skeletal muscle, kidney, liver, heart, lung, prostate, uterus, testis, ovary, pancreas, bone marrow, thymus, spleen and tonsils. The corresponding cDNAs were purchased from Clontech Laboratories, Inc.

PCR was performed at a final concentration of 0.2 ng cDNA in 20 µl PCR buffer (20 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.01% gelatin), 50 µM dNTP, 3 mM MgCl<sub>2</sub>, 0.1 µCi/µl [ $\alpha$ -<sup>32</sup>P]dCTP, 0.25 µM each of 5' and 3' primers, 0.025 U/µl Ampli Taq<sup>+</sup> DNA Polymerase (Perkin Elmer-Cetus). The mixture was amplified with the Perkin Elmer-Cetus 9600 thermal cycler. The amplification profile involved denaturation at 95°C for 20 s, primer annealing at 60°C for 30 s, and extension at 75°C for 20 s. PCR assays were carried out in duplicate. The primers used were: CB1 sense primer, 5'-catcatcatccacacgtctg-3'; CB1 antisense primer, 5'-atgctgttatccagaggctg-3'; CB2 sense primer, 5'-tttccactgatcccaatg-3'; CB2 antisense primer, 5'-agttgatgagcagcagcatg-3';  $\beta_2$  microglobulin sense primer, 5'-ccagcagagaatggaagtc-3';  $\beta_2$  microglobulin antisense primer, 5'-gatgctgctcatctgctcg-3'. The expected size of the amplicons were 330 bp and 337 bp for CB1 and CB2, respectively, and 268 bp for  $\beta_2$  microglobulin ( $\beta_2$ m). Southern blotting on the PCR products was performed as described [5]. Quantification of cannabinoid receptor expression levels was performed in the exponential phase of amplification, in which the amount of PCR products is proportional to the initial amount of template [5, 23]. In practice, the exponential phase of amplification was determined by analysing the amount of amplicon synthesized at various numbers of cycles using a fixed quantity of cDNA. After electrophoresis of 10 µl PCR reaction mixture on a 10% acrylamide gel in 0.089 M Tris/HCl, 0.089 M borate, 0.002 M EDTA, pH8, the amount of the specific amplicon synthesized was determined by measuring the radioactivity recovered in the specific PCR products, excised from the gel after ethidium bromide staining. Negative controls, in which reverse transcriptase or cDNA was omitted, were run in parallel in each experiment.

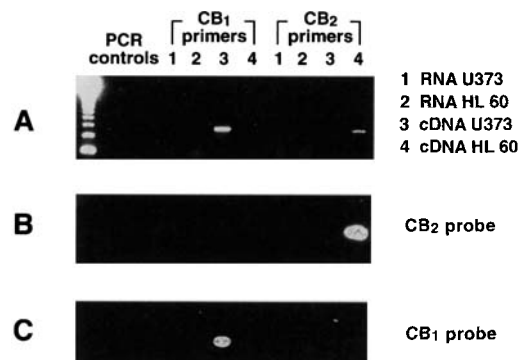
To correct for any variation in the RNA content and cDNA synthesis in the different preparations, each sample was normalized on the basis of the house-keeping gene content, which was also evaluated, in parallel, in the exponential range. For comparative purposes, the CB1 or CB2 mRNA contents were expressed relative to the  $\beta_2$ m RNA content in a sample. Amplification efficiencies, close to 70%, that were identical for the different sets

of primers used and for the various samples analyzed (data not shown), made the normalization possible.

**Northern-blot analysis.** Multiple-tissue Northern-blot-containing poly(A)-rich RNA from various human tissues (heart, brain, placenta, lung, liver, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, kidney and pancreas) was obtained from Clontech. Membranes were prehybridized for 15 min at 68°C in hybridization solution (Quickhyb™ rapid hybridization solution, Stratagene), then hybridized for 4 h at 68°C using radiolabeled CB2 probe ( $4 \times 10^5$  cpm/ $\mu$ l) in hybridization solution containing 100  $\mu$ g/ml salmon sperm DNA. Blots were washed twice with  $2 \times \text{NaCl/Cit}/0.1\%$  SDS for 30 min at 37°C ( $\text{NaCl/Cit} = 0.015$  M sodium citrate, 0.15 M NaCl, pH 7.0),  $0.5 \times \text{NaCl/Cit}/0.1\%$  SDS for 15 min at 60°C, and finally with  $0.1 \times \text{NaCl/Cit}/0.1\%$  SDS for 10 min at 60°C before being autoradiographed using Kodak X Ray film for 7 days at  $-70^\circ\text{C}$ . The cDNA CB2 probe was prepared from plasmid pCANN2 (Sanofi Labège) and radiolabeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by random priming using the random-primed DNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

**Anti-CB2 IgG production and immunohistological analysis.** CB2 C-terminal peptide (Y-P-D-S-R-D-L-D-L-S-D-C) and BSA-conjugated peptide, used as immunogens were from Neosystem. Rabbits were injected subcutaneously with 2 mg BSA-peptide in 250  $\mu$ l water and 250  $\mu$ l complete Freund's adjuvant; animals were boosted monthly under the same conditions. Blood was taken 10 days after the second and subsequent injections. The IgG fraction was prepared by affinity chromatography on a protein-A-Sepharose column as described [24] and stored at  $-80^\circ\text{C}$ . The animal (designated 4-AI), having the higher antibody titer level, determined by ELISA against both the free peptide and CHO-CB2 cells, was selected for immunohistological analysis. For anti-CB2 IgG immunofluorescence analysis in CHO-wt, CHO-CB1 and CHO-CB2, cells were grown for 3 days on 12-mm glass coverslips (Prolabo) prior to analysis. Cells were washed twice in 10 mM sodium phosphate, pH 7.4, 140 mM NaCl ( $\text{NaCl/P}_i$ ) and fixed for 5 min with 4% paraformaldehyde in  $\text{NaCl/P}_i$ . Washed cells were permeabilized for 10 min with 0.1% saponin and 1% BSA in  $\text{NaCl/P}_i$ . 100  $\mu$ l rabbit anti-CB2 IgG (20  $\mu$ g/ml in  $\text{NaCl/P}_i$ /saponin/BSA) was applied and the reaction mixture was incubated for 30 min at room temperature. Cells were washed and incubated with a 1:200 dilution of CY3-conjugated anti-rabbit IgG (Sigma Immuno Chemicals). After washing, coverslips were inverted and mounted on glass microscope slides using glycerol montant containing 1,4-diazabicyclo[2.2.2]octane as anti-quenching agent. Slides were viewed on a laser-scanning confocal microscope equipped with a 543-nm Helium-Neon-laser (LSM 410, Zeiss).

CB2 expression was investigated by immunohistological analysis on human tonsils. Surgical specimens were stored at  $-80^\circ\text{C}$  prior to analysis. Frozen tissue sections of 8  $\mu$ m were obtained using a cryotome (Slee), fixed with acetone for 5 min at  $4^\circ\text{C}$ , before staining using a standard three-stage biotin-avidin immunoperoxidase method as described [25]. Briefly, sections were incubated with 20  $\mu$ g/ml rabbit anti-CB2 IgG for 1 h, with a 1:200-diluted biotinylated anti-rabbit IgG (Sigma) for 30 min, then with a 1:50-diluted streptavidin-biotin-peroxidase complex for 20 min (Amersham). Excess antibody was removed by several washes with  $\text{NaCl/P}_i$  at each step. Sections treated under the same conditions with an irrelevant antibody (rabbit anti Growth-releasing factor IgG) were used as negative controls. Enzymic revelation was performed using hydrogen peroxide and aminoethylcarbazole, and nuclei were counterstained with hematoxylin before observation by light microscopy (Dialux 22,



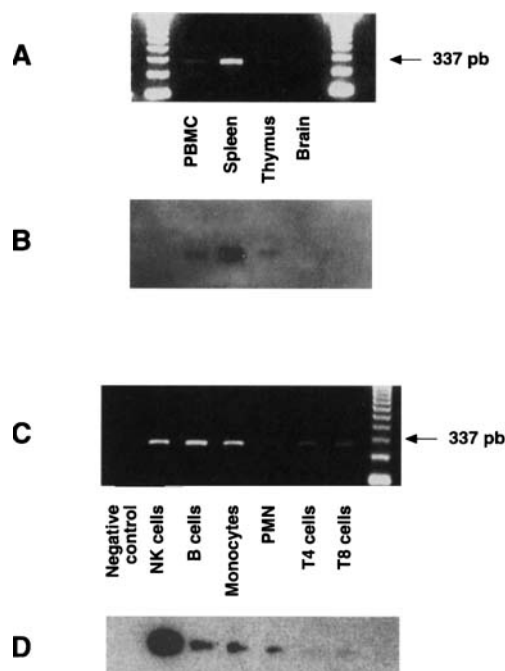
**Fig. 1.** PCR amplification of CB1 and CB2. RNA from the U373 MG and HL60 cell lines was (lanes 3 and 4) or was not (lanes 1 and 2) reverse transcribed into cDNA before amplification for CB1 or CB2. Negative PCR controls were amplification assays with CB1 or CB2 primers without template. PCR products were fractionated by electrophoresis on a 2% agarose gel and revealed by ethidium bromide staining (A). The gel was transferred to nylon membranes and hybridized with either a CB2-specific (B) or a CB1-specific (C) probe.

Leitz). Color photomicrographs were obtained using Gold 100 film (Kodak) on Orthomat (Leitz).

## RESULTS

**Expression of the cannabinoid receptors in immune tissues and immune cell subpopulations.** The expression of cannabinoid receptors in human tissues was analysed by the RT-PCR technique. The specificities of amplification for CB1 and CB2 were primarily established using cDNAs from U373 MG and HL60 cell lines as templates, which only express either CB1 (U373 MG cells) or CB2 (HL60 cells) transcripts [9] (Fig. 1A). The absence of contaminating genomic DNA in cDNA samples was assessed by a direct amplification reaction on RNA samples. The specificity of the bands was also confirmed by the specific signals obtained after Southern blotting of the gels and hybridization with CB1 or CB2 oligonucleotide probes (Fig. 1B and C).

We have already shown that CB1 transcripts are particularly abundant in human brain and that they could also be detected, but at a much lower level, in human lymphoid tissues [5]. In contrast, we observed that CB2 mRNA was not detectable in the brain but rather in spleen, thymus and PBMC (Fig. 2A). Control of these PCR products by probing samples with CB2 probe after Southern blotting showed a specific signal which identified the PCR product as an amplified cDNA segment encoding the CB2 receptor (Fig. 2B). It is noteworthy (Fig. 2A) that the signal from spleen is much more intense than that from thymus or from PBMCs. As we observed notable variations between the different tissues, which might be attributed to heterogeneous expression of the CB2 gene in the different cell types composing the tissue, we further examined isolated T4-cell, T8-cell, monocytes, PMN, B-cell and NK-cell subpopulations. Leukocyte subset purification was performed using both density gradient and immunomagnetic separation methods. T4 cells, T8 cells, NK cells, monocytes and PMN were isolated from PBMC and a B-lymphocyte subset from human tonsils. The purities determined by three-colour immunofluorescence flow-cytometry analysis were 95% for PMN, 90% for T4 cells, 75% for T8 cells, 90% for NK cells, 95% for B cells and 91% for monocytes. All cell types contained detectable transcripts but at variable levels (Fig. 2C). Signals could be classified as NK > B cells > macrophages > PMN > T4 cells > T8 cells. The amplicon of the



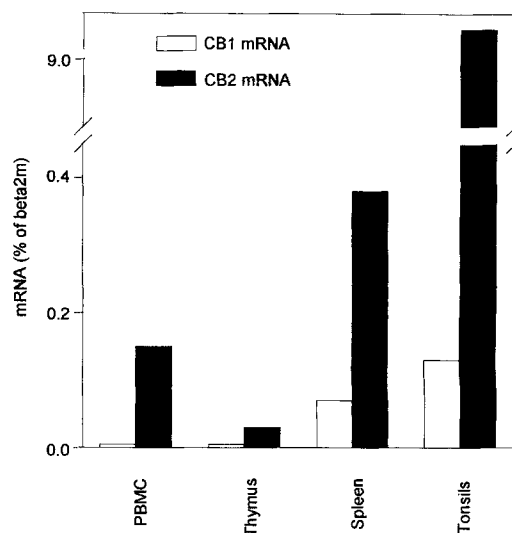
**Fig. 2. RT-PCR analysis of CB2 in lymphoid tissues and purified cellular subtypes.** cDNA-derived mRNA from human PBMC, spleen, thymus and brain were used as templates for CB2 amplification. (A) PCR products were separated by 2% agarose-gel electrophoresis and revealed by ethidium-bromide staining. (B) Southern blotting of the gel and hybridization with a specific CB2 probe. (C) cDNA-derived mRNAs from purified human cell subpopulations were used as templates for CB2 amplification and analyzed on an agarose gel. (D) Southern blotting of the gel and hybridization with a specific CB2 probe.

predicted size hybridized specifically to the CB2 probe (Fig. 2D). Interestingly, these results are similar to those previously obtained for CB1 [5]. This prompted us to perform simultaneous quantitative analyses of both cannabinoid receptor expression.

The expression levels of CB1, CB2 and  $\beta_2m$  genes in each sample were determined by quantitative measurements of mRNA-derived cDNA by RT-PCR (see Materials and Methods). The  $\beta_2m$  mRNA content was used as a reference to normalize variations in initial mRNA contents. The quantification of PCR products was performed in the exponential phase of the amplifications, where similar amplification efficiencies for the three sets of primers were obtained (data not shown). This made it possible to compare the expression of the three genes. The mRNA levels of CB1 and CB2 were expressed relative to the  $\beta_2m$  mRNA level, thus allowing comparison of both CB1 and CB2 mRNA levels in each sample and also from sample to sample.

We first focused on the analysis of immune tissues (spleen, thymus and PBMC) but also of tonsils because of their high B-cell content (Fig. 3). The CB2 transcripts were 10–100-fold more abundant than those of CB1 in all tissues analysed, and the highest amount of CB2 mRNA was detected in tonsils (9.3% of the  $\beta_2m$  content). The rank order for CB2 mRNA expression was tonsil  $\gg$  spleen  $>$  PBMC  $>$  thymus. The rank order for CB1 mRNA was tonsils  $\gg$  spleen  $>$  PBMC = thymus.

When studies were performed on cell subsets, we also found a dominant level of CB2 mRNA and major expression in B cells and NK cells. Contrary to what was observed in a detection experiment (Fig. 2C), CB2 expression was comparable in B and NK cells (3% and 1.7% of the  $\beta_2m$  mRNA level, respectively; Fig. 4A), thus emphasizing the requirement for normalization of



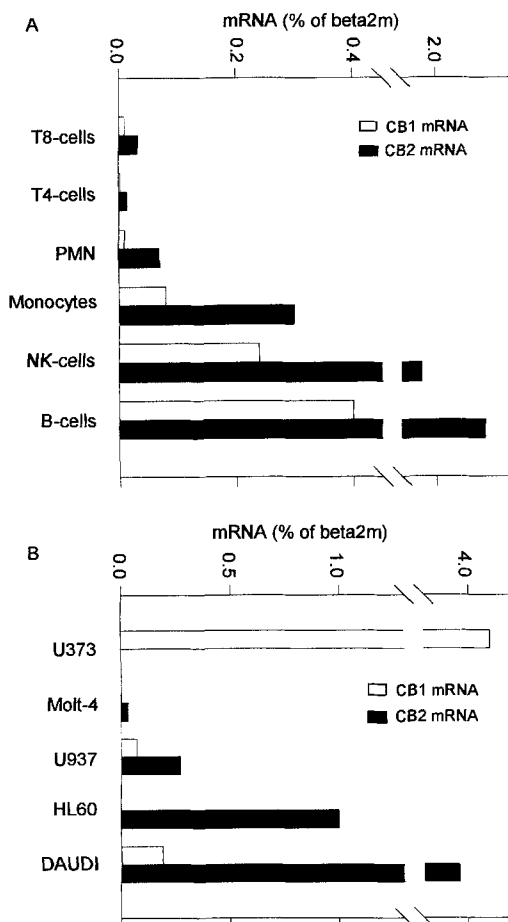
**Fig. 3. Quantitative analysis of CB1 and CB2 transcripts in human immune tissues.** The level of mRNA in PBMC, thymus, spleen and tonsils was quantified by RT-PCR (see Materials and Methods). CB1 mRNA and CB2 mRNA contents were normalized with that of  $\beta_2m$  and expressed relative to the  $\beta_2m$  mRNA level.

the samples with the house-keeping  $\beta_2m$  gene for an accurate quantification. Modulations of CB1 gene expression were in total agreement with our previous results [5]. Extending the analysis to a panel of cell lines from the myeloid, monocytic and lymphoid lineages, we observed that (a) the rank order for the CB2 mRNA content was B-cell (line DAUDI)  $>$  myeloid cell (lines HL60, U937)  $>$  T cell (line Molt-4), and (b) the CB2 gene expression was higher than that of CB1 gene expression in all cases. Together, these results are in accordance with those obtained using purified immune cell subpopulations (Fig. 4B). Used as a control, the U373 MG cell line derived from brain astrocytoma tumor expressed CB1 mRNA exclusively.

We next investigated whether the higher CB2 expression (as compared to CB1 expression) in immune tissues could also be observed in other human tissues. Among all the tissues examined (Fig. 5), high CB2 gene expression is restricted to tissues of the immune system (tonsils, spleen). CB1 mRNA was found expressed at a far lower level in these tissues. Contrastingly, tissues that display the highest CB1 gene expression are from the central nervous system; cerebellum (7.1% of  $\beta_2m$ ), cortex (2% of  $\beta_2m$ ), total brain (1.2% of  $\beta_2m$ ) and pituitary gland (0.9% of  $\beta_2m$ ). In other tissues (Fig. 5), CB1 and CB2 mRNA levels were either extremely low (adrenal gland, heart, lung, prostate, uterus, pancreas, ovary and testis, for which they represented less than 0.08% that of  $\beta_2m$ ), or undetectable (thyroid, retina, skeletal muscle, kidney, liver and placenta).

Heterogeneous expression of the CB2 gene among tissues was confirmed by Northern-blot analysis (Fig. 6). Two distinct bands of approximately 5 kb and 2 kb, corresponding to CB2 transcripts, were shown only in spleen and PBL. This agrees with what was observed in quantitative RT-PCR analysis, even though not all the same tissues were analysed.

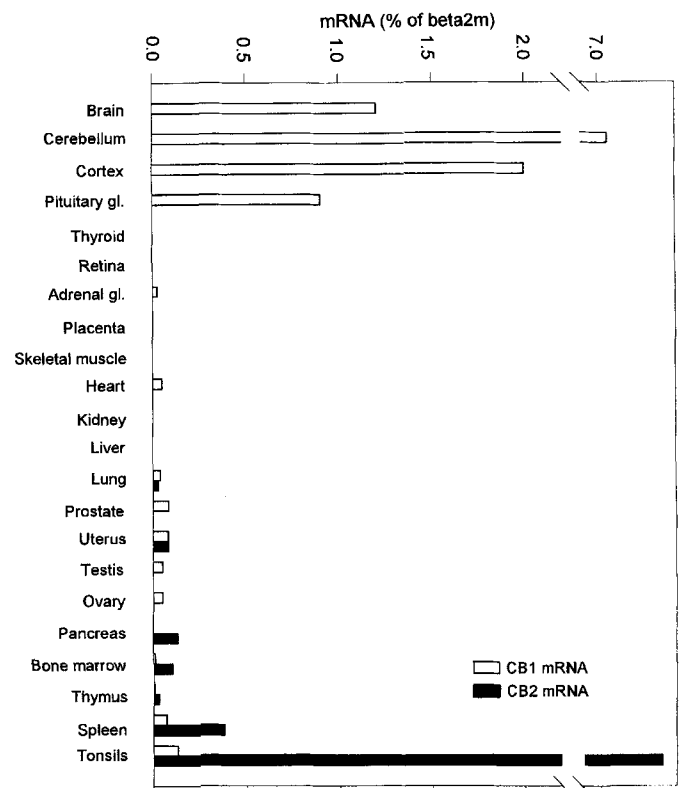
**Expression of the CB2 receptor protein in tonsils.** Finally, to investigate whether or not the results obtained at the mRNA level for CB2 could be extended to the protein level and thus represent a physiological reality, we carried out an immunohistochemical analysis. An anti-CB2 polyclonal antibody (4-AI) was produced against the C-terminal fragment of the CB2 receptor (see Materials and Methods). CHO cells transfected with the



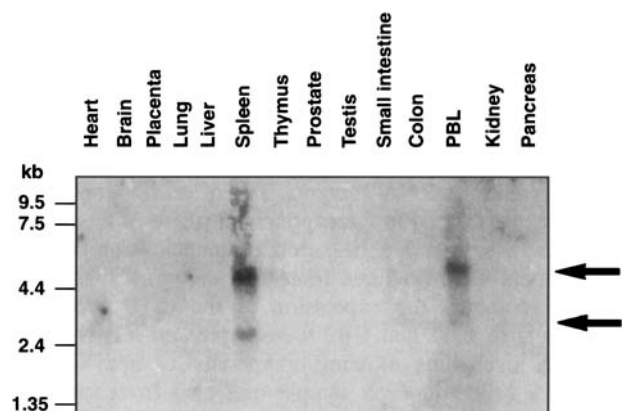
**Fig. 4. Quantitative analysis of CB1 and CB2 transcripts in human immune cell subsets and in human hematopoietic cell lines.** (A) Distribution patterns of CB1 and CB2 mRNAs in purified immune cell subpopulations of B cells, T4 cells, T8 cells, NK cells, monocytes and PMN. (B) Distribution patterns of CB1 and CB2 mRNAs in myeloid (U937, HL60), T (Molt-4) and B (DAUDI) leukemia cell lines. The human astrocytoma cell line U373 MG was used as a positive control for CB1 gene expression. Quantification was performed as described in Fig. 3. CB1 mRNA in Molt-4 and HL60 was undetectable, as was CB2 mRNA in U373 MG.

human CB2 cDNA receptor (CHO-CB2) were used as a positive control in immunofluorescence-staining experiments with this antibody and analysed by confocal microscopy (Fig. 7). The specificity of the antibody was assessed by the absence of labelling on CHO-wt or CHO-CB1 (Fig. 7) and also by the displacement of labelling on CHO-CB2 by the addition of an excess of the C-terminal peptide (data not shown). The staining localization shows that CB2 receptors are associated with the plasma membrane in permeabilized CHO-CB2 cells (Fig. 7). When CHO-CB2 cells were not permeabilized, no labelling was observed, strongly suggesting that the C-terminal fragment of CB2 is intracellular (data not shown).

The availability of anti-CB2 IgG made it possible to further analyse CB2 expression in tonsils, selected for their high CB2 mRNA content. Microscopic observations of tonsil sections showed the typical structure of lymphoid organs with numerous B-cell follicles, included in T-cell areas (Fig. 8A). An indirect immunoperoxidase method using anti-CD19 or anti-CD3 monoclonal antibodies confirmed the tissular localization of B and T lymphocyte subsets, respectively (data not shown). Using the rabbit anti-CB2 IgG, specific labelling, which seemed to be restricted to B lymphocytes and not to T-lymphocyte-enriched



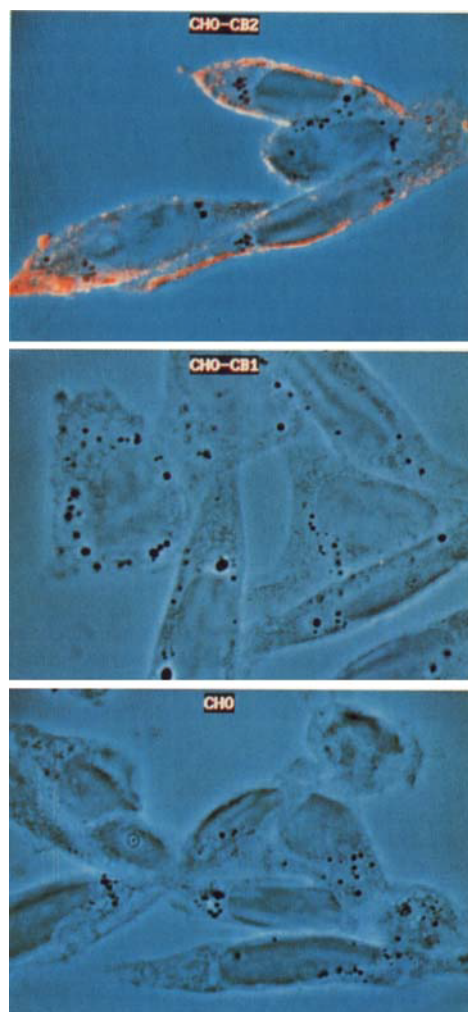
**Fig. 5. Quantitative analysis of CB1 and CB2 transcripts in human organs.** Analysis was performed on cDNA from various human organs as indicated and quantification was performed as described in Figs 3 and 4. Both CB1 and CB2 were undetectable in thyroid, retina, placenta, skeletal muscle, kidney and liver. CB1 was undetectable in pancreas. CB2 was undetectable in total brain, cerebellum, cortex, pituitary gland, adrenal gland, heart, prostate and ovary. gl., gland.



**Fig. 6. Northern-blot analysis of CB2 gene expression in human organs.** 2 µg poly (A)-rich RNA from various organs, as indicated, were loaded onto each lane. Hybridization of the Northern blot was performed with a specific CB2 cDNA probe (see Materials and Methods).

areas, was observed. Anti-CB2 receptor-positive B cells were mainly located in the mantle of secondary lymphoid follicles, which contain immature B cells (Fig. 8D). In the germinal centers of secondary follicles which contain mature B cells, scarce and isolated positive cells also appeared (Fig. 8C). These results, which demonstrate the expression of CB2 as a protein in B lymphocytes, further suggest heterogeneous distribution of CB2 protein over B-cell maturation stages.



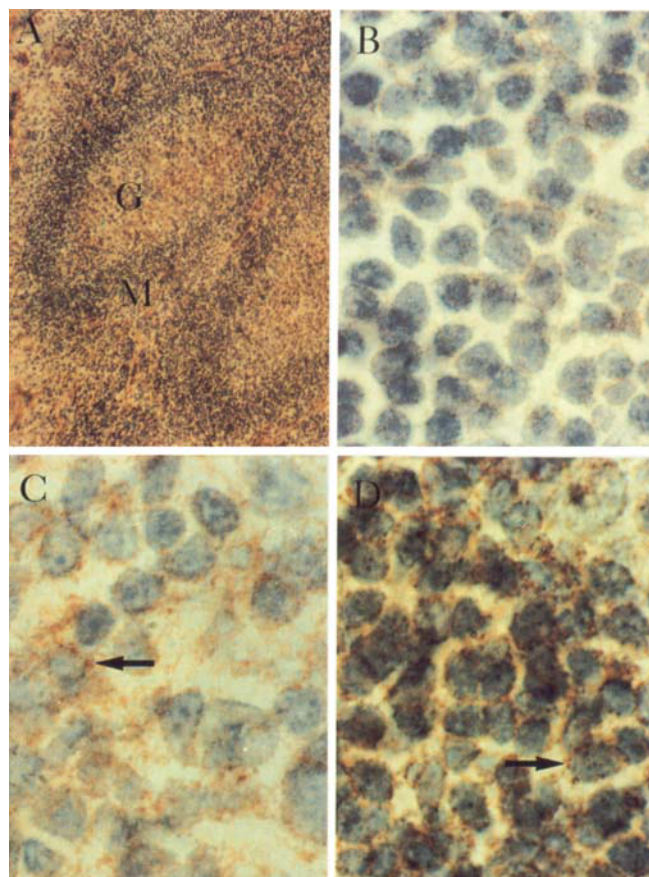


**Fig. 7. Immunofluorescence analysis of CB2 expression.** CHO cells transfected with human CB2 cDNA (CHO-CB2) or with human CB1 cDNA (CHO-CB1), or wild-type cells (CHO) were labelled with the rabbit anti-CB2 IgG (4-AI) as detailed (Materials and Methods) and analysed by light-scanning confocal microscopic analysis.

## DISCUSSION

In addition to their effects on the central nervous system, cannabinoids and related cannabinoid compounds are well known to possess immunosuppressive properties. Several studies have suggested that the mechanism of this inhibitory effect is mediated by specific receptors [26]. We have previously demonstrated the presence of mRNA for the central cannabinoid receptor (CB1) in human immune tissues (spleen, tonsils and PBL) and we have also reported a heterogeneous distribution pattern of expression among the cell subsets of human peripheral blood. As a peripheral receptor subtype for cannabinoids (CB2) has been described, we investigated whether or not CB2 is also expressed in immune tissues in order to determine whether cannabinoid-induced immunosuppression can be assigned to CB1 or CB2 or both. Our first approach consisted of comparing distribution patterns of the expression of the cannabinoid receptor genes in different human organs and among immune tissues for purified cell subsets, as well as for their corresponding human cell lines.

First, we observed highly heterogeneous expression of CB1 and CB2 genes among the tissues examined, these being classified in three subsets as follows.



**Fig. 8. Immunohistological analysis of CB2 expression in tonsil sections.** Tonsil sections were labelled with the anti-CB2 IgG (A, C, D) or a control antibody (B). In (A), the germinal center and the mantle of lymphoid follicle are indicated by the letters G and M, respectively. Arrows show CB2-receptor-positive cells in the germinal center (C) or in the mantle of lymphoid follicle (D). Original magnification is X63 (A), X1000 (B–D).

Subset 1, tissues rich in CB1 mRNA and devoid of CB2 mRNA. This subset concerns tissues of the central nervous system (brain and cerebellum), in which the high expression of CB1 observed is in agreement with previous studies [27, 28] and is consistent with the effects of cannabinoids on equilibrium and motion.

Subset 2, tissues rich in CB2 mRNA and (almost) devoid of CB1 mRNA. We provide here, to our knowledge for the first time, evidence that immune tissues and immune cell subpopulations mainly express the CB2 gene, and that CB1 mRNA levels represent only 1–10% of the CB2 content. Among immune cell subpopulations, B cells and NK cells exhibited particularly high levels of CB2 mRNA. We also observed a good correlation of expression of the CB2 gene between monocytic, T and B leukemia cell lines and their normal tissue counterparts. It is noteworthy that the rank order of CB2 mRNA levels in the immune tissues examined (tonsils > spleen > PBMC > thymus) could be attributed to their B cell content (60–80%, 40–60%, 5–10%, 0–1%, respectively). Compared to other immune tissues, the thymus displayed a relatively low CB2 mRNA level. Such a difference is probably due to its high T cell content, T-cells being the immune-cell subset that expresses the lowest CB2 mRNA level.

Subset 3, contains other peripheral tissues where both CB2 and CB1 mRNA expressions were either low (adrenal gland, heart, lung, prostate, uterus, pancreas and testis) or undetectable

(thyroid, retina, skeletal muscle, mature kidney, mature liver, placenta and ovary).

Importantly, when we compared the expression of the CB1 gene in the central nervous system and the CB2 gene in immune tissues, we noted that levels were within the same order of magnitude, strongly suggesting that, by analogy with what is known for CB1, CB2 can be considered as a tissue-selective antigen and important physiological functions should be expected for the CB2 receptor in immunity.

To analyse cannabinoid receptor expression at the protein level, we developed specific antibodies for CB2 which enabled us to demonstrate that CB2 is present at the surface of CHO cells transfected with human CB2 cDNA. The immunohistological analysis of CB2 receptor localization on tonsils revealed the staining of only B cells in the mantle of follicles and not of T cells from such tissues. Such a restricted expression of the protein to the B lymphocytes is in agreement with the recently published work of Lynn and Herkenham who, using the cannabinoid ligand [<sup>3</sup>H]CP-55,940, showed that the predominant cell type bearing cannabinoid receptors is the B lymphocyte [29]. Germinal centers in tonsils are the generation sites of memory B cells undergoing somatic mutations for Ig gene synthesis and isotype switching. B cells from the mantle of the follicle are considered as precursor cells, bearing IgD<sup>+</sup> on their surface, and which become IgD<sup>-</sup> within the germinal center [30, 31]. Since we observed that the highest amount of the CB2 receptor was localized in the mantle of follicles, and scarce labelling occurred in the germinal center containing mature B cells, this may suggest a decrease in the expression of CB2 with memory-B-cell generation.

The high CB2 expression restricted to cells of the immune system and particularly in B cells and NK cells may be related to the well-established alterations of NK and B-cell functions by cannabinoids. Even though one cannot exclude a contribution of CB1 to cannabinoid immunosuppressive effects, the results reported here provide evidence for a role of CB2 in the immune system.

Cellular functions of CB2 are unknown and are under active research. The similarity between CB1 and CB2 amino-acid sequences allowed us to speculate that the functions of these two receptors could be related. CB1 functions have been partially elucidated; it is coupled to several biological responses including the inhibition of both adenylyl cyclase and N-type calcium channel inhibition, as well as to the induction of the immediate-early gene *Krox-24* [7–9]. As intracellular level variations of cyclic AMP and calcium occurred during immune response modulation, one may suppose that the peripheral cannabinoid receptor mediates immunosuppression via the inhibition of adenylyl cyclase or the calcium channels.

However, pharmacological differences between CB1 and CB2 have been observed [11], suggesting the possible development of subtype-selective drugs. Drugs specific for the CB2 receptor should have great therapeutic applications, for instance, immunosuppression induced by a cannabinoid compound devoid of central activity could have a positive therapeutic value in autoimmune disorders.

We are grateful to Dr M. Portier for critical review of the manuscript.

## REFERENCES

- Dewey, W. L. (1986) Cannabinoid pharmacology, *Pharmacol. Rev.* **38**, 151–178.
- Devane, W. A., Dysarz III, F. A., Johnson, M. R., Melvin, L. S. & Howlett, A. C. (1988) Determination and characterization of a cannabinoid receptor in rat brain, *Mol. Pharmacol.* **34**, 605–613.
- Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA, *Nature* **346**, 561–564.
- Gérard, C. M., Mollereau, U. C., Vassart, G. & Parmentier, M. (1991) Molecular cloning of a human cannabinoid receptor which is also expressed in testis, *Biochem. J.* **279**, 129–134.
- Bouaboula, M., Rinaldi, M., Carayon, P., Carillon, C., Delpech, B., Shire, D., Le Fur, G. & Casellas, P. (1993) Cannabinoid receptor expression in human leukocytes, *Eur. J. Biochem.* **214**, 173–180.
- Kaminski, N. E., Abood, M. Y., Kessler, F. K., Martin, B. R. & Schatz, A. R. (1992) Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation, *Mol. Pharmacol.* **42**, 736–742.
- Howlett, A. C., Qually, J. M. & Khachatrian, L. L. (1986) Involvement of G<sub>i</sub> in the inhibition of adenylyl cyclase by cannabimimetic drugs, *Mol. Pharmacol.* **29**, 307–313.
- Mackie, K. & Hille, B. (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells, *Proc. Natl Acad. Sci. USA* **89**, 3825–3829.
- Bouaboula, M., Bourrié, B., Rinaldi-Carmona, M., Shire, D., Le Fur, G. & Casellas, P. (1995) Stimulation of cannabinoid receptor CB1 induces *Krox-24* expression in human astrocytoma cells, *J. Biol. Chem.* **270**, 13973–13980.
- Devane, W. A., Hanus, L., Breuer, A., Peertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. & Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor, *Science* **258**, 1946–1949.
- Rinaldi-Carmona, M., Barth, F., Heaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Neliat, G., Caput, D., Ferrara, P., Soubrie, P., Breliere, J. C. & Le Fur, G. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor, *FEBS Lett.* **350**, 240–244.
- Munro, S., Thomas, K. L. & Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids, *Nature* **365**, 61–65.
- Pross, S. H., Klein, T. W., Newton, C. A. & Friedman, H. (1987) Differential effects of marijuana components on proliferation of spleen, lymph node and thymus cells *in vitro*, *Int. J. Pharmacol.* **9**, 363–370.
- Luo, Y. D., Patel, M. K., Wiederhold, M. D. & Ou, D. W. (1992) Effects of cannabinoids and cocaine on the mitogen-induced transformations of lymphocytes of human and mouse origins, *Int. J. Immunopharmacol.* **14**, 49–56.
- Klein, T. W., Kawakami, Y., Newton, C. & Friedman, H. (1991) Marijuana components suppress induction and cytolytic function of murine cytotoxic T cells *in vitro* and *in vivo*, *J. Toxicol. Environ. Health* **32**, 465–477.
- Arata, S., Klein, T. W., Newton, C. & Friedman, H. (1991) Tetrahydrocannabinol treatment suppresses growth restriction of *Legionella pneumophila* in murine macrophage cultures, *Life Sci.* **49**, 473–479.
- Blanchard, D. K., Newton, C., Klein, T. W., Stewart, W. E. II & Friedman, H. (1986) *In vitro* and *in vivo* suppressive effects of delta-9-tetrahydrocannabinol on interferon production by murine spleen cell, *Int. J. Immunopharmacol.* **8**, 819–824.
- Kaminski, N. E. (1993) Evidence for a cannabinoid receptor in immunomodulation by cannabinoid compounds, *Adv. Exp. Med. Biol.* **335**, 115–120.
- Kaminski, N. E., Koh, W. S., Yang, K. H., Lee, M. & Kessler, F. K. (1994) Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylyl cyclase by a pertussis toxin-sensitive G-protein coupled mechanism, *Biochem. Pharmacol.* **48**, 1899–1908.
- Canat, X., Carayon, P., Bouaboula, M., Cahard, D., Shire, D., Roque, C., Le Fur, G. & Casellas, P. (1993) Distribution profile and properties of peripheral-type benzodiazepine receptors on human hemopoietic cells, *Life Sci.* **52**, 107–118.
- Carayon, P., Bord, A. & Raymond, M. (1991) Simultaneous identification of eight leukocyte subsets of human peripheral blood using three-colour immunofluorescence flow cytometric analysis, *J. Immunol. Methods* **138**, 257–264.

22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, *Biochemistry* 18, 5294–5299.
23. Murphy, L. D., Herzog, C. E., Rudick, J. B., Fojo, A. T. & Bates, S. E (1990) Use of the polymerase chain reaction in the quantitation of *mdr-1* gene expression, *Biochemistry* 29, 10351–10356.
24. Ey, P. L., Prowse, S. J. & Jenkin, C. R. (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose, *Immunochemistry* 15, 429–436.
25. Hsu, S. M., Raine, L. & Fanger, H. (1981) Use of avidin-biotin peroxidase complex (ABC) in Immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PA) procedures, *J. Histochem. Cytochem.* 29, 577–580.
26. Kaminski, N. E. (1994) Mechanisms of immune modulation by cannabinoids, in *Immunotoxicology and Immunopharmacology* (Dean, J., Luster, M. I., Munson, A. E. & Kimber, I., eds) 2nd edn, pp. 349–362, Raven Press, New York.
27. Mailleux, P. & Vanderhaeghen, J. J. (1992) Distribution of cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and *in situ* hybridization histochemistry, *Neuroscience* 48, 655–668.
28. McLaughlin, C. & Abood, M. E. (1993) Developmental expression of cannabinoid receptor mRNA, *Dev. Brain Res.* 76, 75–78.
29. Lynn, A. B. & Herkenham, M. (1994) Localization of cannabinoid receptors and nonsaturable high-density cannabinoid binding sites in peripheral tissues of the rat: implications for receptor-mediated immune modulation by cannabinoid, *J. Pharm. Exp. Ther.* 268, 1612–1623.
30. Thorbecke, G. J., Amin, A. R. & Tsiagbe, V. K. (1994) Biology of germinal centers in lymphoid tissue, *FASEB J.* 8, 832–840.
31. Hardie, D. L., Johnson, G. D., Khan, M. & MacLennan, I. C. M. (1993) Quantitative analysis of molecules which distinguish functional compartments within germinal centers, *Eur. J Immunol.* 23, 997–1004.