THE ROLE OF CROSS-REACTIVITY BETWEEN MYCOBACTERIA AND JOINT TISSUES IN THE AETIOLOGY OF RHEUMATOID ARTHRITIS WITH PARTICULAR REFERENCE TO TERMINAL N-ACETYLGUCOSAMINE AND 65 KILOCALTON HEAT SHOCK PROTEIN

BY

MOHAMMED SHARIF

From
The Department of Medical Microbiology,
University college and Middlesex School of Medicine,
London.

This thesis is submitted to the University of London
for the Degree of Doctor of Philosophy
in the Faculty of Science
June 1991.
To my parents......
ABSTRACT

Several historical and recent reports have implicated mycobacteria or autoantigens cross-reactive with mycobacteria in the aetiology of rheumatoid arthritis (RA). Thus adjuvant arthritis can be transferred to naive rats with a T cell clone responsive to the mycobacterial 65 kDa heat shock protein (hsp65). Moreover agalactosyl IgG [Gal(0)], of which the oligosaccharides lack terminal galactose and terminate in N-acetylgalactosamine (GlcNAc), occurs with increased frequency in RA and some mycobacterial infections. The cell walls of bacteria including mycobacteria are rich in terminal GlcNAc and the hsp65 is one of the immunodominant antigens of mycobacteria. The present study was undertaken to establish whether GlcNAc and/or hsp65-directed autoimmunity could occur in RA.

Using a monoclonal antibody (mAb) which binds to terminal GlcNAc of Gal(0) the presence of large quantities of immunoreactive GlcNAc has been demonstrated in the joints of RA patients. It was also discovered that a subset of monocytes from both RA and normal donors transiently expresses membrane GlcNAc in culture. Affinity-purified anti-GlcNAc from both pooled normal and RA sera binds to IgG heavy chains on western blots.

A murine mAb raised against mycobacterial antigen and a rabbit polyclonal antibody to hamster hsp65 were used to document the presence of a major band of around 65 kDa in synovial fluid (SF) from inflamed joints and immune complexes from RA patients. This protein was thought to be the human homologue of the mycobacterial hsp65. However, this molecule is about 7 kDa larger than the affinity-purified hsp65 from human placenta and it appeared unlikely that this molecule is of bacterial origin. This observation therefore casts doubt on several earlier reports where anti-mycobacterial antibodies were used to show increased expression of hsp65 in rheumatoid joints. The latter antibodies bind to several different components of human tissue and were not
selected for specificity to human tissue. Therefore, mAbs were made to the human hsp and it has been shown that the major band at 65 kDa in SF is not the human homologue of the bacterial hsp65. Human hsp is present in the joint but at very low concentration and in SDS PAGE runs at about 58 kDa. Moreover, the previous reports showing increased expression of the hsp in joint tissues are inaccurate. First, it is clear that the presence of the hsp in rheumatoid tissues is not RA specific and secondly the distribution is also rather different from that described using the anti-mycobacterial antibodies.

The levels of IgA and IgG antibodies to mycobacterial hsp65 are raised in RA sera and at least some of these IgG antibodies appeared to be autoantibodies. Moreover in RA SF a significant rise in the levels of IgA and IgG antibodies to the human hsp58 was found.

There appears to be a correlation between raised levels of Gal(0) and antibody to hsp65 in mice, however no such correlation was found in RA sera or synovial fluid. Moreover, anti-GlcNAc antibody was not associated with raised Gal(0), and neither anti-GlcNAc nor antibody to hsp65 appear to lack galactose.
<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Table of contents</td>
<td>5</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>6</td>
</tr>
<tr>
<td>List of tables</td>
<td>7</td>
</tr>
<tr>
<td>List of figures</td>
<td>8</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 1: General introduction</td>
<td>14</td>
</tr>
<tr>
<td>Chapter 2: A Study of the Distribution of the Terminal N-Acetylglucosamine in Normal and RA Synovial Tissues</td>
<td>32</td>
</tr>
<tr>
<td>Chapter 3: Expression of Membrane N-Acetylglucosamine by Cells in Rheumatoid Synovial fluid, and by Pre-Cultured Monocytes</td>
<td>57</td>
</tr>
<tr>
<td>Chapter 4: The Role Antibody to Terminal N-Acetylglucosamine in the Pathogenesis of Rheumatoid Arthritis</td>
<td>77</td>
</tr>
<tr>
<td>Chapter 5: Preliminary Evidence for the Presence of a Human Homologue of the <em>Mycobacterium Bovis</em> 65 Kilodalton Heat shock Protein in the Synovial Fluid and Antibodies to it in Rheumatoid Arthritis</td>
<td>99</td>
</tr>
<tr>
<td>Chapter 6: Monoclonal Antibodies to the Human Homologue of the Mycobacterial 65 Kilodalton Heat Shock Protein</td>
<td>124</td>
</tr>
<tr>
<td>Chapter 7: Further Characterization of the Monoclonal Antibodies and the presence of Human hsp58 in Inflamed Tissues</td>
<td>154</td>
</tr>
<tr>
<td>Chapter 8: Galactosylation State of the Anti-GlcNAc and Anti-hsp65 Antibodies: A Possible Link Between Immune Responses to hsp and Terminal GlcNAc</td>
<td>183</td>
</tr>
<tr>
<td>Chapter 9: Final Discussion</td>
<td>192</td>
</tr>
<tr>
<td>References</td>
<td>200</td>
</tr>
<tr>
<td>Publications from the thesis</td>
<td>215</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I am greatly indebted to Dr. G. A. W. Rook for his expert supervision and critical reading of this manuscript. I would like to express my deep appreciation to all those in the Medical Microbiology laboratory at the University college and Middlesex School of Medicine (UCMSM) who have helped throughout the project, and in particular Mrs. J. Edge who taught me the trade of making monoclonal antibodies.

I am also grateful to Dr. J. C. W. Edwards of the Rheumatology Department (UCMSM) who supplied all the tissues used for histological studies and for allowing me to carry out some of the histological studies in his department. I gratefully acknowledge the help and advice of Dr. J. Worrall and Ms. L. S. Wilkinson of the same department. I must also thank Dr. D. Isenberg and his clinical staff at the department of Rheumatology, UCMSM, who were generous enough to collect and provide synovial fluids and sera from rheumatoid arthritis patients.

I am also indebted to Professor R. S. Gupta of McMaster University, Canada, who supplied the purified and the recombinant forms of the human homologue of the 65 kDa hsp. My thanks are also to Dr. P. Lydyard of the Immunology department, Dr. C. Lambert and Dr. C. A. Pilkington of the Medical Microbiology Department, UCMSM, for useful discussion. Many thanks to Mr. C. Green for typing part of the thesis into a word processor. Finally I am grateful to the Wellcome Trust and the Arthritis and Rheumatism Council for financial support.
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.</td>
<td>Patterns of staining with the mAb GN7 on synovial tissues and rheumatoid nodules.</td>
<td>36</td>
</tr>
<tr>
<td>4.1.</td>
<td>Details of the columns used for the affinity purification of the human anti-GlcNAc antibodies.</td>
<td>82</td>
</tr>
<tr>
<td>4.2.</td>
<td>Details of the fractions eluted with 0.5 M GlcNAc from the columns.</td>
<td>85</td>
</tr>
<tr>
<td>4.3.</td>
<td>Antibody binding to glycoproteins with terminal GlcNAc assayed by ELISA.</td>
<td>91</td>
</tr>
<tr>
<td>4.4.</td>
<td>Serum concentrations of the IgA, IgG and IgM anti-GlcNAc antibodies from pooled normal and RA sera.</td>
<td>94</td>
</tr>
<tr>
<td>5.1.</td>
<td>The effect of exposure of the P1 protein, RA synovial fluid and hsp70 to hypochlorite treatment before SDS PAGE, on subsequent binding of SF8.</td>
<td>112</td>
</tr>
<tr>
<td>6.1.</td>
<td>The results of initial screening of culture supernatants by ELISA and immunoblots for fusion plate 1.</td>
<td>141</td>
</tr>
<tr>
<td>6.2.</td>
<td>The results of initial screening of culture supernatants by ELISA and immunoblots for fusion plate 2.</td>
<td>142</td>
</tr>
<tr>
<td>6.3.</td>
<td>The results of initial screening of culture supernatants by ELISA and immunoblots for fusion plate 3.</td>
<td>143</td>
</tr>
<tr>
<td>6.4.</td>
<td>The results of initial screening of culture supernatants by ELISA and immunoblots for fusion plate 4.</td>
<td>144</td>
</tr>
<tr>
<td>7.1.</td>
<td>Screening of the monoclonal antibodies on western blots of heat shock proteins and cell lysates.</td>
<td>163</td>
</tr>
<tr>
<td>7.2.</td>
<td>Sequence of the peptide S2 from <em>M. leprae</em> hsp65.</td>
<td>165</td>
</tr>
<tr>
<td>7.3.</td>
<td>Epitope group according to results of screening of the mAbs on various deletions of the P1 protein.</td>
<td>167</td>
</tr>
<tr>
<td>8.1.</td>
<td>Percentage of agalactosyl IgG in the affinity-purified human anti-GlcNAc antibodies.</td>
<td>188</td>
</tr>
<tr>
<td>8.2.</td>
<td>Percentage of agalactosyl IgG in the affinity-purified human anti-hsp65 antibodies from RA and tuberculosis sera.</td>
<td>189</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Normal synovial tissue stained with GN7 and rabbit anti-mouse peroxidase conjugate, counterstained with haematoxylin. No significant staining.</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Normal tonsil stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing strong staining of mucosal and crypt epithelial cells.</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Normal salivary gland stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing staining of mucus-secreting acinar epithelial cells.</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>Myelinated nerve trunk stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing intracellular staining of Schwann cells.</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>Synovial tissue from an OA patient stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing moderate diffuse granular cytoplasmic staining of the synovial lining cells.</td>
<td></td>
</tr>
<tr>
<td>2.6</td>
<td>Synovial tissue from RA patient stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing reticular extracellular staining around cells near the tissue surface.</td>
<td></td>
</tr>
<tr>
<td>2.7</td>
<td>A high power view of superficial synovial tissue from RA patient stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing a reticular net work of extracellular staining.</td>
<td></td>
</tr>
<tr>
<td>2.8</td>
<td>A high magnification of RA synovial tissue similar to the one shown in Figure 2.6 showing dense granular cytoplasmic staining by GN7 of macrophage like cells in the deep synovial tissue.</td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>Superficial RA synovial tissue double stained with GN7 and rabbit anti-mouse IgM TRITC conjugate (red) and with EBM11 and rabbit anti-mouse IgG FITC (green).</td>
<td></td>
</tr>
<tr>
<td>2.10</td>
<td>Synovial tissue from RA patient stained GN7 and rabbit anti-mouse Ig alkaline phosphatase conjugate, with no counterstain.</td>
<td></td>
</tr>
</tbody>
</table>
3.1. RA synovial fluid cells stained in suspension under non-capping conditions with the anti-GlcNAc mAb GN7 and rabbit anti-mouse FITC.

3.2. Monocytes cultured for two days and stained with GN7 by the peroxidase method. (a) Staining without pretreatment with β-hexosaminidase. (b) Staining after treatment with the enzyme β-hexosaminidase.

3.3. The time course of the percentage of monocytes expressing membrane GlcNAc in culture.

3.4. Non-specific esterase stained GN7 positive cell.

3.5. Double staining of monocytes pre-cultured on plastic for 20 hours to maximise the number of GN7 positive cells.

3.6. Immunoblots of fresh or cultured monocytes from blood or RA synovial fluid stained with anti-GlcNAc, GN7.

4.1. Inhibition of binding of the affinity-purified human anti-GlcNAc antibodies to GlcNAc-phe-BSA by increasing concentrations of N-acetylgalactosamine or N-acetylglucosamine.

4.2. Binding of the affinity purified anti-GlcNAc antibodies to IgG heavy chain on western blots.

5.1. Immunoblots of purified 65 kDa protein from human placenta, and recombinant 65 kDa hsp from *M. bovis*.

5.2. Immunoblots of denatured synovial fluid and PEG-precipitated immune complexes from rheumatoid synovial fluid.

5.3. Discrepancy in the molecular weight of the 65 kDa protein recognised by SF8 in the RA synovial fluid, compared to pure hsp65 from human placenta, mycobacteria and *E. coli*.

5.4. Binding to the human and mycobacterial hsp65 of IgG from RA sera eluted from an affinity column of recombinant mycobacterial hsp65.

5.5. Screening of the affinity-purified human antibodies to *M. bovis* hsp65 from RA sera on western blots of hsps and RA synovial fluid.

5.6. Antibody response to P1A in RA and control synovial fluids.

5.7. Antibody response to *M. bovis* hsp65 in RA and control synovial fluid.

5.8. Correlation between IgG antibody to P1A and mycobacterial hsp65 in RA synovial fluid.
6.1. Screening of the monoclonal antibodies on preparative western blots of P1A using Miniblotter.

6.2. Screening of the monoclonal antibodies on preparative western blots of P1D using Miniblotter.

6.3. Screening of the monoclonal antibodies on preparative western blots of RA synovial fluid using Miniblotter.

6.4. HEP-2 cell line stained with the IgG mAb, 4B9/89, and rabbit anti-mouse FITC conjugate.

6.5. HEP-2 cell line stained with the IgM mAb, 3C8/65, and rabbit anti-mouse FITC conjugate.

7.1. Immunoblots of cell lysates from PBMC and JRT probed with the monoclonal antibodies to human hsp58, 3C8/65 and 4B9/89.

7.2. Deletions of the P1 protein used for estimating the mAb epitopes.

7.3. Results of cross-inhibition studies with the mAb to human hsp58.

7.4. Capture assay for quantitatively demonstrating the presence of human hsp58 in RA serum and synovial fluid.

7.5. Western blots of immunoprecipitated hsp58 from RA synovial fluid probed with the biotin-labelled IgG mAb to this hsp.

7.6. Normal synovial tissue stained with 3C8/65 by the APAAP method, developed with Fast-Red and counterstained with haemotoxylin. Showing intense staining of the synovial lining cells.

7.7. Synovial tissue from a RA patient stained with 3C8/65 by the APAAP method, developed with Fast-Red and counterstained with haemotoxylin. Showing moderately strong staining of large mononuclear cells near the tissue surface.

7.8. Synovial tissue from an RA patient similar to the one shown in Figure 7.7 showing cytoplasmic staining of scattered cells and blood vessel media by 3C8/65 in the deep synovial tissue.

7.9. Synovial tissue from an OA patient stained with 3C8/65 by the APAAP method, developed with Fast-Red and counterstained with haemotoxylin. Showing intense cytoplasmic staining of almost all cells in the deep tissue.

7.10. RA synovial tissue stained with the IgG mAb, 4B9/89, by the APAAP method, developed with Fast-Red and counterstained with haemotoxylin. Showing a weak cytoplasmic staining of large mononuclear cells and a patchy extracellular staining in the deeper layers of the synovium.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Adjuvant Arthritis</td>
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<tr>
<td>ABTS</td>
<td>2', 2'- Azinobis (3-ethylbenzthiazoline sulphonic acid)</td>
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<td>APAAP</td>
<td>Alkaline Phosphatase-anti-Alkaline Phosphatase</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>B cell</td>
<td>B lymphocyte</td>
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<tr>
<td>BCG</td>
<td><em>Bacillus Calmette-Guérin</em></td>
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<td>BLL</td>
<td>Boardline Leprosy</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CD4</td>
<td>Helper/Inducer T cells</td>
</tr>
<tr>
<td>CD5</td>
<td>Human Leukocyte Differentiation Antigen</td>
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<tr>
<td>CD8</td>
<td>Suppressor/Cytotoxic T cells</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EBNA-1</td>
<td>Epstein-Barr Nuclear Antigen-1</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-acetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ENL</td>
<td>Erythema Nodosum Leprosum</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<tr>
<td>ETF</td>
<td>Enzyme Treated Fetuin</td>
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<tr>
<td>FCA</td>
<td>Freund's Complete Adjuvant</td>
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<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
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<tr>
<td>FIA</td>
<td>Freund's Incomplete Adjuvant</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>GABA</td>
<td>Gamma Aminobutyric Acid</td>
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<td>Gal(0)</td>
<td>Agalactosyl IgG</td>
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<td>GalNAc</td>
<td>N-Acetylgalactosamine</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>HEP-2</td>
<td>Human Epithelial cell line</td>
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<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>HRP</td>
<td>Horse Radish Peroxidase</td>
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<td>HSA</td>
<td>Human Serum Albumin</td>
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<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>IDDM</td>
<td>Insulin-Dependent Diabetes Mellitus</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
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<tr>
<td>JRT</td>
<td>Jurkat T cell line</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<td>LL</td>
<td>Lepromatous Leprosy</td>
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<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MCP</td>
<td>Magnetic Particule Concentrator</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MPL</td>
<td>Monophosphoryl Lipid A</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<tr>
<td>NSE</td>
<td>Non-Specific Esterase</td>
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<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>P1</td>
<td>Affinity-purified hsp58 from human placenta</td>
</tr>
<tr>
<td>P1A</td>
<td>Recombinant human hsp58</td>
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<tr>
<td>P1Ab</td>
<td>Rabbit polyclonal antibody to P1</td>
</tr>
<tr>
<td>P1D</td>
<td>Recombinant human hsp58 with 20 amino acid deleted</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PAP</td>
<td>Peroxidase Anti-Peroxidase</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>---------------------------------------</td>
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<tr>
<td>RANA</td>
<td>Rheumatoid Arthritis Nuclear Antigen</td>
</tr>
<tr>
<td>rINF-γ</td>
<td>Recombinant gamma Interferon</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered Saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose Dimycolate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
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<td>VBS</td>
<td>Veronal-Buffered Saline</td>
</tr>
</tbody>
</table>
CHAPTER 1

GENERAL INTRODUCTION

1.1. PATHOGENESIS OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic recurrent, systemic inflammatory disease primarily involving the joints. RA is a relatively common disease affecting about 1 per cent of the population worldwide with a female to male ratio of 4:1. The aetiology of RA is unknown although considerable progress has been made in understanding the pathogenesis of the disease. The clinical course of RA appears to be variable; in some cases there is rapid progression to severe disability, and in others, a prolonged benign course with little or no joint deformities.

The major immunological features of the disease include formation of anti-IgG immunoglobulins (rheumatoid factor) by the synovial fluid lymphocytes. Elevated levels of IgM, IgG, and IgA rheumatoid factors are found in the serum and synovial fluid of majority of the RA patients. However a large proportion of the circulating rheumatoid factors are relatively low affinity antibody and their apparent ability to give rise to no more than rather small, readily dissociable, non-complement fixing complexes has raised doubt regarding a pathogenetic role for the anti-IgG response. Nevertheless the unique ability of the rheumatoid factors to self-associate and to form immune complexes is of crucial significance in RA. A decreased complement level in synovial fluid but normal in serum is also regarded as an immunological feature of RA. The disease is often divided into sero-positive and sero-negative cases. In sero-positive cases IgM rheumatoid factors are readily demonstrable by agglutination tests. In sero-negative cases the IgM rheumatoid factor is absent and the IgG rheumatoid factors are not detected by conventional agglutination tests used in routine diagnostic laboratories. Rheumatoid
factor is present in over 70% of RA patients but it is also present in 30% of systemic lupus erythematosis (SLE) patients and as many as 90% of patients with Sjogren's syndrome and in some chronic infectious diseases such as leprosy and tuberculosis. Epidemiological studies have shown that a small number of normal people also have rheumatoid factor. Moreover, a large proportion of elderly people usually give positive latex agglutination tests suggesting that the rheumatoid factor is not fundamental to the pathogenesis of RA. Rather, it may be normal to have a minimum amount of rheumatoid factors which may have the physiological role of clearing immune complexes.

An unidentified initial stimulus, failure to regulate the autoimmune response to IgG or some environmental influences, results in the excessive autosensitization of the IgG in the joint and formation of rheumatoid factors by the synovial fluid lymphocytes. The presence of IgG rheumatoid factor complexes in the synovial fluid stimulate complement components. The activation of the complement system results in a number of inflammatory phenomena including release of cytokines, production of chemotactic factors for polymorphonuclear leukocytes and mononuclear cells. The secretion of these mediators amplifies immune responses and damages neighbouring cells. For example interleukin 1 (IL-1) can be found in RA lesions together with inhibitors of its action. Tumour necrosis factor (TNF) is another monokine able alone and in conjunction with IL-1 to stimulate bone resorption and inhibit proteoglycan synthesis. The presence of these stimulatory substances, together with invading leukocytes, and other genetic and environmental factors, keep the rheumatoid inflammatory response simmering. The earliest change seen in RA synovium is the inflammation of small blood vessels (vasculitis). This inflammation is accompanied by increased capillary permeability and results in oedema of the synovium due to cellular infiltration initially by polymorphonuclear leukocytes and later by plasma cells. The inflamed synovium becomes hypertrophied due to proliferation of the lymphocytes and plasma cells in the synovial lining and forms granulation tissue (pannus) over the articular cartilage. The pannus produces collagenase and elastase that gradually destroy the joint cartilage. The pannus may progress to form a scar in the joint that leads to loss of mobility and deformities.
1.2. AUTOIMMUNE DISEASE

There are many diseases known or thought to be autoimmune in nature, and susceptibility to almost all is influenced by genes within the major histocompatibility complex (MHC), particularly Class I and Class II. Autoimmune diseases as a group, affect 5-7% of the population, with a peak incidence at or shortly after puberty, often with a second peak of incidence in the forties and fifties. Many of them involve immune responses against self molecules that are expressed in anatomically privileged extrathymic sites (organ specific e.g. Hashimoto’s thyroiditis and insulin dependent type I diabetes). Others appear to be due to immune response to ubiquitous nuclear and cytoplasmic antigens (non-organ specific e.g. SLE and Scleroderma). MHC Class II associated autoimmune diseases show female preponderance (RA, SLE, hyperthyroidism etc.) while MHC Class I associated autoimmune diseases show male preponderance (ankylosing spondylitis, Reiter’s syndrome etc).

1.3. GENETIC SUSCEPTIBILITY TO RHEUMATOID ARTHRITIS

A genetic susceptibility to RA was first implicated by family studies showing that the incidence of RA among twins was far higher than in the population at large (Thymann 1957; Harvald & Hauge 1965). However, a precise genetic factor was not identified until 1976 when Stastny demonstrated that HLA-DR4 individuals are more susceptible to RA. This was generally true for RA in Caucasians, Japanese and Negroes. However, in some populations (e.g. Jews and Indians) the DR1 molecule is present in greater frequency (Stastny 1980; Woodrow et al., 1981; Schiff et al., 1982). The functions of the human leukocyte antigen (HLA) Class II molecules (of which DR is a subset) is to bind to antigenic peptides so that they can be recognized by T cells with the appropriate receptor. The DR subset is made up of alpha and beta chains. The DR beta
chain consists of 3 major regions of variability called hypervariable regions and it is the third hypervariable region in both DR4 and DR1 molecules that determines genetic susceptibility to RA (Nepom et al., 1986; Bell et al., 1987; Gregersen et al., 1987). Differences in the amino acid sequence of the third hypervariable region give rise to various DR4 subsets. The sequence QRRAA and QKRAA at residues 70-74 are found on the third hypervariable region of DR1 and 3 subgroups of DR4 (Dw4, Dw14 and Dw15) which have been associated with RA, but not Dw10 or Dw13 subgroups of DR4 which are not associated with RA (Gregersen et al., 1987 & Vaughan et al., 1988). These observations have led to the hypothesis that the DR molecules that are characteristic of RA bind selected peptides which predispose the individual to the development of autoimmunity based on the homologies between the bound peptides and peptides within self proteins (Vaughan et al., 1988).

1.4. AETIOLOGY OF RHEUMATOID ARTHRITIS

The aetiology of RA is unknown. Over the last three decades many factors have been implicated in the initiation and perpetuation of the disease. Such factors have often evaded as well as intrigued investigators. During the 1960's the rheumatoid factors were studied extensively in search of clues into the aetiology of RA. In the 1970's most of the research interests were focused around autoimmunity, and in the 80's immunoregulation received a great deal of interest. Judging by the present trend of research into the aetiopathogenesis of RA, the 1990's will probably be dominated by studies of microbes as inciting or perpetuating factors in the pathogenesis of RA.

An infectious aetiology for RA has always been suspected although investigators differ considerably in their choice of the infectious agents. Thus many unrelated viruses and bacteria have been associated with RA largely because (a) RA patients show abnormal serological or cellular responses to these organisms, (b) the presence of the agents is found in synovial membrane, (c) there is cross-reactivity between microbial
antigens and host tissues, and (d) involvement of known agents or their antigens in animal models of arthritis (Phillips 1988 & Venables 1989).

1.4.1. Viruses and arthritis

Many viruses have been implicated in RA: Epstein-Barr virus (EBV), Parvovirus, rubella, human T cell leukaemia virus and cytomegalovirus are thought to be the most credible ones. However, as the experimental techniques improved over the years, the evidence implicating most of these viruses has become less impressive. Thus, the IgM antibodies to parvovirus in RA may be due to non-specific binding of IgM rheumatoid factor to pre-existing IgG anti-viral antibodies (Venables 1989). Similarly Fc receptors expressed on cytomegalovirus infected fibroblasts could be mistaken for cytomegalovirus early antigens (Venables 1989). Moreover, rubella isolates from chronic arthritides including RA have been reported to be due to technical artefact (Phillips et al., 1988). EBV however, still remains a potential aetiological agent. It is a polyclonal B cell activator and can induce rheumatoid factor synthesis (Slaughter et al., 1978). Patients with RA have raised levels of antibody to rheumatoid arthritis nuclear antigen (RANA) (Alspaugh et al., 1978) and peripheral blood lymphocytes from these patients have an increased proportion of circulating B cells infected with EBV (Slaughter et al., 1978). Recent discoveries of sequence homologies between EBV antigens and host proteins have led to renewed interest in the possibility that EBV antigens could be important in the generation of autoimmunity in RA. In 1986 Fox et al., demonstrated a 62 kDa antigen in the synovial membrane which cross-react with Epstein-Barr nuclear antigen-1 (EBNA-1). EBNA-1 is the most antigenic of the EBNA's and nearly one third of this molecule is made up of a glycine-alanine repeat sequence (Rumpold et al., 1987 & Vaughan et al., 1988). RA patients have raised levels of antibody to this glycine-alanine repeat region of EBNA-1 (Rhodes et al., 1985 and 1987; Rumpold et al., 1987). Subsequent study has shown that these antibodies are autoantibodies to RANA (Venables et al., 1988).
Another interesting homology between EBV and host antigens has been described by Roudier et al., (1988). The haplotype DR1 and Dw4, Dw14 and Dw15 all share the almost identical amino acid sequences EQKRAA and EQRRAA. These sequences are also found on a capsid antigen of EBV known as gp110 and a small number of RA patients but not normal donors have antibodies to this peptide (Roudier et al., 1988).

1.4.2. Bacteria and arthritis

A number of bacteria have been associated with arthritis. For example, Ebringer et al., (1988) indicated that RA and ankylosing spondylitis are forms of reactive arthritis to Proteus and Klebsiella respectively. Reactive arthritis have also been reported to occur following gastrointestinal infections with Shigella, Salmonella or Campylobacter (Aho et al., 1985). Similarly lyme arthritis has been associated with Borrelia burgdorferi (Golding & Jericho 1986). Individuals are commonly exposed to these organisms and many of these bacteria are thought to be constituent members of the normal bowel flora. Mycobacteria on the other hand, which have been implicated in the aetiopathogenesis of RA since the nineteenth century are saprophytic organisms and they are not considered to be part of the normal commensal flora of man (Rook et al., 1990a). They are abundant in some environments, for example Africa while they are rare in others, e.g. the developed countries. Interestingly, the incidence of RA appears to show an inverse relationship with the abundance of mycobacteria.

1.5. MYCOBACTERIA AND RHEUMATOID ARTHRITIS

Mycobacteria have been implicated in the aetiology of RA by several historical observations. Thus the introduction of gold therapy for RA followed the demonstration that the chronic disease process of tuberculosis resembled the chronic synovitis seen in RA (Forestier 1934). In 1897 Poncet described an RA-like syndrome occurring in
tuberculosis patients known as tuberculosis rheumatism or Poncet’s disease and isolated cases of Poncet’s disease still occur (Issacs & Sturrock 1974). In India, Papua New Guinea and Egypt similar syndromes have been reported in leprosy patients especially during episodes of erythema nodosum leprosum (Ramu & Balakrishnan 1968; Atkin et al., 1987).

Arthritic symptoms have also been described in *Bacille Calmette-Guerin* (BCG) immunotherapy (Torisu et al., 1978). Serum from patients with mycobacterial infections such as tuberculosis and leprosy contain rheumatoid factor (Lindquist et al., 1970). Moreover, mycobacteria are also involved in Pearson’s animal models of RA. Pearson induced arthritis in genetically susceptible rats by injecting *Mycobacterium tuberculosis* (*M. tuberculosis*) in oil. The arthritis induced (adjuvant arthritis) resembled human RA including rheumatoid factor formation (Pearson 1956 & 1964). There are also reports suggesting that susceptibility of germ-free rats to adjuvant arthritis can be increased or decreased by appropriate reconstitution of the bowel flora with various bacterial species (Kohashi et al., 1985). Similarly the bowel flora appears to play a role in the resistance of some rat strains to the arthritis induced by streptococcal cell walls (ven den Broek 1989b) and pristane arthritis in mice can be blocked by *M. vaccae* in a route dependent manner (Thompson et al., 1990 & Thompson et al., in preparation). Finally, when arthritic rats are fed on a diet containing mycobacteria, their arthritis improves (Dr. H. Weiner, Boston; personal communication to Dr. Rook).

Recent studies have unveiled more striking links between mycobacteria and RA and have led to renewed interest in the possibility that mycobacterial components or antigens cross-reactive with mycobacteria may be involved in the pathogenesis of RA. Thus RA patients have been reported to show both cell mediated and humoral responses to these organisms which differ from the responses of normal donors. Moreover, skin test studies have shown that RA patients respond poorly to common mycobacterial antigens as do patients with tuberculosis or leprosy. Tuberculin skin tests also showed significant correlation with HLA haplotypes in RA, but not in normal controls or in patients with
tuberculosis (Bahr et al., 1989). Thus RA patients with HLA-DR4 (a haplotype associated with increased risk of developing RA) showed increased skin-test responses while those patients with HLA-DR7 (a haplotype associated with reduced risk of developing RA) showed diminished skin-test response to tuberculin. A similar HLA-correlation was found with the antibody binding to mycobacterial sonicates (Bahr et al., 1988a & Tsoulfa et al., 1989a).

1.6. HEAT SHOCK PROTEINS

Application of heat or other physical or chemical stress results in the inhibition of the synthesis of normal proteins and instead the cell produces proteins known as heat shock proteins (hsp) or stress proteins (The terms hsp or stress protein are used interchangeably in this study). This was first observed in the salivary gland chromosomes of Drosophila larvae (Ritossa et al., 1962) where areas of DNA transcription show up as swellings known as puffs. If the temperature of the larvae is raised to about 35 °C, normal puffs start to shrink and new puffs appear. These are the heat shock puffs which encode the heat shock genes that are responsible for the synthesis of specific proteins known as hsp. Following this original study by Ritossa in 1962, the heat shock response has been demonstrated in all cells examined, from bacteria to man, with considerable homology between genes and proteins of different species.

The hsp are usually classified and named according to their molecular mass in kilodaltons. This has led to grouping of hsp into families of different sizes. Members of a given family share several features in addition to the size, and similar groups of families have been described in different species. There are at least five different gene families responsible for the production of proteins at approximately 90 kilodalton (kDa), 70 kDa, 65 kDa, 40 kDa and 20 kDa (Lindquist 1986). Of these 5 hsp gene families, the 70 kDa family is the most conserved and best characterized. The functions of hsp are
under intense scrutiny at present and are gradually becoming understood. They are thought to be ATP-dependent enzymes which fold, unfold, translocate, assemble subunits, or bind and inactivate other proteins (Goloubinoff \textit{et al.}, 1989; Hemmingsen \textit{et al.}, 1988; Reading \textit{et al.}, 1989; Cheng \textit{et al.}, 1989; Osterman \textit{et al.}, 1989; Chirico \textit{et al.}, 1988; Deshaies \textit{et al.}, 1988). Thus hsps are said to have ‘nurse maid’, ‘house-keeping’ or chaperone functions which are essential for cell survival. Many hsps are synthesized under normal condition (constitutive hsps) and their production is up-regulated following physiological or chemical stress such as heat, viral infections, oxygen free radicals, pH changes etc (Poll 1988).

1.7. HEAT SHOCK PROTEINS IN AUTOIMMUNITY

The role for hsps in autoimmunity is based on the characteristic features of hsps. First they are highly immunogenic. They show significant sequence conservation and they are found in all cells. These features of hsps have led investigators to propose that there may be a link between infection and autoimmunity (Lamb \textit{et al.}, 1989 & Young \textit{et al.}, 1990). The immune response generated during bacterial and parasitic infections is directed primarily to the variable epitopes but sometimes, depending upon the nature of the infection and genetic make up of the host an immune response to conserved epitopes may also be induced. A subsequent localized increase in the synthesis of the corresponding hsp by stimuli such as inflammation or viral infection may lead to the activation of autoreactive lymphocytes and the generation of autoimmune diseases like RA.
1.7.1. Antibodies to the hsps in autoimmune diseases

In several autoimmune diseases the presence of autoantibodies has been reported. Thus, histone H2B is a well known autoantigen in SLE and related disorders (Tan et al., 1988). In addition a raised level of IgG antibody to hsp90 and IgM antibody to the members of the hsp70 family are found in SLE (Minota et al., 1988a and 1988b; Winfield et al., 1989). Antibodies to hsp 90 in ankylosing spondylitis have also been reported (Lakomek et al., 1984). IgG and IgA antibody binding to mycobacterial hsp70 and hsp65 have been reported in RA but not in normal individuals or SLE (Bahr et al., 1988b; Tsoulfa et al., 1989a and 1989b). Moreover antibodies to a 64 kDa antigen have been reported in type I insulin-dependent diabetes mellitus (IDDM) (Jones et al., 1990 & Christie et al., 1990) and they may have a diagnostic value because the levels of these antibodies fall just before the clinical onset of the disease (Baekkeskov et al., 1987 & Christie et al., 1988). This 64 kDa autoantigen generated a lot of interest because it was thought that it could be the mammalian homologue of the mycobacterial hsp65 (Jones et al., 1990). However, subsequent studies by Baekkeskov et al., (1990) has identified this autoantigen as glutamic acid decarboxylase, an enzyme responsible for the biosynthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and it does not appear to be associated with autoimmunity to islet cell hsp.

1.7.2. T cell responses to hsps in autoimmune diseases

T cell lines which respond to hsp65 purified from either bacteria or human cells have been isolated from healthy individuals with no history of tuberculosis and also from individuals with no history of mycobacterial or autoimmune disease (Lamb et al., 1989). Moreover, several groups have demonstrated that T cells from the synovial fluid of RA patients respond in vitro to mycobacterial hsp65 (Res et al., 1988; Gaston et al., 1988 and 1989). However the donors were not tested for tuberculin positivity, and it is not
clear that the frequency of such cells was any higher than that would be expected in normal donors. There are also such cells in cord blood, though they may be responding to contaminants derived from *Escherichia coli* (*E. coli*) (Dr. C. A. Pilkington, personal communication). In addition, CD8+ MHC class I restricted T cell lines which recognize both bacterial and human hsp65 and hsp70 have also been isolated from tuberculosis patients (Rees *et al.*, 1988). Moreover, in mice CD8+ MHC Class I restricted T cells responsive to mycobacterial hsp65 recognize and lyse macrophages which have been activated (stressed) by gamma interferon (Koga *et al.*, 1989).

### 1.8. THE ROLE OF HSP 65 - RESPONSIVE T LYMPHOCYTES IN RODENT MODELS OF ARTHRITIS

The evidence for mycobacterial hsp65 responsive T cells in autoimmunity, particularly in RA, comes from studies of animal models of RA. In early 1985 van Eden *et al.*, demonstrated that arthritis can be transferred to naive irradiated rats with T cell clones responsive to mycobacteria and to a cross-reactive antigen present in human cartilage. Later a T cell line isolated from the draining lymph node of a arthritic rat and propagated *in vitro* by repeated re-stimulation with *M. tuberculosis* antigen caused a transient form of the disease in irradiated rats but protected normal rats from subsequent induction of adjuvant arthritis (van Eden *et al.*, 1987). Subsequently it became clear that the T cell line from the arthritic rat contained two helper T cell lines, one arthritogenic and the other a protective or suppressor clone. Both clones recognized a short seven amino acid residue (amino acids 180-188) on the mycobacterial hsp65 (van Eden *et al.*, 1988). Immunization with mycobacterial hsp65 in soluble form does not induce adjuvant arthritis but protects normal rats when subsequently challenged with the whole adjuvant (van Eden *et al.*, 1988). Moreover, the induction of other rodent models of arthritis including the streptococcal cell wall model (van den Broek 1989a), pristane-induced arthritis in mice (Thompson *et al.*, 1990), and arthritis induced by a synthetic mycobacteria-free adjuvant (alkyl diamine CP-20961)(Billingam *et al.*, 1990) can be blocked by
pretreatment with this hsp in soluble form. Like adjuvant arthritis, IDDM can also be transferred from non-obese diabetic (NOD) mice (these mice have an inherited predisposition to autoimmune disease resembling human IDDM) to pre-diabetic NOD mice using T cell clones from diabetic mice which respond to mycobacterial hsp65 (Elias et al., 1990). Moreover, mycobacterial hsp65 can either induce or vaccinate against diabetes in NOD mice depending upon the form of administration (Elias et al., 1990).

1.9. HSP65 AND γδT LYMPHOCYTES

The T cells mentioned above are either CD4+ or CD8+ and express αβ heterodimeric T cell receptors (TCR) which recognize hsp65-derived peptides bound in the cleft of MHC molecules. In 1986 Brenner et al., found an additional subset of T cells which express a distinct TCR composed of γ and δ chains and are CD4- CD8-. These double negative or γδT cells appear to respond preferentially to mycobacterial antigens (Janis et al., 1989 & Modlin et al., 1989) particularly to mycobacterial hsps (O’Brien et al., 1989 & Born et al. 1990). γδT Cell hybridomas generated from thymocytes of new born mice expressing TCR have been shown to be autoreactive (O’Brien et al., 1989) and self-reactivity can be increased by heat shock (Rajasekar et al., 1990). These observations led to the suggestion that γδT cells may be responsible for the elimination of autologous stressed cells. Moreover, γδT cells can induce B lymphocytes to produce IgG autoantibodies to DNA in several SLE mouse strains (Datta et al., 1987) and can break oral tolerance in mice (Kitamura et al., 1987). But as yet there are no evidence suggesting a role for γδT cells in human autoimmune diseases. Nevertheless, γδT cells responsive to mycobacteria have been isolated from RA synovial fluid and one of these T cell clones recognized mycobacterial hsp65 (Holoshitz et al., 1989) and
although there is no increase in the frequency of such cells in RA, differences in the usage of Vδ genes has been reported (Smith et al., 1990). Using monoclonal antibodies (mAbs) to Vδ1 and Vδ2 Smith et al., (1990) showed that the Vδ1+ population was more frequent in the synovial fluid and membrane compared to peripheral blood in RA patients.

The observations discussed above have led to the hypothesis that γδT cells which recognize bacterial hsp provide first line of defence against infections and that cross-reactivity with autologous hsp leads to autoimmunity (Rajasekar et al., 1990). However double negative T cells have also been isolated from a normal individual and shown to respond to mycobacterial hsp65 (Haregewoin et al., 1989) suggesting that these T cells may also have a protective role.

1.10. AGALACTOSYL IgG

Mycobacteria are also implicated in RA by a parallel line of study of the N-glycosylation pattern of the Fc region of serum IgG. In 1985 Parekh et al., demonstrated that RA patients have an increased proportion of an IgG glycoform in which the usual terminal sugar galactose is missing from both arms of the covalently attached complex-biantennary oligosaccharides, and as a consequence, N-acetylglucosamine (GlcNAc) becomes the terminal sugar. This glycoform of IgG is known as agalactosyl IgG or Gal(0) and it varies with age (Parekh et al., 1988a) and severity of both adult and juvenile RA (Parekh et al., 1988b). The production of agalactosyl IgG is believed to be due to a decreased activity of the enzyme β-galactosyltransferase in B lymphocytes of patients with RA (Axford et al., 1988). Raised levels of agalactosyl IgG relative to age-matched controls, were later found in juvenile arthritis (Parekh et al., 1988b), tuberculosis, and SLE with Sjogren’s syndrome, and Crohn’s disease (Rademacher et al., 1988). It is also
raised in leprosy during episodes of erythema nodosum leprosum (Filley et al., 1989). The raised levels in Crohn’s disease are particularly interesting since mycobacterium-like organisms have been suspected of being the causative agent (Burnham et al., 1978 & Chiodine et al., 1984). Thus agalactosyl IgG could be a marker for the immune responses induced by mycobacteria as it is not raised in other types of infection.

Changes in the levels of agalactosyl IgG occur in all rodent models of arthritis and a recent study of leprosy and Crohn’s disease by Rook et al., (1990b) suggest that Gal(0) correlates with T cell-mediated tissue damaging pathology. More recently vanZeben et al., (in preparation) have shown that raised percentage Gal(0) early in the disease correlates very strongly with severity of disease and joint destruction assessed by number of erosions 8 years after disease onset. However, patients with streptococcal induced acute rheumatic fever have normal levels of agalactosyl IgG (Bahr et al., 1990) and during pregnancy the levels have been reported to go down (Rook et al., 1991b).

1.11. THE ROLE OF AGALACTOSYL IgG IN RA

Although it is now more than five years since the presence of agalactosyl IgG was first demonstrated in RA sera and its value as a correlate has become clear, its role in RA, if any, still remains speculative. Rademacher et al., (1988) suggested several mechanisms through which IgG self-association could occur. First, through the insertion of a Fab-linked oligosaccharide from an IgG molecule into the vacant lectin-like site of agalactosyl IgG. Secondly, the exposed terminal GlcNAc may be recognized by naturally occurring or induced anti-GlcNAc antibodies. The antigenic site of the IgG molecule is believed to be located within the Fc region (Nardella et al., 1981), but there is no evidence of any changes of amino acids in the Fc region. The loss of galactose from the CH2 domain could theoretically lead to autosensitization and IgG self-association (Rademacher et al., 1988). The loss of a galactose and the subsequent exposure of a GlcNAc on the oligosaccharide of the IgG molecule may result in an imbalance of normal
interactions between the Fc region and cellular receptors for these sugar epitopes and thus provoke pathological changes (Haltiwanger & Hill 1986).

1.12. THE TERMINAL GlcNAc

Under normal conditions GlcNAc is usually masked by other carbohydrate moieties. Therefore, when GlcNAc becomes terminal as in agalactosyl IgG, it may have important regulatory or signalling functions as well as being a target for autoimmune reactions.

Cells bearing GlcNAc receptors have been reported to play a role in the recognition of peptidoglycan structures of the bacterial cell walls (Munson & Glaser 1981) and in natural killer cell-mediated cytolysis of tumour cells (Arhens & Ankel 1987). The binding of macrophages to thymocytes undergoing apoptosis (programmed death) can be blocked by GlcNAc (Duvall et al., 1985). Terminal GlcNAc is also involved in cell-cell recognition and cell substrate adhesion during fertilization and embryonic development (Bayna et al., 1986).

In the 1960's bee venom was routinely used in Russia as a traditional remedy for arthritis. The efficacy of this treatment was dependent on the total quantity of the bee venom injected (Zurier et al., 1973) and subsequent studies had shown that the bee venom contains terminal GlcNAc (Weber et al., 1986) and can block the induction of adjuvant arthritis in rats (Chang & Bliven 1979; Zurier et al., 1973).

1.12.1. Immunogenicity of terminal GlcNAc

The peptidoglycan/polysaccharide (PG/PS) complex of bacteria including mycobacteria is rich in GlcNAc and such bacterial component can induce antibody
formation to GlcNAc. For example, rabbits immunized with *M. smegmatis* in oil developed antibody to highly branched N-linked oligosaccharides terminating in GlcNAc residues (Chechik *et al.*, 1987). Moreover patients with rheumatic fever, which follows infection with Group A streptococci, have raised levels of antibody to the GlcNAc-rich PG/PS complex of these organisms. RA patients also have raised levels of antibody to Group A streptococci (Johnson 1984 & Bahr *et al.*, 1988a). Patients with mycobacterial infections particularly tuberculosis also have raised levels of antibody to terminal GlcNAc and these antibodies can be blocked by free GlcNAc (Bahr *et al.*, 1988a).

Thus GlcNAc appears to be highly immunogenic when in terminal position and both Group A streptococci and mycobacteria which have been associated with arthritic syndromes can evoke antibody to GlcNAc. In view of the presence of terminal GlcNAc on a glycoform of IgG which is raised in RA, autoimmunity to this sugar epitope may be relevant to the pathogenesis of RA.

### 1.13. POSSIBLE RELATIONSHIP BETWEEN HSP65 AND RAISED PERCENTAGE AGALACTOSYL IgG

There have been speculations that an immune response to hsps may induce the changes in galactosylation of IgG leading to autosensitization of the immunoglobulin molecule (Roitt *et al.*, 1988) but as yet there is no evidence suggesting such a link. However, in diseases where agalactosyl IgG is raised there is also increased antibody binding to mycobacterial hsp65 (Rook *et al.*, 1990b). Moreover, a correlation between raised levels of agalactosyl IgG and antibody to mycobacterial hsp65 has been demonstrated in mice injected with BCG, Schistosomiasis or intraperitoneal pristane (Rook *et al.*, 1990b). Thus whatever, if any, the relationship between the two phenomena might be, mycobacteria appear to be involved in both. Hsp65 is one of the immunodominant antigens of mycobacteria (Young *et al.*, 1987) and GlcNAc is a major constituent of their cell wall. Mycobacteria are able to induce cross-reactive responses to
both of these potential autoantigens and in view of the links between mycobacteria and arthritic conditions discussed in this chapter such cross-reactivities may be relevant to the pathogenesis of RA.

1.14. AIMS OF THE PRESENT STUDY

In view of the evidence linking mycobacteria, or autoantigens cross-reactive with mycobacteria, in the aetiology of RA, the present study was undertaken to investigate the cross-reactivity between the organisms and human material, concentrating on the two antigens discussed above namely terminal GlcNAc and hsp65. The distribution and antibody to these antigens are studied in RA, normals and diseased controls using existing and new mAbs.
CHAPTER TWO

A STUDY OF THE DISTRIBUTION OF TERMINAL N-ACETYLGUCOSAMINE IN NORMAL AND RA TISSUES
CHAPTER 2

A STUDY OF THE DISTRIBUTION OF TERMINAL N-ACETYLGUCOSAMINE IN NORMAL AND RA SYNOVIAL TISSUES

2.1. INTRODUCTION

Mycobacteria have been increasingly implicated as the triggers of RA. Their cell wall PG/PS complex is known to be rich in GlcNAc (Esser et al., 1985) and animals immunized with *M. smegmatis* in oil develop antibodies to highly branched N-linked oligosaccharides terminating in GlcNAc residues (Chechik et al., 1987). In 1988 Rook et al., used a similar cell wall preparation from group A streptococci as immunogen to make monoclonal antibodies (mAbs) to terminal GlcNAc. One of these mAbs GN7 is being routinely used for the immunoassay of agalactosyl IgG. This glycoform of IgG bears an N-linked oligosaccharide which terminate in GlcNAc, and occurs with increased frequency in rheumatoid sera and synovial fluid as well as in some mycobacterial infections (Parekh et al., 1985 & Rademacher et al., 1988). Moreover, antibody to the GlcNAc-rich PG/PS complex is raised in rheumatic fever (which develop following infection with group A streptococci) and in RA (Johnson et al., 1984).

GlcNAc is a component of many complex sugars and glycoproteins. It occurs on alternate sugar residues in bacterial cell wall peptidoglycan. It is present in many human glycoproteins and accounts for almost half the sugar residues in hyaluronic acid (West 1986 & Hadler 1981). However, it is unusual for GlcNAc to be a terminal sugar, thus GlcNAc may not normally be exposed to the immune recognition system.

The oligosaccharides on many glycoproteins are believed to be unrelated to the primary function of the protein, acting non-specifically as lubricants and stabilizers, and
conferring negative charge (West 1986). However, it is becoming clear that they may also have specific roles in the localization of glycoproteins within or outside cells (West 1986 & Lernmark 1987). In addition, oligosaccharides have regulatory functions; interacting with hormone receptors, cytokines and lectin-like molecules in cell membranes. A range of receptors has been described which recognize different sugars including mannose, fructose, and GlcNAc (Duvall et al., 1985; Ezekowitz & Gordon 1982; Mokoena & Gordon 1985; Imber et al., 1982; Leoni & Dean 1984). Recognition of GlcNAc has been implicated in cellular uptake of lysosomal enzymes, removal of effete or ageing proteins, removal of cells undergoing programmed death (apoptosis) and as a signal for tissue remodelling (West, 1986; Duvall et al., 1985; Stahl et al., 1976).

In view of the possible antigenic role of terminal GlcNAc and increased levels of agalactosyl IgG in RA, the present chapter looks at the distribution of terminal GlcNAc, as recognized by the mAb GN7, in normal and diseased synovial tissue.
2.2. MATERIALS AND METHODS

2.2.1. TISSUES

Synovial tissues from patients with RA and osteoarthritis (OA) were obtained from diagnostic biopsy, synovectomy or joint replacement. Rheumatoid nodules were obtained by excision biopsy. Patients were documented using a computer-based stratified indexing system (Edwards et al., 1987).

Patients were considered to have OA if joints showed load bearing cartilage loss with bone overgrowth with a normal erythrocyte sedimentation rate (ESR) negative serum rheumatoid factor and no evidence for a cause of synovitis other than mechanical irritation from damaged bone and cartilage. Patients with RA satisfied the American Rheumatism Association criteria for definite or classical rheumatoid arthritis (with widespread synovitis, not attributable to trauma, sepsis or crystal deposition). The details of patients are given in Table 2.1 including the presence or absence of serum IgM rheumatoid factor, bone erosions and nodules for rheumatoid cases.

Normal human muscle, skin, nerve tendon and joint synovium were obtained from each of four limbs amputated for localized proximal sarcomata. Tissues were taken from areas at least 20 cm distant from the tumour and were macroscopically normal. Human tonsil was obtained from eight routine tonsillectomies. Normal brain, obtained post mortem was provided by Dr M. Griffiths. Histologically normal minor salivary gland was kindly provided by Dr. S. Tinkler from routine diagnostic material from the University College Dental Hospital.
# Table 2.1 Patterns of staining with the mAb GN7 on synovial tissues and rheumatoid nodules

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Site</th>
<th>Staining patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GRAN</td>
</tr>
<tr>
<td>Synovia</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JD</td>
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<td>53</td>
<td>RA (E-, N-, RF-)</td>
<td>Knee</td>
<td>+</td>
</tr>
<tr>
<td>AB</td>
<td>F</td>
<td>54</td>
<td>RA (E+, N-, RF+)</td>
<td>Knee</td>
<td>+</td>
</tr>
<tr>
<td>WG</td>
<td>F</td>
<td>55</td>
<td>RA (E+, N+, RF+)</td>
<td>Elbow</td>
<td>+</td>
</tr>
<tr>
<td>CH</td>
<td>F</td>
<td>51</td>
<td>RA (E+, N+, RF+)</td>
<td>Wrist</td>
<td>+</td>
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<tr>
<td>JP</td>
<td>F</td>
<td>43</td>
<td>RA (E+, N-, RF+)</td>
<td>Knee</td>
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<tr>
<td>JU</td>
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<td>Knee</td>
<td>+</td>
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<td>HW</td>
<td>F</td>
<td>73</td>
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<td>Wrist</td>
<td>+</td>
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<tr>
<td>CP</td>
<td>M</td>
<td>69</td>
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<td>Knee</td>
<td>+</td>
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<td>F</td>
<td>61</td>
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<td>Wrist</td>
<td>+</td>
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<tr>
<td>AC</td>
<td>F</td>
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<td>Knee</td>
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<td>81</td>
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<td>Knee</td>
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<td>+</td>
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<td>-</td>
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<tr>
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<td>F</td>
<td>81</td>
<td>OA</td>
<td>Hip</td>
<td>-</td>
</tr>
<tr>
<td>EM</td>
<td>F</td>
<td>70</td>
<td>OA</td>
<td>Knee</td>
<td>+ (SLC)</td>
</tr>
<tr>
<td>IB</td>
<td>F</td>
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<td>OA</td>
<td>Knee</td>
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<tr>
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<td>77</td>
<td>OA</td>
<td>Hip</td>
<td>-</td>
</tr>
<tr>
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<td>F</td>
<td>71</td>
<td>OA</td>
<td>Knee</td>
<td>+ (SLC)</td>
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<tr>
<td>EG</td>
<td>F</td>
<td>78</td>
<td>OA</td>
<td>Knee</td>
<td>+ (SLC)</td>
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<td>RA (E+, N+, RF+)</td>
<td>Elbow</td>
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<td>M</td>
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<td>RA (E+, N+, RF+)</td>
<td>Elbow</td>
<td>-</td>
</tr>
<tr>
<td>EA</td>
<td>F</td>
<td>61</td>
<td>RA (E+, N+, RF+)</td>
<td>Elbow</td>
<td>+</td>
</tr>
<tr>
<td>CPR</td>
<td>M</td>
<td>61</td>
<td>RA (E+, N+, RF+)</td>
<td>Foot</td>
<td>-</td>
</tr>
</tbody>
</table>

GRAN, granular; EC, extracellular; CS, cell membrane; RA, rheumatoid arthritis; OA, osteoarthritis; E, erosive; N, nodular; RF, rheumatoid factor; +, present; -, absent; SLC, synovial lining cells.
2.2.2. MONOCLONAL ANTIBODIES

The mAb to terminal GlcNAc (GN7) was raised by Rook et al., (1988a) in order to develop an immunoassay for agalactosyl IgG. The authors immunized Balb/c mice with the PG/PS complex of group A streptococci and selected the mAb by screening for binding to asialo-agalactosyl-fetuin, prepared by enzymatic digestion of native fetuin to expose terminal GlcNAc. It binds to biantennary oligosaccharides carrying terminal GlcNAc on human glycoproteins, including heat denatured agalactosyl IgG heavy chain, but not to other serum glycoproteins. It also binds to neoglycoproteins constructed by conjugating the diazonium derivative of p-aminophenyl GlcNAc onto bovine serum albumin (BSA). Binding of GN7 to proteins on nitrocellulose blots and monocyte cytospins is abolished by preincubation with specific N-acetylglucosaminidase and not with buffer alone. Binding is inhibited by free GlcNAc but not galactose. The mAb GN7 is an IgM antibody, thus two other IgM mAbs SF1 and SF5 which were raised against mycobacterial antigen, with affinity for human endothelium and neutrophil nuclei respectively were used as isotype matched controls.

The other monoclonal antibodies used were :-

1. EBM 11. Murine IgGl. Stains all macrophages and monocytes (DAKO M718) (Theaker et al., 1985 & Kelly et al., 1988)

2. RFD1, an IgG binding to a determinant associated with MHC class II as it is expressed on dendritic cells, and a subset of monocytes (Poulter et al., 1986).

3. RFD7, an IgGl binding to a 77 kDa antigen on mature macrophages, and some monocytes (Poulter et al., 1986).

4. UCHM1, an IgG2a binding to a 52 kDa antigen on most monocytes, and endothelial cells (Hogg et al., 1984).

37
5. RFT1, an IgG1 which identifies human T cells by binding to the CD5 determinant (Janossy & Prentice, 1982).

6. Several mAbs recognizing CD20 and CD22 on B lymphocytes (Stashenko et al., 1980; Campana et al., 1985) were also used.

RFD1, RFD7, and RFT1 were kindly provided by Dr. L. Poulter, Royal Free Hospital and UCHM1 was a generous gift from Dr. P. Beverley, University College Hospital, London.

2.2.3. IMMUNOCHEMICAL STAINING

Five-micrometre frozen sections were cut, air dried overnight and fixed in acetone for 10 minutes before staining. Staining was performed using GN7 as ascites diluted 1/200 in Tris buffered saline (TBS) at pH 7.6 for 30 minutes, followed by 30 minutes wash in TBS. Then either enzyme or fluorochrome-labelled rabbit anti-mouse immunoglobulin (Ig) was added at 1/40 diluted in TBS and incubated for 30 minutes. For peroxidase staining, the endogenous enzyme (peroxidase) in the sections was blocked with 2% hydrogen peroxide in methanol for 30 seconds after incubation of the sections with the primary antibodies. The peroxidase conjugate was developed in a solution of 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) (BDH 13033) and 10 μl/ml 1% hydrogen peroxide and counterstained with Harris haematoxylin.

2.2.3.1. Double staining

Double staining for comparison of GlcNAc and IgG distribution was performed as follows: sections were fixed and blocked for endogenous peroxidase as described above and sections were washed for 30 minutes in TBS between each incubation. Following
incubation with GN7, sections were incubated with a goat anti-mouse IgM alkaline phosphatase conjugate (Sigma A-7784) diluted 1/40 in TBS and developed with fast blue, followed by a rabbit anti-human IgG polyclonal antiserum (DAKO A090) diluted 1/4000 in TBS and swine anti-rabbit immunoglobulins (DAKO Z196) diluted 1/200 in TBS and peroxidase-antiperoxidase complexes (PAP; DAKO Z113) diluted 1/1000 in TBS. Peroxidase was developed with DAB as above. Double immunofluorescence with GN7 and cell specific antisera was performed by simultaneous incubation with GN7 and a cell specific IgG mAb and simultaneous incubation with tetramethylrhodamine isothiocyanate (TRITC) conjugated rabbit anti-mouse IgM (Seralab SBA 1020-03) diluted 1/10 and fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Seralab SBA 1030-02) diluted 1/40.

2.2.3.2. Comparative staining

Staining with GN7 was compared with staining with three other mouse mAbs at the same dilution. GN6, with similar affinity for terminal GlcNAc, was used as a positive control. SF1 and 5, raised against mycobacterial components, with affinity for human endothelium and neutrophil nuclei respectively, but not for GlcNAc, were used as negative controls.

Staining with GN7 was compared with staining using biotinylated Bandeiraea simplificolia agglutinin II (a GlcNAc specific lectin) and an avidin peroxidase conjugate as described by Capaldi et al., (1985).

2.2.4. IDENTIFICATION OF CELL POPULATIONS

Cell populations were identified using mouse IgG monoclonals RFD7 (1/10), RFD1 (1/4), RFT1 (1/5) and mAbs to B-cell determinants (1/10).
2.3. RESULTS

2.3.1. Normal tissues

The mAb GN7 produced a faint nuclear staining in all tissues detectable at dilutions of GN7 no greater than 1/100. At the 1/200 dilution which was used throughout the study it was minimal and appeared as very weak ring shadows (see for example Figure 2.10). This observation is explained by the recent reports that the nuclear pores have terminal GlcNAc (Starr & Hanover 1990).

At least four sections were examined from each block of tissues. Sections of normal human connective tissue, including synovial tissue from 12 normal joints, fascia from six areas and subcutaneous tissue from four areas, showed no staining (Figure 2.1). Normal muscle (six samples) and brain (two samples) showed no staining. In normal human tonsil (eight samples) GN7 bound strongly to mucosal and crypt epithelial cells (Figure 2.2). Immunofluorescence indicated both cytoplasmic and cell surface staining. Paraffin sections stained with GN7 by the immunoperoxidase method showed localization of cytoplasmic staining to vesicles in the Golgi area. Membrane staining of very occasional cells (<2%) occurred in lymphocytic areas and did not co-distribute with staining for IgG or IgM on double stained preparation using peroxidase and alkaline phosphatase conjugates. Normal human salivary gland showed GN7 staining of cytoplasm only (Figure 2.3). Normal skin keratinocyte cytoplasm (four specimens) stained moderately strongly.

Myelinated nerve trunks (two blocks of ulnar and two of peroneal nerve) showed staining of Schwann cell cytoplasm (Figure 2.4). Schwann cell staining was strictly intracellular and did not include myelin sheaths.
Figure 2.1. Normal synovial tissue stained with GN7 and rabbit anti-mouse peroxidase conjugate, counterstained with haematoxylin. No significant staining is present. (x150).
Figure 2.2. Normal tonsil stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing strong staining of mucosal and crypt epithelial cells. (x200).
**Figure 2.3.** Normal salivary gland stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin. Showing staining of mucus-secreting acinar epithelial cells. (x300).
2.3.2. DISEASED TISSUES

Synovial tissue from RA patients showed three patterns of staining with GN7: (1) granular cytoplasmic staining, (2) extracellular reticular staining and (3) surface staining of isolated cells.

2.3.2.1. Granular cytoplasmic staining

Figure 2.4. Myelinated nerve trunk stained with the mAb GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing intracellular staining of Schwann cells. (x200).
2.3.2. DISEASED TISSUES

Synovial tissue from RA patients showed three patterns of staining with GN7: (1) granular cytoplasmic staining, (2) extracellular reticular staining and (3) surface staining of isolated cells.

2.3.2.1. Granular cytoplasmic staining

In tissues from patients with OA the only staining seen with GN7 was a weak to moderate diffuse granular cytoplasmic staining of synovial lining cells (Figure 2.5). These samples did not show evidence of recent tissue damage in terms of fibrin deposition or cell death.

In tissues from patients with RA dense reticular extracellular staining of GlcNAc was seen close to the tissue surface (Figure 2.6). The intensity of this extracellular staining was very striking at high power (Figure 2.7). Moreover a granular cytoplasmic staining could also be seen in groups of cells in the deep synovial tissue (Figure 2.8). These cells were of macrophage morphology and in some cases belonged to groups containing haemosiderin or red cell debris. Comparison with cell specific markers using double immunofluorescence showed these cells to be a sub-population of RFD7 positive cells. Staining using fluorochromes appeared as intracytoplasmic rings of variable sizes, possibly representing material at the periphery of phagolysomes.
Figure 2.5. Synovial tissue from an OA patient stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing moderate diffuse granular cytoplasmic staining of the synovial lining cells. (x200).
Figure 2.6. Synovial tissue from RA patient stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin showing reticular extracellular staining around cells near the tissue surface. (x150).
Figure 2.7. High power view of superficial synovial tissue from a RA patient stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin. A reticular net of extracellular staining is seen close to the tissue surface. (x400).
Figure 2.8. RA synovial tissue similar to the one shown in Figure 2.6 showing dense granular cytoplasmic staining by GN7 of macrophage like cells in the deep synovial tissue. (x300).
2.3.2.2. Extracellular reticular staining

Tissues from patients with OA showed no extracellular staining. Tissues from patients with definite RA showed extracellular staining in a reticular or "chicken wire" pattern (Figure 2.7). Reticular staining occurred as a band either between synovial lining cells, or where there was surface fibrin. The fibrin deposits themselves did not stain. When intermingled with lining cells, staining did not follow the contour of cell membranes, as seen with cell surface markers. However, the staining was consistent with material shed or otherwise restricted to a halo around individual cells. Reticular staining also occurred in isolated patches in the deep tissue matrix where no other cellular or matrix abnormality was evident.

Double indirect immunofluorescence with GN7 and the macrophage marker EBM11 (Figures 2.9) confirmed staining specificity and showed that many of the cells on the surface of and intermingled with GN7-positive material were macrophages. Rheumatoid nodules showed no staining of cells or areas of fibrinoid necrosis, but showed similar isolated patches of reticular staining. The extracellular matrix at these sites appeared unremarkable, but nearby cells were pyknotic, suggesting an early stage of necrosis. The isolated patches of reticular staining were similar in pattern to the staining in superficial bands of synovium.
Figure 2.9. Superficial RA synovial tissue double stained with GN7 and rabbit anti-mouse IgM TRITC conjugate (red) and with EBM11 and rabbit anti-mouse IgG FITC (green). The GN7 staining is intimately associated with the lining cell layer but does not correspond to either the membranes or cytoplasm of individual cells. (x400).
2.3.2.3. Cell surface staining

In two samples of synovium from patients with RA in which there were dense lymphocyte infiltrate, ring (membrane) staining was seen around isolated large mononuclear cells in the deep tissue, usually scattered amongst lymphocytes (Figure 2.10). These cells had an open chromatin pattern, high nuclear/cytoplasmic ratio and one or two short processes extending between neighbouring cells. They could not otherwise be identified using available cell markers. Their position and shape is consistent with that of macrophage-like antigen presenting cells. Work on peripheral blood monocytes has shown that a proportion of monocyte derived cells demonstrate terminal GlcNAc on their cell membranes after a period of maturation (Sharif et al., 1989), suggesting that the cells exhibiting ring staining in tissue may be of similar monocyteoid origin.

2.3.2.4. Comparative staining

Staining with GN7 was more intense than, but otherwise identical to staining with GN6. Neither of the two anti-mycobacterial mAbs gave staining patterns as seen with GN7. The distribution of GN7 staining on normal tonsil matched staining with Bandeiraea simplicifolia agglutinin II.

The relationship between binding of GN7 and the presence of IgG was studied using several indirect immunochemical methods for detecting IgG, including double fluorescence and double enzyme techniques (peroxidase/PAP and alkaline phosphatase/APAAP) on single sections and immunoperoxidase on serial sections. With all techniques the distribution of GN7 staining was totally separate from IgG, which was present on scattered cells within lymphocyte clusters and at low levels in the connective tissue stroma, away from the sites of maximum GN7 staining.
Figure 2.10. Synovial tissue from a RA patient stained with GN7 and rabbit anti-mouse Ig alkaline phosphatase conjugate, with no counterstain. Two isolated cells (one shown with an arrow) amongst a cluster of lymphocytes show ring staining. Lymphocytes nuclear membranes show faint staining. (x400).
2.4. DISCUSSION

These results indicate that terminal GlcNAc is not normally exposed in the extracellular matrix of connective tissue or supporting matrix elements in non-epithelial organs. GlcNAc was found on certain epithelial cells and their secretions, but appears to be separated from connective tissue and cells of the immune system by basal laminae. This observation is consistent with the view that GlcNAc rarely occurs at the terminal positions on glycoprotein oligosaccharides (West 1986). Nuclear membrane staining is explained by the recent discovery of GlcNAc attached to proteins of the nuclear pore (Hanover et al., 1987; Starr & Hanover 1990). Staining of normal epithelial cells may represent binding to glycoproteins undergoing glycosylation in the Golgi apparatus or binding to mucopolysaccharide secretory products such as mucus.

Several mechanisms may lead to abnormal exposure of GlcNAc residues on proteins and glycosaminoglycans. First, defects in synthesis of the glycoprotein, such as the reduced galactosyltransferase activity which has been described in lymphocytes from patients with RA (Axford et al., 1988), may lead to failure of the addition of terminal galactose and sialic acid, leaving GlcNAc in the terminal position. Secondly, GlcNAc exposure may result from post-synthetic removal of galactose and sialic acid by enzymes or by other inflammatory mediators such as oxygen free radicals (Griffiths & Lunec et al., 1989).

In the diseased tissue studies, terminal GlcNAc was found in the cytoplasm of a proportion of cells. In OA, cytoplasmic staining was never intense and was confined to cells on the synovial surface. In RA, intense granular staining occurred in macrophages in the deeper tissue, suggesting that these cells are actively involved in processing GlcNAc-bearing material.

Extracellular material bearing GlcNAc was found only in rheumatoid synovium, suggesting a more specific role for this material in RA. Such material may be derived
from the circulation, or may be of local origin. IgG does not appear to contribute to the stainable GlcNAc-bearing material. However, GN7 does not bind to agalactosyl IgG unless the immunoglobulin has been denatured so the results do not preclude the coexistence of agalactosyl IgG in the tissue. Bacterial fragments might be expected to carry terminal GlcNAc, and could conceivably be responsible for the staining, although no structures resembling bacteria were seen. GlcNAc can inhibit the binding of the complement degradation product iC3b to its receptor CR3 on the macrophage membrane (Ross et al., 1985). Thus iC3b may bear terminal GlcNAc and the observed staining may represent deposition of complement components. Connective tissue matrix molecules contain GlcNAc within their polysaccharide chains. Oxygen free radicals have been shown to cause the partial degradation of hyaluronic acid (McNeil et al., 1985) which may expose GlcNAc in the terminal position. In addition some of the extracellular GlcNAc could be derived from nearby cell membranes, since some cells which appeared to have GlcNAc on their cell membrane were seen in deeper layer of the synovial tissues. However the extracellular staining pattern of GlcNAc bearing material does not coincide with that of membrane-bound proteins such as Fc receptors or Class II antigens.

The absence of terminal GlcNAc from established fibrinoid foci in rheumatoid nodules is somewhat surprising, in view of the suggestion that cellular events in the palisading layer (thin layer of columnar, macrophage-like epithelial cells) may be similar to those occurring at the synovial surface in rheumatoid arthritis. However, the presence of GlcNAc close to small foci of cell pyknosis (densely stained small nuclei of dying cells) suggests that exposure of terminal GlcNAc may be an early or transient phenomenon, and may not be detectable in the more fibrous chronic lesions.

GlcNAc expression may simply be a marker of tissue damage. However, there are two reasons for considering the possibility that terminal GlcNAc may contribute to the propagation of inflammation. First, raised antibody levels to the GlcNAc-rich PG/PS complex of group A streptococci in RA and rheumatic fever (Johnson et al., 1984) suggest an immune response to GlcNAc-bearing material in both these diseases. Second, there is evidence that terminal GlcNAc can act as a signal for cells to take up material such
as lysosomal enzymes, effete or ageing proteins, cell undergoing programmed death and tissue elements during developmental remodelling (West 1986; Duvall et al., 1985; Stahl et al., 1976). At sites of inflammation, uptake of GlcNAc-bearing material may act in conjunction with mechanisms such as antibody-antigen complexing and complement fixation to promote the release of inflammatory mediators or cytokines. A number of such cytokines, including tumour necrosis factor, Interferons, interleukin-1, 2 and 6 have been isolated from rheumatoid synovial fluid (Westacott et al., 1990 & Guerne et al., 1990).

The significance of terminal GlcNAc residues may vary with associated sugars in the oligosaccharide chain, particularly if chains are branched (bi- or tri-antennary). It is not yet clear how closely reagents such as GN7 or Bandeiraea agglutinin II resemble the lectin-like molecules on the macrophage surface.
CHAPTER THREE

EXPRESSION OF MEMBRANE N-ACETYLGLUCOSAMINE BY CELLS IN RHEUMATOID SYNOVIAL FLUID, AND BY PRE-CULTURED MONOCYTES
3.1. INTRODUCTION

The work described in Chapter 2 has demonstrated the presence of large quantities of immunoreactive GlcNAc in synovial tissue from RA patients. This abnormal exposure of terminal GlcNAc in RA tissues appeared to show disease specificity since little or no immunoreactive GlcNAc was found in inflamed joint tissues from OA. Moreover, in some samples of synovial tissues from RA patients with dense lymphocyte infiltration, a small number of isolated large mononuclear cells in the deep tissue showed membrane staining (Chapter 2, Figure 2.10). Since this might indicate selective expression of GlcNAc on cell membrane by some cells, the mAb GN7 was used to screen cell populations from synovial fluid and peripheral blood for the presence of terminal GlcNAc.

3.2. MATERIALS AND METHODS

Monoclonal antibodies.

In addition to the mAb GN7 a series of mouse mAbs were also used for identification of the cell populations. The detail of all these mAbs have been provided in Chapter 2 (Section 2.2.2)
3.2.1. CULTURE OF HUMAN MONOCYTES

Sixty millilitres of blood from normal donors were defibrinated by shaking with glass beads in a glass universal, and the mononuclear cells were separated by centrifugation over lymphocyte separation medium (Flow Laboratories, Irvine, Ayrshire, Scotland). The cells were washed, suspended in RPMI 1640 supplemented with 10% autologous serum, and incubated in 80 cm² flasks which had been pretreated with undiluted autologous serum. After 2 hours non-adherent cells were removed by rinsing three times with RPMI 1640. Then the monocyte-enriched population was detached by incubation at 37 °C with RPMI containing 10% autologous serum and 3.3 mg/ml disodium EDTA. After 45 minutes cells were removed by gentle pipetting, centrifuged once, and resuspended in RPMI at 10⁶ cells/ml. In some experiments cytospin preparations were made from part of this suspension to obtain 2 hours cultured monocytes. The remaining cells were supplemented with 20% autologous serum, and this suspension was plated at 10⁵ cells/well in tissue culture wells formed by clamping a silicone rubber culture chamber (Bellco Glass Co., Vineland, NJ) onto tissue-culture quality plastic slides cut from 80 cm² culture flasks similar to those used for the first 2 hour culture. For a few experiments glass slides were also used. Any further additions to the wells were added in RPMI, to give a final volume of 0.2 ml, with 20% serum. These cultures were maintained for varying periods, then rinsed gently five times with RPMI 1640, dried at room temperature overnight, and stained within 24 hours.

3.2.2. AGENTS USED TO ACTIVATE THE CULTURES

The vitamin D3 metabolite (1,25-(OH)₂ cholecalciferol) was kindly provided by Dr. M. Uskokovic, Hoffman La Roche, Nutley, NJ, U.S.A. It was stored as a solution in ethanol at -20 °C and diluted before use so that ethanol was present in all cultures at a
final concentration of 1%. The final concentration was $10^{-7}$. Recombinant gamma interferon (rINF-γ) was obtained from Boehringer Ingelheim, Vienna, Austria and recombinant GM-CSF was obtained from Biogen. For both these cytokines the units referred to in the text are those indicated by the suppliers. Indomethacin was purchased from Sigma, dissolved in ethanol, and added to appropriate wells at a final concentration of 1 or 10 µg/ml.

### 3.2.3. IMMUNOCHEMISTRY

Monocyte preparations were fixed in methanol for 10 minutes. They were then washed in 10 mM phosphate buffered saline (PBS, pH 7.4) for 10 minutes with constant agitation. Excess PBS was removed and 50 µl of monoclonal antibody appropriately diluted inPBS was applied to each preparation and incubated in a humid chamber for 40 minutes. All incubation for immunocytochemistry was carried out at room temperature. The preparations were then washed and treated with 1% hydrogen peroxide in methanol for 30 seconds to block endogenous peroxidase. Then after further washes the cell monolayers were incubated for 35 minutes with peroxidase-labelled, rabbit anti-mouse immunoglobulin (DAKO P260) diluted 1/40 in PBS. The preparations were washed, and incubated with substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride, (BDH, 13033) and 0.01% hydrogen peroxide in PBS for 5-10 minutes. The slides were then washed and counterstained with Harris's haematoxylin, washed in running tap water, mounted in Apathy's media or dehydrated (glass slides only) in graded alcohol, cleared in CNP 30 (Penetone, Northumberland) and mounted in histomount. For all experiments controls included the isotype-matched IgM monoclonal SF5, and a second layer control to which no monoclonal had been added.
3.2.4. NON-SPECIFIC ESTERASE

Monocyte preparations were incubated with diazotised pararosaniline and α-naphthyl acetate at pH 7.1 for 5 minutes at 37 °C. The preparations were then washed in PBS and stained for GlcNAc using the peroxidase method above with the addition of 15 μl/ml of 8% nickel chloride to the developing solution to produce a black reaction product. Haematoxylin counterstaining was omitted.

3.2.5. IMMUNOFUORESCENCE OF FIXED CELLS

Monocyte preparations were fixed in methanol for 10 minutes, washed in PBS for 10 minutes and then incubated with appropriately diluted monoclonal antibodies in PBS for 30 minutes in a moist chamber. After further washes they were incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO F261) for 30 minutes, washed thoroughly in PBS and mounted in CITIFLUOR glycerol/PBS solution containing an antifading agent AF1. Double staining was performed by simultaneous incubation with anti-GlcNAc and one of a range of mouse IgG mAbs followed by goat anti-mouse IgM rhodamine conjugate (Sera-Lab SBA 1020-03) and goat anti-mouse IgG FITC-conjugate (Sera-lab SBA 1030-02). In each experiment it was checked to make sure that cells stained with GN7 showed no positivity when the anti-mouse IgG was used as the second layer, and conversely that the anti-IgM did not bind to IgG. Controls using SF5 rather than GN7 were also included.
3.2.6. IMMUNOFLUORESCENT STAINING OF LIVE CELLS IN SUSPENSION

Cell suspensions were incubated with monoclonal GN7 diluted 1/200 in RPMI 1640, containing 0.1% sodium azide at 4 °C, in order to minimise capping. Suspensions were washed three times in the same medium by centrifugation. The second layer was the FITC-conjugated rabbit anti-mouse immunoglobulins. The mAb, SF5 was used as an isotype matched negative control, and anti-Class II as a positive control.

3.2.7. PREPARATION OF WESTERN BLOTS FROM LYSATES OF CULTURED MONOCYTES

Lysates of cultured monocytes were prepared in two ways. In some experiments monolayers of monocytes in tissue culture flasks were washed repeatedly with RPMI 1640 and then lysed with 0.1 M citric acid containing 100 μl of Zaponin (Coulter Electronics Ltd, Northwell drive, Luton, Beds, U.K.) per 25 ml. This procedure avoided lysis of nuclei and consequent contamination with DNA. Nuclei were subsequently removed from the preparation with high speed centrifugation. In other experiments cells were detached from the plastic, pelleted by centrifugation, and lysed by the addition of 3% sodium dodecyl sulphate (SDS) and 5% urea in order to eliminate any possibility of autolysis. DNA was broken up with brief sonication. Protein content of the lysate was measured with the Biorad protein estimation kit.

Lysate (approximately 25 μg for Aurodye or Ink staining, and 40-80 μg for staining with mAbs) was loaded into 6 mm wells and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels (1.5 mm thick) of 12.5% (vol/vol) acrylamide according to the discontinuous buffer system of Laemmli (1970). Electrophoresis was performed on a vertical slab gel unit (Hoefer SE600) overnight at 4
0°C at constant current using 6 mA per gel. A mixture of standard protein markers (MW-SDS-200, Sigma) were used for the determination of molecular mass. Proteins were transferred from gels to nitrocellulose membranes (0.2 μm, BA 83 Schleicher and Schuell, FRG) using a semi-dry electroblotter as described by Kyhse-Andersen (1984). The standard blotting time of 60 minutes was prolonged to 80 minutes to ensure penetration of protein into the membranes. In order to visualise protein bands, the nitrocellulose membranes were washed with excess PBS supplemented with 0.05% (vol/vol) Tween 20 once for 120 minutes at 32 °C, and three times for 30 minutes at room temperature with constant agitation. To visualise bands, blots were then rinsed in water and incubated overnight either in Aurodye, a colloidal gold stain (Janssen Life Sciences Products, Belgium), or in India Ink (Pelikan type Fount India) diluted 1/1000 in PBS containing 0.05% Tween 20. Stained blots were air-dried between Whatman 1 paper sheets and kept at -20 °C in aluminium foil.

3.2.8. IDENTIFICATION OF PROTEIN BANDS BY MONOCLONAL ANTIBODIES

Unstained nitrocellulose membranes were soaked in 1% (wt/vol) BSA in PBS (10mM, pH 7.4) with 0.05% (vol/vol) Tween 20 to block any free protein binding sites. After blocking for 2-4 hours, nitrocellulose strips were incubated overnight at 4 °C with mAbs diluted between 1/200 and 1/1000. Binding was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO P260) at a 1/800 dilution for 2 hours at room temperature. IgG heavy chains on Western blots were also identified with a peroxidase-conjugated rabbit anti-human gamma chain (DAKO P214). Peroxidase labelling was visualised with 4-chloro-1-naphthol (Sigma C-8890) made up in 5 mM Tris-HCL (pH 7.6) at a final concentration of 50 μg/ml containing 18% methanol AR (v/v) and 1 μl/ml of hydrogen peroxide (BDH; 100 vol; v/v). The reaction was stopped by washing the blots in distilled water.
In order to check the specificity of the binding of mAb GN7, cytospins, or fragments of Western blots, were incubated for overnight at 37 °C in citrate/phosphate buffer at pH 5.0 containing 1 unit/ml of β-hexosaminidase. (This was a generous gift from Dr. G. Jacobs and T. Rademacher, Department of Biochemistry, Oxford, U.K.). Replicate samples were incubated in buffer alone. Subsequently all samples were washed, and then incubated with GN7 as described above.
3.3. RESULTS

3.3.1. Staining of cells in cytospins of rheumatoid synovial fluid with the anti-GlcNAc monoclonal antibody GN7

Freshly prepared cytospins of rheumatoid synovial fluid showed GlcNAc positive cells with granular cytoplasmic staining and a small percentage (2-8%) of these cells also showed membrane staining. The membrane location of this staining was most clearly seen when using immunofluorescence to stain live cells in suspension under non-capping conditions by immunofluorescence (Figure 3.1). Occasional cells of similar appearance have also been seen in synovial tissue (Chapter 2).

3.3.2. Staining of monocytes with GN7

Freshly prepared monocytes, or monocytes cultured for up to 5 hours showed no staining with anti-GlcNAc. However, when monocytes from the same donors were cultured in RPMI 1640 with 20% autologous serum for 21-48 hours, a subset of the cells developed intense membrane staining (Figure 3.2a). Incubation of the cells in pure β-hexosaminidase almost eliminated the binding of the monoclonal, whereas incubation in the buffer alone did not (Figure 3.2b). No staining of cultured cells (or sections) was observed when GN7 was added in the presence of 0.1 M GlcNAc, whereas staining was not affected by Galactose at 0.5 M. Similarly there was no staining with SF5 or with the anti-mouse Immunoglobulins alone. Immunofluorescent studies of unfixed cells confirmed the membrane distribution of this staining. Cultured cells occasionally also showed mild to moderate granular staining in their cytoplasm.

The time-course of the percentage of cultured cells expressing the intense membrane GlcNAc is shown in Figure 3.3. After peaking at about 21 hours, the percentage of positive cells declined progressively and positive cells were not seen by 72 hours.
Figure 3.1. RA synovial fluid cells stained in suspension under non-capping conditions with the anti-GlcNAc mAb GN7. Fluorescein-conjugated rabbit anti-mouse Ig was used as the second layer to demonstrate membrane staining of these cells. Second layer controls, and controls with an IgM mAb to an intracellular determinant, showed no staining.
Figure 3.2. Monocytes cultured for two days stained with GN7 by the peroxidase method.
a) Staining without pretreatment with β-hexosaminidase. A subpopulation of these monocytes shows intense staining, revealed as membrane staining by parallel fluorescent staining of live cells (not shown). (x300).
b) Staining after treatment with the enzyme. Staining is almost eliminated. (x500).
Figure 3.3. The time course of the percentage of monocytes expressing membrane GlcNAc in culture. Cells from control donors (■), or RA patients (○).
The time courses and percentages seen in cultures of cells from ten patients with rheumatoid arthritis did not differ significantly from those seen in cultures from ten control donors (Figure 3.3). Further analysis of the data in relation to sero-positivity, age or sex revealed no significant correlations.

The expression of this membrane GlcNAc was not significantly changed by culture for 24 hours in $10^{-7}$ M 1,25(OH)2 vitamin D3, 1 μg/ml indomethacin, 200 U/ml GM-CSF, or 200 U/ml rINF-γ

3.3.3. Correlation of the expression of membrane GlcNAc with other markers

The cells expressing membrane GlcNAc were strongly positive for non-specific esterase suggesting that they are macrophages. Figure 3.4 shows a typical GlcNAc positive cell first stained for non-specific esterase (NSE) and subsequently stained with GN7.

The monoclonal EBM 11 stains all macrophages and monocytes (Third Workshop and Conference on Leucocyte Differentiation, and Dr. L.W. Poulter personal communication to Dr. Rook) and stained essentially all the cells in cultures, therefore contaminating cell types were rare (Figure 3.5). Double staining with EBM 11 and GN7 showed that GN7 positive cells are a subset of EBM 11 staining macrophages (Figure 3.5a & b). Similarly these cells were non-specific esterase positive (data not shown). Some, but not all RFD7 positive cells were also positive for GN7 (Figures 3.5c & d) though RFD7+ cells were too rare to allow us to find RFD7+/GN7+ and RFD7+/GN7- cells in the same field. Double staining was also seen with GN7 and UCHM1 (Figure 3.5e & f). Double staining with GN7 and RFD1 or RFT1 was never seen.
Figure 3.4. Non-specific esterase stained GN7 positive cell. Monocyte preparation was first stained for non-specific esterase (red) and then with the mAb GN7 using nickel enhanced DAB (black). The cell showing black reaction product is also positive for NSE. (x500).
Figure 3.5. Double staining of monocytes precultured on plastic for 20 hours to maximise the number of GN7+ cells. Double staining was performed by simultaneous incubation with anti-GlcNAc GN7 (an IgM monoclonal antibody) and EBM 11 or RFD7 or UCHM1 as indicated (all IgG monoclonal antibodies). Binding was revealed by goat anti-mouse IgM TRITC conjugate and goat anti-mouse IgG FITC-conjugate.
3.3.4. Partial characterisation of the GlcNAc-bearing molecule by SDS-PAGE.

Immunoblots of two day cultured monocytes, from rheumatoid and normal donors, when stained with anti-GlcNAc, showed the agalactosyl IgG heavy chain and a band of molecular weight 70-80 kDa (see Figure 3.6). The band due to agalactosyl IgG was anticipated since the monocytes were cultured in autologous sera. Even in normal serum approximately 20% of IgG are agalactosyl therefore contamination with agalactosyl was inevitable. When immunoblots of fresh monocytes (2 hours), 2 day cultured monocytes and 4 day cultured monocytes from the same donors were stained with anti-GlcNAc the 70-80 kDa band was seen clearly in the lysates of 2 day cultured monocytes (Figure 3.6) and occasionally, but weakly in the fresh monocytes. The intensity of this band was similar in cells from RA and normal donors, though as expected the band attributable to the agalactosyl IgG heavy chain was always stronger for RA donors. Immunoblots of rheumatoid synovial fluid cells showed both bands even when fresh cells were used (Figure 3.6). Like the 21-48 hours cultured peripheral blood monocytes, treatment of immunoblots with pure N-acetylhexosaminidase eliminated all staining by the anti-GlcNAc mAb GN7.
Figure 3.6. Immunoblots of fresh or cultured monocytes from blood or RA synovial fluid stained with anti-GlcNAc, GN7. A= unidentified band bearing terminal GlcNAc. B= IgG heavy chain. Lane 1 shows molecular weight markers. Lanes 2, 3, 4 & 5 show SDS PAGE of lysates of normal monocytes from the same donor, cultured in autologous serum for 2 hours, 2 days, 2 days and 4 days respectively. Lane 4 is a replica of lane 3 which was incubated with β-hexosaminidase before staining with GN7. Incubation with buffer alone did not diminish staining. Lane 6 shows monocytes from an RA patient cultured for 2 days, and lane 7 synovial fluid adherent cells cultured for only 2 hours.
3.4. DISCUSSION

The cells expressing membrane GlcNAc are a subset of macrophages since they are positive for non-specific esterase and stained by the macrophage-specific mAb EBM 11. They also appear to be a subset of both UCHM1 positive and RFD7 positive cells. The antibody UCHM1 stains 60-90% of monocytes cultured on plastic for 24 hours, while RFD7 stains approximately 10%. Cells positive for both markers are known to occur (Dr. L. W. Poulter personal communication to Dr. Rook) and the GlcNAc-bearing cells may fall within this overlapping population, though it has not been confirmed. It is likely that these cells result from a reduction in terminal galactose, leading to increased exposure of GlcNAc. It is also possible that a glycoprotein bearing terminal GlcNAc becomes selectively adsorbed onto this small subpopulation of cells. If so, the glycoprotein in question must be present in very low concentration, since Western blots of cell free synovial fluid stained with GN7 reveal only one molecule bearing terminal GlcNAc, and this is the IgG heavy chain.

Cell membranes bearing glycoproteins with terminal GlcNAc appear to play an important role in interactions between different cell types, and between cells and biologically active molecules. Thus GlcNAc is found on the membranes of many tumour cell lines, and oligosaccharides containing this sugar epitope in terminal position will block destruction of tumour cells by natural killer cells as well by antibody dependent cell mediated cytotoxicity (Dennis & Laferte 1985). In addition, reversion of a non-metastatic, GlcNAc-expressing tumour cell line to a metastatic phenotype can be induced by selecting variants resistant to the GlcNAc-binding lectin from Bandeiraea semplicifolia (Dennis et al., 1986). Another group has studied correlations between expression of various cell membrane lectins and sugars, and the selective binding and homing of tumour cells to different organs, cells and substrates (Gabius et al., 1987). GlcNAc is also involved in cell-cell recognition and cell-substrate adhesion during fertilisation and embryonic development (Bayna et al., 1986) and addition of GlcNAc to the medium will inhibit the selective binding of macrophages to thymocytes undergoing...
apoptosis induced by the glucocorticoid hormone methyl-prednisolone (Duvall et al., 1985). GlcNAc is also a ligand for the serum "mannose binding protein" (Taylor & Summerfield 1987) which has actin-like properties. For example the calcium ion dependent binding of bovine conglutinin to C3 degradation product iC3b can be blocked by GlcNAc (Baatrup et al., 1987). Thus GlcNAc is not usually terminal, but when it is, it has signalling functions.

The significance of the new cell type described here is at present unknown. Moreover until the GlcNAc-bearing membrane component has been formally identified from the cells which express it following 21-48 hours in culture and confirmed that the same component is present in the cells seen in tissue sections and synovial fluid, no specific role of this new cell type could be envisaged in RA.

The monoclonal was raised against the GlcNAc-rich cell wall/polysaccharide complex of Group A streptococci. It has been shown that both rheumatic fever and RA patients have raised levels of antibody to this preparation (McCarty 1958 & Johnson et al., 1984). Therefore auto-reactivity to complex-type oligosaccharides bearing terminal GlcNAc remains a real possibility. However, most of the hybridomas induced by the streptococcal cell wall preparation secrete antibodies which bind to a synthetic epitope of GlcNAc formed by conjugating the diazonium derivative of p-aminophenyl GlcNAc to BSA, but fail to bind to GlcNAc-terminating complex-type oligosaccharides (Rook et al., 1988a). It will therefore be of interest to discover whether the proportion of the anti-GlcNAc response which has this cross-reactivity is higher in arthritic patients. Particularly, in view of the reports that Mycobacteria, which are increasingly implicated as triggers of RA (Rook et al., 1990a; Bahr et al., 1988a and 1988b; Tsoulfa et al., 1989a and 1989b; Winfield 1989) are also able to provoke a response to β-linked GlcNAc-terminating complex-type oligosaccharides (Chechik et al., 1987).
The joints of RA patients appear to contain several potential targets for such a response. There is terminal GlcNAc on agalactosyl IgG (Parekh et al., 1985) and the presence of clusters of macrophage-like cells containing strongly GlcNAc-positive granular material, and a reticulate pattern of GlcNAc staining between the synoviocytes (Chapter 2 & Sharif et al., 1990). In addition the experiments carried out in the present chapter demonstrated the presence of a sub-set of macrophages with strong membrane staining for GlcNAc. Such membrane GlcNAc may provide a further site for anti-GlcNAc-mediated damage in RA.
CHAPTER FOUR

ANTIBODY TO

THE ROLE OF TERMINAL GlcNAc IN THE PATHOGENESIS OF
RHEUMATOID ARTHRITIS
CHAPTER 4

THE ROLE OF ANTIBODY TO TERMINAL GlcNAc IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

4.1. INTRODUCTION

In 1966 Boyden described a group of antibodies in normal human serum as natural antibodies. He defined these antibodies as "antibodies present in physiologically healthy individuals capable of binding specifically to potential antigens but not with immunologically acceptable molecules". Since then the existence of such natural antibodies in normal human serum has been widely reported (Guilbert et al., 1982; Lutz et al., 1984; Evans 1988). These antibodies display various degrees of cross-reactivity and the antigenic structures they recognize are usually poorly defined. Moreover, their serum level is usually very low (<1 μg/ml of serum) and there is little or no evidence of their involvement in any pathological conditions. In contrast to other natural antibodies, the anti-carbohydrate antibodies occur in high titres and quantities (50-100 μg/ml of serum) and display a more subtle specificity (Galili et al., 1984 and 1985). For example, natural antibodies to blood group A and B bind specifically to GalNAcα1-3 (Fucα1-2) Gal and Galα1-3 (Fucα1-2) Gal respectively (Kabat 1976 & Watkins 1966). Other natural antibodies with anti-carbohydrate specificity include the anti-Thomson-Friedenreich antibody, which binds to Galβ1-4GalNAc residues, an antigen associated with human breast cancer (Bray et al., 1981), and anti-Gal antibodies which appear to mediate the lysis of normal and pathological (thalassemic and sickle cell) red blood cells by interacting with α-galactosyl residues on these cells (Galili et al., 1983, 1984 and 1985). These anti-carbohydrate antibodies are constantly produced in normal serum.
possibly as a immune response to cross-reactive epitopes present on normal gastrointestinal and bronchial flora. It is not known whether mycobacteria are truly commensal but group D strep is a member of the normal bowel flora. Both of these organisms are able to induce arthritis in animal models and can induce anti-carbohydrate antibodies with specificity for terminal GlcNAc (McCarty 1958 & Bahr et al., 1988a). Work described in the preceding chapters has established the existence of immunoreactive GlcNAc in RA joints (Sharif et al., 1990) and demonstrated the presence of a novel macrophage subset with transient but strong expression of membrane GlcNAc (Sharif et al., 1989). Therefore a GlcNAc bearing autoantigen or bacterial antigen could be relevant to the pathogenesis of RA.

The aim of the present chapter is to determine the class, titre and specificity of anti-GlcNAc antibodies in the sera of patients with RA, rheumatic fever, tuberculosis, leprosy, Crohn's disease and healthy controls. The ability of affinity-purified anti-GlcNAc antibody from these sera to bind to agalactosyl IgG and immunoreactive GlcNAc in RA joints was also investigated.

4.2. MATERIALS AND METHODS

The following glycoproteins were selected for use in the ELISA because they all bear terminal GlcNAc, they constitute potential autoantigens, or they have been reported to be the target of anti-carbohydrate antibodies in preliminary studies.

1. GlcNAc-phe-BSA - A synthetic epitope of GlcNAc prepared by conjugating GlcNAc to bovine serum albumin (BSA) via the diazonium derivative of p-aminophenyl GlcNAc (Sigma) as described by Zopf et al., (1978).

2. Human orosomucoid - High performance liquid chromatography (HPLC) purified, fully sialylated starting material and the enzyme treated agalactosyl preparation with
exposed terminal GlcNAc were used. These preparations were kindly provided by Dr T. Rademacher (Dept of Biochemistry, University of Oxford). The latter preparation contains bi-, tri-, and tetra-antennary GlcNAc, not bisected.

3. Hen ovomucoid (from chicken egg white, type III-O; Sigma) - bi-, tri-, tetra-, and penta-antennary sugars with bisecting GlcNAcs.

4. Ovalbumin (from chicken egg, grade V, Sigma) - hybrid series of sugars believed to be similar to sugars in bee venom.

5. Asialo-, agalacto-fetuin (enzyme treated fetuin; ETF) and fully sialylated HPLC purified preparation, as used to screen monoclonal antibodies to terminal GlcNAc. These preparations were also provided by Dr. Rademacher.

6. Bee venom phospholipase A2 (Sigma) - Included because bees lack galactosyltransferase and the glycoprotein in their venom appears to have an anti-arthritic effect (Chang & Bliven 1979). Ovalbumin and bee venom phospholipase were HPLC purified before they were used as antigens in the ELISA assay.

4.2.1. ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

The glycoprotein antigens were coated at 5 μg/ml for GlcNAc-phe-BSA, ovomucoid, ovalbumin, and at 2.5 μg/ml for orosomucoid, ETF and bee venom phospholipase in carbonate-bicarbonate buffer (0.05 M, pH 9.6) onto the wells of microtitre ELISA plates (Nunc) and incubated overnight at 4 °C. After coating wells were washed with PBS, pH 7.4 containing 0.05% (v/v) Tween 20 (PBS/Tween). Test sera were then applied at dilutions of 1/50; 1/100 and 1/200 in PBS/Tween in duplicate
and incubated for 2 hours at room temperature. After further washes horseradish peroxidase (HRP) conjugated rabbit anti-human immunoglobulins (DAKO P212) was added at 1/1000 in PBS/Tween and incubated for 3 hours at room temperature. The washing process was repeated again to wash off any unbound conjugate and the assay was developed with 0.5 mg/ml of 2,2'-azino-bis 3-ethylbenzthiazoline sulphonic acid (ABTS; Sigma A-1888) in citrate phosphate buffer (0.1 M, pH 4.1) with 0.35 μl/ml hydrogen peroxide (H₂O₂) vol. 20 (6% w/v). The reaction was stopped after 30 minutes with 9.6 mg/ml sodium fluoride (Sigma S 1504) in distilled water and absorbance values read immediately at 650 nm with a Titertek multiscan ELISA reader (Flow). The volume of reagent added throughout the assay was 100 μl/well, each wash step repeated three times with three minutes incubation between washes at room temperature. A normal serum was included in each run as a standard control so that a correction factor could be derived for each plate. Antibody binding was expressed as a ratio of this correction factor. The serum dilution used for all calculations was 1/100 which was within the linear part of the antibody binding curve.

4.2.2. AFFINITY PURIFICATION OF ANTI-GlcNAc ANTIBODIES

To define the fine specificity of the anti-GlcNAc antibody and to generate reagents for subsequent phases of this work, pools of sera from RA and normal controls were subjected to affinity purification on columns of immunoadsorbent containing glycoprotein with terminal GlcNAc (See Table 4.1). Serum was run sequentially through the columns starting with the affigel 10-bee venom column. This sequence was chosen because from the ELISA assays it appeared that all sera which responded to the synthetic epitope of GlcNAc (GlcNAc-phe-BSA; structurally similar to the immunoadsorbent in the fourth column) also had some antibody to the other glycoproteins used.
Table 4.1. Details of the columns used for the affinity purification of the anti-GlcNAc antibodies

<table>
<thead>
<tr>
<th>Sequence of columns</th>
<th>Immunoadsorbents</th>
<th>Oligosaccharide structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Affigel 10-Bee venom</td>
<td>Structures of the oligosaccharides in bee venom is not known but it is believed to be similar to ovalbumin</td>
</tr>
<tr>
<td>2</td>
<td>Affigel 10-Ovalbumin</td>
<td>There are at least five different structures of the asparagine-linked oligosaccharides in ovalbumin. The simplest structure is shown here:</td>
</tr>
<tr>
<td>3</td>
<td>Ovomucoid-Agarose (Sigma O-0129)</td>
<td>The oligosaccharides in ovomucoid has many structures. The structure which is found commonly in all penta-antennary sugar chains is shown here:</td>
</tr>
<tr>
<td>4</td>
<td>GlcNAc-Agarose (Sigma A-2530)</td>
<td>GlcNAc-Agarose</td>
</tr>
</tbody>
</table>

R = GlcNAc 1-4GlcNAc-Asn.
4.2.3. PREPARATION OF AFFIGEL COLUMNS

Ten milligrams of the HPLC purified bee venom were coupled to 2 ml of Affi-gel 10 (Biorad) following the manufacturer's instructions; the vial containing affi-gel 10 was gently shaken and the slurry transferred to a small Buchner funnel. The supernatant solvent was drained and the gel was washed with 20 bed volumes of cold (4 °C) deionised water. Care was taken to ensure that the gel bed did not dry during the washing process. The moist gel cake was then transferred to a glass test tube and 1 ml of the ligand (HPLC purified bee venom phospholipase, 10 mg/ml in PBS) solution was added to 2 mls of gel and agitated to make a uniform suspension. Gentle agitation continued for 2 hours at room temperature on an orbital shaker. Then to block any active esters, 0.1 ml of ethanolamine-HCl (1 M, pH 8) per ml of gel was added and agitated for a further hour. The gel was then transferred to a column (bed volume was approximately 2 mls) and washed with PBS until the gel was free of reactants. The column was then washed with 0.5 M GlcNAc solution which was subsequently used to elute anti-GlcNAc specifically bound to the column. The column was also equilibrated with Glycine-HCl (0.2 M, pH 2.9) and finally the column was washed again with PBS prior to application of serum sample. 20 milligrams of purified ovalbumin was coupled to 2 ml of Affi-gel 10 in the same way.

4.2.4. ISOLATION OF ANTI-GlcNAc FROM RA AND NORMAL SERUM

50 ml of heat inactivated pooled RA serum was run sequentially through all four columns starting with the affi-gel 10 bee venom column at a flow rate of 20 mls per hour. The columns were then washed extensively with PBS until the absorbance of the effluent was zero at 280 nm. Antibody bound specifically to the column was eluted first with 0.5 M GlcNAc (N-Acetyl-D-Glucosamine, Sigma A-8625), and then with glycine/HCl (0.1 M; pH 2.8). This was to remove any anti-GlcNAc antibodies with complex epitopes.
which may not have been removed by 0.5 M GlcNAc and also to remove anti-protein antibodies. Fractions eluted with glycine/HCl were quickly neutralised with 1 M sodium hydroxide to pH 7. The columns were washed with PBS until the base-line came to zero. The procedure was then repeated for 50 mls of pooled normal serum.

All eluates from the columns were kept separated and dialysed for 48 hours against PBS with three changes, and concentrated to approximately 5 ml (1/10th of the original volume of serum run through the columns). The protein contents of all the fractions were first measured spectrophotometrically by the method of Warburg & Christian (1941), using UV absorbance values at 260 and 280 nm, and then by the Folin method (Lowry et al., 1951) to obtain more accurate values. The fractions eluted with 0.5 M GlcNAc were coded as shown in Table 4.2.

4.2.5. SPECIFICITY OF THE ANTI-GlcNAc ANTIBODIES ELUTED FROM THE COLUMNS

Specificity of the eluates from columns was checked by ELISA. Microtitre ELISA plates were coated with the six glycoproteins as described earlier. Eluates were applied at 10, 5 and 1 μg/ml in PBS/Tween incubated for 2 hours at room temperature and after washing the plates, HRP conjugated rabbit anti-human immunoglobulins was added at 1/1000 and incubated for 3 hours at room temperature. The ELISA was then developed in the usual way. Fractions which were at least weakly positive on the corresponding glycoproteins at 10 μg/ml were selected for the next phases of study.
Table 4.2. Details of the fractions eluted with 0.5 M GlcNAc from the columns

<table>
<thead>
<tr>
<th>Immunoadsorbents</th>
<th>Eluates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From the normal pool</td>
</tr>
<tr>
<td>Affigel-Bee venom</td>
<td>HGN1</td>
</tr>
<tr>
<td>Affigel-Ovalbumin</td>
<td>HGN3</td>
</tr>
<tr>
<td>Ovomucoid-Agarose</td>
<td>HGN5</td>
</tr>
<tr>
<td>GlcNAc-Agarose</td>
<td>HGN7</td>
</tr>
</tbody>
</table>
4.2.6. INHIBITION ASSAYS

All the anti-GlcNAc antibodies bound very strongly to GlcNAc-phe-BSA. Therefore, for inhibition studies ELISA plates were coated with with 5 μg/ml of GlcNAc-phe-BSA as before. The anti-GlcNAc antibodies were incubated with either GlcNAc or N-acetylgalactosamine (GalNAc) at concentrations of 0.0 M, 0.01 M, 0.02 M, 0.05 M, 0.10 M and 0.25 M, for 30 minutes before addition to the antigen coated wells. Antibodies were applied at a final concentration of 0.5 μg/ml. The anti-GlcNAc antibodies were detected with peroxidase-labelled anti-human immunoglobulins in the usual way.

4.2.7. ISOTYPE ANALYSIS OF THE AFFINITY-PURIFIED HUMAN ANTI-GlcNAc IMMUNOGLOBULINS

All eluates which were positive at 10 μg/ml on their corresponding antigens were strongly positive on GlcNAc-phe-BSA. Therefore, for the analysis of the immunoglobulin types, ELISA plates were coated with 5 μg/ml of this synthetic epitope of GlcNAc as described earlier. Eluates were added at 10 μg/ml in PBS/Tween and after 2 hours incubation, plates were washed in the usual way, and the immunoglobulin classes detected using rabbit heavy chain specific anti-sera to human IgA, IgG and IgM (DAKO P214, DAKO P215 and DAKO P216 respectively).

4.2.8. CAPTURE ASSAY TO ESTIMATE THE CONCENTRATION OF IgA, IgG AND IgM ANTI-GlcNAc ANTIBODIES IN THE ELUATES

Microtitre ELISA plates were coated with goat anti-human IgA (Sigma 1-0884) at 1 μg/ml and with goat anti-human IgG (Sigma 1-338) and IgM (Sigma 1-10759) at 0.5 μg/ml. After washing the plates, human IgA, IgG and IgM standards (Behring, OCDT
07;) were added at 10 μg/ml and diluted 1/2 across the ELISA plate. Test samples (eluates from columns) were applied at 1/50 dilution and diluted 1/2 across the ELISA plate as for the standard. After 2 hours incubation at room temperature, plates were washed and goat heavy chain specific anti-human IgA, IgG and IgM HRP conjugate (Sigma A 7032, A 6029 and A 4290 respectively) were applied at 1/1000 and incubated for 3 hours at room temperature. Finally the ELISA was developed in the usual way and a calibration curve was constructed by plotting optical density of the standards at 650 nm against their concentrations in μg/ml. IgA, IgG and IgM concentrations of the test samples were then read off the standard curves.

4.2.9. BIOTINYLATION OF HUMAN ANTI-GlcNAc ANTIBODIES

Aliquots of the affinity purified human anti-GlcNAc were freeze-dried and resuspended in PBS at 1 mg/ml. Biotin (N-Hydroxy-succinimido-biotin; Sigma H-1759) was made to 1 mg/ml in dimethyl sulfoxide (DMSO, Sigma D-5879) in a glass container. Then the antibody solution was mixed with biotin in a ratio of 10:1. Biotin was added drop wise with constant agitation. The antibody was left at room temperature and in dark overnight. After this incubation, the biotinylated antibody was run through NAP-10 (Sephadex G-25; Pharmacia 17-0854-01) desalting columns to remove any free biotin. Biotinylated antibodies were stored at 4 °C and used within 2 weeks of preparation.

4.2.10. ASSAYS FOR BINDING OF HUMAN AFFINITY-PURIFIED ANTI-GlcNAc ANTIBODIES TO AGALACTOSYL IgG

The possibilities that the human anti-GlcNAc antibodies from the RA and normal sera could be autoantibodies to agalactosyl IgG or that they may contain high proportion of this glycoform of IgG were investigated.
For the latter eluates with total immunoglobulin concentrations of more than 10 μg/ml was assayed for Gal(0) using a ELISA based techique, developed in this department and now routinely used for estimation of Gal(0) (see Chapter 8).

4.2.10.1. **ELISA assay to check whether the affinity-purified anti-GlcNAc antibodies bind to intact agalactosyl IgG**

Microtitre ELISA plate was coated with 2 μg/ml of IgG from a patient with tuberculosis [70 per cent of this was Gal(0)] and 2 μg/ml of IgG from a normal individual [only 30 percent this IgG was Gal(0)]. Biotinylated human anti GlcNAcs were added at 5 μg/ml and their binding was detected with peroxidase conjugated streptavidin (DAKO P397).

4.2.10.2. **Immunoblot assay for binding of human affinity-purified anti-GlcNAc to agalactosyl IgG**

2 μg of normal and RA IgG was loaded into 6 mm wells and resolved by SDS PAGE on slab gels (1.5 mm thick) of 10% acrylamide as described in Chapter 3. After transferring the protein from gels to nitrocellulose membrane one track was cut and stained with aurodye and the remaining nitrocellose membrane was blocked with PBS/Tween containing 1% BSA (wt/vol) for 2 hours. After blocking nitrocellulose strips were incubated with the biotinylated human anti-GlcNAcs and GN7 at 5 μg/ml. Binding was detected with peroxidase conjugated streptavidin (DAKO P397) at 1:5000 dilution for 2 hours at room temperature. IgG heavy chains were also identified with a peroxidase-conjugated rabbit anti-human gamma chain (DAKO P214).
4.2.11. IMMUNOHISTOLOGY ON RA AND NORMAL TISSUES

Biotinylated human anti-GlcNAcs were used for immunohistological studies on biopsies of rheumatoid, Osteoarthritis and normal synovium as well as cytospins of cells from synovial fluid. Normal peripheral blood monocytes cultured for 21 hours were also stained by an indirect immunoperoxidase procedure. The protocol used for staining is essentially as described in Chapter 2. Biotinylated human anti-GlcNAcs and GN7 were applied at 10-70 μg/ml and binding was detected using HRP-conjugated streptavidin (DAKO P397) at 1: 500.
4.3. RESULTS

4.3.1. Antibody levels to glycoproteins with terminal GlcNAc measured by ELISA

Patients with RA, tuberculosis (TB), Crohn's disease, leprosy (erythema nodosum leprosum, ENL; borderline leprosy, BL and lepromatous leprosy, LL), sarcoidosis and SLE as well as normal controls all have antibody to the panel of the six glycoproteins with terminal GlcNAc used in the present study (Table 4.3). The levels of these anti-GlcNAc antibodies do not show any disease specificity. Nevertheless, RA patients have significantly reduced binding to bee venom glycoprotein compared to the other disease groups and normal controls (Mann Whitney test P<0.001). It is also interesting to note that leprosy patients have raised levels of antibody to all six glycoproteins, however since the controls were not matched for geographical and ethnic origin, it could not be established whether this rise in antibody levels was due to specific response to the glycoproteins tested.

4.3.2. Specificities of the anti-GlcNAc antibodies

All fractions eluted from columns were tested on their corresponding antigens as well as on all the remaining glycoprotein antigens mentioned in Section 4.2. Those fractions eluted with 0.2 M glycine/HCl did not contain any immunoglobulins. Among fractions eluted with 0.5 M GlcNAc (see Table 4.2) only those from the ovomucoid-Agarose column (HGN5 and HGN6) and GlcNAc-Agarose column (HGN7 and HGN8) contained specific anti-GlcNAc antibodies detectable by ELISA. All four antibodies bound very strongly to GlcNAc-phe-BSA but not to any of the other antigens. Ironically even those antibodies eluted from the ovomucoid-agarose column bound more strongly to the above synthetic epitope of GlcNAc than to ovomucoid. Moreover there were no
Table 4.3 Antibody binding to glycoproteins with terminal GlcNAc assayed by ELISA

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=15)</th>
<th>RA (n=23)</th>
<th>TB (n=23)</th>
<th>Leprosy Between episodes of ENL (n=14)</th>
<th>Leprosy During episodes of ENL (n=14)</th>
<th>Crohn's (n=24)</th>
<th>Sarcoidosis (n=22)</th>
<th>SLE (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc-phe-BSA</td>
<td>0.89±0.12</td>
<td>0.74±0.18</td>
<td>0.77±0.15</td>
<td>1.05±0.11</td>
<td>0.96±0.11</td>
<td>1.06±0.14</td>
<td>0.97±0.17</td>
<td>0.60±0.19</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.57±0.15</td>
<td>0.49±0.11</td>
<td>0.40±0.09</td>
<td>0.64±0.13</td>
<td>0.60±0.17</td>
<td>0.62±0.17</td>
<td>0.44±0.13</td>
<td>0.47±0.23</td>
</tr>
<tr>
<td>Bee Venom</td>
<td>0.50±0.10</td>
<td>0.27±0.10*</td>
<td>0.35±0.12</td>
<td>0.59±0.13</td>
<td>0.52±0.13</td>
<td>0.56±0.16</td>
<td>0.41±0.12</td>
<td>0.41±0.16</td>
</tr>
<tr>
<td>Agalactosyl fetuin**</td>
<td>0.31±0.06</td>
<td>0.30±0.05</td>
<td>0.29±0.07</td>
<td>0.52±0.12</td>
<td>0.49±0.15</td>
<td>0.54±0.15</td>
<td>0.32±0.09</td>
<td>0.35±0.16</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>0.33±0.15</td>
<td>0.36±0.11</td>
<td>0.30±0.15</td>
<td>0.53±0.12</td>
<td>0.50±0.06</td>
<td>0.51±0.006</td>
<td>0.29±0.16</td>
<td>0.34±0.23</td>
</tr>
<tr>
<td>Agalactosyl orosomucoid**</td>
<td>0.52±0.12</td>
<td>0.47±0.13</td>
<td>0.37±0.13</td>
<td>0.60±0.10</td>
<td>0.55±0.10</td>
<td>0.55±0.10</td>
<td>0.37±0.10</td>
<td>0.44±0.206</td>
</tr>
</tbody>
</table>

Results expressed as mean absorbance ± s.d.

n = number of individuals

* = significantly lower than the corresponding normal value (p=0.001 by Mann-Whitney U-test)

** = binding to fully sialylated HPLC purified material was subtracted before calculating mean values and s.d.
differences in the specificity of the normal and RA anti-GlcNAc antibodies. The binding of all of these antibodies to GlcNAc-phe-BSA can be blocked with free GlcNAc, whereas GalNAc at the same molarity had no effect (Figure 4.1).

4.3.3. **Immunoglobulin isotypes of the human anti-GlcNAc antibodies and their serum concentrations**

The percentage of IgA, IgG and IgM anti-GlcNAcs in pooled normal and RA sera was shown in Table 4.4. IgG and IgM antibodies make up most of the immunoglobulin contents of the isolated anti-GlcNAc antibodies with only a small percentage of the total anti-GlcNAcs being IgA. HGN6, the anti-GlcNAc isolated from pooled RA sera off the ovomucoid-agarose column, consists of IgG anti-GlcNAc antibodies only whereas the corresponding antibody from normal sera (HGN5) contains anti-GlcNAc antibodies of all three major classes.

4.3.4. **Binding of the anti-GlcNAc antibodies to IgG heavy chain on western blots**

On ELISA assays the isolated anti-GlcNAcs did not bind to intact agalactosyl or normal IgG but on western blots of denatured RA synovial fluid they bound to IgG heavy chains and to two low molecular weight bands at 35 kDa and 25 kDa (Figure 4.2) The data shown is for HGN7 and HGN8 only because there were not enough of the other anti-GlcNAc antibodies to put on western blots.
Figure 4.1. Inhibition of binding of the affinity purified human anti-GlcNAc antibodies HGN5, HGN6, HGN7 and HGN8 to GlcNAc-phe-BSA by increasing concentrations of N-acetylgalactosamine (o) or N-acetylglucosamine (●).
Table 4.4. Serum concentrations of the IgA, IgG and IgM anti-GlcNAc antibodies from pooled RA (8 patients; 5 females and 3 males, mean age 57.3 years, range 34-80 years) and normal (6 individuals; 4 females and 2 males, mean age 54.4 years, range 30-64 years) sera.

<table>
<thead>
<tr>
<th></th>
<th>IgA (µg/ml)</th>
<th>IgG (µg/ml)</th>
<th>IgM (µg/ml)</th>
<th>Total anti-GlcNAc (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGN5</td>
<td>5.0</td>
<td>1.3</td>
<td>2.0</td>
<td>3.8</td>
</tr>
<tr>
<td>HGN6</td>
<td>ND</td>
<td>3.2</td>
<td>ND</td>
<td>3.2</td>
</tr>
<tr>
<td>HGN7</td>
<td>1.2</td>
<td>9.2</td>
<td>5.3</td>
<td>15.7</td>
</tr>
<tr>
<td>HGN8</td>
<td>0.8</td>
<td>15.8</td>
<td>11.5</td>
<td>28.1</td>
</tr>
</tbody>
</table>

ND = Not detected
Figure 4.2. Binding of the affinity-purified anti-GlcNAc antibodies to IgG heavy chain on western blots. Lanes 1-4 show western blots of denatured RA synovial fluid stained with anti-human IgG (lane 1), the mAb GN7 (lane 2), affinity-purified anti-GlcNAc from the pooled normal sera, HGN 7, (lane 3) and affinity-purified anti-GlcNAc from the RA pool, HGN8, (lane 4). The positions of the molecular weight markers are shown with horizontal bars.
4.3.5. Immunohistological characterization of the affinity purified anti-GlcNAc antibodies

The isolated anti-GlcNAcs are capable of interacting with human joint tissues and cellular constituents as shown by the immunoperoxidase staining of normal and RA synovium (result not shown). The staining pattern of the human anti-GlcNAcs is similar to that given by the mAb GN7 (Chapter 2) except that the former antibodies gave very weak staining.
Patients with RA have normal levels of antibody to a range of glycoproteins bearing terminal GlcNAc as complex or hybrid type oligosaccharides in bi-, tri-, or tetra antennary forms, with or without bisecting GlcNAc. It is unlikely that all the antibody measured by ELISA will be directed towards the GlcNAc, or even towards the oligosaccharides, since the protein constituents of the glycoproteins are immunogenic. However, two of the glycoproteins used (human orosomucoid and fetuin) were available both in fully sialylated and enzyme-treated agalactosyl forms. Therefore, the binding to the fully sialylated form was subtracted from the binding seen with the agalactosyl preparations. The remaining binding is likely to have been due to exposure of GlcNAc, as bi-, tri-, and tetra-antennary GlcNAc in the agalactosyl preparations of orosomucoid and fetuin. Even after this calculation there was no evidence of an increase in antibody levels in RA. However, RA patient did show significantly reduced binding to bee venom glycoprotein compared to the other disease groups and normal controls. This is an interesting observation since bee venom had been reported to have an anti-arthritic effect in man (Maberly 1910 & Beck 1935) and can suppress adjuvant arthritis in rats (Chang & Bliven 1979).

No attempt was made to analyse in detail the fine specificities of the anti-GlcNAc antibodies using inhibition studies with sugars and control glycoproteins. Instead anti-GlcNAcs from pools of RA and normal controls were affinity-purified on selected columns of glycoprotein immunoadsorbents. The eluates from the first two columns (affigel-bee venom phospholipase and affigel-ovalbumin columns) did not contain any anti-GlcNAc antibodies and only a small amount of anti-GlcNAc bound to the 3rd column, the ovomucoid-agarose column. However, relatively large quantities of anti-GlcNAc bound to the fourth column, the GlcNAc-agarose column, suggesting that most of the anti-GlcNAc in both normal and RA serum are directed towards the simplest of all the GlcNAc epitopes used. These affinity-purified antibodies can be blocked easily by small concentrations of free GlcNAc, but not by GalNAc.
Further analysis of the affinity-purified antibodies using the panel of glycoproteins in ELISA assays did not show any differences between the anti-GlcNAcs from pools of RA and normal serum. However, anti-GlcNAcs from both normal and RA serum bound to agalactosyl IgG on immunoblots, and to RA synovial tissue with a distribution similar to that seen with the control mAb, GN7. It is therefore possible that anti-GlcNAc contributes to the immunopathology in RA, but there was no evidence for an augmented immune response to terminal GlcNAc in RA sera. The total yield of the anti-GlcNAc from the pooled RA sera was considerably higher than that obtained from the same volume of the pooled normal sera, but since in the ELISA study using crude glycoproteins no significant increase in the levels of anti-GlcNAc antibodies in RA was observed the increased concentration of anti-GlcNAc in the RA pool may be due to inadequate matching and small pool of the sera used.

Anti-GlcNAc antibodies may provoke immunopathological changes by damaging cells bearing GlcNAc or simply by interfering with their signalling functions (Haltiwanger & Hill 1986). A further possibility is that interaction of these antibodies with agalactosyl IgG may lead to IgG self-association (Rademacher et al., 1988), but this would require at least partial denaturation of the IgG in vivo to expose the terminal GlcNAc. Such a denaturation could occur through free radical damage as demonstrated for the joint-derived IgG (Blake et al., 1989). However, anti-GlcNAc antibodies may also have a protective role. For example, they might be involved in the removal of damaged or aged tissues or they may play a role in self tolerance by acting as blocking antibodies (Hellestrom et al., 1972). Finally the anti-GlcNAc antibodies may form part of the complex immune network system and therefore may have a role in the immunoregulatory mechanisms.
CHAPTER FIVE

PRELIMINARY EVIDENCE FOR THE PRESENCE OF A HUMAN HOMOLOGUE OF THE *Mycobacterium bovis* 65 KILODALTON HEAT SHOCK PROTEIN IN SYNOVIAL FLUID AND ANTIBODIES TO IT IN RHEUMATOID ARTHRITIS PATIENTS
5.1. INTRODUCTION

There is now convincing evidence for the involvement of mycobacterial hsp65 in the animal models of human RA. (This protein belongs to the hsp60 family and in SDS PAGE runs at about 65 kDa, hence denoted hsp65). Thus adjuvant arthritis can be transferred to naive rats with T cell clones responsive to mycobacterial hsp65 (van Eden et al., 1988) and all the rodent models of arthritis tested including the adjuvant arthritis model can be blocked by pretreatment with this protein in soluble form (van Eden et al., 1988; van den Broek et al., 1989a; Billingham et al., 1990; Thompson et al., 1990). Moreover recent studies demonstrated both humoral and cell mediated immune responses to mycobacterial hsp65 in RA (Bahr et al.; 1988b; Tsoulfa et al., 1989a and 1989b; Holoshitz et al., 1989; Res et al., 1988). The M. bovis hsp65 is one of the immunodominant antigens of mycobacteria (Young et al., 1987) and it shows a remarkable degree of sequence conservation with other species. The human homologue of this hsp shows 47% identical residues, and an additional 20% conservative changes (Jindal et al., 1989; Dudani & Gupta 1989). If molecular mimicry between bacterial hsp65 and the human gene product is responsible for the autoimmune reaction in RA then the latter should be demonstrable in RA joints. Monoclonal antibodies raised against M. bovis have been shown to bind to human tissues including collagen and several heavily glycosylated proteins such as fetuin and transferrin (Morris et al., 1985; Thorns & Morris 1985). Moreover, a monoclonal antibody raised against M. leprae hsp65 protein has
been reported to bind preferentially to joint tissues from RA patients (Karlsson-Parra et al., 1990). In 1986 to investigate the role of mycobacterial antigens as potential triggers of autoimmune reactions in RA Dr. G. A. Rook and Mrs. J. Edge (Department of Medical Microbiology, UCMSM) made mAbs to an acetone precipitable fraction of *M. tuberculosis* which contained the relevant epitope recognized by the arthritogenic rat T-cell clones. They selected eleven monoclonal antibodies all of which bound to urea denatured synovial fluid from RA patients. In this chapter, using one of these monoclonal antibodies, SF8, and a rabbit polyclonal antibody to hamster hsp58 (the mammalian homologue of the bacterial hsp60 family which runs at about 58 kDa in SDS PAGE, hence denoted hsp58), the presence of large quantities of a 65 kDa protein in the synovial fluids and immune complexes from RA patients is reported. Evidence for the presence of autoantibodies to the mycobacterial hsp65 is also presented.
5.2. MATERIALS AND METHODS

**Antigens:** The purified recombinant 65kDa protein from *M. bovis* was provided by Dr. J. van Embden, Bilthoven, Netherlands (Thole et al., 1988). The human homologue of this protein was obtained from Professor Gupta (McMaster University, Canada) who has partially purified it from human placental tissue (from here on this protein may be referred to as P1 or P1 protein) by affinity chromatography using a polyclonal rabbit antiserum (Gupta & Dudani 1987; Lydyard et al., 1990a). Recombinant mycobacterial hsp70 was kindly provided by Dr. D. Young (Hammersmith Hospital, London).

**Antibodies to hsp65:** A polyclonal rabbit anti-serum to hamster hsp58 which reacts specifically with the corresponding antigen in human cells (Dudani & Gupta 1989; Gupta & Dudani 1987) was kindly provided by Professor Gupta (McMaster University, Canada). A mouse IgG mAb raised against *M. bovis* hsp65, Tb78, was obtained from Professor Ivanyi, Hammersmith Hospital, London.

5.2.1. MONOCLONAL ANTIBODY TO MYCOBACTERIAL ANTIGEN

*M. tuberculosis* (H37Rv) (DIFCO) was prepared by grinding the organisms with water in a mortar and pestle. Balb/c mice received 2 intramuscular injections of 50 μg of this material emulsified in 0.2 ml of incomplete Freunds adjuvant on Day 0, and day 20. On day 38 they received a third intravenous dose of 50 μg which was ground with squalene, trehalose dimycolate and monophosphoryl lipid A (Ribi Immunopharmaceuticals Ltd., Montana, USA) following the instruction from the manufacturer (50 μg of antigen to 2 μl of squalene). The mixture was then emulsified in an excess of 0.2% Tween 80 in PBS (1 ml to 10 μl of squalene). Four days after this injection the spleens were harvested and hybridomas prepared as described by Kohler & Milstein (1975). Supernatants were screened by ELISA for the ability to bind both to the aqueous extract of *M. tuberculosis*, and to synovial fluid obtained from patients with...
active rheumatoid arthritis. In order to eliminate any possible rheumatoid factor activity these were denatured in 12 M urea with 0.5 M 2-mercaptoethanol, and dialysed against 0.2 M iodoacetamide. The monoclonal SF8 used in the present study is an IgM, and was used as ascites generated in pristane-treated Balb/c mice. The mAbs to mycobacterial antigen were prepared by Mrs. Edge and Dr. Rook for the present project. The detail of the preparation of these mAbs are included here because they were made for the present project and have not been published elsewhere.

5.2.2. PRECIPITATION OF IMMUNE COMPLEXES FROM RHEUMATOID SYNOVIAL FLUID

The separation of the immune complexes from synovial fluid was based on their decreased solubility in polyethylene glycol (PEG) solutions (Male & Roitt 1979). Synovial fluid samples were spun at 400 g for 20 minutes at room temperature to remove cells and particulate material. They were then either used immediately, or stored at -20 °C. Immune complexes were precipitated in a 2% solution of PEG 6000 (Sigma, Poole Dorset, U.K. Approx molecular weight 6000). 500 µl of synovial fluid was mixed in 10 ml round bottomed tubes with 100 µl of 12% PEG in veronal-buffered saline (VBS, pH 7.6). The tubes were incubated overnight at 4 °C, centrifuged at 2000 rpm for 20 minutes at 4 °C to collect the precipitated immune complexes, and the precipitates were resuspended in 6 ml of 2% PEG in VBS containing 0.01 M EDTA. The tubes were then centrifuged again at 4 °C to wash the complexes, which were then redissolved in 500 µl of VBS and incubated at 37 °C to ensure complete solubilisation. At this stage the complexes were stored at -20 °C.
5.2.3. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE) AND WESTERN BLOTTING

Synovial fluids, immune complexes, purified and recombinant hsp were run on SDS PAGE as described in Chapter 3. For antibody staining 50 μg/lane of protein for synovial fluid or immune complexes, 5 μg/lane for purified hsps, and 2 μg/lane for recombinant hsps were used. Half the above amounts of proteins were used for Aurodye staining.

For immunoblotting unstained nitrocellulose membranes were blocked with BSA as in Chapter 3 and incubated overnight at 4 °C with the monoclonal antibody SF8 diluted 1/5000 for staining blots of rheumatoid synovial fluid, and 1/500 for the affinity-purified human placental hsp, and for the recombinant mycobacterial protein. The polyclonal rabbit antibody to the human homologue was used at 1/400. Binding of SF8 was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO P260) at 1/800 dilution for 2 hours at room temperature. Binding of the rabbit polyclonal was detected with peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO P217). IgG heavy chains on blots of denatured synovial fluid were identified with a peroxidase-conjugated rabbit anti-human gamma chain (DAKO P214). Peroxidase labelling was visualised with 4-chloro-1-naphthol.

5.2.4. THE EFFECT OF FREE RADICAL DAMAGE ON BINDING OF THE MONOCLONAL ANTIBODY SF8

Since free radical damage to proteins occurs in rheumatoid joints (Blake et al., 1989), the effects of exposure of the affinity-purified placental protein to hydroxyl radicals or hypochlorite was tested. Human hsp70 was used as a control antigen and a mAb GN7, as an isotype matched control.
5.2.4.1. Generation of hydroxyl radicals

Hydroxyl radicals were generated by the method of Halliwell et al., (1987): the procedure involved the reaction of an iron-EDTA complex with H$_2$O$_2$ in the presence of ascorbic acid. The reaction mixture was made up in 20 mM phosphate buffer (pH 7.4) using the following reagents (all purchased from BDH Chemicals Ltd.) at the final concentrations stated: Iron chloride (FeCl$_3$; 100 μM), EDTA (104 μM), and ascorbic acid (100 μM). Finally 100 μl of P1 containing approximately 20 μg of the protein was added and incubated at 37 °C for 1 hour. The final volume of the reaction mixture (1 ml) was reduced to zero by freeze drying and the protein was subjected to SDS PAGE under denaturing condition.

5.2.4.2. Sodium hypochlorite treatment

Sodium hypochlorite (BDH 23039) is a powerful bleach and can damage proteins through oxygen-derived species. It was used at concentrations between 50 μM and 20 mM adjusted to pH 6.2 (Green et al., 1985) with dilute sulphuric acid. The placental hsp was incubated in the hypochlorite for 30 minutes at 37 °C and run on SDS PAGE as above. Synovial fluid from RA patients was also subjected to similar treatment and then run on gel. Control aliquots of all the antigens were treated similarly, but without hypochlorite.

5.2.5. AFFINITY PURIFICATION OF ANTIBODY TO THE MYCOBACTERIAL HSP65

Two milligrams of the recombinant *M. bovis* hsp65 were coupled to 2 ml of Affigel-10 (Biorad), following the manufacturer's instructions as described in Chapter 4.
Sera from two RA patients with high levels of IgG binding to this antigen were used in these experiments. 1 ml of a 1/100 dilution of each serum in PBS was passed over the column. Bound antibody was eluted with glycine/HCl (pH 2.8, 0.1 M) and neutralised immediately with dilute sodium hydroxide. After dialysing against PBS the eluate was reconstituted to 1 ml. The original diluted serum, and the bound and unbound fractions were then tested by ELISA for IgG binding to the human and M. bovis hsp65. The bound fraction was also tested on western blots of mycobacterial hsp65, E. coli hsp65, human hsp58 and RA synovial fluid.

5.2.6. MEASUREMENTS OF THE ANTIBODY LEVELS TO THE MYCOBACTERIAL HSP65 AND THE HUMAN HOMOLOGUE IN SYNOVIAL FLUID BY ELISA

Control synovial fluid: Sixteen samples from inflammatory controls made up of 8 OA, 4 psoriatic arthritis, 2 Gout and 2 dialysis arthropathy. There were 8 males and 8 females with a mean age of 40.3 years (range 18-79). These synovial fluids were obtained from Dutch nationals and kindly provided by Dr. R.R.P. de Vries, Leiden, Netherlands.

RA synovial fluid: Samples from twenty five patients all of which satisfied the American Rheumatism Association criteria for definite or classical RA were also provided by Dr. de Vries who has collected them from dutch nationals. There were 10 females and 15 males with a mean age of 58.6 years (range 21-84).

The levels of IgA, IgG and IgM antibodies to the mycobacterial hsp65 and the human homologue were determined in synovial fluids from RA and inflammatory controls. The ELISA used was similar to the one described in chapter 4 (Section 4.2.1). Microtitre ELISA plates were coated with the recombinant forms of the hsps at 2 μg/ml. The synovial fluids were applied at 1/50, 1/100 and 1/200 and the bound antibodies were detected with the appropriate peroxidase-labelled rabbit anti-human antibodies.
5.3. RESULTS

5.3.1. Demonstration of cross-reactivity between the mycobacterial hsp65 and the human homologue

The partially purified human placental protein was run in SDS PAGE on three adjacent tracks. These were stained with Aurodye, monoclonal SF8, and the polyclonal antibody respectively (Figure 5.1). Both antibodies picked up a band of approximately 58 kDa corresponding to the calculated molecular mass of the human homologue (Jindal et al., 1989). Similarly both antibodies picked out the same lines in the preparation of recombinant mycobacterial hsp65 (Figure 5.1). This preparation contains a form which runs higher than the human placental molecule, although the sequence is 7 amino acids shorter. The presence of multiple bands on P1 is due to contaminations particularly with IgG and other proteins. The multiple bands on the recombinant mycobacterial hsp65 is attributable to the break down of the main protein into smaller fragments. Tb78, bound to all of these fragments whereas SF8 only bound to the main band at 65 kDa.

5.3.2. Detection of a 65 kDa protein in western blots of synovial fluid and immune complexes from patients with rheumatoid arthritis.

Rheumatoid synovial fluid and immune complexes from RA patients were run on adjacent tracks (Figure 5.2), and stained with aurodye, SF8, rabbit polyclonal antibody to P1, and antibody to gamma chains. SF8 and the polyclonal antibody both bound to a band of molecular mass about 65 kDa in the synovial fluid and also in the immune complexes from RA patients. This molecule is larger than the hsp in the placental extract. The molecular mass difference was confirmed in further gels where the placental protein, RA synovial fluid, mycobacterial hsp65 and E. coli hsp65 were run on adjacent tracks before staining with the mAb SF8 (Figure 5.3).
Figure 5.1. Immunoblots of purified 65 kDa protein from human placenta, and recombinant 65 kDa hsp from *M. bovis*. Lane 1: Molecular weight markers. Lanes 2, 3, and 4: P1 protein stained with Aurodye, SF8 and the polyclonal antibody respectively. Lanes: 5, 6, and 7: Recombinant mycobacterial 65 kDa hsp stained with aurodye, SF8 and the rabbit polyclonal antibody respectively.
Figure 5.2. Immunoblots of denatured synovial fluid and PEG precipitated immune complexes from rheumatoid synovial fluid. Lane 1: Molecular weight markers. Lanes 2,3,4, and 5: Rheumatoid synovial fluid stained with Aurodye. SF8, the polyclonal anti-hsp65, and anti-IgG heavy chains respectively. Lanes 6,7,8, and 9: Immune complexes from RA synovial fluid stained with Aurodye. SF8, the polyclonal anti-hsp65, and anti-IgG heavy chains respectively.
Figure 5.3. Discrepancy in the molecular weight of the 65 kDa protein recognised by SF8 in the RA synovial fluid, compared to pure hsp65 from human placenta, mycobacteria and *E. coli*. Lane 1: Molecular weight markers. Lanes 2 and 3 RA synovial fluid; lanes 4 and 5 P1 protein; lanes 6 and 7 mycobacterial hsp65 and lanes 8 and 9 *E.coli* hsp65. Lanes 2, 4, 6, and 8 were stained with Aurodye and lanes 3, 5, 7 and 9 were stained the mAb SF8.
It was also noted that SF8 tends to bind weakly to the IgG heavy chains (compare tracks 3 and 5, Figure 5.2), and that the placental preparation contained some contaminating gamma chain. This point is discussed below. SF8 also bound weakly to a higher molecular weight component (approximately 70 kDa) which has not been identified (Figure 5.3, track 3).

5.3.3. The effects of free radical damage on the binding of SF8 to PI and other proteins

The two methods of free radical damage produced identical results. Therefore the results of the simplest method used (hypochlorite treatment) are shown and discussed. Although precise quantitation of the 65 kDa protein in synovial fluid was not possible, it appeared that SF8 bound consistently more strongly to this protein than to the placental preparation. The former protein is likely to be exposed to free radical damage in the RA joint, and RA joint fluid was used to screen and select SF8, therefore the monoclonal was tested on the placental protein which had been exposed to hydroxyl radicals or hypochlorite before separation by SDS PAGE. Exposure to hypochlorite lead to increased binding, and at concentrations readily achieved close to triggered polymorphonuclear cells. Nearly doubled intensity of binding of SF8 was consistently detected both visually and with a laser scanner at 1 mM hypochlorite (Table 5.1). Binding of SF8 to synovial fluid was unaffected by hypochlorite treatment as was its binding to the control antigen hsp70 to which SF8 does not normally bind. Moreover, the isotype matched control mAb GN7, which normally bind to terminal GlcNAc of IgG gamma chain on western blots, showed no increased binding to the sugar epitope following similar treatment. The gradual decrease in the intensity of binding of the mAb from 4-20 mM is probably due to the denaturation of the protein.
Table 5.1. The effect of exposure of the P1 protein, RA synovial fluid and hsp70 to hypochlorite treatment before SDS PAGE, on subsequent binding of SF8

<table>
<thead>
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<th>Molarity of sodium hypochlorite</th>
<th>Laser scanner units</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1 protein</td>
<td>RA synovial fluid</td>
<td>Hsp70</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.12</td>
<td>0.28</td>
<td>0.0</td>
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<tr>
<td>50 µM</td>
<td>0.124</td>
<td>0.25</td>
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<tr>
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<td>0.115</td>
<td>0.29</td>
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<tr>
<td>1 mM</td>
<td>0.214</td>
<td>0.24</td>
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<tr>
<td>4 mM</td>
<td>0.103</td>
<td>0.24</td>
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<tr>
<td>20 mM</td>
<td>0.089</td>
<td>0.20</td>
<td>0.0</td>
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</table>
5.3.4. Is the presence of this 65 kDa protein in synovial fluid disease specific?

Similar gels to that shown in Figure 5.2 were run using synovial fluid from 10 cases of RA, 8 cases of osteoarthritis (OA), and 1 case of dialysis arthropathy. In every case the antibodies identified the same band at 65 kDa, and there was no consistent difference in relative intensity between the RA and the control synovial fluid samples.

5.3.5. The presence of antibody capable of binding to the human homologue of the mycobacterial hsp65.

Antibody derived from RA sera, and eluted from an affinity column of the *M. bovis* hsp65, bound to ELISA wells coated with the human placental protein, from which the contaminating gamma chains had been removed by affinity-chromatography on an Affigel-10 column bearing anti-gamma. (Figure 5.4). These antibodies also bound to *M. bovis* hsp65 and *E. coli* hsp65 and to the human homologue on western blots (see Figure 5.5).

5.3.6. Antibodies to the hsp60 family in RA synovial fluid

The levels of the IgA, IgG and IgM antibodies to the human hsp58 and the corresponding mycobacterial hsp is shown in Figures 5.6 and 5.7 respectively. There appear to be a generalised rise of antibodies of all classes to the mycobacterial hsp65 in RA (mean age 58.6 ±14.6) synovial fluid compared to the inflammatory controls (mean age 40.3 ±19.3) but not statistically significant (Mann Whitney U-test). However, the levels of IgA and IgG antibodies to the human hsp58 was raised significantly (P<0.05 for both IgA and IgG by Mann Whitney U-test) in RA synovial fluid.
Figure 5.4. Binding to the human and mycobacterial hsp65 of IgG from RA sera eluted from an affinity column of recombinant mycobacterial hsp65.
Figure 5.5. Western blots of recombinant *M. bovis* hsp65 (lanes 2 and 3), E. coli hsp65 (lanes 4 and 5), RA synovial fluid (lanes 6 and 7) and the P1 protein (lanes 8 and 9). Lanes 2, 4, 6, and 8 were stained with Aurodye to show all protein bands. Lanes 3, 5, 7 and 9 were stained with the affinity purified antibodies to mycobacterial hsp65 from RA sera. Lane 1 shows molecular weight markers.
Figure 5.6. Antibody response to P1A in RA and control synovial fluids. The horizontal line represent the mean absorbance values for each group.
Figure 5.7. Antibody response to *M. bovis* hsp65 in RA and control synovial fluid. The horizontal lines represent the mean absorbance values for each group.
Moreover when the correlation between the levels of antibodies to mycobacterial hsp65 and the human hsp58 was checked only the IgG antibodies showed significant correlation (R= 0.595). see Figure 5.8.
Figure 5.8. Correlation between IgG antibody to P1A and mycobacterial hsp65 in RA synovial fluid

\[ y = 116.67 + 0.31395x \quad R^2 = 0.595 \]
5.4. DISCUSSION

The mycobacterial hsp65 has been identified as the target of a T cell clone capable of causing autoimmune disease in a rat model of adjuvant induced arthritis (van Eden et al., 1988), and T cells from the RA synovial infiltrates proliferate in vitro in response to the same antigen (Res et al., 1988). Moreover, unlike the antibody responses to the hsp90 and hsp70 proteins families, the IgG activity to mycobacterial hsp65 was observed specifically in RA (Tsoulfa et al., 1989b). These observations raise the possibility that mycobacterial hsp65 has an uniquely important cross-reactivity with a human antigen. The most likely antigen is the human homologue of the bacterial hsp65.

Experiments carried out in this chapter have demonstrated that RA synovial fluid and immune complexes contain relatively large quantities of a 65 kDa protein which is recognised by the rabbit polyclonal antibody and the mAb SF8. The presence of the protein is not RA-specific, since it was also present in fluid from OA joints. However this 65 kDa protein does not appear to be the human homologue of the bacterial hsp65 for several reasons. First, the 65 kDa synovial fluid protein has a molecular mass approximately 7 kDa higher than the placental P1. Secondly, the mAb SF8 binds very poorly to the mycobacterial hsp65 and is not monospecific on western blots of RA synovial fluid. Similarly the rabbit polyclonal antibody used is not monospecific and binds to human serum albumin (HSA) on western blots (which could explain the 65 kDa band in Figure 5.2, lane 4). Thus either the identified 65 kDa band is an artefact, or there is more than one hsp65 homologue, or the human hsp65 homologue is present in an altered form in RA synovial fluid. The last two points are discussed below.

It is not yet known how many forms of the hsp65 are found in man. There is evidence for different isoforms (Welch 1991) and for the existence of two alleles (Waldinger et al., 1988). It is unlikely that the greater molecular weight of the
protein seen in RA synovial fluid and immune complexes is due to release of precursor forms of the molecule from dying cells, since such forms are present in minute quantities. A more possible explanation is that there has been some proteolytic degradation of the placental protein during purification, and indeed the apparent molecular weight of the protein in synovial fluid is in closer agreement with the sequence described by Jindal et al., (1989). Similarly it is probable that the molecule in the joint has undergone free radical damage, as already shown for joint-derived IgG (Blake et al., 1989). This can lead to changes in the binding of antibodies to IgG (Griffiths personal communication to Dr. Rook). The mAb, SF8 binds more strongly to the placental hsp after it has been exposed to 1 mM hypochlorite for 30 minutes at 37 °C but its binding to the molecule identified in synovial fluid from RA patients is unaffected by such treatment. It is therefore possible that the cross-reactivity between the unidentified 65 kDa human molecule and mycobacterial molecules is enhanced by free radical damage to the former. Moreover the complete absence of binding of the mAb to hsp70 before and after hypochlorite treatment indicate that the enhanced binding to P1 following hypochlorite treatment is not nonspecific. It is possible that most of the protein in the joint including the 65 kDa protein is in a free radical damaged form.

A further peculiarity of the monoclonal SF8 is its ability to bind to IgG heavy chains as well as to the hsp (Figure 5.2). In view of the role of autoimmunity to IgG in RA this finding is provocative, and similar phenomena have been observed by others. Thus another monoclonal with specificity for mycobacterial hsp65 (Tb78) has also been found to bind to SDS-denatured human immunoglobulins (I.L. McLean, J.R. Archer and A.H. Cherrie personal communication to Dr. Rook). Moreover, it may not be a coincidence that the placental hsp, which had been affinity-purified using a rabbit antiserum to it, was contaminated with human IgG. Although unexpected, such a cross-reactivity would have clear implications for the pathogenesis of RA and deserves further analysis particularly in view of the observation that a
constant domain of the rat immunoglobulin delta chain show highly significant homology with a 62 amino acid sequence of the human hsp58 (Dudani & Gupta 1989).

Synovial fluid from patients with RA show a generalised rise in antibody levels of all classes to mycobacterial hsp65. However when the antibody levels to the human homologue was looked at, there was a significant rise in the levels of IgA and IgG antibodies. The levels of the latter IgG antibody in RA patients correlated (R= 0.595) with the IgG antibody to mycobacterial hsp65 suggesting that these antibodies may have been produced in response to the latter antigen. In addition the finding that affinity-purified antibody to the mycobacterial hsp65 was able to bind to the human molecule by ELISA and immunoblots suggest that these antibodies are autoantibodies. Apart from the formation of immune complexes such autoantibodies may also interfere with the normal physiological functions of the hsp58 and other cross-reactive antigens. Several recent reports claimed that some cells express hsp58 on their surface (Jarjour et al., 1990 & Fisch et al., 1990). Although the function(s) of hsp58 on cell membrane is as yet unknown, antibody to this molecule would have profound effects on such function(s). Indeed it has been reported recently that antibody to the bacterial hsp65 and the human homologue can block γδT cells responses (Fisch et al., 1990) although it is unusual for antibody to block such a response to processed antigen associated with MHC molecules on antigen-presenting cells.

In a collaborative study with Dr. P. Lydyard and Mr. M. Smith of our Immunology Department the level of antibody binding to the mycobacterial hsp65 homologue was found to be significantly lower in synovial fluid than in serum of 23 paired samples tested by ELISA (unpublished observation) indicating that the antibody detected in the affinity column elution experiment could be complexed with the human homologue in synovial fluid. Thus the presence of the 65 kDa protein in synovial fluid and in immune complexes, suggests that the human hsp65 is one of the antigens
involved in autoimmune responses in RA. Moreover, there is antibody to the mycobacterial hsp65 in RA synovial fluid, and at least some of this is autoantibody capable of binding to the human homologue by ELISA and immunoblots.

In spite of the arguments put forward to suggest that the heavy 65 kDa band in RA synovial fluid could be hsp58 in a modified form, this point remaind doubtful at this stage. The rabbit antibody to hamster hsp65 is a polyclonal and the mAb, SF8, does not appear to be absolutely monospecific. Thus, these antibodies can not be used to identify the human homologue of the bacterial hsp65 beyound reasonable doubt. Therefore, mAbs were made to the human hsp58 for precise identification of this molecule in joint tissues and synovial fluid from RA patients (next chapter).
CHAPTER SIX

MONOCLONAL ANTIBODIES TO THE HUMAN HOMOLOGUE OF THE MYCOBACTERIAL 65 KILODALTON HEAT SHOCK PROTEIN
6.1. INTRODUCTION

There are large quantities of a protein of molecular mass approximately 65 kDa in synovial fluid from RA and other inflammatory conditions (Chapter 5). This is an intriguing observation but raises several crucial questions. First, does this protein represent the human homologue of the mycobacterial hsp65? Secondly, could the synovial fluid molecule be the product of a different gene belonging to the hsp65 family or is it a completely different protein which just happens to share a key epitope with the mycobacterial hsp65. The human homologue of the mycobacterial hsp65 has recently been cloned (McMullin & Hallberg 1988; Jindal et al., 1989) and both the affinity-purified form from placenta (P1), and the recombinant form (P1A) run at about 58 kDa in polyacrylamide gel electrophoresis whereas the corresponding mycobacterial protein runs at about 65 kDa. The presence of the synovial fluid molecule was demonstrated using a rabbit polyclonal to P1 and a mAb to a water soluble extract of M. tuberculosis. Both of these reagents are inadequate to answer the questions raised earlier because of the discrepancies in the molecular mass of P1 and the protein in synovial fluid, and also because the mAb SF8 binds to more then one band on western blots of denatured RA synovial fluid. Similarly, other existing monoclonals are also unsuitable because they were not selected for binding to human material eg. Tb78 (Coates et al., 1981) and ML30 (Ivanyi et al., 1983). ML30 was raised against M. leprae antigen and is specific for mycobacterial hsp65 but, on western blots of RA synovial fluid it appears to bind to IgG light chains and other unidentified components. Therefore it was essential to make mAbs to the P1 protein. This reagent would not only help in the identification of the protein in
synovial fluid but would also be invaluable in histological studies to show the distribution of the human homologue of the hsp65. Moreover, such mAbs would also be useful in the immunopurification of the native protein for use as potential antigen in animal models of arthritis and T cell work. These mAbs would also be useful in the investigation of anti-hsp65 function in vivo and in the modulation of arthritis in animal models.
6.2. MATERIALS AND METHODS

At the time of immunization neither recombinant nor the purified form of the P1 protein was available. Therefore, a crude extract of P1 from human placenta was used. This preparation and all other forms of the P1 protein was provided by Professor Gupta, McMaster University, Ontario, Canada.

6.2.1. PREPARATION OF FREUND'S INCOMPLETE ADJUVANT (FIA)

6.5 mls of liquid paraffin (BDH 29436) was mixed with 1.5 mls of Arlacel-A (Sigma 8009). Equal volume of this preparation was added to equal volume of the crude P1 protein in PBS. The mixture was then subjected to brief sonication and mixed with a syringe until a homogenous emulsion was obtained.

6.2.2. THE RIBI ADJUVANT SYSTEM

The Ribi Adjuvant System (Ribi ImmunoChem Research, Inc.) is a new adjuvant system which replaces the classical Freund's water-in-oil emulsion with a stable oil-in-water emulsion. The tubercle bacilli contained in Freund's complete Adjuvant (FCA) has been replaced by Trehalose Dimycolate (TDM), a highly purified adjuvant with minimal toxicity or allergenicity. Monophosphoryl lipid A (MPL), detoxified endotoxin of gram-negative bacteria is added to the system to further enhance the immune response. The advantages of the Ribi Adjuvant System over the FCA are that the former involves no mycobacteria, it is more stable and can be given intravenously.
6.2.3. PREPARATION OF THE RIBI ADJUVANT SYSTEM

Into a clean, dry and sterile 10 ml tissue grinder fitted with a teflon pestle (Potter-Elvehjem type) 250 μl (about 100 μg) of the affinity-purified P1 protein was added and freeze dried. Then 90 μl of TDM and 90 μl of MPL which had been solublized in C:M (chloroform:methanol; 4:1) were added at concentrations of 2 mg/ml each. The C:M solvent was evaporated by placing the grinder tube under a laminar flow hood. Then 20 μl of oil (Squalene; Sigma S-3626) was added so that the oil was in contact and soaked into the dry antigen-adjuvant mixture. The teflon pestle rod was clamped in the chuck of a power drill and the teflon end was inserted into the antigen-containing grinding tube. The oil and antigen-adjuvant mixture was ground into a paste on the wall of the lower 1/3 of the grinding tube. Grinding was continued for 2-4 minutes at room temperature using a drill speed of 800-1200 rpm. Then 1 ml of 0.2 % Tween 80 (Sigma P-1754) in PBS (PBS/Tween 80) was added. The oil-mixture paste was emulsified into the PBS/Tween 80 by slowly moving the pestle up and down in the grinding tube over a period of 4 minutes with the drill speed at 800-1200 rpm. Finally the pestle was removed and the emulsion was poured from the grinding tube into a vial. Immediately before removing aliquots of emulsion for injections, the vial was gently vortexed to mix the contents, since some separation of the oil from the aqueous phase could occur upon standing.

6.2.4. IMMUNIZATION PROTOCOL

Balb/c mice were given two intramuscular injections (given at four sites) of 50 μg of the crude P1 protein each, in 0.2 ml of FIA on day 0, and day 5. Seven weeks after the first injection they received 20 μg of the affinity purified P1 protein each intravenously in 0.2 ml of Ribi adjuvant. Four days after this injection spleens were harvested for fusion. The mice were bled by cardiac puncture at the same
6.2.5. PREPARATION OF GROWTH MEDIUM AND MEDIUM CONSTITUENTS

6.2.5.1. Basic growth medium for cell line and hybridoma.

Cells were grown in RPMI 1640 with 15% foetal calf serum (FCS, Gibco, 011-6290M) and 5% horse serum (Gibco, 034-6040M), supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, 061-5145). Fungizone (Amphotericin B 250 µg/ml, Gibco, 061-5295D) at a final concentration of 2 µg/ml was also added to the growth medium.

6.2.5.2. Preparation of HAT medium

For HAT medium 1x10⁻⁴ M hypoxanthine, 4x10⁻⁷ M aminopterin and 1.6x10⁻⁵ M thymidine (Flow, 16-808-49) was made up in the above basic growth medium. HAT constituents were stored frozen (-20 °C) at 50 times the working concentration.

6.2.5.3. Polyethylene glycol solution

Polyethylene glycol (PEG, MW 1500, BDH 29575) was melted by autoclaving for 15 minutes at 15 lbs. When cooled to 50 °C it was mixed 1:1 with RPMI making 50 % solution which was aliquotted and stored at -20 °C.
6.2.6. CELL LINE USED FOR FUSION

The mouse myeloma cell line chosen for fusion was the JK (P3-X63-Ag8, 653), a clone derived from P3-X63-Ag8 (Kearney et al., 1979) and obtained from Dr. A. Thakur, Nuclear Medicine, UCMSM, London. This myeloma cell line had irreversibly lost the ability to express detectable amounts of immunoglobulin heavy or light chains and was sensitive to 'HAT'. It was shown to have a high fusion rate compared with other myeloma lines and a fast growth rate (Mrs. J. Edge, personal communication).

6.2.7. COLLECTION OF FEEDER CELLS

Feeder cells were collected either from mouse peritoneum or from spleen. To collect feeder cells from mouse peritoneum, a syringe was filled with 5 mls of RPMI, injected into the peritoneum of an ether anaesthetised (Anaesthetic ether B.P., May and Baker Ltd.,) mouse, massaged gently and as much volume as possible removed. This procedure was repeated and the cells were washed with RPMI resuspended in HAT medium and plated into microtitre wells at 5x10^4 cells/wells, either immediately or just before use. Feeder cells collected from mouse peritoneum had little or no red blood cells, therefore, they were not treated with ammonium chloride lysing buffer. One mouse peritoneum was found to give approximately 5x10^6 cells which was sufficient for one microtitre plate (96 wells, Nunc FB 1-67-008) when plated at 5x10^4 cells/well.

When a large number of feeder cells was needed (eg. before cloning), feeder cells were collected from mouse spleen. An average mouse spleen produced enough feeder cells for at least 6 microtitre plates at 5x10^4 cells/well. To collect feeder cells
from spleen, a mouse was killed by placing it in a glass container filled with vapour form anaesthetic ether. The spleen was removed aseptically and placed into a 7 ml bijou bottle (Sterilin 129A) containing approximately 5 mls of RPMI preincubated to 37 °C. Using a pair of sterile scissors the spleen was cut into small pieces and allowed to stand for a few minutes. After the clumps and pieces of the connective tissues had sedimented out the supernatant was transferred with a pasteur pipette into a sterile 30 ml universal container (Sterilin 128A). The volume of RPMI was replaced with fresh RPMI and more feeder cells were squeezed-out from the spleen using a pair of forceps. This procedure was repeated several times until no more spleen cells can be extracted. The splenocytes were washed with RPMI at 1200 rpm for about 8 minutes. After spinning, the red blood cells were lysed with ammonium chloride solution.

6.2.8. LYSING OF THE RED BLOOD CELLS WITH AMMONIUM CHLORIDE SOLUTION.

The pellet of spleen cells was gently tapped to loosen the cells and 5 mls of ammonium chloride solution was added. After gentle shaking for 3-4 minutes, volume was made up to about 25 ml and spun at 1200 rpm for about 8 minutes. After 2 further washes, feeder cells were counted in a Neubauer haemocytometer (Depth 0.1 mm, 1/400 mm² Hawksley, England) and resuspended in the HAT medium. Feeders were either plated out at 5x10⁴ cells/wells or kept in a universal for up to a week in the growth medium. Feeders were always collected one day before they were required so that they can be checked for infections prior to use.
Spleen cells were fused with JK myeloma cells using standard procedures (Kohler & Milstein 1975; Galfre & Milstein 1981). One of the immunized mice was killed and the spleen was removed aseptically, cells teased out and suspended in RPMI. After pelleting, red blood cells were lysed by adding 5 ml of ammonium chloride lysing buffer for 3 minutes. Cells were then washed three times in RPMI and resuspended at $10^8$ cells/ml.

Meanwhile, JK cells which had been growing for over a week, were washed and resuspended at $10^8$ cells/ml in RPMI. The JK and spleen cells were then mixed at 1:5 ratio JK:spleen cells. The mixed cells were pelleted by spinning at 1000 rpm for 8 minutes. The pellet was broken with gentle tapping and the cells were fused by adding 1 ml of 50% PEG solution (preincubated to 37°C) dropwise over a period of 1 minute with constant agitation and mixing. Mixing was continued for a further minute. Over the third minute 1 ml of RPMI (prewarmed to 37°C) was added gently and slowly. Followed by a further 20 ml of RPMI at 37°C added dropwise over 4 minutes. The universal was inverted several times to mix the cells gently and spin at 800 rpm for 8 minutes. Finally, the pellet of mixed cells was broken with very gently tapping and resuspended in HAT medium at 1.5x$10^6$ cells/ml. The cells were then plated into 4x96 well microtitre plates at 100 μl/well (i.e. 1.5x$10^5$ cells/well) which already contained 100 μl of HAT medium with feeders.

The plates were then incubated at 37°C in a humid CO₂ incubator. Medium in the wells was half changed on day 3, and 7, by removing 100 μl and replacing with 100 μl of fresh HAT medium. Growth began at 7-10 days, this was earlier than expected. The number of wells with growth were counted and the
fusion rate calculated. Supernatants from growing wells were tested by ELISA and by immunoblots on miniature preparative western blots.

6.2.10. SCREENING OF CULTURE SUPERNATANTS

Initial screening was done on culture supernatants from all growing wells by ELISA and immunoblots. By this time Professor Gupta was able to supply two recombinant forms of the P1 protein (P1A and P1D). P1A is the full length human P1 protein and gives a single band at approximately 58 kDa on SDS PAGE whereas P1D has a proportion of the N-terminal sequence missing and on SDS PAGE gives a single band of about 40 kDa. Both preparations were obtained by electroelution from SDS PAGE gels. Culture supernatants were screened by ELISA on P1, P1A and a mixture of two negative control antigens, and by immunoblot on P1A and RA synovial fluid. It was important to exclude selection of any clones which may bind to proteins with similar molecular mass as P1 or the 65 kDa protein in RA synovial fluid. Thus human IgG and HSA which are abundant in body fluids and in SDS PAGE run at about 56 kDa and 65 kDa respectively were chosen as negative control antigens.

6.2.10.1. Screening of the culture supernatants by ELISA

Microtitre ELISA plates were coated with 2 μg/ml of the proteins in carbonate-bicarbonate buffer (pH 9.6). Coating was carried out at 37 °C for 2 hours. Plates were washed 3 times with PBS/Tween 20 and 50 μl of culture supernatant was transferred to each of the wells in the ELISA plates with a multichannel pipette using sterile tips. Plates were incubated at 37 °C for 1 hour 30 minutes, washed 3 times and then incubated overnight at 4 °C with peroxidase conjugated rabbit anti-human
immunoglobulins diluted 1:1000 in PBS/Tween 20. Finally the ELISA was developed in the usual way.

6.2.10.2. Screening of the culture supernatants by western blots

6.2.10.2.1. Preparation of Miniature western blots

Preparative western miniature blots were prepared using the 'mighty small' miniature slab gel electrophoresis unit, SE200 (Biotech Instrument Limited).

10 µg of P1A, P1D or 150 µg of RA synovial fluid was loaded into each preparative wells formed by using a teflon comb with one reference well (Biotech Instrument Limited; SE211-R-.75). The proteins were resolved by SDS PAGE on slab gels (0.75 mm thick) of 12.5% (vol/vol) acrylamide according to the discontinuous buffer system of Laemmli (1970). Electrophoresis was carried out on a vertical slab gel unit (Hoefer SE200) at room temperature at constant current using 18 mA per gel. A mixture of standard protein markers (MW-SDS-200, Sigma) was included in each run for the determination of molecular mass. Subsequently, the resolved antigens were transferred electrophoretically to nitrocellulose paper in Tris-glycine-methanol buffer using a wet blotter (Hoefer Mini Transphor TE 22) at a constant voltage of 50 V for 1 hour or 10 V overnight, at room temperature (Towbin et al., 1979). Nitrocellulose membranes were blocked with 1% (wt/vol) BSA in PBS with 0.05% (vol/vol) Tween 20 for 2 hours at room temperature. The preparative blots were then stored between filter papers at -20°C until used.
6.2.10.2.2. Screening of the culture supernatants by Miniblotter.

The Miniblotter used was the Biometra Miniblotter MN 28. Each preparative blot covered 22 channels, therefore to use the full width of the MN 28 Miniblotter, it was necessary to use more than one preparative blots for each set of the Miniblotter channels. The upper Miniblotter plate was turned over and a wet nitrocellulose membrane was positioned against the channel area, with the antigen-bearing face of the membrane facing the channels. The membrane was aligned so that the full length of each of the channels to be used was covered. A dry white plastic cushion was then placed over the nitrocellulose membrane. The back plate of the Miniblotter was set on the inverted top and the entire unit was turned over. All the screws were then inserted, tightened by hand making sure that there were no gaps between the top and the bottom plates. The excess liquid from the channels were drained out using a pipette tip connected to a vacuum pump (Millipore U.K. Ltd).

Culture supernatants (50 µl) were then introduced through the numbered holes with a semi-automatic pipettor using standard disposable plastic pipette tips. The Miniblotter was then incubated on a rocker table (Taab Laboratories) overnight at 4°C. After this incubation period, the nitrocellulose membrane was removed from the Miniblotter and incubated with the secondary antibodies in large plastic weighing boats. Binding of mAbs and the rabbit polyclonal antibody to P1 was detected as described in Chapter 5. The screening procedure was repeated once ascites fluid of all the selected clones were available.
Hybrids were selected for cloning on the basis that (1) they were strongly positive on P1A by both ELISA assay and by immunoblots, (2) they did not bind to the negative control antigens used, and (3) they did not bind to multiple bands on western blots of RA synovial fluid.

Cells to be cloned were counted and resuspended at 40 cells/10 ml of growth medium. Then 100 µl of this was added to each well of a microtitre plate which already contained healthy feeders in 100 µl of growth medium. Growth was observed 7-10 days later and the culture supernatants tested for anti-P1A specificity.

Once clones were known to be positive, and growing well, they were transferred into 24 well plates (Cel-Cult, 33F24L) which already contained 5x10⁵ healthy feeders in 500 µl of growth medium. Once the costa wells were confluent, clones were transferred into small flasks (25 cm²; Nunc 1-63371). Feeder cells were no longer required. A large quantity of culture supernatant containing mAb could thus be produced. When the clones in the small flasks were confluent, they were either frozen in liquid nitrogen or given to pristane treated mice for generation of more concentrated mAbs.
6.2.13. GENERATION OF ASCITES FLUID

Cells from the positive clones were counted and resuspended at $10^8$ cells/100 μl in PBS. This was injected intraperitoneally into female Balb/c mice which had been treated with 0.5 ml of pristane (2,6,10,14-Tetramethyl-pentadecane, Sigma T-7640) 10 days prior to injecting the hybridoma cells. When the peritoneal cavity of the mouse became swollen (usually 10-15 days) the mouse was killed and ascites fluid drained with a syringe. Ascites fluids were spun at 3000 rpm to discard the cells. The floating pristane oil was sucked out with a pasteur pipette and the ascites fluid aliquotted and stored at -70 °C.

6.2.14. FREEZING AND THAWING CELLS

Freezing solution was made of 50% FCS, 40% RPMI and 10% dimethyl sulfoxide (DMSO; Sigma D5879) and used at 4 °C. Healthy cells were pelleted by spinning at 1000 rpm, resuspended into the freezing solution and aliquotted into freezing vials (Nunc-363401) at 1 ml/vial. The vials were placed in polystyrene box at -70 °C overnight and transferred to liquid nitrogen the next day.

For thawing cells, vials were removed from liquid nitrogen, thawed quickly at 37 °C. After pelleting the cells, they were transferred to small flasks with growth medium. Cells were allowed to grown for at least 4-7 days before they were injected into pristane treated mice.
6.2.15. DETERMINATION OF THE IMMUNOGLOBULIN SUB-CLASSES OF THE MONOCLONAL ANTIBODIES

Microtitre ELISA plates were coated with 2 μg/ml of P1A in coating buffer (carbonate-bicarbonate buffer, pH 9.6) either overnight at 4 °C or 2 hours at 37 °C. After washing, the plates were blocked with 2% BSA in PBS/Tween 20 for 1 hour at 37 °C. Each of the subsequent steps were in the presence of M. vaccae sonicates (0.25 mg/ml) to neutralize non specific anti-mycobacterial activity since the reagents used were raised using FCA. After washing the plates with PBS/Tween 20, the goat anti-mouse isotypes (Southern Biotechnology Associates, Inc.) were added at 1/5000 and incubated for 1 hour at 37 °C. Then peroxidase-labelled donkey anti-goat antibody (Jackson Immunoresearch Laboratories, Inc.) was added to the wells at 1/5000 and incubated overnight at 4 °C. The ELISA was then developed in the usual way.

* Culture supernatants were added to the wells and incubated for 1 hour at 37 °C. The plates were washed again and....

6.2.16. CAPTURE ASSAY TO ESTIMATE THE PROTEIN CONCENTRATIONS OF THE IgG AND IgM MONOCLONAL ANTIBODIES

This assay was essentially similar to the capture assay used in Chapter 4 for the estimation of human anti-GlcNAc antibodies, except that the reagents used were different. Microtitre ELISA plates were coated with goat anti-mouse IgG and IgM (Sigma M-1397 and M-8644 respectively). Mouse IgG standard was purchased from Sigma (1-5381) and IgM standard from Binding Site. The secondary antibody used was peroxidase-conjugated goat anti-mouse IgG (Sigma A-3673) and IgM (Sigma A-8786).
6.2.17. STAINING OF THE HEP-2 CELL LINE BY INDIRECT IMMUNOFLUORESCENT TECHNIQUE

A human epithelial cell line called HEP-2 was grown on glass cover slips and provided by Dr. Cambridge, Department of Rheumatology, UCMSM. Before staining the cells they were washed in PBS and fixed in acetone. After further washes in PBS cells were incubated with 50-70 μg/ml of the ascites fluid of the mAbs. The staining protocol was similar to the indirect immunofluorescent technique described in Chapter 3.
6.3. RESULTS

6.3.1. Fusion rate

Growth was first observed on day 7 and fusion rate scored on day 10. From the four fusion plates with a total of 384 wells, 90 wells showed growth, giving a fusion rate of 23.4%.

6.3.2. Screening of the culture supernatants

The initial screening was done on culture supernatants from all the growing wells by ELISA and immunoblots. The results are shown in Tables 6.1-6.4. Hybrids which were strongly positive on P1A by both assays and which did not bind to the negative control antigen used in ELISA assays were selected for cloning. On western blots of RA synovial fluid, some hybrids did not bind to any bands (pattern 0), some bound very strongly to two bands at 96 kDa and 54 kDa (Pattern 1). A third pattern of binding (Pattern 2) was seen with some clones which bound to the two bands above and somewhat weakly to a band at 66 kDa (see Key in Tables 6.1-6.4). At this stage the criteria used for the selection of hybrids were (a) that they were positive on P1 and P1A by both ELISA and western blots, (b) they did not bind to the IgG or HSA and (c) that they did not give multiple bands on western blots of RA synovial fluid.
Table 6.1 The results of initial screening of culture supernatants by ELISA and immunoblots

<table>
<thead>
<tr>
<th>Fusion Plate 1</th>
<th>A3</th>
<th>A5</th>
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<th>A11</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
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<th>G7</th>
<th>G10</th>
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<td><strong>Screening by</strong></td>
<td><strong>P1A</strong></td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
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<tr>
<td>ELISA</td>
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<td>±</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>±</td>
<td>++</td>
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<td>±</td>
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<td>+</td>
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</table>
Table 6.2 The results of initial screening of culture supernatants by ELISA and immunoblots

| Fusion Plate 2          | A1 | A8 | A12 | B8 | B9 | C1 | E2 | E3 | E4 | E10 | G3 | G4 | G7 | G8 | G9 | H1 | H2 | H3 |
|------------------------|----|----|-----|----|----|----|----|----|----|-----|----|----|----|----|----|----|----|
| Screening by ELISA     | ++ | ++ | ++  | +  | +  | ++ | ++ | ++ | ++ | ++   | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| HSA + IgG              | -  | -  | -   | ±  | -  | -  | -  | -  | -  | -    | -  | -  | -  | -  | -  | -  | -  | -  |
| Screening by immunoblots| ±  | ++ | ++  | +  | -  | -  | ++ | ++ | ±  | ++   | +  | ±  | +  | ++ | ±  | +  | ±  | ±  |
| Synovial fluid         | 0  | 0  | 2   | 0  | 0  | 0  | 1  | 0  | 0  | 2    | 1  | 0  | 0  | 0  | 2  | 0  | 2  | 0  |

**Key**

- 0
- 1
- 2
- 96K
- 66K
- 54K (γ-chain)

The binding patterns of the culture supernatants on RA synovial fluid
Table 6.3 The results of initial screening of culture supernatants by ELISA and immunoblots

<table>
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<td>+</td>
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<td>++</td>
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</tr>
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<td>HSA + IgG</td>
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<td>±</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Screening by immunoblots</td>
<td>P1A</td>
<td>±</td>
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Key:

- 96K
- 66K
- 54K (γ-chain)

The binding patterns of the culture supernatants on RA synovial fluid
Table 6.4 The results of initial screening of culture supernatants by ELISA and immunoblots

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</tbody>
</table>

Key

The binding patterns of the culture supernatants on RA synovial fluid

- 0: 96K
- 1: 66K
- 2: 54K (γ-chain)
A total of 29 wells were cloned and following a second screening of culture supernatants from the wells only 10 clones were kept (Shaded in Tables 6.1-6.4). These clones were coded from here on as:


To avoid any confusion, clones were coded according to the position of the clones in the original fusion plates. Thus 1A8/20 means it came from the fusion plate 1 from row number A8 of the microtitre plate. The number after the slash represents a serial number given to the clone for screening. This number was also used to distinguish between clones which came from the same parent clone.

6.3.3. Isotype analysis of the monoclonal antibodies

All the clones selected were IgM except clone 4B9/89 which was IgG2a.

6.3.4. Generation of ascites fluid and estimation of their immunoglobulin concentrations.

Ascites fluid of all the selected clones except 4B11/6 were generated in Balb/c mice (5-20 mls of each). The immunoglobulin concentrations of these ascites fluid were estimated using a capture assay for mouse immunoglobulins.
6.3.5. Screening of the ascites fluid of selected clones on miniature blots of P1A, P1D and RA synovial fluid.

Figures 6.1 and 6.2 shows the results obtained on P1A and P1D respectively. All the mAbs (except 4B11/6 for which no ascites fluid was available) bound very strongly to both of these recombinant forms of the human hsp58 even when they were used at a concentration of 1 μg/ml (see Figure 6.1 and 6.2). However none of the mAbs bound to RA synovial fluid. When the monoclonals were used at very high concentrations (50-100 μg/ml) and left in the substrate solution for an hour, they appear to bind very weakly to 3 bands (Figure 6.3). This pattern of staining was thought to be non-specific since some of the culture supernatants which also bound to the negative control antigens used for screening gave similar pattern of immunostaining on western blots of RA synovial fluid. This result is very odd and unexpected since the rabbit polyclonal anti-serum (P1Ab) which binds to both the recombinant and the native form of the human hsp58 picked up a band of approximately 65 kDa on western blots of RA synovial fluid (See Chapter 5).

6.3.6. Indirect immunofluorescent staining of HEP-2 cell line

The IgG mAb, 4B9/89, gave a strong granular cytoplasmic staining of all cells (Figure 6.4). This pattern of staining is consistent with mitochondrial staining. The IgM mAbs gave a diffuse cytoskeletal staining of the HEP-2 cells (Figure 6.5). In addition, granular cytoplasmic staining of some cells were also seen with the IgM mAbs, particularly 3C8/65 and 1A8/20.
Figure 6.1. Screening of the monoclonal antibodies on preparative western blots of P1A using a Miniblotter. Lane 1 was stained with the rabbit polyclonal antibody and Lanes 2-9 were stained with the mAbs. Lanes 10 and 11 had culture supernatants from negative clones.
Figure 6.2. Screening of the monoclonal antibodies on preparative western blots of P1D using a Miniblotter. Lane 1 was stained with the rabbit polyclonal antibody and Lanes 2-9 were stained with the mAbs. Lane 10 had culture supernatants from a negative clone.
Figure 6.3. Screening of the monoclonal antibodies on preparative western blots of RA synovial fluid using a Miniblotter. Lanes 1-9 were stained with the mAbs. None of these mAbs bound to RA synovial fluid even when used at high concentrations and pushed to the limit. Lanes 10 and 11 had culture supernatants from negative clones.
Figure 6.4. HEP-2 cell line stained with the IgG mAb, 4B9/89, and rabbit anti-mouse FITC conjugate. Showing granular cytoplasmic staining which is typical of mitochondrial staining pattern. (x1000).
Figure 6.5. HEP-2 cell line stained with one of the IgM mAb, 3C8/65, and rabbit anti-mouse FITC conjugate showing a diffuse cytoskeletal staining. This mAb also produced granular cytoplasmic staining similar to the one shown in Figure 6.4 on some HEP-2 cells. (x1000).
The P1 protein appear to be highly immunogenic in mice since a large number of B cells had been stimulated by immunizing mice with a crude extract of this protein. The mammalian hsps are highly conserved protein with a considerable degree of sequence homology, thus P1 represent a potential cross-reactive autoantigen. All the anti-P1 mAbs bound to the affinity-purified native protein as well as to the recombinant forms, P1A and P1D. Moreover the mAbs are highly specific since they did not bind to any of the large numbers of different proteins present in the RA synovial fluid. This absence of binding on synovial fluid also indicates that the synovial 65 kDa protein (Chapter 5) may not be the same as P1. The P1 protein is a mitochondrial protein (Jindal et al., 1989) and the pattern of immunofluorescent staining given by some of the mAbs is consistent with this observation. Moreover the IgM mAbs gave a diffuse cytoskeletal staining in addition to the granular mitochondrial staining. This may be because the IgM monoclonals cross-reacted with another cytoplasmic protein or an isoform of the P1 protein. It is interesting to note that a cytoplasmic protein called TCP-1 (Silver et al., 1979 and 1983) has been shown to have 60% similar amino acid sequence (20% identical residues and about 40% conserved substitutions) with the mammalian P1 protein (Gupta 1990). It is not yet known whether any of the mAbs would bind to TCP-1 but is possible that the cytoskeletal staining seen on HEP-2 cell line could well be due to the binding of the monoclonals to this molecule. It would be important to check the monoclonals on TCP-1 since this protein is encoded by the genes located at the MHC region (Gupta 1990) which plays a major role in immune recognition and susceptibility to autoimmune deseases (eg. HLA DR4 in RA). The mAbs are characterized further in the following chapter which may help to illuminate the problem of the nature of the hsp65 in RA joints and provide further evidences suggesting a role for the human hsp58 in the aetiopathogenesis of RA.

In conclusion, this study shows that if the mitochondrial hsp58 is present in RA
synovial fluid, it is at a very low concentration, and cannot be picked up on western blots with mAbs which bind strongly to the recombinant or purified placental hsp58.
CHAPTER SEVEN

FURTHER CHARACTERIZATION OF THE MONOCLONAL ANTIBODIES AND 
THE PRESENCE OF HUMAN HSP58 IN INFLAMED TISSUES
CHAPTER 7

FURTHER CHARACTERIZATION OF THE MONOCLONAL ANTIBODIES AND THE PRESENCE OF HUMAN HSP58 IN INFLAMED TISSUES

7.1. INTRODUCTION

Having obtained a number of mAbs binding to the human hsp58, the next phase of the work was to define the epitope(s) these mAbs recognize on this hsp and to establish the range of cross-reactivity with the bacterial, particularly mycobacterial, hsp65. The members of the hsp60 and hsp 70 families appear to fulfill similar functions. Both families are involved in folding, unfolding and translocation of polypeptides as well as in the assembly and disassembly of oligomeric protein complexes (Reading et al., 1989; Cheng et al., 1989; Hemmingsen et al., 1988; Osterman et al., 1989; Goloubinoff et al., 1989; Chirico et al., 1988; Deshaies et al., 1988). Also it had been reported that the population of T cells that respond to mycobacterial hsp65 also respond to E. coli hsp65 and hsp70 and, most interestingly human hsp70 purified from heat shocked macrophages (Lamb et al., 1989). Thus it would be interesting to see if any of the mAbs cross-react in the same way. Moreover several groups have attempted to demonstrate the endogenous expression of the mammalian homologue of the bacterial hsp65 using a mAb raised against mycobacterial antigen (Karlsson-Parra et al., 1990 & Kleinau et al., 1991). In the latter study the authors claimed that there is increased exposure of the hsp in the joint tissue from rats with AA and collagen induced arthritis though the antibody they used binds to several components of mammalian tissues. In the present study mAbs to the human hsp58 are used to show the presence and distribution of the human homologue in joint tissues from normal, RA and inflammatory control.
7.2. MATERIALS AND METHODS

**Antibodies:** A panel of control monoclonal and polyclonal antibodies with well established specificities were used. These included a rabbit polyclonal anti-serum to hamster hsp58 (P1Ab), SF8 and Tb78. The sources and specificities of these antibodies have been described in the preceding chapters. A rabbit anti-serum to mycobacterial hsp70 (CosII) and an anti-serum to *E. coli* hsp65 raised in rabbit were also used. These anti-sera were kindly provided by Dr. D. Young, Hammersmith Hospital, London.

7.2.1. PREPARATION OF CELL LYSATES

Cell lysates of a mouse fibroblast cell line (L929), a Jurkat T cell line (JRT) and peripheral blood monocellular cells from a SLE patient were prepared as follows: Cells were washed several times with PBS. After pelleting the cells they were lysed with Laemmli buffer containing SDS and mercaptoethanol. The lysates were then subjected to brief sonication to break up the DNA.

7.2.2. PREPARATION AND IMMUNOSTAINING OF THE MINIATURE POLYACRYLAMIDE GELS OF HEAT SHOCK PROTEINS AND CELL LYSATES

Preparative miniature blots of mycobacterial hsp65, hsp70 and *E. coli* hsp65 was prepared as described in Chapter 6. For the hsps 10 µg of the recombinant protein was used per preparative blot and for the cell lysates approximately 300 µg (5 x 10⁶ cells) of the proteins were loaded into each preparative blot. After blocking the nitrocellulose membrane with 1% BSA/PBS/Tween 20, the blots were stained with the mAbs at 50 µg/ml using the Miniblotter 28 as described in the previous chapter.
7.2.3. SCREENING OF THE MONOCLONALS ON SYNTHETIC PEPTIDES OF M. LEPRAE

All the mAbs were screened by ELISA on the whole M. leprae hsp65 and M. bovis hsp65. Monoclonals which gave positive signals in this assay were then screened on 105 overlapping synthetic peptides of between 15 and 20 amino acids in length and corresponding to the complete sequence of the M. leprae hsp65 using a specially developed ELISA assay. This ELISA assay was developed by Dr. D. B. Lowrie and Dr. P. Jenner (National Institute for Medical Research, Mill Hill, London). The assay was carried out at Dr. Lowrie's laboratory, and is as follows:- Two of the mAbs (1A8/20 and 3G9/92) were used in this assay, thus two sets of peptides were prepared with duplicate wells of each peptide in each of the two sets of plates. All the peptides were made up in trifluoroacetic acid (TFA; PIERCE, 28903) at 1 mg/ml. Then 2 μl of each of the peptides was pipetted in duplicate into the appropriate wells. This procedure was carried out very quickly since TFA evaporates very rapidly at room temperature. Each peptide was added to the microtitre wells in turn leaving empty wells to receive recombinant whole peptide later and adding just TFA to the blank wells. The plates were then stacked on top of one another with small gaps in between and placed in a large vacuum desiccator containing sodium hydroxide as desiccant. After about 30 minutes plates were removed from the desiccator and left at room temperature overnight. The next day some M. leprae 65kDa protein was diluted with 0.25 M (pH 9.6) carbonate-bicarbonate buffer to give a final concentration of 1 μg/ml and 100 μl was applied into the appropriate wells as positive controls and incubated for 1 hour at 37 °C. After this incubation period, 100 μl of 0.25 M carbonate-bicarbonate buffer was added to the remaining wells and incubated for a further hour at 37 °C. The wells were tapped out and blocked with 200 μl of washing buffer (45g of sodium chloride in 5 liters of deionized water with 2.5 mls of Tween 20) supplemented with 5% powdered milk (w/v) and incubated at 37 °C for 3-4 hours or overnight at 4 °C. The blocking buffer was discarded from the plates and the mAbs were
added at 1/200 dilution made up in the blocking buffer. The plates were then incubated overnight at 4 °C or 2-3 hours at 37 °C. After washing the plates 4 times with washing buffer rabbit anti-mouse alkaline phosphatase conjugate (DAKO D314) was added at 1/2000, made up in the milk blocking buffer, and incubated for 2 hours at 37 °C. After this incubation, the plates were washed 6 times with washing buffer and developed with phosphatase substrate (Sigma 104-0). Finally the plates were read at 405 nm.

7.2.4. CROSS-INHIBITION ASSAYS

Microtitre ELISA plates were coated with 1 μg/ml of P1A in carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight. After washing the plates 3 times with PBS/Tween 20, the blocking antibodies were applied at concentrations of 0, 1, 10, 25, 50 and 200 μg/ml. Following a 2 hours incubation period at room temperature, the plates were washed again and freshly biotinylated mAbs were added to the ELISA plate wells at dilutions corresponding to the linear portion of the dose response curve of these monoclonals on P1A. Plates were then incubated for 2 hours at room temperature, washed again and peroxidase-labelled streptavidin (DAKO P397) was added at 1/10,000. After overnight incubation at 4 °C the ELISA was developed in the usual way.

7.2.5. CAPTURE ASSAY TO DETECT FREE HSP58 IN RA SYNOVIAL FLUID AND SERUM.

A capture assay was set up using P1A. Microtitre ELISA plates were coated with 10 μg/ml of the mAbs in usual way. P1A was then applied at 10 μg/ml made up in 1% BSA PBS/Tween 20 and diluted across the ELISA plate. After 3 hours incubation at room temperature, plates were washed and biotin-labelled mAbs to P1A were added at 1 μg/ml and incubated for 2 hours at room temperature. After further washes peroxidase-
labelled streptavidin was added at 1/10,000 and incubated overnight at 4 °C. Finally, the plates were washed and developed in the usual way. The mAb which produced the best results in capturing P1A (2E1/53) was chosen to capture the endogenous hsp58 from synovial fluid and RA serum. ELISA plates were coated with this antibody at 10 µg/ml and after washing the plates RA serum and synovial fluid were added at a dilution of 1/2 made up in 1% BSA PBS/Tween 20 and diluted 1/2 across the ELISA plates. The captured antigen was detected with the biotin labelled IgG mAb to hsp58, 4B9/89. This mAb was used because it appeared to be most efficient in detecting the captured antigen.

7.2.6. IMMUNOPRECIPITATION OF THE HUMAN HSP58 FROM RA SYNOVIAL FLUID.

It appeared from the results (section 7.3.4) of the above capture assay that there were small quantities (<1.5 µg/ml) of the endogenous hsp58 in the RA body fluids tested, thus immunoprecipitation was carried out to confirm the results of the capture assay. Immunoprecipitation of human hsp58 from RA synovial fluid was carried out using both conventional assay and Dynabeads M-450 (Dynal U.K. Ltd..) coated with the mAb 2E1/53.

7.2.6.1. Immunoprecipitation

Freshly collected RA synovial fluid was spun down at 3000 rpm for 8 minutes and the cells were discarded. Then 2 mls of synovial fluid was diluted 1/25 with PBS/Tween 20. The mAb (2E1/53) was added at a final concentration of 1 µg/ml and incubated for 48 hours with constant agitation at 4 °C. After this incubation period rabbit anti-mouse IgM (Sigma M-8644) was added at a dilution of 1/1000 and incubated for 24 hours at 4 °C on an orbital shaker. To collect the immunoprecipitate, the synovial fluid was spun at
1000 (these precipitates contain most of the debris), 3000 and 10,000 rpm. Immunoprecipitates from these three samples were washed several times with PBS and run on 12% SDS PAGE under denaturing condition. After transferring the protein onto nitrocellulose membrane, the blots were blocked and stained with biotin-labelled mAb, 4B9/89.

7.2.6.2. **Immunomagnetic isolation of hsp58 using Dynabeads M-450.**

One of the IgM mAbs (2E1/53) was purified on a Sepharose gel filtration column (Sigma CL-6B-200) and the Dynabeads were coated with this mAb by direct physical adsorption. The beads were washed in sterile distilled water and a uniform suspension of beads obtained by brief shaking. The purified mAb was dissolved in 0.2 M borate buffer (pH 9.5) to a concentration of 150 μg/ml. Then an equal volume of the homogenous suspension of the dynabeads M-450 was added to the antibody solution so that an antibody to bead ratio of 75 μg/15 mg was achieved. After 24 hours incubation at room temperature by slow end-over-end rotation, the dynabeads were collected with a magnetic particle concentrator (MPC; MPC-1, Dynal). The supernatants discarded while the tube was in the MPC, and the coated beads were then washed sequentially starting with 5 mls of 0.01 M PBS/Tween 20 for one hour, followed by a 12 hours wash with 5 mls of 0.01 M sodium chloride, 0.01% merthiolate, 0.1% Tween 20 (pH 7.5). Finally the coated beads were washed with PBS and resuspended in PBS to a concentration of approximately 4x10^8 dynabeads/ml (30 mg/ml). Coated dynabeads were stored at 4 °C with either merthiolate (0.01%) or sodium azide (0.02%) as a bacteriostatic agent.

For immunoprecipitation 5 μl of the homogenous suspension of the coated beads was mixed with 200 μl of synovial fluid in 2 mls of PBS. After 24 hours incubation by slow end-over-end rotation at 4 °C the dynabeads were collected using the MPC as before. Dynabeads were washed several times with PBS, concentrated using the MPC
and subjected to SDS PAGE. The immunoprecipitated hsp58 was subsequently detected on western blots using a biotin-labelled IgG mAb, 4B9/89.

7.2.7. IMMUNOHISTOCHEMISTRY

For immunohistochemical staining synovial tissues were prepared as described in detail in Chapter 2 and stained by the Alkaline Phosphatase-anti-Alkaline Phosphatase (APAAP) procedure. The mAbs were applied at concentration of 100 μg/ml in TBS on frozen and acetone fixed sections of synovial tissues from normal, RA and OA patients, and incubated for 30 minutes at room temperature. Sections were then washed in TBS and incubated with the rabbit anti-mouse Ig (DAKO Z 259) at a dilution of 1/50 for 30 minutes. After further wash the sections were incubated for 30 minutes with the APAAP immunocomplexes (DAKO D 651) diluted 1/50 in TBS. The sections were washed again and to enhance the intensity of staining incubation with the rabbit anti-mouse Ig and the APAAP immunocomplexes were repeated but with a reduced incubation period of 10 minutes each. After a further wash in TBS, sections were incubated with the Fast Red substrate. The Fast Red substrate was prepared by dissolving 2 mg of naphthol AS-MX (Sigma N 4875) in 0.2 ml of dimethylformamide in a glass tube, and then adding 9.8 mls of 0.1 M Tris buffer, pH 8.2. Immediately before use, Fast Red TR salt (Sigma F1500) was dissolved in the above solution at a concentration of 1 mg/ml and filtered directly on to the slides. The sections were incubated for approximately 10 minutes with the substrate and then rinsed in tapwater. Finally the sections were counter-stained with Mayer’s haemotoxylin and mounted in histomount.
7.3. RESULTS

7.3.1. Screening of the mAbs on hsps and cell lysates

All mAbs bound to a band of approximate molecular mass 58 kDa on western blots of cell lysates from PBMC and a jurkat T cell line. The rabbit polyclonal to the P1 protein also bound to this band (Table 7.1). The reactivity of two of the mAbs on the above cell lysates is also shown in Figure 7.1. On blots of L929 cell lysates 5 of the mAbs bound weakly to a band of about 58 kDa, however, the rabbit polyclonal antibody bound very strongly to this band. The mAbs did not bind to any bands on western blots of human hsp70 or mycobacterial hsp70. Two of the monoclonals, however, bound to mycobacterial hsp65 and \emph{E. coli} hsp65 (1A8/20 and 3G9/92 only). See Table 7.1.

7.3.2. Screening of monoclonals on synthetic peptides of the \emph{M. leprae} hsp65 and various deletions of the human P1 protein

Although 2 of the mAbs gave relatively strong positive signals when screened on the full length \emph{M. bovis} hsp65, only 1A8/20 was positive on a synthetic peptide of \emph{M. leprae} (P52). This peptide corresponds to amino acid residues 258-273 on \emph{M. leprae} hsp65 and 282-299 on P1 protein. (Table 7.2). The minimum epitope for 1A8/20 was estimated on the basis that the monoclonal did not bind to peptide 51 and peptide 53 (overlapping peptides from either sides of peptide 52). As part of a collaborative study, Professor Gupta (MacMaster University, Canada) checked the reactivity of the mAbs against several deletions of the human P1 protein by immunoblots (see Figure 7.2) but his results are not compatible with the epitope estimated above for 1A8/20, possibly because it binds to discontinuous epitopes. According to Figure 7.2 the epitopes recognised by the mAbs is contained within the amino acid residues 211-288 for clones 2E1/52, 2E1/53 and 3G9/92 (group 1); 288-366 for clones 3C8/65, 3C8/67, 4B11/4 and 4B11/6 (group 2); and 335-366 for clones 1A8/20 and 4B9/89 (group 3). See Table 7.3.
Table 7.1 Screening of the monoclonal antibodies on western blots of heat shock proteins and cell lysates

<table>
<thead>
<tr>
<th>Panel of control antibodies</th>
<th>Monoclonal antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1 Ab</td>
</tr>
<tr>
<td><strong>Heat shock proteins</strong></td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> 65K</td>
<td>±</td>
</tr>
<tr>
<td><em>E. Coli</em> 65K</td>
<td>+</td>
</tr>
<tr>
<td>Human 70K</td>
<td>-</td>
</tr>
<tr>
<td><em>M. bovis</em> 70K</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cell lysates</strong></td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>++</td>
</tr>
<tr>
<td>JRT</td>
<td>++</td>
</tr>
<tr>
<td>L929</td>
<td>++</td>
</tr>
</tbody>
</table>

Binding of the mAbs to hsp and cell lysates on western blots: - = negative, ± = weak, + = moderate, ++ = strong reactivity, ND = not determined.

PBMC = Peripheral blood mononuclear cells from SLE patient.
JRT = Jurkat T cell line.
L929 = Mouse modified fibroblasts.
Figure 7.1. Immunoblots of cell lysates from PBMC and Jurkat T cell line probed with the mAbs 3C8/65 and 4B9/89. Lane 1 shows molecular weight markers. Lanes 2, 3 and 4: cell lysates of PBMC from an SLE patient stained with Aurodye (lane 2), 3C8/65 (lane 3) and 4B9/89 (lane 4). Lanes 5, 6 and 7: cell lysates of Jurkat T cell line stained with Aurodye (lane 5), 3C8/65 (lane 6) and 4B9/89 (lane 7). Lane 8 showing P1A stained with the mAb 4B9/89 was used as a positive control.
Table 7.2. Sequence of the peptide 52 from *M. leprae* hsp65

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>258</th>
<th>273</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. leprae</em></td>
<td>ALSTLVNIRGTFKSV</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>ALETLVLNRLKVGLQV</td>
<td></td>
</tr>
</tbody>
</table>

Amino acid sequence of the *M. leprae* hsp65 using the single letter amino acid code. Residues conserved in both *M. leprae* and the human hsp58 are underlined.
Figure 7.2. Deletions of the P1 protein used for estimating the mAb epitopes

* All the mAbs reacted with these peptides
Table 7.3. Epitope group according to results of screening of the mAbs on various deletions of the PI protein

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Peptides</th>
<th>Antibody group</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E1/52, 2E1/53 and 3G9/92</td>
<td>211-288</td>
<td>1</td>
</tr>
<tr>
<td>3C8/65, 3C8/67, 4B11/4 and 4B11/6</td>
<td>288-366</td>
<td>2</td>
</tr>
<tr>
<td>1A8/20 and 4B9/89</td>
<td>335-366</td>
<td>3</td>
</tr>
<tr>
<td>3A4/9</td>
<td>?</td>
<td>4</td>
</tr>
</tbody>
</table>
7.3.3. Cross-inhibition assay

The results of the cross-inhibition study is shown in Figure 7.3. Some of the mAbs produced identical results in this assay indicating that they recognised similar epitopes. These include 2E1/52 and 2E1/53; 3C8/65 and 3C8/67; 4B11/4 and 4B11/6 (the data for only the first mAb in the pairs are shown in Figure 7.3). This results are compatible with the pattern of binding on the P1 deletion peptides (Figure 7.2). However there were several discrepancies which may be explained by overlap between epitopes seen by group 1 and group 2 antibodies, and by group 2 and group 3 antibodies. It is not clear why some group 1 antibody (e.g. 3G9/92) blocked group 2 and 3 antibodies. One possibility is that these mAbs bind to discontinuous epitopes. The binding of the mAbs 2E1/52 and 2E1/53 to P1A was blocked 100% by all the other mAbs but they themselves could not inhibit the binding of the others. The most obvious explanation for this would be that the binding of the mAbs was non specific but, it seems unlikely because of the stringent screening procedure used for selecting these antibodies. However they may have poor affinity for P1A and therefore be displaced easily by the other mAbs. The reactivity of the mAb 3A4/9 has not been checked on the P1 deletion peptides and the binding of this mAb to P1A was not inhibited significantly by any of the other mAbs suggesting that this antibody probably recognizes an epitope with little or no overlapping with the epitopes seen by the other mAbs. Therefore, 3A4/9 belongs to a different group (group 4). However, this antibody itself inhibited some of the group 2 antibodies (2E1/52 and 2E1/53) and one of the group 3 antibodies, 1A8/20 (see Figure 7.3) suggesting that it too recognizes discontinuous epitopes.
Figure 7.3. Results of cross-inhibition studies with the mAbs.

Antibody group according to Figure 7.2

Inhibiting antibodies at 200 μg/ml

169
7.3.4. **Capture assay and immunoprecipitation of hsp58**

The capture assay set up did not turn out to be very sensitive, its lower detection limit was approximately 0.75 µg/ml. In normal sera (8 samples) no free hsp58 was detected, however small quantities of the antigen was present in 2/6 RA sera and 1/3 RA synovial fluids tested. The results of 1 RA serum and 1 RA synovial fluid is shown Figure 7.4. The endogenous hsp58 was successfully immunoprecipitated from RA synovial fluid (Figure 7.5) using both conventional precipitation technique and Dynabeads coated with mAb. The latter gave similar results but was more efficient and produced cleaner results therefore only these results are shown in Figure 7.5. From the conventional immunoprecipitates, those collected at 3000 rpm contained most of the immunoprecipitated hsp58.
Figure 7.4. Capture assay for quantitatively demonstrating the presence of human hsp58 in RA serum and synovial fluid.
Figure 7.5. Western blots of immunoprecipitated hsp58 from RA synovial fluid probed with the biotin-labelled IgG mAb to this hsp. Lane 1: 4 μg of the recombinant hsp58 (P1A), lane 2: 10 μg of P1A in PBS immunoprecipitated with the mAb 2E1/53 and lane 3: hsp58 immunoprecipitated from 200 μl of RA synovial fluid with the mAb 2E1/53.
7.3.5. Immunohistochemistry

The IgM mAbs used in the immunohistochemical study (1A8/20 and 3C8/65) produced similar pattern of staining on the sections of normal and diseased synovial tissues. In normal synovium 3C8/65 showed intense staining of fibroblasts and macrophage-like cells in the surface layers (Figure 7.6). In RA synovium 3C8/65 showed widespread cellular staining of variety of morphological cell types throughout the tissue predominantly large mononuclear cells possibly macrophages or fibroblasts (Figure 7.7). In the deeper layers of RA synovial tissue 3C8/65 showed moderately strong cytoplasmic staining of scattered cells and a patchy staining of the blood vessel walls (Figure 7.8). In synovial tissues from OA patients 3C8/65 showed similar staining as on RA synovium except that in the deep tissue intense cytoplasmic staining was seen in almost all cells (Figure 7.9).

The IgG mAb, 4B9/89, showed a broadly similar pattern of staining as the IgM mAb above on normal and diseased synovium. However the staining of 4B9/89 was considerably weaker in all tissues compared to the IgM mAb, and unlike the IgM mAb, no staining of blood vessel walls was seen with the IgG mAb (Figure 7.10). Moreover in the deeper layers of RA synovium a weak cytoplasmic staining of large mononuclear cells and a patchy extracellular staining was seen with the IgG mAb (Figure 7.10).
Figure 7.6. Normal synovial tissue stained with 3C8/65 by the APAAP method, developed with Fast-Red and counterstained with haematoxylin. Showing intense staining of the synovial lining cells. (x400).
Figure 7.7. Synovial tissue from a RA patient stained with 3C8/65 by the APAAP method, developed with Fast-Red and counterstained with haematoxylin. Showing moderately strong staining of large mononuclear cells near the tissue surface. (x400).
Figure 7.8. Synovial tissue from an RA patient similar to the one shown in Figure 7.7 showing cytoplasmic staining of scattered cells and blood vessel media by 3C8/65 in the deep synovial tissue. (x200).
Figure 7.9. Synovial tissue from an OA patient stained with 3C8/65 by the APAAP method, developed with Fast-Red and counterstained with haematoxylin. Showing intense cytoplasmic staining of almost all cells in the deep tissue. (x200).
Figure 7.10. RA synovial tissue stained with the IgG mAb, 4B9/89, by the APAAP method, developed with Fast-Red and counterstained with haematoxylin. Showing a weak cytoplasmic staining of large mononuclear cells and a patchy extracellular staining in the deeper layers of the synovium. (x400).
7.4. DISCUSSION

(a) Specificities of the new monoclonal antibodies.

All the new mAbs reacted with murine cells giving a mitochondrial pattern of staining indicating that they are all mouse autoantibodies. They also reacted with a 58 kDa protein on western blots of several cell lines and peripheral blood mononuclear cells from a SLE patient. This finding is in agreement with earlier observation that the human homologue of the mycobacterial hsp65 has a molecular mass of approximately 58 kDa under denaturing conditions (Lamb et al., 1989; McMullin & Hallberg 1988). Some of the mAbs also reacted with a protein of similar molecular mass on western blots of a mouse cell line (L929) suggesting that the mAbs recognized conserved epitopes in mammalian species. Screening of the monoclonals on various deletion peptides of P1 had shown that majority of the antibodies recognize epitopes between the amino acid residues 211-366. Thus the most immunogenic fragment of the P1 protein appears to be located within this region, and the fact that the monoclonals reacted with the deletion peptides indicates that they are directed to the linear determinants on the P1 protein, possibly because they were selected by immunoblots on denatured protein.

The monoclonals did not cross-react with hsp70 from either human or bacteria but two of the mAbs reacted with hsp65 from mycobacteria and E. coli. These 2 antibodies recognize conserved epitopes and since hsp65 can be produced at sites of inflammation (Polla 1988), a T cell response triggered by bacterial antigens could conceivably mediate autoimmune destruction in RA joints. Moreover, when the antibody epitopes were mapped within the M. leprae hsp65 one of the cross-reactive monoclonal, 1A8/20, reacted with peptide 52 (Table 7.2) in which 9/18 amino acid residues are conserved in both M. leprae and the human homologue. This monoclonal was also able to block the binding of all the mAbs to P1A except 3A4/9 (Figure 7.3). Therefore it was anticipated that this cross-reactive antibody could be used as a way of locating the relevant epitopes on the human protein. Unfortunately the mapping on the mycobacterial peptides is not
compatible with the pattern of binding on the deletions of the human protein. Therefore the homologous sequence in the corresponding human protein can not be assumed to be the epitope in question.

The epitopes recognised by the mAbs could not be estimated accurately because the screening of the mAbs on the human deletion peptides is incomplete and also because several of the mAbs (e.g. 1A8/20, 3A4/9 and 3G9/92) appeared to bind to discontinuous epitopes.

(b) Expression of hsp58 in tissues.

The results of capture assay show that there are minute quantities of the hsp58 present in the synovial fluid and serum from RA patients (Figure 7.4). This might explain why the mAbs failed to bind to the western blots of RA synovial fluid (Chapter 6). Synovial fluid from RA joints is very concentrated and therefore had to be diluted many fold (at least 50 times) before it could be run in SDS PAGE. Thus it is not surprising that none of the mAbs picked up the hsp58 on western blots of RA synovial fluid. Nevertheless the presence of the hsp58 in synovial fluid was confirmed in immunoprecipitation assays and work is currently underway to confirm the presence of this molecule in RA sera. Moreover it is now clear that the heavy protein band at 65 kDa demonstrated in RA synovial fluid (Chapter 5) is not the human homologue of the bacterial hsp65.

In contrast to the low concentration of P1 in synovial fluid, there appears to be large quantities of the protein in RA joint tissues. Using mAbs cross-reactive with human and mycobacterial hsp65 a broad intracellular distribution of hsp65 had been reported both in rats with experimentally induced arthritis and in human RA (De Graeff-Meeded et al., 1990). In another study by Karlsson-Parra et al., (1990) showed that a mAb to mycobacterial hsp65 (ML 30) reacts with material from RA joints but not from normal joint tissues or inflamed tissues, suggesting that the expression of hsp65 in the joint is
RA specific. In the present study using the new mAbs to human hsp58, endogenous hsp58 is demonstrated in synovial tissues from RA, OA as well as in normal. Moreover there does not appear to be an increased expression of the hsp in RA. On the contrary the inflammatory control (OA) synovial tissues used in the present study consistently showed more intense cytoplasmic staining of large mononuclear cells in the deep tissue. This observation is also inconsistent with another recent report by Kleinau et al., (1991) who had claimed to have shown increased expression of rats hsp60 in the joint tissues from rat with AA and collagen induced arthritis. Kleinau et al., (1991) used the mAb ML 30 which does not appear to be monospecific since the authors showed an immunoblot where it bound to at least three bands on western blots of lysates of inflamed synovia from collagen induced arthritis. Moreover an irrelevant isotype matched control used by this group also bound to one of the three bands picked up by ML 30. The new mAbs used in the present study have not yet been checked on lysates from inflamed synovia, but on cell lysates from PBMC and a Jurkat T cell line they only bind to a single band at 58 kDa. And since they gave a similar pattern of staining on both RA and OA synovial tissues, the expression of hsp58 in joint tissues appears to be an indication of inflammation rather than RA. The previous reports using ML 30 (Karlsson-Parra et al., 1990 & Kleinau et al., 1991) are likely to be due to lack of specificity, though it remains interesting that a monoclonal to a mycobacterial antigen binds to RA tissue, even if the epitope in question is not confined to the hsp58.

It has been claimed that 10-20% of the T cells which respond to M. tuberculosis are specific for hsp65, (Kaufmann 1990), and T cells from sites of inflammation show more augmented immune response to hsp65 than those from peripheral blood (Res et al., 1988; Gaston et al., 1988 and 1989). This may be because of the high expression of the endogenous hsp65 in inflamed joints which leads to accumulation and constant re-stimulation of the T cells at the site of inflammation. Stressed host cells can serve as targets of T cells against hsp65 and macrophages pulsed with gamma interferon or hsp65 can be lysed with T cells with specificity for hsp65 (Koga 1989). This cytotoxic effect of the T cells may extend to other cells (e.g. chondrocytes near the pannus in joints) bearing
autologous hsp65. Thus an autoimmune response to the bacterial hsp65 could result in tissue restricted inflammatory disease like RA. However, as discussed in Chapter 1, much doubt now surrounds the claim that there is increased T cell responsiveness to hsp in RA joint, and the frequency of such T cells may be normal, or simply exaggerated due to impurities in the mycobacterial hsp65.
CHAPTER EIGHT

GALACTOSYLATION STATE OF THE ANTI-GlcNAc AND ANT-HSP65 ANTIBODIES: A POSSIBLE LINK BETWEEN IMMUNE RESPONSES TO HSP AND TERMINAL GlcNAc.
CHAPTER 8

GALACTOSYLATION STATE OF THE ANTI-GlcNAc AND ANT-HSP65 ANTIBODIES: A POSSIBLE LINK BETWEEN IMMUNE RESPONSES TO HSP AND TERMINAL GlcNAc.

8.1. INTRODUCTION

Agalactosyl IgG is raised in RA (Parekh et al., 1985) and RA patients have raised levels of IgG antibody to mycobacterial hsp65 (Bahr et al., 1988b & Tsoulfa et al., 1989b). These two phenomena do not appear to be linked. However, in several rodent models of arthritis there appears to be a correlation between an increase in the levels of agalactosyl IgG and antibody to mycobacterial hsp65. Thus mice with pristane induced arthritis show evidence of antibody response to mycobacterial hsp65 (Thompson et al., in preparation) and this antibody response rises in parallel with the levels of agalactosyl IgG (Rook et al., 1991a). A similar situation arises in mice infected with BCG (Rook et al., 1990b). Therefore, the correlation between the raised levels of agalactosyl IgG and antibody to mycobacterial hsp65 may not be a coincidence. It is possible that the glycosylation defect leading to the formation of agalactosyl IgG, which is believed to be due to a decrease in the levels of the enzyme β-galactosyltransferase (Axford et al., 1988), is induced by a cross-reaction involving the mycobacterial hsp65 or an autoantigen cross-reactive with this hsp. In the present chapter the glycosylation state of the affinity-pruified anti-GlcNAc and anti-hsp65 was determined, and the possibility that the anti-hsp65 or the new mAbs to the human homologue of this hsp may cross-react with β-galactosyltransferase was also explored.
Sources of anti-GlcNAcs and anti-hsp65

The anti-GlcNAc antibodies assayed for agalactosyl IgG are those obtained from Chapter 4. Human anti-hsp65 antibodies were obtained from an affigel column of *M. bovis* hsp65 as described in Chapter 5 (Section 5.2.5). Three samples of Anti-hsp65 were used, two from RA patients and one from a tuberculosis patient.

8.2.1. IMMUNOASSAY FOR AGALACTOSYL IgG.

An earlier version of this assay which involved the detection of agalactosyl IgG on nitrocellulose membrane has been described elsewhere in detail (Filley et al., 1989). Following the binding of the IgG molecules to protein A coated membrane they are denatured to expose terminal GlcNAc and subsequently detected with the anti-GlcNAc mAb GN7. The assay used in the present study is a much improved ELISA based method developed by Dr. Rook and now in routine use in his department.

A stock solution of protein A (Sigma P-6031) was prepared at 2.5 μg/ml in PBS (pH 7.3) and 50 μl of the solution was placed into each well of the ELISA plate (Nunc-immunoplate maxisorp F-96) and incubated overnight at 4 °C. The protein A solution was then discarded and the wells were blocked with 100 μl of 1% BSA/PBS/Tween 20 for 1 hour at 37 °C. The plate was washed three times (approximately 10 minutes for each wash) with PBS/Tween 20, and then 50 μl of the affinity-purified antibodies were added to the wells in duplicate at concentrations of 50 μg/ml, 100 μg/ml and 200 μg/ml. Standard sera of known agalactosyl IgG values were also added in duplicate in each plate. The affinity-purified antibodies and standard sera were diluted in a buffer containing 0.75 g of glycine per 100 ml and 0.935 g of sodium chloride per 100 ml,
adjusted to pH 7.0 with sodium hydroxide. Following a 2 hours incubation the plate was washed (the final wash was in PBS only) and 50 μl of PBS was added to all wells in the plate. The plate was then placed carefully (making sure no air bubbles were trapped underneath the plate) in a waterbath at 85 °C for approximately 8 minutes to denature the protein A bound IgG. After cooling the plate to room temperature, PBS was discarded from the wells and 50 μl of the biotin-labelled anti-GlcNAc mAb, GN7, was added at 2 μg/ml in 1% BSA/PBS/Tween 20 and incubated overnight at 4 °C. The plate was washed and peroxidase-labelled streptavidin (DAKO P397) added at 1 μg/ml. Following a one 1 hour incubation at 37 °C the ELISA was developed with ABTS substrate in the usual way. A standard curve was constructed using the Gal(0) values of the standard sera and their corresponding optical density values. The agalactosyl IgG values of the affinity-purified human antibodies were then read off this standard curve. Almost identical values of Gal(0) were obtained for the affinity purified antibodies at 100 μg/ml and 200 μg/ml, indicating that at these concentrations protein A on the ELISA wells was completely saturated.

8.2.2. ELISA TO CHECK CROSS-REACTIVITY BETWEEN THE ENZYME β-GALACTOSYLTRANSFERASE AND THE HSP60 FAMILY.

Microtitre ELISA plates were coated with β-galactosyltransferase from bovine milk (Sigma G-5507) at 0.25 U/ml as described in earlier chapters. The affinity-purified anti-hsp65 from the affigel column of M. bovis hsp65 (Chapter 5) was biotin labelled and placed at 20 μg/ml and diluted across the ELISA plate. The mAbs to human hsp58 were also applied at 20 μg/ml and diluted as above. After a 2 hours incubation period at room temperature, the binding of the affinity-purified anti-hsp65 from RA sera was detected with peroxidase-labelled streptavidin (DAKO P397) and the binding of the mAbs was detected with peroxidase-labelled anti-mouse immunoglobulins (DAKO P260). The ELISA was developed in the usual way.
8.3.1. RESULTS

The percentage agalactosyl IgG of the anti-GlcNAc antibodies obtained from pooled normal and RA sera is shown in Table 8.1. The proportion of agalactosyl IgG in the anti-GlcNAc antibodies is similar to that found in the original sera and the unbound sera from the column suggesting that the IgG anti-GlcNAc antibodies were galactosylated to the same extent as normal IgG. A similar result was obtained for the anti-hsp65 antibodies from RA and tuberculosis patients (Table 8.2). Neither the affinity-purified nor the mAbs to the human hsp58 bound to the enzyme β-galactosyltransferase in ELISA assays.
Table 8.1. Percentage agalactosyl IgG in the affinity-purified human anti-GlcNAc antibodies

<table>
<thead>
<tr>
<th>Anti-GlcNAc/Sera</th>
<th>Percentage agalactosyl IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled normal serum</td>
<td>28.13</td>
</tr>
<tr>
<td>HGN5</td>
<td>27.00</td>
</tr>
<tr>
<td>HGN7</td>
<td>26.40</td>
</tr>
<tr>
<td>Unbound from pooled normal serum</td>
<td>27.20</td>
</tr>
<tr>
<td>Pooled RA serum</td>
<td>37.12</td>
</tr>
<tr>
<td>HGN6</td>
<td>36.00</td>
</tr>
<tr>
<td>HGN8</td>
<td>37.30</td>
</tr>
<tr>
<td>Unbound from pooled RA serum</td>
<td>35.08</td>
</tr>
</tbody>
</table>
Table 8.2. Percentage agalactosyl IgG in the affinity-purified anti-hsp65 antibodies from RA and tuberculosis sera.

<table>
<thead>
<tr>
<th>Anti-hsp65 antibodies/Sera</th>
<th>Percentage agalactosyl IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original RA serum no. 1</td>
<td>47.00</td>
</tr>
<tr>
<td>Anti-hsp65 from serum no. 1</td>
<td>47.00</td>
</tr>
<tr>
<td>Unbound from serum no. 1</td>
<td>43.00</td>
</tr>
<tr>
<td>Original RA serum no. 2</td>
<td>39.15</td>
</tr>
<tr>
<td>Anti-hsp65 from serum no. 2</td>
<td>37.00</td>
</tr>
<tr>
<td>Unbound from serum no. 2</td>
<td>37.00</td>
</tr>
<tr>
<td>Original tuberculosis serum</td>
<td>51.38</td>
</tr>
<tr>
<td>Anti-hsp65 from tuberculosis serum</td>
<td>ND</td>
</tr>
<tr>
<td>Unbound from tuberculosis serum</td>
<td>51.24</td>
</tr>
</tbody>
</table>

ND= Not determined. There was not enough of affinity-purified anti-hsp65 from the tuberculosis patient to evaluate the percentage agalactosyl IgG.
The percentage of agalactosyl IgG in the affinity-purified human anti-GlcNAc antibodies in similar to that found in the original sera. Therefore the IgG anti-GlcNAc antibodies do not contain a higher proportion of agalactosyl IgG than the original sera. Similarly, the affinity-purified anti-hsp65 from RA sera were also galactosylated to the same extent as the other IgG antibodies from the same serum. Moreover, the mAbs to the human hsp58 and the affinity-purified anti-hsp65 did not bind to the enzyme β-galactosyltransferase thus discounting any cross-reactivity which could lead to a decreased level of this enzyme. Therefore it is likely that the two phenomena: the glycosylation defect leading to the formation of agalactosyl IgG and immune response to hsp65, are not directly related to each other but independantly contribute to the pathogenesis of RA. However, a recent report by Rook et al., (1990b) suggested that a down regulation of the enzyme β-galactosyltransferase may occur due to dysregulation of B cells by cytokines released during T cell mediated tissue damaging pathology, and that the association between the levels of agalactosyl IgG and antibody response to hsp65 is probably due to increased production of the hsp by stressed host cells or organisms in the damaged tissue. Such a view is consistent with another recent finding that there is a concurrent rise in the levels of agalactosyl IgG in transgenic mice which over express human IL-6 (Rook et al., 1991a). Another possible explanation for the correlation might be that this glycoform of IgG, which can bind to a variety of glycoproteins (Rademacher et al., 1988), is cross-linked onto hsp65 by one of these glycoproteins or that the agalactosyl IgG is able to bind directly to this hsp. Nevertheless, a cross-reactivity between hsp65 and an enzyme involved in the glycosylation of the IgG molecule (other than β-galactosyltransferase) might still be relevant. In this context, it is interesting to note that the 9 amino acid residues (180-188) of the mycobacterial hsp65 which is recognized by the arthritogenic rat T-cell clone show considerable sequence homology (6/9 amino acids are identical) with a peptide of the enzyme α-galactosidase which
hydrolyses galactose residues (Professor Gupta, unpublished observation). The significance of this similarity remains to be determined. It would be interesting to see if any of the mAbs to the human hsp58 or the affinity-purified anti-hsp65 bind to this enzyme.
CHAPTER NINE

FINAL DISCUSSION
FINAL DISCUSSION

Using a mouse mAb which binds to terminal GlcNAc the distribution of terminal GlcNAc residues in normal and diseased synovial tissues has been studied. In normal tissues including synovium there is little or no staining of GlcNAc. In contrast, widespread staining of terminal GlcNAc occurs in synovial tissues from RA patients. Moreover, a unique reticular extracellular staining of GlcNAc was seen only in synovial tissues from patients with definite RA suggesting a specific role for GlcNAc in RA (Chapter 2 & Sharif et al., 1990). Materials bearing terminal GlcNAc may act as an inflammatory stimulus in RA, either by acting as an autoantigen or by interacting with lectin-like receptors in macrophage membranes (Ross et al., 1985) which may also recognise GlcNAc on bacterial material. Thus an immune response to GlcNAc moieties on bacterial PG/PS complex may lead to recognition of self-antigen bearing GlcNAc.

The possible sources of terminal GlcNAc in the inflamed synovium have been discussed in Chapter 2. Although it seemed unlikely that this material is of bacterial origin, it cannot be ruled out completely, since bacterial components have been identified in the joints of rats following an intraperitoneal injection of streptococcal cell wall fragments (Cromartie et al., 1977; Lehmann et al., 1983 and 1984).

Double labelling experiments using GN7 and mAbs to bacterial sonicates including mycobacterial, on RA joint tissues may resolve this question.

The presence of a subset of macrophages which transiently express membrane GlcNAc in culture and the presence of similar cells in cytospins of fresh RA synovial fluid (Chapter 3 & Sharif et al., 1989) provides further evidence for GlcNAc-directed autoimmunity in RA. The possible functions of this novel subset of macrophages have been discussed in Chapter 3. It is unusual for GlcNAc to be expressed without having
some sort of signalling function but in the case of these cells the signalling function is not
known. It is remotely possible that these cells are involved in macrophage-lectin
interactions on some other cell surface and so the effect of anti-GlcNAc on T cell
proliferation in response to various antigens was tested. However the results of these
experiments are not presented because they could not be interpreted. In addition since it
was not possible to modulate the amount of terminal GlcNAc expressed with a variety of
mediators, no conclusion can be drawn as to the function of these cells from the present
study. Nevertheless, the presence of these cells in fresh synovial fluid from RA patients
represents an extra site for GlcNAc or anti-GlcNAc mediated damage in RA.

There was no evidence of a raised level of antibody to terminal GlcNAc in RA
suggesting that this sugar molecule is not inherently immunogenic in RA (Chapter 4).
The specificity of the anti-GlcNAc antibodies from both normal and RA pools appeared
to be similar and antibodies from both pools bind to denatured agalactosyl IgG but not to
intact native IgG molecules. In vivo damage to agalactosyl IgG by proteolytic enzymes,
free radicals or other oxidative species may lead to partial degradation of the IgG
molecule (Blake et al., 1989) exposing terminal GlcNAc and therefore formation of
immune complexes between agalactosyl IgG and anti-GlcNAc antibodies remains a real
possibility.

The investigation of cross-reactivity between the highly conserved mycobacterial
hsp65 and RA tissues has revealed interesting data. There are large quantities of a 65
kDa protein in synovial fluid and immune complexes from RA patients, which cross-
react with mycobacterial antigens as demonstrated by the reactivity of a mAb raised to
mycobacterial antigen (SF8) and a polyclonal antibody to hamster hsp65 (Chapter 5).
However, although the mAb SF8 was raised against mycobacterial antigens it bound
very strongly to the 65 kDa molecule on western blots of RA synovial fluid but rather
poorly to the recombinant M. bovis hsp65 or purified human hsp58. In addition the
mAbs raised against the human homologue of the bacterial hsp65 do not bind to the 65
kDa protein in synovial fluid suggesting that this protein is not the same as human hsp58
or that it is an artefact. Nevertheless, it is possible that the synovial fluid molecule has undergone structural changes e.g. through glycosylation (this may account for the increased molecular mass) or it may be the product of a different gene of the hsp60 family (Waldinger et al., 1988).

The human homologue of the mycobacterial hsp65 appeared to be highly immunogenic since it was possible to evoke antibodies to this protein by immunizing Balb/c mice using FIA for primary immunization and MPL and TDM as adjuvants for the final intravenous boost (Chapter 6). All the mAbs produced (9 IgM and 1 IgG) reacted with mouse tissues suggesting that they are autoantibodies and picked out a single band of 58 kDa on lysates of murine as well as human cells. It has not been possible to estimate the precise epitopes recognised by the mAbs on the P1 protein using the limited resources available. Further studies using Pepscan may be useful in mapping the mAb epitopes. This procedure allows simultaneous synthesis of small quantities of up to several hundred peptides on activated polyethylene rods arrayed in a microtiter plate pattern. After synthesis the peptides remain attached to the rods and their reactivity with the mAbs can then be analysed in simple ELISA assays. Pepscanning has not been carried as part of the present study for several reasons. First, it is an expensive procedure and secondly the importance of the mAb epitopes in RA should be established prior to their precise identification. The epitopes appeared to be highly autoimmunogenic in mouse and it is likely that these epitopes may also be autoimmunogenic in man. This may be checked using inhibition assays with RA sera and if RA sera block the binding of the mAbs then the identification of the mAb epitopes on the P1 protein would be extremely useful.

Minute quantities of free hsp58 in both synovial fluid and serum was found in some RA patients in capture assays using the new mAbs to this hsp. The presence of the free hsp58 in RA synovial fluid has been confirmed in immunoprecipitation assays. This finding is interesting because no free hsp58 could be detected in the small numbers of normal sera tested. In future studies large numbers of sera and synovial fluid from RA,
disease controls and normal controls should be tested for the presence of free hsp58. The presence of a mycobacterial hsp65 cross-reactive molecule has been reported in the sera of NOD mice just before the clinical onset of IDDM (Elias et al., 1990). However these investigators used a rabbit polyclonal antibody to the mycobacterial hsp65 and in view of identification of the 64 kDa beta-cell autoantigen in IDDM as glutamic acid decarboxylase (Baekkeskov et al., 1990), this study ought to be repeated using well defined mAbs such as the ones used in the present study.

Immunohistochemical studies with the new mAbs led to a need to review and reinterpret earlier claims by Karlsson-Parra (1990) of an increased expression of the hsp58 in RA synovial tissue. The present study have exposed several errors. First, the protein band at 65 kDa in RA synovial fluid usually regarded as the human hsp is not the human homologue of the bacterial hsp65. Secondly, essentially all cells express the hsp58, as expected since it is a constitutively expressed mitochondrial protein. Finally there is no relative increase in the expression of this hsp in RA joint tissues, though the distribution is somewhat different. The implications of the presence of hsp58 in the joint tissues have been discussed in Chapter 7. It will now be important to discover whether this altered distribution of the hsp is a primary mechanism, or whether oxidative or inflammatory stress secondary to the disease lead to the extracellular release of the hsp (Chapter 7, Figure 7.10), and so to the chronic stimulation of the lymphoid tissue characteristic of RA. That responses to mycobacterial hsp65 may be associated with the primary event is suggested by the finding that T cell responses to it are present in the joint (Holoshitz et al., 1989) particularly in early disease (Rees et al., 1988). However, these authors have used mycobacterial hsp65 which was contaminated with lipopolysaccharides, and with E. coli GroEL. In addition the frequency of T cells responding to hsp65 has not been looked at nor has the response been related to tuberculin positivity. Future study should be aimed at documenting the frequency of such T cells (using contaminant free hsp65) and their ability to recognize the human hsp58, or other antigens of the joint. The γδT cells are believed to be biased toward
mycobacterial antigens (Janis et al., 1989 & Modlin et al., 1989) particularly to hsp65 (O’Brien et al., 1989). The arthritogenic rat T cell clones and neonatal thymic γδT cells from mice recognize overlapping hsp65 sequences 180-188 and 180-196 respectively (Born et al., 1990). Moreover αβT cell responses to the latter sequence of *M. leprae* hsp65 have been found in all species studied (Watson 1989). The rat hsp65 has not been sequenced and the corresponding human protein shows little homology with the adjuvant arthritis T cell epitope (amino acid residues 180-188). Nevertheless, it will be interesting to see if γδT cells responsive to mycobacterial hsp65 also respond to human hsp58 or to some quite different cross-reactive autoantigen, such as the cartilage proteoglycan implicated by van Eden et al., (1987) in the rat adjuvant arthritis model.

There is some evidence of membrane expression of hsp58 or of cross-reactive molecules of similar molecular mass (Jajour et al., 1989 and 1990). The mAbs generated in the present study can be used to confirm surface expression of hsp58. If membrane expression is confirmed, the identification of cell types and events leading to surface expression should be a priority. The functions of the membrane bound hsp58 is not yet clear, but there are several possibilities. First, it may interact with γδT cells. Secondly, since hsp70 and hsp60 families have similar chaperonin functions and a constitutive member of the hsp70 family, found on surface membranes of macrophages and B cells, has been shown to be involved in presentation of cytochrome c to T cells (Vanbuskirk et al., 1989), hsp58 may also have a role in antigen presentation.

A raised level of IgG antibodies to mycobacterial hsp65 in RA sera has been documented by two separate studies (Bahr et al., 1988b & Tsoulfa et al., 1989b) and at least some of these IgG antibodies are autoantibodies (Chapter 5; Lydyard et al., 1990a and 1990b). In the present study a generalized rise in antibody levels of all three major classes to mycobacterial hsp65 was found in RA synovial fluid. However, the IgA and IgG antibodies to the human hsp58 showed a statistically significant rise in RA synovial
fluid and there appeared to be a correlation between the levels of IgG antibody to mycobacterial hsp65 and IgG antibody to the corresponding human protein (Chapter 5). Therefore it is likely that the formation of these IgG antibodies may have been triggered by mycobacterial antigen or antigens cross-reactive with mycobacteria.

The T cell and antibody responses to hsp65 discussed above have been looked at using recombinant hsp which is unlikely to be present in the same conformation as the corresponding protein in vivo. This view is supported by the finding in Chapter 7 that the mAb 1A8/20 which bound to a M. leprae peptide 52 but did not bind to the corresponding amino acid residues on the P1 protein. Therefore, both the T cell and antibody responses to the hsp should be repeated using affinity-purified hsp58 from the joint where it would be somewhat modified but correctly folded.

Apart from the formation of immune complexes, antibody to hsp58 may damage cells expressing this molecule on their surface or interfere with their regulatory function(s). It is unusual for antibody to block T cell responses. However, in view of the recent reports that the responses of murine T cells (Dr. D.B Lowrie personal communication to Dr. Rook) and γδ T cells (Fisch et al., 1990) to antigens of the hsp60 family can be blocked by antibody, it is possible that these molecules are presented without being fully degraded or processed as peptides (i.e. hsp58 may be a superantigen). In this case, the antibodies to hsp58 may block or modify T cells responses to these hsps. The new mAbs to the human hsp58 will be invaluable in checking such phenomena.

The two cross-reactivities investigated appeared to be distinct and may contribute to the pathogenesis of RA independently. The hypothesis that immune responses to hsps may result in the reduced activity of the enzyme β-galactosyltransferase and hence increased production of the IgG molecule with terminal GlcNAc (Roitt et al., 1988) seems unlikely in view of the results obtained in Chapter 8. However, further studies
may uncover close association between the two. The effect, if any, of either hsp58 or mAb to it on the galactosylation of the IgG should be checked. The human B cells secreting IgG and IgM rheumatoid factors are often CD5 positive and occur with increased frequency in cord blood and RA (Caseli et al., 1987; Maini 1988; Plater-Zyberk et al., 1985). Therefore, it is interesting to note that though the percentage of agalactosyl IgG is low in cord blood it rises suddenly between 3 and 6 months, and there is also a burst of production of antibody to hsp65 at this time (Dr. C. A. Pilkington, personal communication). This may be due to a burst of antibody production by CD5+ cells at this time. Moreover, the polypeptide chain of a chaperone protein, PapD, which mediates the assembly of pili in *E. coli* shows 26% amino acid sequence homology with the human lymphocyte differentiation antigen (CD5 or Leu-1) and it is structurally similar to the immunoglobulin superfamily (Holmgren & Bränden 1989). Therefore an interesting proposition would be that CD5 positive B cells produce agalactosyl IgG, and express hsp58 or a cross-reactive antigen on their surface. Future study should look for sequence similarities between PapD, CD5 and hsp58 and test CD5 positive B cells for surface expression of hsp58. The effect of mAbs to hsp58 on CD5 positive B cells producing IgG should also be investigated to see whether the percentage of IgG with terminal GlcNAc is affected.

Finally passive immunization of mice with purified mAbs to hsp58 may alter disease susceptibility. An infection by mycobacteria or mycobacterium-like organisms can lead to the availability of both terminal GlcNAc and hsp65. Abnormal exposure of both of these potential autoantigens have been demonstrated in RA joint tissues. Therefore, mycobacteria-directed cross-reactivity involving terminal GlcNAc and hsp65 may initiate and perpetuate the autoimmune responses in RA.

This study has opened up a wide area of research and also generated suitable reagents to facilitate such investigations. The further studies outlined above may lead to better understanding of the aetiopathogenesis of RA and possible development of novel immunotherapeutic approaches for effective prevention and treatment of RA.
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213


PUBLICATIONS FROM THE THESIS


Membrane N-acetylglucosamine: expression by cells in rheumatoid synovial fluid, and by pre-cultured monocytes


Departments of Medical Microbiology and *Rheumatology, University College and Middlesex School of Medicine, London, UK

Received for publication 18 July 1988
Accepted for publication 2 June 1989

Summary. After 21–48 h in culture, 2–8% of human peripheral blood monocytes strongly express terminal N-acetylglucosamine (GlcNAc) on their membranes. This can be detected with a monoclonal antibody selected for binding to asialo-agalacto-fetuin, and is eliminated by incubating the cells in pure N-acetylglucosaminidase. Expression of GlcNAc is transient, and can no longer be detected by day 4. These cells are a subset of macrophages since they are positive for non-specific esterase and stained by the monoclonal antibody EBM11. GlcNAc-positive cells showing double staining with monoclonal antibodies UCHM1 and RFD7 were detected. Their numbers were not influenced by the addition of GM-CSF, IFN-γ, 1,25-(OH)2 cholecalciferol or indomethacin. Macrophages which give membrane staining for terminal GlcNAc were also found in rheumatoid synovial fluid, and in synovial tissue, though in the peripheral blood their frequency was the same in samples from normal donors and from patients with rheumatoid arthritis.

Immunoblots of 24–48-h monocyte cultures or of fresh synovial fluid cells using the anti-GlcNAc monoclonal, show the anticipated agalactosyl IgG heavy chains, and an additional band of 70–80kDa.

Keywords: N-acetylglucosamine, rheumatoid arthritis, monocytes

Recently we selected a monoclonal antibody binding to asialo-agalacto-fetuin (Rook et al. 1988) from mice immunized with the cell wall/polysaccharide complex of Group A streptococci. This monoclonal binds to oligosaccharides bearing terminal N-acetylglucosamine. It was raised because of the observation that in patients with rheumatoid arthritis (RA) the conserved N-linked biantennary oligosaccharide on asparagine 297 of the CΗ2 domain of the IgG molecule tends to lack the terminal galactose (Parekh et al. 1985). Therefore in RA this oligosaccharide terminates with GlcNAc more frequently than is normal, and our monoclonal can be used for the rapid immunoassay of this glycoform of IgG (Filley et al., 1989). We selected Group A streptococci as the immunogen because it is rich in GlcNAc (Esser et al. 1985), has adjuvant properties, and is associated with another arthritic condition, rheumatic fever. These patients have raised levels of antibody to GlcNAc (McCarty 1958).

In view of the possible link between immunoreactive GlcNAc and arthritic conditions...
we have used this monoclonal to screen cell populations from synovial fluid and peripheral blood for the presence of terminal GlcNAc. We report here that a monoclonal to terminal GlcNAc (GN7) binds very strongly to some cells in rheumatoid synovial fluid, and also to a small subset of peripheral blood monocytes which appears transiently after 21-48 h in culture. This is an unusual finding because exposed terminal GlcNAc is rare except in intracellular sites. It is usually masked by galactose residues, which may themselves be sialylated. We suggest that the transient ‘flashing’ of terminal GlcNAc is likely to constitute a signal to other cells, the significance of which is not yet clear. Evidently Group A streptococci can evoke antibody to these cells, and autoimmunity to oligosaccharides terminating in β1-linked GlcNAc may have pathogenetic significance.

Materials and methods

Monoclonal antibodies

The monoclonal antibody to terminal GlcNAc (GN7) has been described elsewhere (Rook et al. 1988). Briefly, Balb/c mice were immunized with the peptidoglycan/polysaccharide complex of Group A streptococci. Supernatants of hybridomas were initially screened by ELISA on a neoglycoprotein formed by conjugating the diazonium derivative of p-aminophenyl-GlcNAc (Sigma) onto bovine serum albumin. Then the antibodies were screened on HPLC purified fetuin which had been digested with neuraminidase from Arthrobacter ureafaciens (Boehringer Mannheim) and 6 units/ml of Jack Bean β-galactosidase in 0.1 M sodium acetate buffer, pH 4.0, in order to expose terminal GlcNAc on the biantennary oligosaccharides. GN7 is an IgM antibody, and another IgM monoclonal (SF5) binding to neutrophil nuclei was used as a control.

The other monoclonal antibodies used were:

2. RFD1, an IgG binding to a determinant associated with MHC class II as it is expressed on dendritic cells, and a subset of monocytes (Poulter et al. 1986).
3. RFD7, an IgG1 binding to a 77kDa antigen on mature macrophages, and some monocytes (Poulter et al. 1986).
4. RFT1, an IgG1 which identifies human T cells (Janossy & Prentice 1982).
5. UCHM1, an IgG2a binding to a 52kDa antigen on most monocytes, and endothelial cells (Hogg et al. 1984).
6. SF5 is an IgM antibody binding to neutrophil nuclei raised in this laboratory (unpublished observations). This was used throughout as an isotype matched control for GN7.

RFD1, RFD7, & RFT1 were gifts from Dr L. Poulter, Royal Free Hospital, and UCHM1 was a gift from Dr P. Beverley, University College Hospital, London.

Culture of human monocytes

Sixty millilitres of blood from normal donors were defibrinated by shaking with glass beads in a glass universal, and the mononuclear cells were separated by centrifugation over lymphocyte separation medium (Flow Laboratories, Irvine, Ayrshire, Scotland). The cells were washed, suspended in RPMI 1640 supplemented with 10% autologous serum, and incubated in 80-cm² flasks which had been pretreated with undiluted autologous serum. After 2 h, non-adherent cells were removed by rinsing three times with RPMI 1640. Then the monocyte-enriched population was detached by incubation at 37°C with RPMI containing 10% autologous serum and 3.3 mg/ml disodium EDTA. After 45 min, cells were removed by gentle pipetting, centrifuged once, and resuspended in RPMI at 10⁵ cells/ml. In some experiments cyto spun preparations were made from part of this suspension to obtain 2-h cultured monocytes. The remaining cells were supplemented with 20% autologous serum and this suspension was plated at 10⁵
Membrane N-acetylglucosamine

569

cells/well in tissue culture wells formed by clamping a silicone rubber culture chamber (Bellco Glass Co., Vineland, NJ) onto tissue-culture quality slides cut from 80-cm² culture flasks similar to those used for the first 2-h culture. For a few experiments, glass slides were also used. Any further additions to the wells were added in RPMI, to give a final volume of 0.2 ml, with 20% serum. These cultures were maintained for varying periods, then rinsed gently five times with RPMI 1640, dried at room temperature overnight, and stained within 24 h.

Agents used to activate the cultures

The vitamin D₃ metabolite (1,25-(OH)₂ cholecalciferol) was a gift from Dr M. Uskovovic, Hoffman La Roche, Nutley, NJ, USA. It was stored as a solution in ethanol at −20°C and diluted before use so that ethanol was present in all cultures at a final concentration of 1%. The final concentration was 10⁻⁷ M. Recombinant gamma interferon (rIFN-γ) was a gift from Boehringer Ingelheim, Vienna, Austria. Recombinant GM-CSF was a gift from Biogen. For both these cytokines the units referred to in the text are those indicated by the suppliers. Indomethacin was obtained from Sigma, dissolved in ethanol, and added to appropriate wells at a final concentration of 1 or 10 μg/ml.

Immunochemistry

Monocyte preparations were fixed in methanol for 10 min. They were then washed in 10 mM phosphate buffered saline (PBS, pH 7.4) for 10 min with constant agitation. Excess PBS was removed and 50 μl of monoclonal antibody appropriately diluted in PBS was applied to each preparation and incubated in a humid chamber for 40 min. All incubation for immunocytochemistry was carried out at room temperature. The preparations were then washed and treated with 1% hydrogen peroxide in methanol for 30 s to block endogenous peroxidase. Then after further washes the cell monolayers were incubated for 35 min with peroxidase-labelled, rabbit anti-mouse immunoglobulin (DAKO P260) diluted 1/40 in PBS. The preparations were washed, and incubated with substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (BDH, 13033), and 0.01% hydrogen peroxide in PBS for 5-10 min. The slides were then washed and counterstained with Harris’s haematoxylin, washed in running tap water, mounted in Apathy’s media or dehydrated (glass slides only) in graded alcohol, cleared in CNP 30 (Penetone, Northumberland) and mounted in histomount. For all experiments controls included the isotype-matched IgM monoclonal SF5, and a second layer control to which no monoclonal had been added.

Non-specific esterase

Monocyte preparations were incubated with diazotized pararosaniline and α-naphthyl acetate at pH 7.1 for 5 min at 37°C. The preparations were then washed in PBS and stained for GlcNAc using the peroxidase method above with the addition of 15 μl/ml of 8% nickel chloride to the developing solution to produce a blue-black reaction product. Haematoxylin counterstaining was omitted.

Immunofluorescence of fixed cells

Monocyte preparations were fixed in methanol for 10 min, washed in PBS for 10 min and then incubated with appropriately diluted monoclonal antibodies in PBS for 30 min in a moist chamber. After further washes they were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (DAKO F261) for 30 min, washed thoroughly in PBS and mounted in CITTIFLUOR glycerol/PBS solution containing an anti-fading agent AF1. Double staining was performed by simultaneous incubation with anti-GlcNAc and one of a range of mouse IgG monoclonal antibodies followed by goat anti-mouse IgM.
rhodamine conjugate (Sera-Lab SBA 1020-03) and goat anti-mouse IgG FITC-conjugate (Sera-lab SBA 1030-02). In each experiment, we checked that cells stained with GN7 showed no positivity when the anti-mouse IgG was used as the second layer, and conversely that the anti-IgM did not bind to IgG. Controls using SF5 rather than GN7 were also included.

**Immunofluorescent staining of live cells in suspension**

Cell suspensions were incubated with monoclonal GN7 diluted 1/200 in RPMI 1640, containing 0.1% sodium azide at 4°C, in order to minimize capping. Suspensions were washed three times in the same medium by centrifugation. The second layer was the FITC-conjugated rabbit anti-mouse Ig. An irrelevant IgM monoclonal (SF5, binding to neutrophil nuclei) was used as a negative control, and anti-la as a positive control.

**Preparation of Western Blots from lysates of cultured monocytes**

Lysates of cultured monocytes were prepared in two ways. In some experiments monolayers of monocytes in tissue culture flasks were washed repeatedly with RPMI 1640 and then lysed with 0.1 M citric acid containing 100 µl of Zaponin (Coulter Electronics Ltd, Northwell Drive, Luton, Beds, UK) per 25 ml. This procedure avoided lysis of nuclei and consequent contamination with DNA. Nuclei were subsequently removed from the preparation with high-speed centrifugation. In other experiments cells were detached from the plastic, pelleted by centrifugation, and lysed by the addition of 3% SDS and 5% urea in order to eliminate any possibility of autolysis. DNA was broken up with brief sonication. Protein content of the lysate was measured with the Biorad protein estimation kit.

Lysate (approximately 25 µg for Aurodye or Ink staining, and 40-80 µg for staining with monoclonal antibodies) was loaded into 6 mm wells and resolved by SDS-PAGE on slab gels (1.5 mm thick) of 12.5% acrylamide according to the discontinuous buffer system of Laemmli (1970). Electrophoresis was performed on a vertical slab gel unit (Hoefer SE600) overnight at 4°C at constant current using 6 mA per gel. A mixture of standard protein markers (MW-SDS-200, Sigma) were used for the determination of molecular mass. Proteins were transferred from gels to nitrocellulose membranes (0.2 µm, BA83 Schleicher and Schuell, FRG) using a semi-dry electroblotter as described by Kyhse-Andersen (1984). The standard blotting time of 60 min was prolonged to 80 min to ensure penetration of protein into the membranes. In order to visualize protein bands, the nitrocellulose membranes were washed with excess PBS supplemented with 0.05% (vol/vol) Tween 20 once for 120 min at 32°C, and three times for 30 min at room temperature with constant agitation. To visualize bands, blots were then rinsed in water and incubated overnight either in Aurodye, a colloidal gold stain provided by Janssen Life Sciences Products (Belgium), or in India Ink (Pelikan type Fount India) diluted 1/1000 in PBS containing 0.05% Tween 20. Stained blots were air-dried between Whatman 1 paper sheets and kept at −20°C in aluminium foil.

**Identification of protein bands by monoclonal antibodies**

Unstained nitrocellulose membranes were soaked in 1% (wt/vol) bovine serum albumin in phosphate-buffered saline with 0.05% (vol/vol) Tween 20 to block any free protein-binding sites. After blocking, nitrocellulose strips were incubated overnight at 4°C with monoclonal antibodies diluted between 1/200 and 1/1000. Binding was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO P260) at a 1/800 dilution for 2 h at room temperature. IgG heavy chains on Western blots were also identified with a peroxidase-conjugated rabbit anti-human gamma chain (Dako P214).
Treatment of cells or Western blots with \( \beta \)-hexosaminidase

In order to check the specificity of the binding of monoclonal antibody GN7, cytospins, or fragments of Western blots, were incubated overnight at 37\(^\circ\)C in citrate/phosphate buffer at pH 5.0 containing 1 unit/ml of \( \beta \)-hexosaminidase. (This was a gift from Dr G. Jacobs and T. Rademacher, Department of Biochemistry, Oxford, UK.) Replicate samples were incubated in buffer alone. Subsequently all samples were washed, and then incubated in GN7 as described above.

**Results**

**Staining of cells in cytospins of rheumatoid synovial fluid with anti-GlcNAc monoclonal antibody**

Freshly prepared cytospins of rheumatoid synovial fluid showed GlcNAc positive cells with granular cytoplasmic staining. We have previously reported cells with a similar appearance in biopsies of rheumatoid synovial tissue (Sharif et al., 1989). However, RA synovial fluid also contained cells with membrane staining. The membrane location of this staining was most clearly seen when using immunofluorescence to stain live cells in suspension under non-capping conditions by immunofluorescence (Fig. 1). Occasional cells of similar appearance have also been seen in synovial tissue (unpublished observation).

**Staining of monocytes with anti-GlcNAc monoclonal**

Freshly prepared monocytes, or monocytes cultured for up to 5 h, showed no staining with anti-GlcNAc. However, when monocytes from the same donors were cultured in RPMI 1640 with 20\% autologous serum for 21–48 h, a subset of the cells developed intense membrane staining (Fig. 2a). Incubation of the cells in pure \( \beta \)-hexosaminidase eliminated the binding of the monoclonal, whereas incubation in the buffer alone did not (Fig. 2b). No staining of cultured cells (or sections) was observed when GN7 was added in the presence of 0.1 M GlcNAc, whereas staining was not affected by galactose at 0.5 M. Similarly, there was no staining with SF5 or with the anti-mouse Ig alone. Immunofluorescent studies of unfixed cells confirmed the membrane distribution of this staining. Cultured cells occasionally also showed mild to moderate granular staining in their cytoplasm.

The time-course of the percentage of cultured cells expressing the intense membrane GlcNAc is shown in Fig. 3. After peaking at about 21 h, the percentage of positive cells declined progressively and positive cells were not seen by 72 h. The time courses and percentages seen in cultures of cells from ten patients with rheumatoid arthritis did not differ significantly from those seen in cultures from ten control donors (Fig. 3). Further analysis of the data in relation to sero-positivity, age, or sex, revealed no significant correlations.

The expression of this membrane GlcNAc was not significantly changed by culture for 24 h in \( 10^{-7} \) M 1,25 (OH) \(_2\) vitamin D3, 1 \( \mu \)g/ml indomethacin, 200 U/ml GM-CSF, or 200 U/ml gamma interferon.

**Correlation of the expression of membrane GlcNAc with other markers**

The monoclonal EBM 11 stains all macrophages and monocytes (Third Workshop and Conference on Leucocyte Differentiation, and L.W. Poulter, personal communication) and stained essentially all the cells in our cultures; therefore, contaminating cell types were rare (Fig. 4b). Double staining with EBM 11 and GN7 showed that GN7-positive cells are a subset of EBM 11-staining macrophages (Figs 4a and b). Similarly, these cells were non-specific esterase positive (data not shown). Double staining was also seen with GN7 and UCHM1 (data not shown). Some, but not all, RFD7 + cells were also positive for GN7 (Figs 4c and d) though RFD7 + cells
Fig. 1. RA synovial fluid cells stained in suspension under non-capping conditions with the monoclonal anti-GlcNAc antibody GN7. Fluorescein-conjugated rabbit anti-mouse Ig was used as the second layer to demonstrate membrane staining of these cells. Second layer controls, and controls with an IgM monoclonal to an intracellular determinant, showed no staining.

were too rare to allow us to find RFD7+/GN7+ and RFD7+/GN7− cells in the same field. Double staining with GN7 and RFD1 or RFT1 was never seen.

Tentative characterization of the GlcNAc-bearing molecule by SDS-PAGE

Immunoblots of 2-day cultured monocytes, from rheumatoid and normal donors, when stained with anti-GlcNAc, showed the anticipated agalactosyl IgG heavy chain and a band of molecular weight 70–80 kDa (see Fig. 5). When immunoblots of fresh monocytes (2 h), 2-day cultured monocytes, and 4-day cultured monocytes from the same donors, were stained with anti-GlcNAc the 70–80 kDa band was seen clearly in the lysates of 2-day cultured monocytes (Fig. 5) and occasionally, but weakly in the fresh monocytes. The intensity of this band was similar in cells from RA and normal donors.
Membrane N-acetylglucosamine

Fig. 2. Monocytes for 2 days stained with GN7 by the peroxidase method. a. Staining without pretreatment with β-hexosaminidase. A subpopulation of these monocytes shows intense staining, revealed as membrane staining by parallel fluorescent staining of live cells (not shown). b. Staining after treatment with the enzyme. Staining is eliminated.
bands even when fresh cells were used (Fig. 5). Like the 21–48 h cultured peripheral blood monocytes, treatment of immunoblots with pure N-acetylhexosaminidase eliminated all staining by anti-GlcNAc monoclonal.

Discussion

These results indicate that the cells expressing membrane N-acetylglucosamine in the configuration recognized by GN7 are a subset of macrophages. They are also a subset of both UCHM1-positive and RFD7-positive cells. The former antibody stains 60–90% of monocytes cultured on plastic for 24 h, while RFD7 stains approximately 10%. Cells positive for both markers are known to occur (L.W. Poulter, personal communication) and the GlcNAc-bearing cells may fall within this overlapping population, though we have been unable to confirm this. It is likely that these cells result from a reduction in terminal
Fig. 5. Immunoblots of fresh or cultured monocytes/macrophages from blood or RA synovial fluid stained with anti-GlcNAc, GN7. A, unidentified band bearing terminal GlcNAc. B. IgG heavy chain. Lane 1 shows molecular weight markers. Lanes 2–5 show SDS PAGE in lysates of normal monocytes from the same donor, cultured in autologous serum for 2 h, 2, 2 and 4 days respectively. Lane 4 is a replica of lane 3 which was incubated with β-hexosaminidase before staining with anti-GlcNAc. Incubation in buffer alone did not diminish staining. Lane 6 shows monocytes from an RA patient cultured for 2 days, and lane 7 shows synovial fluid adherent cells cultured for only 2 h.

galactose, leading to increased exposure of GlcNAc. It is also possible that a glycoprotein bearing terminal GlcNAc becomes selectively adsorbed onto this small subpopulation of cells. If so, the glycoprotein in question must be present in very low concentration, since Western blots of synovial fluid stained with GN7 reveal only one molecule bearing terminal GlcNAc, and this is the IgG heavy chain.

The glycosylation of membrane components is important for interactions between different cell types, and between cells and biologically active molecules. GlcNAc is expressed on the membranes of many tumour cell lines, and GlcNAc-terminating membrane oligosaccharides will block tumour killing by NK cells (Dennis & Laferte 1985). It is also reported that reversion of a non-metastatic, GlcNAc-expressing tumour cell line to a metastatic phenotype can be achieved by selection of variants resistant to the GlcNAc-binding lectin from Bandeiraea simplicifolia (Dennis et al. 1986). Another group has studied correlations between ex-
pression of various cell membrane lectins and sugars, and the selective binding and homing of tumour cells to different organs, cells and substrates (Gabius et al. 1987). GlcNAc is also involved in cell–cell recognition and cell–substrate adhesion during fertilization and embryonic development (Bayna et al. 1986), and addition of GlcNAc to the medium will inhibit the selective binding of macrophages to apoptotic lymphocytes (Duvall et al. 1985). GlcNAc is also a ligand for the serum 'mannose binding protein' (Taylor & Summerfield 1987) which has conglutinin-like properties (Baatrup et al. 1987).

Thus GlcNAc is not usually terminal, but when it is, it has signalling functions. The significance of the new cell type described here is at present quite unknown. Moreover, until we have formally identified the GlcNAc-bearing membrane component from the cells which evolve in vitro, and confirmed that the same component is present in the cells seen in tissue sections and synovial fluid, we cannot be sure that they play a specific role in RA.

The monoclonal was raised against the GlcNAc-rich cell wall/polysaccharide complex of Group A streptococci. It has been shown that both rheumatic fever and RA patients have raised levels of antibody to this preparation (McCarty 1958; Johnson et al. 1984). Therefore auto-reactivity to complex-type oligosaccharides bearing terminal GlcNAc seems a real possibility. Antibody with this ability is however a minor component of the anti-GlcNAc response evoked by Group A streptococci. Most of the hybridomas induced by this antigen secrete antibodies which bind to a neoglycoprotein formed by conjugating the diazonium derivative of p-aminophenyl GlcNAc to BSA, but fail to bind to GlcNAc-terminating complex-type oligosaccharides (Rook et al. 1988). It will therefore be of interest to discover whether the proportion of the anti-GlcNAc response which has this cross-reactivity is higher in arthritic patients. The joints of RA patients appear to contain several potential targets for such a response. There is terminal GlcNAc on agalactosyl IgG (Parekh et al. 1985) and we have previously described the presence of clusters of macrophage-like cells containing strongly GlcNAc-positive granular material, and a reticulate pattern of GlcNAc staining between the synoviocytes (Sharif et al. 1989). The present report of cells with strong membrane staining for GlcNAc provides a further site for anti-GlcNAc-mediated damage. Moreover, it is interesting that Mycobacteria, which are increasingly implicated as triggers of RA (Bahr et al. 1988, 1989; Tsoulfa et al. 1988) are also able to provoke a response to β-linked GlcNAc-terminating complex-type oligosaccharides (Chechik et al. 1987).

Acknowledgements
We are grateful to the Wellcome trust for financial support.

References
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Antibodies to Heat Shock proteins in Rheumatoid Arthritis

Departments of Immunology, Rheumatology Research and Medical Microbiology, UCMSM., London; MRC TB unit, RPMS, London, University of Kuwait, Kuwait; National Institute of Public Health and Hygiene, Bilthoven, Netherlands and McMaster University, Hamilton, Ontario, Canada.

Abstract
A number of observations have suggested a role for mycobacteria and in particular the 65 kDa heat shock protein (HSP) in an animal model of rheumatoid arthritis (RA).

In this paper, we review our data on antibody responses in RA to 2 different families of HSP of mycobacterial, E. coli and human origin.

Increased serum IgG antibodies to the mycobacterial 65kDa HSP were characteristic of RA patients but not other diseases including systemic lupus erythematosus (SLE), ankylosing spondylitis (AS) and Crohn's disease (CD). Some of these antibodies cross-reacted with human 65kDa HSP (ie. were autoantibodies). IgG and IgA antibodies to the myco 65kDa HSP were decreased in synovial fluids (SF) relative to sera and the presence of the human 65kDa both free and complexed with antigen argues that these antibodies could contribute to pathogenesis in RA. The high levels of these T cell dependent antibodies to the 65kDa HSP might represent a marker of enhanced T cell activity which preliminary evidence suggests is also elevated in RA.
**Introduction**

Heat shock, or 'stress' proteins are a group of mainly cellular 'nurse maid' or 'molecular chaperone' proteins which are highly conserved in structure throughout the plant and animal kingdoms (1). They exist as several gene families including groups controlling production of proteins at approximately 20, 40, 65, 70 and 110 kDa (2). They occur as constitutive proteins which are elevated following stress or as de novo synthesised proteins induced by stress. "Stress" conditions include heat, pH change, virus infection, oxygen reduction products etc (2).

The possible role of heat shock proteins (HSP) in rheumatoid arthritis (RA) was first suggested by experiments showing that T cells reacting with *Mycobacterium tuberculosis* (M.Tb) 65kDa HSP were arthritogenic in a rat model of arthritis (3). Increased T cell reactivity to this protein has also been found in synovial fluid of patients with RA(4 and unpublished observations).

As an approach to understanding the significance of the enhanced immune reactivity to the *M. bovis* 65kDa HSP in RA we have investigated the antibody (IgM, IgG and IgA) responses to this and other HSPs in serum and synovial fluid. The particular relevance of the mycobacterial 65kDa versus *E.coli* homologue was also tested. Whether or not the 65kDa gene family was the only HSP gene family of importance in RA was tested using 70kDa preparations from *M. bovis, E.coli* and a human lymphoblastoid cell line. The particular relevance of these responses to RA was analysed by using several disease controls. These included ; Ankylosing spondylitis (AS); Systemic lupus erythematosus (SLE) ;Crohn's disease(CD) and Tuberculosis (TB).
Methods and Patient groups
Sera were collected from RA patients and other groups with defined diseases as previously described (5,6).

Patients with rheumatoid arthritis (RA) were attending a clinic at UCMSM (n=50; mean age 55 years) and Mubarak teaching hospital in Kuwait (n=40; mean age 30 years).

Systemic lupus erythematosus (SLE) patients (n=18; mean age 42 years) were assessed for disease activity according to the previously published index (7). The SLE patients were studied as another connective tissue disease group.

Sera from Ankylosing spondylitis patients (AS) (n=15; mean age 50 years) were kindly provided by Drs A. Ebringer and F. Yuksel, and were included as a group with another inflammatory joint disease.

Crohn's patients (CD, n=21; mean age 36 years) were provided by Professor Lennard-Jones and colleagues (St. Mark's Hospital, City Road, London) and included since an association with mycobacterial infections has been postulated.

Tuberculosis sera (n=90; mean age 33 years) were collected in Kuwait and were included as a group with mycobacterial disease.

Control sera in London were from healthy laboratory staff (n=45; mean age 35 years) and in Kuwait were from laboratory staff and blood donors (n=79; mean age 31 years).

Details of patients, disease activity, drug treatments etc. are as previously described (6).

Antigens
Recombinant forms of 65 kDa HSP of *M. bovis* (8) and *E.coli* (9) were used. 70 kDa HSP from M.Tb, *E.coli* and a human lymphoblastoid cell were also used (10).

Six synthetic peptides based on the sequence of *M. leprae* (11) were included to determine the fine specificities of antibodies. The sequences of these peptides are shown in table I.
Table 1. Sequences of synthetic peptides used.

Preparation of the human 65 kDa HSP from placenta.

Freshly excised human placenta was cleared of all connective tissue and rinsed several times in homogenisation buffer (10 mM Tris-HCl pH 7.2, 10mM NaCl, 1 mM phenylmethylsulphonyl fluoride). The washed placenta was cut into small pieces and homogenised in 10 volumes of buffer in a blender. The suspension was centrifuged at 2000 rpm for 15 min, followed by another spin at 10,000 rpm for 30 min to remove aggregates. Ammonium sulphate was added to the supernatant and the proteins precipitating between 20-60% saturation were dialyzed against several changes of buffer.

Aliquots of the above preparation were applied onto a Biogel A1.5 column (2.5 cm x 80 cm), pre-equilibrated with buffer and 4 ml fractions collected. The fractions were examined for P1-antibody cross-reactivity by slot blot and western blot analysis, and the peak fractions were pooled and concentrated using an Amicon centrio 30 microconcentrator. Further purification of human P1 for some experiments was carried out by immuno-affinity chromatography using P1-antibody coupled to sepharose 4B as absorbent. The human P1 was depleted of contaminating IgG by an anti human IgG affinity column. The polyclonal rabbit antibody to P1 employed in these studies was raised against hamster 65kDa HSP and has previously been shown to react specifically with the corresponding protein in human cells (12).
**Measurement of antibodies.**

The methods are as previously described (5,6). Briefly, HSP antigens were coated onto plates at 2μg/ml; sera were added at 1/50, 1/100, 1/200 and 1/400 in phosphate buffered saline (PBS) in duplicate. Peroxidase-labelled F(ab')2 antibodies to \(\mu, \gamma\) and \(\alpha\) heavy chains (Sigma) were added. The colour reaction was developed with ABTS and hydrogen peroxide and read on an ELISA microplate reader at 650 nm. Data used was on the linear part of the dose response curve and was standardised from plate to plate by being expressed as a ratio of OD in the sample to that of a standard serum. Negative controls of agammaglobulinaemic or cord sera never gave values >0.004 OD units. Data were analysed Mann-Whitney U rank two tailed test and Spearman rank correlation coefficient.

**Preparation of M. bovis 65kDa HSP affinity column.**

2 mg of recombinant 65kDa HSP were coupled to an Affigel column (13). Sera from 2 RA patients with high levels of IgG antibodies to this 65kDa were used in these experiments. 1 ml. of 1/200 of serum in PBS was put over the column and eluted with glycine/ HCL (0.1M, pH 2.8) The eluate was made up to 1 ml. and samples tested for IgG antibodies to both M. bovis and human 65kDa HSPs.

**Detection of the 65kDa HSP in synovial fluid**

Synovial fluid samples were prepared as described in detail elsewhere (14). Briefly, the samples were spun at 10,000 rpm for 20 mins at room temperature and denatured with 12M urea and 2-mercaptoethanol in a 42 °C water bath for 6 hours. Polyethylene glycol (PEG) precipitated immune complexes from synovial fluid were obtained (15). 30 μg of SF, 50 μg of PEG, 4 μg of myc and 4 μg of human 65kDa were run on a 10% SDS-PAGE overnight at 6mA/gel. Western blots were prepared, reference lanes were gold-stained (Aurodye) (lanes 1-3) and the lanes required for immunoblotting were blocked with phosphate buffered saline (0.1M, pH 7.4)/ 1% BSA/ 0.05% Tween 20. Immunoblotting was carried out using appropriate dilutions of polyclonal rabbit anti-hamster 65kDa antibody P1(12), a peroxidase conjugated anti-rabbit F(ab')2 antibody and 4-chloronapthol as the colour detecting agent.
Results
Increased levels of IgG antibodies to mycobacterial 65kDa HSP in RA but not control diseases.

A summary of our previous data on changes in IgG and IgA antibody levels to different HSP is shown in table 2.

Table 2 indicates significant increases in antibody levels (p<0.005) relative to normal control sera. IgM antibody levels to all of the HSPs tested were not significantly increased above control levels (data not shown; 6).

IgA antibodies to the mycobacterial (myco) version of the 65kDa HSP were significantly increased in all 4 patient groups, although the number of patients with values of >mean +2SD of the control group were 11/45 for RA compared with only1/18 for SLE, 3/15 for AS and 2/21 for CD (data not shown).

In contrast, IgG antibody levels to the Myco 65kDa HSP were significantly increased only in the RA patient group.

<table>
<thead>
<tr>
<th>HSP families</th>
<th>Species</th>
<th>RA</th>
<th>AS</th>
<th>SLE</th>
<th>CD</th>
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<tr>
<td>65kDa</td>
<td>M. bovis</td>
<td>IgG</td>
<td>IgA</td>
<td>IgG</td>
<td>IgG</td>
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<tr>
<td></td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>E. coli</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Summary of IgG and IgA antibody responses to 65kDa (M.bovis, E.coli) and 70kDa (M.Tb, E.coli and human) heat shock proteins in RA and other disease groups.

With regard to the 70kDa HSPs, RA patients showed significant increases in both IgG and IgA antibody levels to myco, E.coli and human versions of this protein. IgA levels to the human homologue (and therefore autoantibodies) were also raised in AS and SLE but not CD.

These data, together with our findings that IgG antibody levels to the myco 65kDa HSP are higher in RA than TB
patients (6) suggest that IgG responses to this version of HSP are characteristic of RA.

There was no evidence that age or sex influenced antibody levels to any of the HSP. In addition, no correlations were seen between antibody levels of any class to different HSPs and serum levels of IgG, rheumatoid factors, disease activity or drug treatments in any of the diseases studied (data not shown).

**Fine specificity of antibodies to myco 65kDa HSP**

As an approach to determining whether or not the fine specificities of elevated IgG antibodies to the mycobacterial 65kDa HSP were different in RA patients compared with controls, a number of synthetic peptides were tested (see table 1). These represent continuous epitope sequences. IgG antibodies were present in control sera to P3, P4 and P5 (Fig.1). Antibodies to these same 2 peptides were increased in RA sera (P3<0.05; P4, p<0.001; P5, p<0.002). Data for the intact 65kDa HSP are included. It is interesting that these peptides derived from the *M.leprae* sequence of 65kDa HSP differ in only a few amino acids from the recently sequenced human 65kDa HSP (16) indicating that at least some of these antibodies could be autoantibodies.

![Increased IgG antibody responses to some synthetic peptides based on M.leprae in RA patients](image)
**Antibodies in RA sera can bind to human 65kDa HSP**

In order to formally demonstrate that at least some of the antibodies reacting with the mycobacterial 65kDa HSP, will also bind to the human homologue, we tested the antibodies eluted from the myco 65kDa column on human 65kDa. Fig 2 shows OD values for binding to myco and human 65 kDa HSP. Starting material, eluate and column depleted serum are shown for a representative experiment. It can be seen that the eluted antibodies bind well to 65 kDa HSPs of both myco and human origin.

![Graph](image)

**Fig 2:** Cross-reactivity of IgG antibodies to myco 65kDa HSP with human 65kDa HSP.

**Antibody levels to myco 65kDa HSP in synovial fluids of RA patients**

If antibodies to the 65kDa HSP were important in the pathogenesis of RA they might be found in high amounts at the site of damage, namely the joint. Analysis of paired serum and synovial fluid samples from 23 RA patients showed that levels of IgA and IgG antibody but not IgM, were low in fluid compared with serum (Table 3). If a significant proportion of the antibody which binds to the mycobacterial 65kDa hsp, is autoantibody, the lower apparent levels of IgG and IgA antibody found in the synovial fluid may be due to complexing with the human homologue.
Table 3: Decreased IgG and IgA antibody levels to myco 65kDa HSP in synovial fluids compared with paired RA sera.

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>SF</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>3.16 ± 2.92</td>
<td>2.05 ± 1.81</td>
<td>0.024</td>
</tr>
<tr>
<td>IgM</td>
<td>1.75 ± 2.43</td>
<td>1.10 ± 1.03</td>
<td>0.076</td>
</tr>
<tr>
<td>IgG</td>
<td>1.75 ± 1.10</td>
<td>1.26 ± 0.92</td>
<td>0.042</td>
</tr>
</tbody>
</table>

Presence of human 65kDa HSP in synovial fluid (fig 3)
The recombinant myco 65kDa is shown gold-stained in lane 2. (molecular weight markers are shown in lane 1) The multiple bands presumably represent proteolysis-derived fragments. The human homologue is shown in lane 3 and staining of this with a polyclonal antibody (PI) indicates that there are several contaminants in this preparation (lane 4). The lower band of 55kDa is probably \( \gamma \) chains since it is removed by an anti-IgG affinity column (data not shown).

In the synovial fluid preparation probed with the PI antibody (lane 5) the 65kDa band is broader. This could be due to accumulation of precursor protein released from dying cells and/or the existence of different molecular weight isoforms (Welch, this volume). Two different allelic forms of this protein have been described in human lymphocytes (17).

Lane 6 shows binding of PI antibody to the solubilised Polyethylene glycol(PEG) precipitated material from synovial fluid.

Although it is theoretically possible that the 65kDa protein band identified in the SF and PEG precipitates is not of human origin, there would have to be a large amount of 'mycobacterial' material to account for this in the joint! However, without definitive proof we favour the notion that some IgG and possibly IgA antibodies to myco 65kDa HSP cross-react and complex with human 65kDa HSP in the
synovial fluid (ie are autoantibodies).

**Discussion**

The presence of elevated levels of IgG antibodies to myco 65kDa HSP but not *E. coli* 65kDa HSP suggests a special relationship in RA with the myco version of this molecule.

This was emphasized by the higher IgG levels to this HSP in RA than in Tuberculosis where the patients are infected with mycobacteria. We did, however, fail to find differences in the specificities of these antibodies in RA groups compared with controls although we only looked at a small number of peptides representing linear epitopes and only about 1/4th of the 540 amino acids in the 65kDa HSP.

What is the significance of these particular HSPs in RA? A number of observations have suggested a role for mycobacteria in RA. These include the following;

a) There is an increase in defectively glycosylated IgG in RA patients. This has been shown to occur in other diseases of known or possible mycobacterial aetiology but not in numerous other conditions. Elevated levels of agalactosyl IgG occur in TB, Crohn's disease and leprosy during episodes of erythema nodosum leprosum (18), but not sarcoidosis, SLE
or viral infections. The possible relationship between this defective glycosylation and elevated levels of antibodies to the myco 65kDa HSP is discussed by Rook et al (this volume).

b) DR4 is strongly associated with RA (19) and the skin test response to tuberculin has been shown to be increased in DR4 positive RA (20) and leprosy (21) patients but not DR4 positive normal individuals.

c) Adjuvant arthritis can be adoptively transferred using T cell clones which recognise the 65kDa HSP derived from mycobacteria (3). Induction of the disease can be blocked by pretreatment with this antigen in soluble form (22). In fact, this same antigen in soluble form can prevent induction of arthritis in other animal models. (See Rook et al, this volume).

With regard to the latter studies, the increase in T cell dependent IgG and IgA antibody responses in RA may be a marker of elevated T cell reactivity to the myco 65kDa HSP as seen in synovial fluid of RA patients (4 and unpublished observations). Tγδ cells have been shown to respond to bacterial antigens (23) and it is conceivable that these cells, which are increased in number in some RA joints (24 and unpublished observations) are important in the disease process (see Rook et al, this volume, for fuller discussion).

The production of the IgG "autoantibodies" against the human 65kDa HSP is presumably through breakdown in tolerance to 'mimicked' epitopes on the HSP's since, as indicated, there is a high degree of structural homology between the HSP's within each family. It is unclear why mycobacterial HSP are more able to elicit autoreactivity than \textit{E. coli} for example.

Our data suggest that cellular breakdown in the joint leads to release of the 65kDa and other HSP into the SF and these can become complexed with antibodies contributing to the chronic inflammation of the joint.

It is also possible that specific IgG antibodies, like the T cell clones in a rat model of arthritis (directed against proteoglycan core protein; 3) will cross-react with other autoantigens in the joint.
Autoantibodies of the IgG and IgA class to the 70kDa HSP might also produce immune inflammatory complexes, not only in RA but also other diseases where they are elevated.

Further studies will focus on studying antibodies to the recombinant form of the human 65kDa and T cell responses to both myco and human homologues of this protein.

References


Immunity to heat shock proteins in rheumatoid arthritis

P.M. Lydyard, G. Tsoulfa, M. Sharif1, B. Broeker2, M. Smith, G.A.W. Rook1

Departments of Immunology, Rheumatology Research and Medical Microbiology1, University College and Middlesex School of Medicine, London, U.K.; Max Planck Institute for Immunology, Erlangen, F.R.G2.

Abstract. Heat shock proteins (hsp) or “stress proteins” are a group of highly conserved proteins which are important in the day to day function of all cells. Early studies by others have indicated that immunity to the 65 kDa hsp of mycobacteria is important in the development of arthritis in the adjuvant arthritis model in rats. In this paper, we review the evidence suggesting that, as for the rat model, immune reactivity to hsp is of importance in the human disease. Elevated levels of IgG antibodies to the 65 kDa hsp of mycobacteria are characteristic of rheumatoid arthritis (RA) patients. Much of this antibody cross-reacts with human 65 kDa hsp and is therefore autoreactive. The 65 kDa hsp is found in synovial fluid and is therefore a potential target for antibody. Antibodies to the 70 kDa hsp (both of mycobacterial and human origin) are elevated, but not specifically, in RA. Increased T cell responses to the 65 kDa hsp are also found in synovial fluid of RA patients. Although γδT cells are present in the synovial joint of RA patients, they do not appear to be particularly increased in frequency although the subset distribution of these cells is clearly different from that seen in the circulation. In fact, the synovium looks like the “gut” with regard to these subsets!

Key words: heat shock proteins, hsp, autoantibodies, rheumatoid arthritis, γδT cells.

Introduction

Interest in the possible role of heat shock proteins (hsp) in the aetiopathogenesis of rheumatoid arthritis (RA) originates from the findings of van Eden et al (1). Using the rat adjuvant arthritis (AA) model of RA (2), these investigators were able to show that T cell clones obtained from arthritic animals could induce the disease when injected into normal rats (1). Furthermore, these T cells recognised a protein which was later identified as the 65 kDa hsp of the mycobacteria used to induce the AA (3). The cross-reactivity observed between the hsp and proteoglycan of cartilage led to the suggestion that RA could be caused by a similar mechanism of ‘molecular mimicry’.

Heat shock or stress proteins are proteins which are constitutively expressed, or more often are preferentially synthesised following environmental stresses of many kinds.

These include heat, viral infections, pH change etc. and their importance is emphasised by their occurrence in conserved form throughout the many animal and plant species (4). Some may represent as much as 1% of the total cellular protein. They exist as several gene families including groups controlling production of proteins at approximately 20, 40, 65, 70 and 110 kDa (5). The various gene family products play somewhat different roles, but the major intracellular role appears to be involvement in folding/unfolding and transport of proteins across intracellular membranes. Many have ATPase activity which probably plays a role in these processes. The hsp are the dominant antigens following immunisation with a variety of different microorganisms leading to both cellular and humoral responses (6). The very high degree of homology between bacterial and self hsp could contribute to the generation of autoimmune diseases through ‘molecular mimicry’.

In this paper, we review the evidence suggesting that, as for the adjuvant arthritis model of rats, immune reactivity to hsp is of importance in the human disease. Antibody responses were measured in RA to the gene products of 2...
Elevated antibody responses to hsp

Using ELISA we have shown that serum IgG and IgA antibody levels to the 70 kDa hsp of both *M. bovis* and *E. coli* are significantly increased in RA and SLE, but not in CD. IgA antibody levels only are increased in AS. This was shown in two different laboratories with sera from patients in the UK and Kuwait (7,8,9). That there should be a response to this hsp is not surprising since hsp of several families appear to be immunodominant antigens in responses to many micro-organisms (6).

Since the 70 kDa hsp show a high level of homology between species (6), we also tested human 70 kDa derived from a human lymphoblastoid cell line. Elevated levels of both IgG and IgA autoantibodies were found in RA, whereas only IgA autoantibodies were increased in SLE and AS. This was not unprecedented for SLE since autoantibodies to the 90 kDa hsp have been previously described (11).

In contrast to the data on the 70 kDa hsp, although there were elevated IgG antibody levels to the mycobacterial 65 kDa hsp in RA, neither IgG nor IgA levels were elevated to the *E. coli* homologue. None of the other patient groups tested showed elevated IgG levels to the mycobacterial 65 kDa hsp. This suggested that elevated responses to the mycobacterial version of the 65 kDa hsp were important and a characteristic of patients with RA. This was further emphasised by the fact that the titres of responses to the mycobacterial 65 kDa hsp in RA patients were even higher than those seen in the TB sera (7). Since some of these antibodies to the mycobacterial 65 kDa hsp might cross-react with the human homologue, i.e. were autoantibodies, the following experiments were carried out. Sera from RA patients with high levels of antibodies were run over a column of affigel linked with mycobacterial 65 kDa hsp and the antibodies eluted. These antibodies showed some binding to an enriched fraction of human 65 kDa hsp, thus indicating that some of them were autoreactive (12). It is conceivable that binding of RA IgG to the mycobacterial 65 kDa hsp could be due, in part, to its low galactose content - see below.

Elevated T cell responses to the mycobacterial 65 kDa hsp

As already indicated, the hsp are immunodominant components of microorganisms (6) and are a major target of T cell responses. For example, up to 30% of T cell clones to *M. leprae* derived from two intensely studied leprosy patients were shown to react with the 65 kDa mycobacterial hsp (15) and a number of T cell epitopes have been defined...
on this particular molecule (16). In addition, delayed skin test responses to tuberculin were higher in DR4 positive than DR4 negative RA patients (17). This is intriguing because of the association of RA with this class II haplotype (18). Elevated T cell responses to the mycobacterial 65 kDa hsp have been shown in the blood and especially in the synovial fluids of patients, particularly those with early disease (19). Our own studies on paired samples of blood and fluids from RA patients have also indicated higher levels of response to the mycobacterial 65 kDa hsp in the fluid relative to the blood (unpublished observations). However, this elevated response in the synovial fluid also appeared to be common in patients with a variety of reactive arthropathies (20) and therefore not restricted to RA. In the case of reactive arthropathies it would be interesting to determine whether the reactivity decreases during resolution of the disease.

Since the hsp are so highly conserved, it is likely that some of the T cells recognising the bacterial hsp can also react with some sequences common to self. In this regard, human CD8+ T cell lines/colonies which recognise both the bacterial and human 65 or 70 kDa hsp have been isolated from TB patients and in the case of the 65 kDa hsp, from a healthy individual (21,22,23). Thus, these potentially autoreactive T cells are present in normal individuals. In mice, it has been shown that T cells directed to the mycobacterial 65 kDa hsp recognise and lyse MHC class I compatible macrophages which have been activated (and stressed) by γ-interferon (24). It has recently been shown that the 70 kDa hsp is present on the surface of activated B cells (25) and can act as an antigen presenting molecule (26). Thus, these molecules could play a role in the normal immune response, and could be the recognition structures for regulatory cells. In fact, it has been suggested that T cells recognising hsp are involved in immune surveillance of autologous stressed cells and may play a role in first line protection against virus infection (24).

The presence of such potentially autoreactive T cells in 'normal' individuals is not unprecedented. T cell clones have been isolated from "normal" mice with specificity to myelin basic protein and shown to produce the disease on injection into susceptible animals (27). The question is still unanswered as to how these T cells are prevented from participating in immune surveillance of autologous stressed cells and may play a role in first line protection against virus infection (24).

The γδT cell - its connection with hsp

T cells in mammals predominantly utilise α and β chains together with CD3 for their T cell receptor but a minor population expresses a receptor with a different heterodimer, namely γ with δ chains (28). Recently, it has been suggested that this T cell subpopulation has a biased repertoire for bacterial, and in particular, heat shock proteins (10). We have therefore examined patients with RA for frequencies of these cells in various cellular compartments. We have used immunocytochemical/histochemical techniques with two monoclonal antibodies that recognise two different subsets, but together identify 99% of the blood γδT cells in man (BB3 and A13). We have shown that there is a significant decrease in frequency in the blood of RA patients compared with controls (Smith et al., submitted). In addition, there was a small increase in frequency of γδT cells in the synovial fluid relative to blood in the RA patients. However, there did not appear to be significantly higher levels of γδT cells within the synovial membranes of RA patients. They were sparsely distributed and found together with the αβT cells both inside and outside the lymphoid follicles. Interestingly, the two γδT cell subsets (defined by the BB3 and A13 monoclonals) were differentially distributed in the RA patients. Whereas the mean ratio of BB3:A13 cells was around 5 in the blood of both control and RA blood patients, it was less than 1 in the synovial fluids and membranes of the RA patients. From studies of V gene usage of γδT cell clones it is possible that BB3+ and A13+ cells have different repertoires (30,31). Thus accumulation of A13+ γδT cells in the synovial tissues could be due to recognition of specific antigens in that site. It is tantalising that this same inverse distribution of BB3/A13+ cells is also found in the gut wall lining (32). It is clearly of importance to define the specificities of the γδT cells for hsp in the synovial joint in order to establish any link between hsp and RA.

Does elevated immune reactivity to (especially self) hsp arise in RA?

The simplest explanation would be that microorganisms carry determinants which mimic self and exposure to some of them, especially mycobacteria, could result in breakdown of tolerance to these self determinants. In fact, mycobacteria are very common microorganisms and are often found contaminating water supplies. Modification of gut flora can produce adverse or beneficial effects on the incidence and severity of adjuvant arthritis and streptococcal wall arthritis (ref. 33 and M. van den Broek, personal commun.). In addition, giving mycobacteria via the oral route has been shown to modulate AA disease (H. Weiner, personal commun.). An intriguing finding which could have implications for therapy is that pretreatment with mycobacterial 65 kDa hsp prevents the development of arthritis in several animal models. These include AA in the rat (3), Streptococcal cell wall (SCW) arthritis (34), and pristane arthritis in mice (35).
It also reduces the magnitude of the arthritis in mice induced by a lipoidal amine CP20961 (36). In the latter two models, no micro-organisms are knowingly administered to induce the arthritis and the mechanism(s) of the induction of arthritis are even more uncertain. Additional factors clearly play a role. One such factor which could have an effect on development of the arthritis is the genetic defect in Lewis rats. This strain is susceptible to both AA and SAC arthritis and fails to mount an endogenous elevated corticosteroid response following injection with IL-1α or the streptococcal cell wall fragments (37). Furthermore, inhibition of this enhanced steroid response in normally non-susceptible (and therefore tolerant) rats resulted in susceptibility to develop the arthritis. Pretreatment with the mycobacterial 65 kDa hsp could conceivably induce the steroid response in the susceptible rats. Whether or not the 65 kDa hsp has this effect, the specificity of the protection mediated by hsp of different gene families clearly needs to be established in all the arthritis models discussed.

In general, the lymphoid cells with specificity for the highly homologous hsp could also be important in maintenance of the idiotypic network, escape from which would result in chronic self-reactivity. This could include early B cells, some of which have specificity for hsp (unpublished observations).

Does reactivity to hsp play a role in pathogenesis?

We have presented evidence for the presence of autologous hsp in soluble form in the synovial tissues. In addition, other studies have indicated that 'normal' T cells can recognise self hsp endogenously expressed on the surface of antigen presenting B cells (26), and interferon-γ-stressed cells (24) or through macrophages binding to exogenously added hsp (22).

Another clue which indicates an association between hsp and RA comes from the observation that IgG from such patients lacks a terminal galactose from an N-linked sugar on the Fc fragment (38). Although this is not peculiar to RA but is also found in TB, it does seem to be associated with certain infections (39). It has not yet be excluded that some binding of IgG to the mycobacterial 65 kDa hsp is due to the low galactose content of antibodies in RA. The degree to which the IgG bears galactose appears to be related to the severity of the disease in RA and in a number of animal models where arthritis is seen and where it is not (40). Thus it appears to be associated with a specific kind of inflammation which probably involves a tissue-damaging cytokine releasing response (40). It is not inconceivable that T cells recognising self hsp produce cytokines which are involved in modifying galactosyl-transferase enzyme levels (41) in B and T cells.

In addition, antibodies to the hsp in the synovial joint would complex with self hsp and the complexes could result in maintenance of the chronic inflammation at that site.

The findings that autoreactive hsp-specific T cells are present in normal individuals and that at least some hsp (70 kDa) are present on antigen presenting B cells and macrophages, suggests that these cells play a role in the normal immune response. These self-reactive cells probably include both classical TCR-2+ CD8+ cells and the TCR-1CD8+ cells. Unlike many other self-antigens, the hsp are present in every cell and can potentially be expressed either with MHC class I or class II molecules (or may be antigen presenting molecules themselves, as in the case of 70kDa hsp). In order to understand the mechanisms by which reactivity to self hsp is controlled, further experiments are required to determine the repertoires of these two cell populations. In addition, analysis of these same populations within the synovial joint early in the disease process might help to shed light on their possible role in pathogenesis.

Acknowledgements

The authors would like to thank the following for their contributions to these studies: D.B. Young, G.M. Bahr, J.D. Van-Embden, D.A. Isenberg, F.C. Hay, R.S. Gupta, C.S. Mayanil, T. Venner, C.E. Grossi, E.Ciccone, L. Moretta, F. Yuksel, B. Yuksel, C. Worman, J. C. W. Edwards, B. Colaco, L. Mackenzie, R. Kinne, G. Wescloh, and K. Gluckert.

M.D. Smith is supported by the Arthritis and Rheumatism Council and M. Sharif by the Wellcome Trust.

References

Heat shock proteins and rheumatoid arthritis


TERMINAL N-ACETYLGLUCOSAMINE IN CHRONIC SYNOVITIS

By M. SHARIF*, G. ROOK*, L. S. WILKINSON†, J. G. WORRALL† and J. C. W. EDWARDS‡

Departments of *Microbiology and †Rheumatology Research, University College and Middlesex Hospital
School of Medicine

SUMMARY

The distribution of terminal GlcNAc residues in normal and diseased synovial tissue has been studied using a mouse monoclonal antibody (mAb) which binds to terminal N-acetylglucosamine (GlcNAc). Normal human connective tissue, including synovium, showed no staining for terminal GlcNAc. Normal epithelial tissues, including tonsillar epithelium, skin, small intestinal epithelium and salivary epithelium showed cellular staining. Synovium from patients with definite rheumatoid arthritis showed dense granular staining of macrophages. In addition, synovium from 9 of 12 patients with definite rheumatoid arthritis showed reticular extracellular staining indicating deposition of material bearing terminal GlcNAc in the connective tissue stroma. The extracellular staining was not seen in synovium from patients with osteoarthritis. Extracellular material bearing terminal GlcNAc may act as an inflammatory stimulus in rheumatoid arthritis, either by acting as antigen or by interaction with receptors on macrophage membranes which also recognize GlcNAc on bacterial material, thus triggering biochemical pathways normally occurring in response to the presence of micro-organisms.

KEY WORDS: Glycosylation, Rheumatoid arthritis, Synovium, Macrophage action.

The stimulus for persistent inflammation in the synovium of patients with rheumatoid arthritis (RA) remains unknown [1]. We report observations which suggest that changes in the terminal sugar residues on glycoproteins or glycosaminoglycans in connective tissue may be involved.

We previously raised mouse monoclonal antibodies (mAbs) to terminal N-acetylglucosamine (GlcNAc) using the GlcNAc-rich peptidoglycan/polysaccharide complex of group A streptococci as immunogen [2]. Antibodies to this material are known to be raised in both rheumatic fever and RA [3]. A small subset of GlcNAc binding antibodies raised in this way will also bind to complex type oligosaccharides bearing terminal GlcNAc, and have proved useful for the immunnoassay of agalactosyl IgG [4]. This glycoform of IgG, which bears an N-linked oligosaccharide with terminal GlcNAc, is present in increased concentrations in rheumatoid sera and it has been suggested that it may encourage self-association of IgG to form immune complexes and, therefore, play a role in the pathogenesis of the disease [5].

GlcNAc is a component of many complex sugars and glycoproteins. It occurs as alternate sugar residues in bacterial cell wall peptidoglycan. GlcNAc occurs in the oligosaccharide chains of human glycoproteins and also in the larger sugar chains of glycosaminoglycans (half the sugar residues in hyaluronic acid) [6, 7]. However, it is unusual for GlcNAc to be present as a terminal sugar residue, so GlcNAc may not normally be exposed to molecular recognition systems.

It has been suggested that oligosaccharides on many glycoproteins are unrelated to the primary function of the protein, acting non-specifically as lubricants and stabilizers, and conferring negative charge [6]. It is becoming clear that they also act as 'address labels' which determine compartmentalization within or outside cells [6, 8]. Moreover, they have regulatory functions, interacting with hormone receptors, cytokines and lectin-like molecules on cell membranes. Changes in terminal sugar residues during synthesis or following partial degradation lead to movement of glycoproteins from one compartment to another, as in the endocytosis of material following binding to macrophage membranes [8, 9]. A range of receptors has been described which recognizes different sugars including mannose, fructose and GlcNAc [10–14]. Receptor specificities may also depend on the arrangement of several sugars in a chain, as is the case with plant lectins. Recognition of GlcNAc has been implicated in cellular uptake of lysosomal enzymes, removal of effete or ageing proteins, removal of cells undergoing programmed death (apoptosis) and as a signal for tissue remodelling [6, 10, 15].

In view of the possible antigenic role of terminal GlcNAc and the increase in levels of agalactosyl IgG in RA, we have undertaken a study of the distribution of terminal GlcNAc, as recognized by the mAb GN7, in normal and diseased synovial tissue.

MATERIALS AND METHODS

Tissues

Synovial tissues from patients with RA and osteoarthritis (OA) were obtained from diagnostic biopsy, synovectomy or joint replacement. Rheumatoid nodules were obtained by excision biopsy. Patients were documented using a computer-based stratified indexing system [16].

Patients were considered to have OA if joints...
showed loadbearing cartilage loss with bone overgrowth with a normal ESR and negative serum rheumatoid factor and no evidence for a cause of synovitis other than mechanical irritation from damaged bone and cartilage. Patients with RA satisfied the American Rheumatism Association criteria for definite or classical rheumatoid arthritis (with widespread synovitis, not attributable to trauma, sepsis or crystal deposition). The details of patients are given in Table I including the presence or absence of serum IgM rheumatoid factor, bone erosions and nodules for rheumatoid cases.

Normal human muscle, skin, nerve, tendon and joint synovium were obtained from each of four limbs amputated for localized proximal sarcomata. Tissues were taken from areas at least 20 cm distant from the tumour and were macroscopically normal. Human tonsil, obtained post mortem, was provided by Dr M. Griffiths. Histologically normal minor salivary gland was provided by Dr D. Isenberg and Dr S. Tinkler from routine diagnostic material from the University College Dental Hospital.

Monoclonal antibody

A mouse IgM mAb, GN7, was raised by immunization to components of streptococcal cell wall [2]. The antibody was selected by screening for binding to asialo-agalactosyl-fetuin, prepared by enzymatic digestion of native fetuin to expose terminal GlcNAc. It binds to biantennary oligosaccharides carrying terminal GlcNAc on human glycoproteins, including heat denatured agalactosyl IgG heavy chain, but not to other serum glycoproteins. It also binds to neoglycoproteins constructed by conjugating the diazonium derivative of p-aminophenyl GlcNAc onto BSA. Binding of GN7 to proteins on nitrocellulose blots and monocyte cytopins is abolished by preincubation with specific N-acetylgalcosaminidase and not with buffer alone. Binding is inhibited by free GlcNAc but not galactose.

Immunohistochemical staining

Five-micrometre frozen sections were cut, air dried overnight and fixed in acetone for 10 min before staining. Staining was performed using GN7 as ascites diluted 1/200 in Tris buffered saline (TBS) at pH 7.6 for 30 min, followed by 30 min wash in TBS, followed by an enzyme or fluorochrome conjugated rabbit anti-mouse immunoglobulin reagent. Routine staining was performed with a rabbit anti-mouse immunoglobulin peroxidase conjugate (DAKO P260) diluted 1/40 in TBS, following blocking of endogenous peroxidase with 2% hydrogen peroxide in methanol for 30 s. The peroxidase conjugate was developed in a solution of 0.5 mg/ml diaminobenzidene tetrahydrochloride (DAB) (Sigma) and 10 µl/ml 1% hydrogen peroxide and counterstained with Harris haematoxylin. Where further information on the precise localization of GlcNAc was wanted, staining was repeated using TRITC conjugated rabbit anti-mouse IgM (Seralab

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GRAN, granular; EC, extracellular; CS, cell membrane; RA, rheumatoid arthritis; OA, osteoarthritis; E, erosive; N, nodular; RF, rheumatoid factor; +, present; −, absent; SLC, synovial lining cells.
with GN7, sections were incubated with a goat anti-described above and sections were washed for 30 min in TBS between each incubation. Following incubation with GN7, sections were incubated with a goat anti-human IgG polyclonal antiserum (DAKO A090) diluted 1/4000 in TBS and swine anti-rabbit immunoglobulins (DAKO Z196) diluted 1/200 in TBS and peroxidase-antiperoxidase complexes (DAKO Z113) diluted 1/1000 in TBS. Peroxidase was developed with DAB as above.

Double immunofluorescence with GN7 and cell specific antisera was performed by simultaneous incubation with GN7 and a cell specific IgG mAb and simultaneous incubation with TRITC conjugated rabbit anti-mouse IgM (Seralab SB A1020-03) diluted 1/40 and FITC conjugated rabbit anti-mouse IgG (Seralab SA1030-02) diluted 1/40.

Cell populations were identified using mouse IgG monoclonals RFD7 (1/10), RFD1 (1/4), RFT1 (1/5) and an anti-B-cell monoclonal cocktail (1/10) provided by Dr L. Poulter of the Royal Free Hospital Medical School. RFD7 recognizes mature tissue macrophages [18]. RFD1 recognizes interdigitating cells in normal tissues [18]. RFT1 recognizes the CD5 determinant on T-lymphocytes [19]. The B-cell cocktail recognizes CD20 and CD22 [20, 21]. Several sections were stained with the macrophage marker EBM11 (1/200) (DAKO M718) which has a wider distribution than RFD7 and recognizes macrophages in all locations so far studied [22].

Staining with GN7 was compared with staining with three other mouse mAbs at the same dilution. GN6, with similar affinity for terminal GlcNAc, was used as a positive control. SF 1 and 5, raised against mycobacterial components, with affinity for human endothelium and neutrophil nuclei respectively, but not for GlcNAc, were used as negative controls.

Staining with GN7 was compared with staining using biotinylated Bandeiraea simplicifolia agglutinin II (a GlcNAc specific lectin) and an avidin peroxidase conjugate as described by Capaldi et al. [23].

RESULTS

Normal tissues

In all tissues GN7 produced a faint nuclear staining detectable at dilutions of GN7 no greater than 1/100. At the 1/200 dilution chosen for routine study it was minimal and appeared as faint ring shadows (see for example Fig. 3).

Four or more sections were examined from each block. Sections of normal human connective tissue, including synovial tissue from 12 normal joints, fascia from six areas and subcutaneous tissue from four areas, showed no staining (Fig. 1). Normal muscle (six samples) and brain (two samples) showed no staining. In normal human tonsil (eight samples) GN7 bound strongly to mucosal and crypt epithelial cells. Immunofluorescence indicated both cytoplasmic and cell surface staining. Paraffin sections stained by immunoperoxidase showed localization of cytoplasmic GN7 to vesicles in the Golgi area. Membrane staining of very occasional cells (<2%) occurred in lymphocytic areas and did not co-distribute with staining for IgG or IgM on double stained preparation using peroxidase and alkaline phosphatase conjugates. Normal human salivary gland showed GN7 staining of mucus and mucus secreting epithelial cell cytoplasm only. Normal skin keratinocyte cytoplasm (four specimens) stained moderately strongly. Myelinated nerve trunks (two blocks of ulnar and two of peroneal nerve) showed staining of Schwann cell cytoplasm.

These findings support the suggestion that terminal GlcNAc is not normally exposed in the extracellular matrix of connective tissue or supporting matrix elements in non-epithelial organs. GlcNAc appears to be expressed on certain epithelial cells and their secretions, but may be separated from connective tissue and cells of the immune system by basal laminae. The proximity between tonsillar crypt epithelial cells expressing terminal GlcNAc and lymphoid cells may be an important exception. Schwann cell staining was strictly intracellular and did not include myelin sheaths.

Diseased tissues

Diseased synovial tissue showed three patterns of staining with GN7: (1) granular cytoplasmic staining, (2) extracellular reticular staining and (3) surface staining of isolated cells.

Granular cytoplasmic staining

In tissues from patients with osteoarthritis the only staining seen with GN7 was a weak to moderate diffuse granular cytoplasmic staining of synovial lining cells. These samples did not show evidence of recent tissue damage in terms of fibrin deposition or cell death.

Fig. 1.—Normal synovial tissue stained with GN7 and rabbit anti-mouse Ig peroxidase conjugate, counterstained with haematoxylin. No significant staining is present. (× 300).
In tissues from patients with RA dense granular cytoplasmic staining occurred in groups of cells in the deep synovial tissue (Fig. 2). These cells were of macrophage morphology and in some cases belonged to groups containing haemosiderin or red cell debris. Comparison with cell specific markers using double immunofluorescence showed these cells to be a sub-population of RFD7 positive cells. Staining using fluorochromes appeared as intracytoplasmic rings of variable sizes, possibly representing material at the periphery of phagolysomes (Fig. 3).

Extracellular reticular staining

Tissues from patients with OA showed no extracellular staining. Tissues from patients with definite RA showed extracellular staining in a reticular or 'chicken wire' pattern (Figs 4, 5 (a)). Reticular staining occurred as a band either between synovial lining cells, or where there was surface fibrin deposition as a band beneath the superficial fibrin. The fibrin deposits themselves did not stain. When intermingled with lining cells, staining did not follow the contour of cell membranes, as seen with cell surface markers. However, the staining was consistent with material shed or otherwise derived from cell membranes and in isolated sites was restricted to a halo around individual cells. Reticular staining also occurred in isolated patches in the deep tissue matrix where no other cellular or matrix abnormality was evident.

Double indirect immunofluorescence with GN7 and the macrophage marker EBM11 (Fig. 5) confirmed staining specificity and showed that many of the cells on the surface of and intermingled with GN7-positive material were macrophages.

Rheumatoid nodules showed no staining of cells or areas of fibrinoid necrosis, but showed similar isolated patches of reticular staining. The extracellular matrix at these sites appeared unremarkable, but nearby cells were pyknotic, suggesting an early stage of necrosis. The isolated patches of reticular staining were similar in pattern to the staining in superficial bands of synovium.

Cell surface staining

In two samples of synovium from patients with RA in which there was a dense lymphocyte infiltrate, ring (membrane) staining was seen around isolated large mononuclear cells in the deep tissue, usually scattered amongst lymphocytes (Fig. 6). These cells had an open chromatin pattern, high nuclear/cytoplasmic ratio and one or two short processes extending between neighbouring cells. They could not otherwise be identified using available cell markers. Their position and shape is consistent with that of macrophage-like antigen presenting cells. Work on peripheral blood monocytes (in preparation) has shown that a proportion of monocyte derived cells demonstrate terminal GlcNAc on their cell membranes after a period of maturation, suggesting that the cells exhibiting ring staining in tissue may be of similar monocytoid origin.

Comparative staining

Staining with GN7 was more intense than, but otherwise identical to staining with GN6. Neither of the two antimycobacterial mAbs gave staining patterns as seen with GN7. The distribution of GN7 staining on normal tonsil matched staining with Bandeiraea simplicifolia agglutinin II.

The relationship between binding of GN7 and the presence of IgG was studied using several indirect immunochromial methods for detecting IgG, including double fluorescence and double enzyme techniques (peroxidase/PAP and alkaline phosphatase/APAAP) on single sections and immunoperoxidase on serial sections. With all techniques the distribution of GN7 staining was totally separate from IgG, which was present on scattered cells within lymphocyte clusters and at low levels in the connective tissue stroma, away from the sites of maximum GN7 staining.

![Fig. 2](image1.png)

**Fig. 2.**—Synovial tissue from a patient with rheumatoid arthritis stained with GN7 and rabbit anti-mouse IgG peroxidase conjugate, counterstained with haematoxylin. Dense granular cytoplasmic staining is present in groups of cells in the deep tissue. (x 300).

![Fig. 3](image2.png)

**Fig. 3.**—Deep synovial tissue from an area similar to that in Fig. 2 from a patient with rheumatoid arthritis stained with GN7 and rabbit anti-mouse IgM TRITC conjugate to show intracellular detail. The cytoplasmic staining surrounds the granular structure, sparing the granular contents. (x 300).
GlcNAc rarely occurs in the terminal position on glycoprotein oligosaccharides [6]. The absence of staining with GN7 on normal connective tissues is consistent with this. Nuclear membrane staining is explained by the recent discovery of GlcNAc attached to proteins of the nuclear pore [24]. Staining of normal epithelial cells may represent binding to glycoproteins undergoing glycosylation in the Golgi apparatus or binding to mucopolysaccharide secretory products such as mucus.

A variety of mechanisms may lead to abnormal exposure of GlcNAc residues on proteins and glycosaminoglycans. Defects in synthesis, such as the reduced galactosyltransferase activity which has been described in lymphocytes from patients with RA [25], may lead to failure of the addition of terminal galactose and sialic acid, leaving GlcNAc in the terminal position. Alternatively, GlcNAc exposure may result from post-synthetic removal of galactose and sialic acid by enzymes or by other inflammatory mediators such as oxygen free radicals [26].

In the diseased tissues studies, terminal GlcNAc was found in the cytoplasm of a proportion of cells. In OA, cytoplasmic staining was never intense and was confined to cells on the synovial surface. In RA, intense staining occurred in granular macrophages in the deeper tissue, suggesting that these cells are actively involved in processing GlcNAc-bearing material.

Extracellular material bearing GlcNAc was found only in rheumatoid synovium, suggesting a more specific role for this material in RA. Such material may be derived from the circulation, or may be of local origin. IgG does not appear to contribute to the stainable GlcNAc-bearing material. However, GN7 does not bind to agalactosyl IgG unless the immunoglobulin has been denatured so the results do not preclude the coexistence of agalactosyl IgG in the tissue. Bacterial fragments might be expected to carry terminal GlcNAc, and could conceivably be responsible for the staining, although no structures resembling bacteria were seen. GlcNAc is known to be intimately involved in the bind-
ing of the complement degradation product iC3b to its receptor on the macrophage membrane [27]. iC3b may bear terminal GlcNAc and the observed staining may represent deposition of complement components. Connective tissue matrix molecules contain GlcNAc within their polysaccharide chains. Oxygen free radicals have been shown to cause the partial degradation of hyaluronic acid [28] which may expose GlcNAc in the terminal position. Studies are in progress comparing the distribution of GlcNAc staining with that of a wide range of matrix components including fibrous proteins and glycosaminoglycans.

Some of the material could be derived from nearby cell membranes. The staining pattern does not coincide with that of membrane-bound proteins such as Fc receptors or Class II antigens, but further double labelling experiments are planned to investigate this possibility.

The absence of terminal GlcNAc from established fibrinoid foci in rheumatoid nodules is somewhat surprising, in view of the suggestion that cellular events in the palisading layer may be similar to those occurring at the synovial surface in rheumatoid arthritis. However, the presence of GlcNAc close to small foci of cell pyknosis suggests that exposure of terminal GlcNAc may be an early or transient phenomenon, and may not be appreciable in the more fibrous chronic lesions.

GlcNAc expression may simply be a marker of tissue damage. However, there are two reasons for considering the possibility that terminal GlcNAc may contribute to the propagation of inflammation. First, raised antibody levels to the GlcNAc-rich peptidoglycan/polysaccharide complex of group A streptococci in RA may be appreciable in the more fibrous chronic lesions.

The significance of terminal GlcNAc residues may vary with associated sugars in the oligosaccharide chain, particularly if chains are branched (bi- or trisaccharide). It is not yet clear how closely reagents such as GN7 or F4/80 in bacillus-Calmette-Guerin-activated mouse macrophages can act as a signal for cells to take up material such as lysosomal enzymes, effete or ageing proteins, cells undergoing programmed death (apoptosis) and tissue elements during developmental remodelling. At sites of inflammation, uptake of GlcNAc-bearing material may act in conjunction with mechanisms such as antibody–antigen complexing and complement fixation to promote the release of inflammatory mediators or cytokines. A number of such cytokines, including tumour necrosis factor and IL-6 have been isolated from rheumatoid synovial fluid [29].

The significance of terminal GlcNAc residues may vary with associated sugars in the oligosaccharide chain, particularly if chains are branched (bi- or trisaccharide). It is not yet clear how closely reagents such as GN7 or Bandeiraea agglutinin II resemble the lectin-like molecules on the macrophage surface. In vitro studies are currently in progress aimed at assessing structure activity relationships of GlcNAc-bearing molecules in terms both of GN7 binding and effects on the function of monocyte-derived cells.

ACKNOWLEDGEMENTS

We are grateful to the Wellcome Trust and the Special Trustees of the Middlesex Hospital for financial support. Tissues were kindly provided by Dr J. Pringle, Dr D. Isenberg and the surgical staff of the Department of Orthopaedics of the Middlesex Hospital and the Royal National Orthopaedic Hospital. Cell specific antisera were kindly provided by Dr L. Poulter of the Royal Free Hospital Medical School.

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NOTICES

35e JOURNÉES ANNUELLES DU CENTRE VIGGO PETERSEN—L’ACTUALITÉ RHUMATOLOGIQUE 1990

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