HSC70 blockade by the therapeutic peptide P140 affects autophagic processes and endogenous MHCII presentation in murine lupus

Nicolas Page, Frédéric Gros, Nicolas Schall, Marion Décossas, Dominique Bagnard, Jean-Paul Briand, Sylviane Muller

ABSTRACT

Background The P140 phosphopeptide issued from the spliceosomal U1-70K small nuclear ribonucleoprotein protein displays protective properties in MRL/lpr lupus-prone mice. It binds both major histocompatibility class II (MHCII) and HSC70/Hsp73 molecules. P140 peptide increases MRL/lpr peripheral blood lymphocyte apoptosis and decreases autoepitope recognition by T cells.

Objective To explore further the mode of action of P140 peptide on HSC70 + antigen-presenting cells.

Methods P140 biodistribution was monitored in real time using an imaging system and by fluorescence and electron microscopy. Fluorescence activated cell sorting and Western blotting experiments were used to evaluate the P140 effects on autophagic flux markers.

Results P140 fluorescence accumulated especially in the lungs and spleen. P140 peptide reduced the number of peripheral and splenic T and B cells without affecting these cells in normal mice. Remaining MRL/lpr B cells responded normally to mitogens. P140 peptide decreased the expression levels of HSC70/Hsp73 chaperone and stable MHCI dimer, which are both increased in MRL/lpr splenic B cells. It impaired refolding properties of chaperone HSC70. In MRL/lpr B cells, it increased the accumulation of the autophagy markers p62/SQSTM1 and LC3-II, consistent with a downregulated lysosomal degradation during autophagic flux.

Conclusion The study results suggest that after P140 peptide binding to HSC70, the endogenous (auto) antigen processing might be greatly affected in MRL/lpr antigen-presenting B cells, leading to the observed decrease of autoreactive T-cell priming and signalling via a mechanism involving a lysosomal degradation pathway. This unexpected mechanism might explain the beneficial effect of P140 peptide in treated MRL/lpr mice.

INTRODUCTION

Systemic lupus erythematosus is a multifactorial (multigenic) and highly polymorphic systemic autoimmune disorder. It affects multiple organs, including skin, muscle, joints and vital internal organs such as kidneys and heart. Current pharmacological treatments are largely palliative and result in non-specific immunosuppression. The goal today is to design more specific and effective treatments.

P140 peptide is a 21-mer fragment of the spliceosomal U1-70K small nuclear ribonucleoprotein protein that significantly ameliorates clinical and biological manifestations in autoimmune patients with systemic lupus erythematosus and enhances survival in lupus-prone mice. This peptide contains a phosphoserine residue at position 140, a modification that specifically occurs at an early stage of apoptosis, before the cleavage of the C-terminal part of the protein by caspase-3 and the dephosphorylation of other serine residues by a PP1 phosphatase-mediated mechanism. Originally thought to act solely via the modulation of intracellular signalling induced by αβ T-cell receptor (TCR) engagement, P140 was unexpectedly found to interact selectively with the constitutive heat-shock HSC70/Hsp75 protein. By a granzyme-B and caspase-dependent mechanism, it induces in Fas-deficient MRL/lpr lupus mice apoptosis of B cells and CD4, CD8 or double negative (DN) αβTCR+CD4/CD8/B220+ T lymphocytes through a regulatory circuit involving γδ T cells. Intravenous P140 peptide administration into young MRL/lpr mice thus causes T- and B-cell egress from peripheral blood but does not affect T-cell priming, and does not interfere with the ability of P140-treated mice to resist to an infectious viral challenge. P140 also downregulates the expression of programmed death 1 (PD-1/CDD279) receptor, a molecule of the CD28 family, which is overexpressed at the surface of MRL/lpr CD4 T cells. P140 peptide thus displays various effects on the immune system that probably interfere with several, yet unidentified, molecular pathways leading finally to a decreased T-cell autoreactivity and to significantly lower levels of autoantibodies to native DNA.

In the postulated P140 mode of action, binding to the chaperone HSC70 protein seems to be central. The implication of or any defect of the HSC70 pathway in regulating immune functions in lupus is not documented. It is not known whether or not HSC70-chaperoned molecules, such as other Hsps and major histocompatibility complex (MHC) molecules, are affected by P140–HSC70 binding. To address these major questions, we evaluated the effect of P140 administration on the expression of HSC70 and MHCII molecules, and studied the possible influence of P140 on autophagosomal pathways implicated in the endogenous MHCII loading. Since HSC70 is involved in certain phases of these pathways, we expected that binding of P140 to HSC70 might alter the delivery of intracellular material into the endosomal/lysosomal pathway and therefore thwart the final presentation of self-antigens to autoreactive T cells.

METHODS

Synthetic peptides and biochemical assays

Peptides were assembled and purified as described. The P140 peptide was also synthesised...
Extended report

with a cysteine residue added at its N-terminus. The luciferase
renaturation assay was as described previously with either intact or
heat-denatured luciferase and rabbit reticulocyte lysate in
the presence of increasing concentrations of P140 peptide.
Luciferase activity was determined using Bright-glo reagent (Promega, Charbomières, France).

In vivo experiments and cell studies
Protection experiments and clinical monitoring have been
described previously. All experimental protocols were carried
out with the approval of the local Institutional Animal Care and
Use Committee (CREMEAS). The renal pathology was evaluated
by measuring proteinuria levels and observing vasculitis corre-
sponding to the area of cell infiltrates. For in vivo imaging, Alexa
Fluor488-labelled P140 was injected through the retro-orbital
venous sinus of anaesthetised mice. Mice were immediately
transferred into the cabinet of the NightOwl Biosmager (Berthold
Technologies, Thoiry, France) and images were taken periodic-
ally for 3 h. Mice were then killed to collect organs for final
fluorescence acquisition and validation of biodistribution. Signal
intensity was evaluated using the Indigo software.

Histology was carried out on frozen section of organs collected
from MRL/lpr mice that received rhodamine-labelled P140 intra-
venously. For co-localisation experiments, spleen sections were
permeabilised and incubated with either fluorescein isothiocya-
nate (FITC)-labelled anti-CD220 antibody or biotin-labelled anti-
CD3e antibodies, followed by Alexa Fluor488-labelled streptavidin,
or biotin-labelled anti-MOMA-1/CD169 antibodies specific for
metallophilic macrophages, followed by Alexa Fluor488-labelled
streptavidin. Other antibodies tested were: biotin-labelled anti-
CD11b and biotin-labelled anti-F4/80 antibodies.

Immunofluorescence and immunoelectron microscopy
experiments were performed on peripheral blood lymphocytes
(PBLs) and splenocytes collected from MRL/lpr mice that had
received Alexa Fluor488-labelled P140 intravenously 1 h before
examination. Some experiments used splenocytes incubated for
1 h at 37°C with 5 μM Alexa Fluor488-labelled P140. HSC70 and
MHCII molecules were revealed with specific antibodies (clone
1B5; Stressgen (Enzo Life Sciences, Villeurbanne, France) and
Clone 2G9; BD Biosciences (Le Pont de Claix, France), respectively).
Fluorescence activated cell sorting was as described previously previously. The antibodies were anti-HSC70 antibody labelled
with R-phycocerythrin (clone 1B5) and anti-IA/IE antibody
labelled with FITC (clone 2G9). For specific analysis of cellular
subtypes, antibodies used were: anti-CD3e-FITC, anti-CD3e-
allophycocyanin, anti-B220-PerCP, anti-B220-allophycocyanin,
antig-CD4-allophycocyanin, anti-CD8-allophycocyanin, anti-
CD11b-PerCP and anti-Gr-1-FITC. HSC70 and MHCII lev-
els were expressed as the ratio of relative mean fluorescence
intensity for specific antibody/mean fluorescence intensity for
isotype-matched control antibody.

For measuring lymphocyte proliferation, MRL/lpr and CBA/J
mice (four to five/group) were given P140 peptide or ScP140
(100 μg/mouse) or saline only, daily for 10 consecutive days
via the intraperitoneal route. Ciclosporin A (20 mg/kg/day) and
prednisolone (feeding at 4 mg/kg/day) were tested in parallel.
A lymphocyte proliferation assay was performed as described previously using 3×10^5 cells/well.

Statistics
Statistical analyses were performed using parametric (Student t
test) and non-parametric (Mann–Whitney U) test. For all tests,
p<0.05 was considered statistically significant.

RESULTS
In vivo P140 peptide effects and biodistribution
We reported previously that intravenous administration of
P140 to MRL/lpr mice significantly improves their clinical
and biological manifestations and prolongs their survival. We
show here that P140 significantly decreases vasculitis with fewer peri-vascular inflammatory infiltrates in kidneys
(figure 1A; p<0.0079), slows down glomerulonephritis (figure
1B; p<0.0001) and reduces the appearance of dermatitis
(figure 1C).

P140 biodistribution after intravenous administration to
MRL/lpr mice was monitored in real time by bioimaging in liv-
ing mice. Alexa Fluor488-labelled P140 accumulated particularly
in the lungs and spleen, a distribution that was not observed
with an unrelated peptide (figure 1D; online supplementary
figure S1; similar results were obtained in 6- and 12-week-old
MRL/lpr mice). Fluorescence in the spleen was detectable 10–15
min after P140 injection and remained for at least 90–120 min.
Similar results were observed in MRL/lpr mice that received
rhodamine-labelled P140 intravenously (supplementary figure
S2A). In the spleen, fluorescence was mainly localised in the
lymphoid white pulp, mostly in the mantle and marginal zones
(supplementary figure S2B). It co-localised essentially with
spleenic B cells and resident macrophages (figure 1E) as well as
antigen is absent from macrophages located in T-cell areas of
the spleen and lymph nodes (LNs). Thus, P140 preferentially
homes in organs enriched in activated B cells and, more genera-
ly, in antigen-presenting cells (APCs).

P140 administration significantly decreased peripheral
hypercellularity in MRL/lpr mice (supplementary figure S3; no
effect was seen with the ScP140 scrambled analogue). B cells,
T cells (including γδ T cells and DN T cells) and granulocytes/
monocytes were affected. In CBA/J control mice, however,
even an overdosage of P140 did not produce any observable
effect while, in contrast, the glucocorticoid prednisolone,
which is active in MRL/lpr mice, and immunosuppressant
ciclosporin A induced a decrease of PBL counts in both CBA/J
and MRL/lpr mice (figure 1F). Peripheral B and T cells from
treated MRL/lpr mice and remaining peripheral B and T
cells from P140-treated mice responded equally well ex vivo to
B-cell mitogen and slightly less well (p=0.024) to T-cell mito-
gen (figure 1G). No effect of P140 was observed in CBA/J mice.
This was not the case for ciclosporin A, which significantly
affected the abilities of peripheral B and T cells from MRL/
lpr and CBA/J mice to respond to mitogens, and prednisolone,
which affected the abilities of CBA/J B and T cells to respond
to mitogens. In the spleen, the proportion of T cells (including
activated T cells) was significantly decreased after P140 treat-
ment (p=0.0022; figure 1H). The percentage of B cells remained
unchanged.

HSC70 and MHCII overexpression in MRL/lpr mice is reduced
after P140 treatment
P140 binds to HSC70 protein both at 25°C and 4°C (figure 2A),
suggesting that P140 recognises both intracellular and cell
surface-expressed HSC70 (result obtained with splenocytes,
LN cells and PBLs). Fluorescent staining of P140 and HSC70
co-localises in the cytoplasm of freshly isolated, permeabi-
lised MRL/lpr PBLs 1 h after intravenous administration of
Alexa Fluor488-labelled P140 (figure 2B1). This co-localisation
was confirmed by immunofluorescence and immunoelectron
microscopy (figure 2B2, C), and was mainly cytoplasmic.
mice appeared to be correlated with an increased HSC70 mRNA expression in MRL/lpr splenocytes (figure 2E; not visualised in LN cells and thymocytes). Interestingly, in MRL/lpr mice that received intravenous injections of 100 μg P140 daily over 6 days, HSC70 expression in splenic B cells was decreased by 32% (figure 2F; expression of inducible Hsp70/Hsp72 protein was not affected).

Interaction of HSC70 with antigen is an important component of the MHCII antigen processing pathway. In accordance HSC70 is present at the surface of non-permeabilised MRL/lpr and CBA/J peripheral B and T cells (notably B220+/TCRβ+ activated T cells and DN T cells) as well as on splenic MRL/lpr B220–/CD11b+/MAC-1/Gr-1– monocytes/macrophages and B220–/CD3/CD11b+/Gr-1+ granulocytes (figure 2D1). In the spleen, surface and intracellular HSC70 expression levels were raised in MRL/lpr mice (figure 2D and supplementary figure S4; observed also in LN cells, notably at the surface). The HSC70 expression increase observed in the spleen of MRL/lpr mice appeared to be correlated with an increased HSC70 mRNA expression in MRL/lpr splenocytes (figure 2E; not visualised in LN cells and thymocytes). Interestingly, in MRL/lpr mice that received intravenous injections of 100 μg P140 daily over 6 days, HSC70 expression in splenic B cells was decreased by 32% (figure 2F; expression of inducible Hsp70/Hsp72 protein was not affected).

Interaction of HSC70 with antigen is an important component of the MHCII antigen processing pathway. In accordance
lymphoblast-like cells (figure 3C; MHCII monomer expression remained unaffected). Thus, P140 associates with both HSC70 and MHCII molecules and decreases directly or indirectly their surface expression on splenic B cells.

P140 alters autophagic processes in MRL/lpr B cells

It has been shown earlier in B-cell lines that manipulating APC expression of HSC70 modifies the presentation of epitopes derived from intracellular proteins but not from exogenous antigens to CD4 T cells. Since HSC70 is involved in autophagy pathways, notably in chaperone-mediated autophagy, leading to endogenous MHCII loading, we explored this mechanism, hypothesising that P140 might disrupt normal functions of HSC70 in this catabolic pathway. First, using a refolding test of heat-denatured luciferase by rabbit reticulocyte lysate, we with observations showing that in B cells HSC70 associates with HLA-DRB1*0401, and that HSC70 and MHCII molecules are associated in B-cell exosomes, for example, we found that HSC70 and MHCII molecules staining co-localises in permeabilised MRL/lpr splenocytes (figure 3A and supplementary figure S5). After in vitro incubation, co-localisation of P140 and MHCII molecules staining was observed in 25% of cells, and co-localisation of P140, HSC70 and MHCII molecules staining was visualised in 5–10% of cells. Interestingly, we found that after daily intraperitoneal injections of P140 into 12-week-old MRL/lpr mice, overexpression of MHCII molecules in spleen B cells significantly decreased (figure 3B). We thus examined further the effect of P140 on the stability of functional MHC molecules using Raji cells. In a dose-dependent manner, P140 hampered the formation of stable HLA-DRαβ dimers in these lymphoblast-like cells (figure 3C; MHCII monomer expression remained unaffected). Thus, P140 associates with both HSC70 and MHCII molecules and decreases directly or indirectly their surface expression on splenic B cells.
examined in vitro whether P140 could hamper folding properties of the HSC70 molecule. At 40 and 80 μM, P140 inhibited luciferase refolding by 50.2% and 100%, respectively (figure 4A; ScP140 showed no effect). This effect was reversed by adding an excess of exogenous HSC70 (figure 4B).

Next, we examined P140-treated cells for altered expression of lysosomal or autophagy pathways markers. We studied the conversion in MRL/lpr cells of the soluble form of microtubule-associated protein-1 light chain 3 (LC3-I), a mammalian homologue of yeast Atg8, into the hydrophobic, mostly membrane-associated form LC3-II, which is enriched in the autophagic vacuole fraction but is rapidly degraded by lysosomal proteases when the autophagic flux is intense. 19 We observed that splenic B cells purified from 8-week-old MRL/lpr mice exhibited almost undetectable levels of LC3-I and rather low LC3-II levels, which were dramatically enhanced in the presence of lysosomal protease inhibitors E64D (calpain and cathepsin B inhibitor) and pepstatin A (cathepsin D inhibitor) (figure 5A). This reflects the existence of a constitutive autophagic flux in resting MRL/lpr B cells that can be blocked by specific inhibitors before the autolysosome formation, at the fused autophagosome–lysosome step. 19 In the presence of P140, the basal accumulation of LC3-II expression was increased in a P140 concentration-dependent manner, and strikingly, the autophagic flux was proportionally less intense (figure 5A). This suggests that in MRL/lpr B cells, P140 downregulates autophagic flux at the autolysosome stage. Interestingly, this stage is also affected by E64D and pepstatin A, for example, as well as by chloroquine, 20 21 an antimalarial drug used in lupus treatment.

This result was confirmed by measuring the levels of p62 (SQSTM1/sequestosome-1), one of the specific substrates that are degraded through the autophagy-lysosomal pathway. p62 is selectively incorporated into autophagosomes via direct interaction with LC3. 22 23 In a peptide dose-dependent manner, p62 accumulated in P140-treated MRL/lpr B cells (figure 5B; the same effect, although at a lower extent, observable in CBA/J B cells), again consistent with an effect of P140 on the steady-state autophagy in these cells.

DISCUSSION

We present evidence here that P140 seems to interfere with a signalling pathway engaged in lysosomal degradation, leading to instability of MHCII molecules supposed to present endogenous antigens to autoreactive T cells in murine lupus. After intravenous injection into MRL/lpr mice, P140 concentrates mainly in the lungs and the spleen, and in the latter it co-localises essentially with B cells and resident macrophages of the lymphoid white pulp. Its localisation (if any) is poor in LN and thymus. This suggests that P140 might possess a certain degree of specificity for targeting some but not all tissues/organs containing HSC70+ cells, and within these tissues and organs, again, it seems to target certain, but not all, HSC70+ cell subsets.

We found that HSC70, which is present at the surface of splenic and LN MRL/lpr and CBA/J B cells, T cells (activated,
treated individual. and class II MHC molecules are destabilised, therefore compromising of antigens into MIIC compartments for class II presentation is altered antigens processing by lysosomal hydrolysis. (4) Subsequent delivery chaperone-mediated autophagy (CMA) leading to the alteration of (auto) complex of co-chaperones involved in the regulation of macroautophagy inhibits chaperone properties of HSC70 protein. P140 (3a) disturbs a on lupus antigen-presenting cells (APC; B cells). (2) P140 hampers/lupus. (1) HSC70 is overexpressed both at the surface and intracellularly properties and infl uences activation of autoreactive lymphocytes in (C) A model by which P140 peptide interacts with HSC70, alters its with P140. Representative results of two independent experiments. Western immunoblotting in MRL/lpr or CBA/J B cells treated in vitro treatment. Representative results of two independent experiments. (B) Accumulation of autophagy-selective substrate p62 visualised by Western immunoblotting in MRL/lpr or CBA/J B cells treated in vitro with P140. Representative results of two independent experiments. (C) A model by which P140 peptide interacts with HSC70, alters its properties and infl uences activation of autoreactive lymphocytes in lupus. (1) HSC70 is overexpressed both at the surface and intracellularly on lupus antigen-presenting cells (APC; B cells). (2) P140 hampers/ inhibits chaperone properties of HSC70 protein. P140 (3a) disturbs a complex of co-chaperones involved in the regulation of macroautophagy (putative) and slows down the autophagic flux, and/or (3b) affects chaperone-mediated autophagy (CMA) leading to the alteration of (auto) antigens processing by lysosomal hydrolysis. (4) Subsequent delivery of antigens into MIIC compartments for class II presentation is altered and class II MHC molecules are destabilised, therefore compromising autoreactive CD4 T-cell response in a beneficial pathway for the P40-treated individual.

Figure 5  P140 peptide alters the autophagic pathway by slowing down the lysosomal activity in MRL/lpr B cells. (A) Western immunoblotting analysis with anti-LC3-II antibodies reveals increased appearance of lipidated LC3-II in B cells after 24 h of in vitro P140 treatment. Representative results of two independent experiments. (B) Accumulation of autophagy-selective substrate p62 visualised by Western immunoblotting in MRL/lpr or CBA/J B cells treated in vitro with P140. Representative results of two independent experiments. (C) A model by which P140 peptide interacts with HSC70, alters its properties and infl uences activation of autoreactive lymphocytes in lupus. (1) HSC70 is overexpressed both at the surface and intracellularly on lupus antigen-presenting cells (APC; B cells). (2) P140 hampers/inhibits chaperone properties of HSC70 protein. P140 (3a) disturbs a complex of co-chaperones involved in the regulation of macroautophagy (putative) and slows down the autophagic flux, and/or (3b) affects chaperone-mediated autophagy (CMA) leading to the alteration of (auto) antigens processing by lysosomal hydrolysis. (4) Subsequent delivery of antigens into MIIC compartments for class II presentation is altered and class II MHC molecules are destabilised, therefore compromising autoreactive CD4 T-cell response in a beneficial pathway for the P40-treated individual.

non-activated and DN T cells) and granulocytes/monocytes is progressively overexpressed in MRL/lpr spleen and LN cells. Intravenous administration of P140 into lupus-prone MRL/lpr mice leads to a decreased expression of HSC70. MHCII molecule levels are also diminished. These data reveal that after downstream P140 binding to HSC70, a series of molecular events take place affecting the APC functions of MRL/lpr B cells, leading finally to a decreased expression or stability of MHCII molecules and a potential reduction of autoreactive T-cell signalling and priming. Importantly, normal lymphocytes seem to be saved from this process, possibly because the density of HSC70 molecules is insufficient at the surface of normal APCs. The number of peripheral cells is not affected in P140-treated CBA/J mice, and B and T cells proliferate normally in response to specific mitogens. We also found that the remaining B and T cells from P140-treated mice responded normally to mitogens. These data add to earlier studies showing that P140-treated and untreated MRL/lpr mice mount equivalent immune responses to a flu virus challenge. HSC70 protein displays numerous housekeeping chaperoning functions. It is notably involved in nascent protein folding, prevention of protein aggregation under stress conditions, modulation of the assembly/disassembly of protein complexes and stabilisation of RNA messengers. HSC70 protein acts with a series of co-chaperones including Nag-4, CHIP, hip, Bcl-2-associated athanogen BAG-1, BAG-3 and Hsp40. Studies have suggested that depending on the cytokine environment, HSC70 preferentially associates with certain of these co-chaperones leading to different effects. We found that P140 inhibits the ability of HSC70 to refold denatured protein. It might therefore disrupt certain interactions of HSC70 with cofactors and chaperones. Future studies should provide insight into this topic and determine if this is relevant to some effects described in this study and lupus phenotype.

HSC70 is central in protein translocation across membranes and lysosomal transport of proteins. It plays a pivotal role in chaperone-mediated autophagy in acting as a chaperone for unfolded substrate protein in association with the membrane receptor Lamp2a. In certain conditions, such as an elevation of co-chaperone BAG-3 level, HSC70 can also associate with p62. Proper turnover of p62 by autophagy is critical, notably to prevent spontaneous aggregate formation. This protein is regarded as a key signalling player in the cell survival/death balance. Thus, certainly the most remarkable information raised in this work, is the striking effect of P140 on lysosomal degradation and class II antigen presentation (figure 5C). P140 directly impairs the molecular chaperoning properties of HSC70 by altering its ability to promote proper folding of polypeptide. In MRL/lpr B cells, P140 increases the accumulation of the autophagy markers p62/SQSTM1 and LC3-II, consistent with a downregulation of lysosomal degradation at the autolysosome stage or with a lysosomal dysfunction. Moreover, the expression of stable MHCII molecules is dramatically affected. This might explain our earlier observations showing a decreased T-cell response to various T-cell autoepitopes in P140-treated MRL/lpr mice and the absence of lupus patient’s PBL proliferation ex vivo in the presence of P140. Autophagy processes have not been explored in lupus. Although much has still to be determined to fully understand the impact of this complex pathway in MHCII presentation, inflammation and central or peripheral tolerance processes, our studies show that a peptide that can modulate lysosomal degradation can be beneficial in MRL/lpr mice by reducing autoreactive T-cell and B-cell activation. Of importance, P140 peptide exerts its effects without affecting normal B and T cells, possibly thanks to the expression levels of HSC70, which seems to act as a sensor receptor on MRL/lpr B cells.

Acknowledgements The authors are indebted to Monique Duval for valuable assistance. The authors acknowledge the Imaging facility (Plateforme Inter-Unités de Microscopie et d’Imagerie Cellulaire Strasbourg-Explanade).

Funding This research was funded by the Centre National de la Recherche Scientifique (CNRS), Région Alsace and ImmuPharma France.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.
REFERENCES


HSC70 blockade by the therapeutic peptide P140 affects autophagic processes and endogenous MHCII presentation in murine lupus
Nicolas Page, Frédéric Gros, Nicolas Schall, Marion Décossas, Dominique Bagnard, Jean-Paul Briand and Sylviane Muller

Ann Rheum Dis published online December 20, 2010

Updated information and services can be found at:
http://ard.bmj.com/content/early/2010/12/20/ard.2010.139832

These include:

Supplementary Material
Supplementary material can be found at:
http://ard.bmj.com/content/suppl/2010/11/15/ard.2010.139832.DC1

References
This article cites 32 articles, 10 of which you can access for free at:
http://ard.bmj.com/content/early/2010/12/20/ard.2010.139832#BIBL

Open Access
This paper is freely available online under the BMJ Journals unlocked scheme, see: http://ard.bmj.com/info/unlocked.dtl

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Open access (604)
Immunology (including allergy) (5144)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/