THE EFFECTS OF SULFASALAZINE, ITS METABOLITES AND RELATED COMPOUNDS ON MITOGEN-INDUCED LYMPHOCYTE PROLIFERATION

BY

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ABSTRACT

Salicylazosulfapyridine (SASP), used in the management of rheumatoid arthritis (RA), comprises sulfapyridine (SP) azo linked to 5-aminosalicylic acid (5-ASA). SASP inhibited mitogen-induced, human, and murine, T- and B-lymphocyte proliferation in vitro. Efficient suppression was attained at supra-physiological levels of SASP; equimolar SP was slightly inhibitory, 5-ASA, negligible. SASP-mediated suppression (SMS) was not due to cytotoxicity, nor to a concerted effect of SP and 5-ASA; the drug appeared to influence lymphocytes rather than macrophages.

The action of SASP and various 5-ASA azo compounds on mouse splenocyte responses to Concanavalin A (Con A), were further analyzed. SMS was dependent on serum, and mitogen, concentrations, and cell numbers, in culture. SMS was abrogated by washing SASP-containing Con A cultures drug-free after 24 hours, perhaps suggesting that SASP interferes with a cellular signal effecting the transition of lymphocytes from G1 to S phase. Interleukin-2 (IL-2) failed to reverse SMS, possibly implying that SASP impedes the binding of IL-2 to its cellular receptor, a mechanism which may be germane to its therapeutic activity in RA.

All SASP analogues tested were much weaker suppressants than SASP; olsalazine (two azo-linked 5-ASA molecules) was the strongest, although its isomers containing 4-ASA were markedly less potent. It was inferred from the findings of several SASP analogue studies, that both the SP and 5-ASA groups within the intact SASP structure, were required for SMS.
SMS of phytohaemagglutinin-induced RA lymphocyte proliferation in vitro, declined in clinical "responders", after 12 weeks of SASP therapy; this change in lymphocyte behaviour was absent in unresponsive RA patients. Possible explanations are discussed.
STATEMENT

This thesis is based on research carried out by the author in the Department of Microbiology at the University of Leicester, U.K., during the period between July 1988 and June 1990.

The work recorded herein is entirely original, unless otherwise acknowledged in the text by references. None of the work has been submitted for another degree in this, or any other, University.

CHRISTOPHER J.A.L.WEBB

15th December 1992
This work is dedicated to all my family
I am very deeply grateful to Mum and Dad for their very
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work could never have been realized. It must be
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(3) Mr J. Fleming, BSc. / Dr J. Ward
(4) Dr Ashe. Samanta (Leicester Royal Infirmary)
(5) Dept of Haematology, Leicester Royal Infirmary
(6) Ms Sue Graham (Dept of Immunopathology)
(7) Reshad Khodabocus, BSc.
(8) Mr J.L. Beckett, BSc. (Computer Centre, Leicester
University)
Bullfight critics ranked in rows
Crowd the enormous Plaza full;
But only one is there that knows-
And he's the man who fights the bull.

Domingo Ortega
(trans. Robert Graves)
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<tr>
<td>AA</td>
<td>adjuvant arthritis</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AO/EB</td>
<td>acridine orange/ethidium bromide</td>
</tr>
<tr>
<td>ARA</td>
<td>American Rheumatism Association</td>
</tr>
<tr>
<td>AS</td>
<td>ankylosing spondylitis</td>
</tr>
<tr>
<td>3-ASA</td>
<td>3-amino salicylic acid</td>
</tr>
<tr>
<td>4-ASA</td>
<td>4- &quot;</td>
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<tr>
<td>5-ASA</td>
<td>5- &quot;</td>
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<tr>
<td>5-ASA-N=N-ABA</td>
<td>5-ASA azo-4 amino benzoic acid</td>
</tr>
<tr>
<td>5-ASA-N=N-BSA</td>
<td>5-ASA azo benzene sulfonic acid</td>
</tr>
<tr>
<td>BALSAL</td>
<td>balsalazide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>collagen arthritis</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>CD4, CD8 etc</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CRL</td>
<td>complement receptor positive lymphocyte</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DHM</td>
<td>dextran/heparin mixture</td>
</tr>
<tr>
<td>d-PEN</td>
<td>d-penicillamine</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>FMLP</td>
<td>N-formyl-l-methionyl-l-leucyl-l-phenylalanine</td>
</tr>
<tr>
<td>GT</td>
<td>galactosyl transferase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salts solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venule</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>$^3$H TdR</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICM</td>
<td>Iscoves' culture medium</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>IM</td>
<td>indomethacin</td>
</tr>
<tr>
<td>IPSAL</td>
<td>ipsalazide</td>
</tr>
<tr>
<td>IU</td>
<td>international unit</td>
</tr>
<tr>
<td>LCDF</td>
<td>lymphocyte-derived monocyte chemotactic factor for monocytes</td>
</tr>
<tr>
<td>LIF</td>
<td>leukocyte inhibition factor</td>
</tr>
<tr>
<td>LPS</td>
<td>(bacterial) lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>leukotriene</td>
</tr>
<tr>
<td>MeSASP</td>
<td>methylsulfasalazine</td>
</tr>
<tr>
<td>MeSP</td>
<td>methylsulfapyridine</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MP</td>
<td>misoprostol</td>
</tr>
<tr>
<td>Mt</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NS</td>
<td>(statistically) non-significant</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>4-OHPAA</td>
<td>4-hydroxyphenylacetic acid</td>
</tr>
<tr>
<td>OLSAL</td>
<td>olsalazine</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMNC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PGLY</td>
<td>proteo(peptido)glycan</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>PHA</td>
<td>phorbol myristic acetate</td>
</tr>
<tr>
<td>PMNL</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PsA</td>
<td>psoriatic arthritis</td>
</tr>
<tr>
<td>PV</td>
<td>plasma viscosity</td>
</tr>
<tr>
<td>PWM</td>
<td>pokeweed mitogen</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>ReA</td>
<td>reactive arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>RN</td>
<td>rheumatoid nodule</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RS</td>
<td>Reiter's syndrome</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RV</td>
<td>rubella virus</td>
</tr>
<tr>
<td>SAARD</td>
<td>slow-acting anti-rheumatic drug</td>
</tr>
<tr>
<td>SASP</td>
<td>salicylazosulfapyridine</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE(M)</td>
<td>standard error (of mean)</td>
</tr>
<tr>
<td>SF</td>
<td>synovial fluid</td>
</tr>
<tr>
<td>SI</td>
<td>stimulation index</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SP</td>
<td>sulfapyridine</td>
</tr>
<tr>
<td>TfR</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analogue scale</td>
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LEGENDS TO FIGURES

FIGURES 1(a)-1(d)
Effect of graded doses (up to 100μg/ml) of SASP, SP and 5-ASA on mouse splenocyte proliferation in response to PHA, Con A, PWM and LPS.

FIGURE 1(e)
Effect of graded doses (up to 400μg/ml) of SASP, SP and 5-ASA on normal human PBL proliferation in response to PHA.

FIGURE 2
Comparison of dose range required for SASP-mediated suppression of mouse splenocyte, and normal human, PBL proliferation in response to PHA (data from Figs. 1(a) and 1(e)).

FIGURE 3
Effect of up to 100μg/ml SASP, SP and 5-ASA on relative Ig production (measured by ELISA) by mouse splenocytes cultured with PWM for 96 hours.

FIGURE 4
Percentage suppression of mouse splenocyte proliferation in response to Con A, attained by 100μg/ml SASP and corresponding molar equivalents of SP and 5-ASA, added separately or in combination.

FIGURE 5(a)
Effect of sub-optimal Con A plus SASP at 25μg/ml on mouse
splenocyte proliferation: relationship between % control CPM and CPM obtained.

FIGURE 5(b)
Effect of sub-optimal Con A plus SASP at 50μg/ml on mouse splenocyte proliferation: relationship between % control CPM and CPM obtained.

FIGURE 6
Effect of up to 50μg/ml SASP on mouse splenocyte proliferation in response to sub-optimal (0.225μg/ml) and optimal (2.5μg/ml) Con A.

FIGURE 7(a)
Effect of variation in cell number on mouse splenocyte proliferation in response to Con A, in the presence of 50μg/ml SASP: relationship between % control CPM and cell number.

FIGURE 7(b)
Effect of variation in cell number on mouse splenocyte proliferation in response to Con A, in the presence of 0 and 50μg/ml SASP: relationship between CPM obtained and cell number.

FIGURE 8(a)
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FIGURE 8(b)
Effect of 0 and 10% FCS on mouse splenocyte proliferation in response to Con A: relationship between % control CPM and CPM obtained in the presence of 50µg/ml SASP.

FIGURE 9
Effect of addition of up to 100µg/ml SASP at 0, 4, 24 and 46 hours of culture, on mouse splenocyte proliferation in response to Con A.

FIGURE 10
FACS printouts for human PBMNC partially depleted of macrophages by carbonyl iron treatment (top figure), and non-depleted (bottom).

FIGURE 11
Standard curve indicating nM hydrogen peroxide present in standard volumes, as determined by a colorimetric method.

FIGURE 12
Effect of increasing concentrations of misoprostol on mouse splenocyte proliferation in response to Con A.

FIGURE 13
Effect of increasing concentrations of indomethacin on mouse splenocyte proliferation in response to Con A.

FIGURE 14
Comparative effects of SASP and various 5-ASA azo
compounds, viz: olsalazine, ipsalazide, balsalazide, 5-ASA azo 4-amino benzoic acid, and 5-ASA azo benzene sulfonic acid, on mouse splenocyte proliferation in response to Con A.

FIGURE 15
Comparative effects of olsalazine and its isomers, 5-ASA-N=N-4-ASA and 4-ASA-N=N-4-ASA, on mouse splenocyte proliferation in response to Con A.

FIGURE 16
Comparative effects of 5-ASA, 4-ASA and 3-ASA on mouse splenocyte proliferation in response to Con A.

FIGURE 17
*In vitro* effect of SASP at 200 and 300 μg/ml on PHA-induced proliferation of PBL obtained from 14 RA clinical responders to SASP therapy: comparison of SASP-mediated suppression at 0 and 12 weeks of treatment.

FIGURE 18
*In vitro* effect of SASP at 200 and 300 μg/ml on PHA-induced proliferation of PBL obtained from 7 RA clinical non-responders to SASP therapy: comparison of SASP-mediated suppression at 0 and 12 weeks of treatment.

FIGURE 19
Suppressibility of 21 individual RA patients' PBL responsiveness to PHA by 200 μg/ml SASP: comparison of Δ CPM at 0 and 12 weeks of SASP therapy between 14 clinical
responders and 7 non-responders.

FIGURE 20
Suppressibility of 16 individual RA patients' PBL responsiveness to PHA by 1mg/ml SP: comparison of A CPM at 0 and 12 weeks of SASP therapy between 12 clinical responders and 4 non-responders.

FIGURE 21
Individual RA patients' PBL responses to PHA: comparison of CPM values at 0 and 12 weeks of SASP therapy, between 14 clinical responders and 7 non-responders.

FIGURE 22
Suppressibility of 10 individual RA patients' PBL responsiveness to PHA by 200μg/ml SASP: comparison of A CPM at 0 and 12 weeks of d-PEN therapy between 7 clinical responders and 3 non-responders.
OLSALAZINE AND ITS ISOMERS (DISODIUM SALTS)

(1) OLSALAZINE (Dipentum [5-ASA-N=N-5-ASA])

(2) 5-ASA-N=N-4-ASA

(3) 4-ASA-N=N-5-ASA
CHEMICAL STRUCTURES OF COMPOUNDS TESTED

SALICYL AZOSULFAPYRIDINE (SASP)

AZO REDUCTASE

SULFAPYRIDINE (SP) + 5-AMINOSALICYLIC ACID (5-ASA)

SASP is cleaved at the azo bond (\(\dagger\)) by azo reductase present in colonic bacteria, yielding SP and 5-ASA.
ADDITIONAL 5-ASA AZO DERIVATIVES TESTED

(1) IPSALAZIDE (5-ASA-4 AMINO BENZOYL GLYCINE [DISODIUM SALT])

(2) BALSALAZIDE (5-ASA-4 AMINO BENZOYL $\beta$-ALANINE [DISODIUM SALT])

(3) 5-ASA-4 AMINO BENZOIC ACID (5-ASA-$\text{N}=\text{H}-4$-ABA [SODIUM SALT])

(4) 5-ASA-BENZENE SULFONIC ACID (5-ASA-$\text{N}=\text{H}$-BZA [DISODIUM SALT])
1 INTRODUCTION

Salicyl-azo-sulfapyridine (sulfasalazine, salazopyrin, SASP) is a well-established therapeutic drug, of considerable ameliorative value in inflammatory bowel disease (IBD). In more recent years, it has gained credence as an effective, alternative second line agent in the treatment of rheumatoid arthritis (RA). There is also mounting evidence of its therapeutic efficacy in the spondyloarthropathies. The in vitro studies presented herein, were undertaken with the ultimate objective of attempting to apply the findings to the in vivo situation of rheumatoid arthritis. Hence, the introduction is chiefly concerned with RA, and the experimental results are discussed in the context of RA.

1/1 REVIEW OF DISEASES FOR WHICH SASP IS BENEFICIAL

1/1/1 RHEUMATOID ARTHRITIS (RA)

RA is an idiopathic, inflammatory pansynovitis, affecting similar joints bilaterally. Extra-articular involvement of several organ systems is frequently a secondary feature. In this respect, RA can be regarded as a systemic disease. In some texts, it is classified as an autoimmune disorder, since there is a very strong correlation between serum levels of autologous antiglobulins (rheumatoid factors, [RFs]), and severity of disease. RA prevalence is widespread, 2-3% of the general...
population being afflicted. The average age of disease onset is about 35 years, although RA can occur at any age. Women of child-bearing age are three times more susceptible than are males of similar age range. However, there is a trend towards equivalent disease incidence between the sexes after the seventh decade. The aetiology of RA remains unknown.

1/1/1/1 HISTORICAL ASPECTS OF RA

The term "rheumatoid arthritis" was first introduced into medical literature by Sir Alfred Baring Garrod, in 1859. In 1890, his son, Sir Archibald Edward Garrod, published A Treatise on Rheumatism and Rheumatoid Arthritis. Under rheumatoid arthritis, he included osteoarthritis, which was subsequently classified as a distinct entity, but his treatise nevertheless presented a lucid clinical description of latter day RA. It was his belief that evidence for the antiquity of RA, was afforded by paleopathological studies of Egyptian, Roman, and Northern European, remains. This view was upheld until 1952, whence it was challenged by Snorrason, who maintained that the incidence of RA amongst Europeans, appeared at, or after, 1800. This claim, corroborated by others, and presumably implying an inauspicious environmental change in the later years of the eighteenth century, can be traced back to a doctoral thesis submitted in 1800, by Augustin-Jacob Landre-Beauvais, a French medical student. The substance of this work, based on case
reports and autopsies, is, in fact, a collation of hitherto unrecognized RA with classic gout. This thesis is generally acknowledged to contain the earliest descriptive account of RA as a separate entity, as based on clinical studies. However, specific features of this disease, such as hyperextension deformities (swan neck deformity) of the finger joints, were formerly described (in 1676) by Thomas Sydenham, a physician whose acute powers of observation facilitated accurate disease description. Thus, the origin of RA in Europe, can be traced as far back as the latter part of the seventeenth century. Studies of human paleopathology, have, nevertheless, failed to produce convincing evidence of the existence of RA in pre-historic times.

1/1/1/2 (IMMUNO)PATHOLOGY OF RA

RA is initiated by a putative immunizing event, strong enough to evoke an inflammatory synovial lesion. The earliest stage of the disease, clinically referred to as the "acute phase", is characterized by immune hyperactivity. Upon localization of the response, macrophages and dendritic cells become involved in presentation of the (unknown) antigen to recruited helper T-lymphocytes. Proliferation of appropriate B-cell clones may occur, with subsequent RF synthesis. RF interaction with autologous IgG molecules generates immune complexes, whose fate is threefold. The majority are either sequestered within the collagenous matrix of articular
cartilage, or phagocytozed by polymorphonuclear leukocytes (PMNLs) and activated macrophages. A further minority migrate to extra-articular blood vessels, causing vasculitis. The synovium becomes infiltrated predominantly with PMNLs, which, during clearance of immune complexes and other debris within the synovial fluid, release proteolytic enzymes such as collagenase and elastase, pro-inflammatory prostaglandins (PGs) and leukotrienes (LTs), and free radicals of oxygen (reactive oxygen species). Non-phagocytozed immune complexes activate the complement cascade; the C5a component in turn stimulates further inflammatory mediator systems, such as the clotting cascade, and fibrinolytic, and kinin, systems. These mechanisms tend to act synergistically, thereby helping to sustain inflammation within the joint space.

The acute phase is also constituted by macrophage activation, with consequent secretion of inflammatory mediators such as interleukin-1 (IL-1), proteolytic enzymes, and most significantly, cell growth and angiogenesis, factors. The latter are responsible for the development of neovascularature, within the synovial tissues. Without this facility the inflammatory process cannot be sustained. Cell growth factors induce synoviocyte proliferation. As a result of this increased cellular turnover, excess synovial fluid (SF) is produced, causing an effusion which is characteristically pale yellow, of low viscosity, and infiltrated with vast numbers of leukocytes, predominantly PMNLs.

If untreated, a chronic synovitis leads to articular
cartilage destruction, and eventual total loss of joint function. Synovial fibroblasts are triggered to synthesize more connective tissue matrix. The hypertrophied synovium with its rapidly increasing capillary network, morphologically resembles an activated lymph node, to the extent of germinal centres containing a cellular infiltrate, mostly comprising T-lymphocytes. Villus formation also occurs. By gradually expanding, the hypertrophied synovium (pannus) encroaches upon joint cartilage and subchondral bone. These tissues are degraded by various enzymes released from the pannus cells. Neutral proteinases disaggregate proteoglycans (PGLYs), which then become solubilized. Cross-linkages among collagen fibrils are cleaved by elastase, and the helical regions by collagenase. Fragmented soluble collagen denatures spontaneously, with further catabolism by neutral proteinases and cathepsins. Collagen fragments are able to attract monocytes by chemotaxis. By this means, even greater levels of arachidonic acid metabolites are attained. These activate osteoclasts to resorb mineral from subchondral bone. Degradation of articular cartilage results in extensive surface ulceration, and articulation causes pain and bone erosion, due to bone-bone contact. Loss of joint integrity also results in fibrosis of tendon sheaths.

Rheumatoid nodules (RNs) are present in about 20-30% of RA cases, and are associated with high RF titers, and thus more severe arthritis. RNs are chronic granulomatous lesions which occur at sites of pressure and movement,
both subcutaneously and internally. Externally, they are most commonly found at the elbow, although their distribution is non-specific; internally, they appear in various organs including the heart and lung. Their provenance is not absolutely clear, although pathologic studies suggest that they develop at capillary sites, and that their surfaces consist of a layer of lymphocytes and plasma cells. It seems likely that RN formation is a consequence of vasculitis, associated with the migration of immune complexes from the synovium. This hypothesis could explain the correlation between RNS and RF.

1/1/1/3 CLINICAL ASPECTS OF RA

The arthritides, or rheumatic disorders, encompass a number of discrete diseases affecting the joints and connective tissues. Clinical manifestations of these disorders often overlap. Furthermore, in the case of RA, the clinical course is highly variable. For instance, an RA patient may present with vague symptoms such as various joint pains and fatigue, in the absence of any clinical evidence of synovitis. In a minority of cases, extra-articular symptoms, e.g., RNS, appear before synovitis.

The clinical course of RA generally falls into three categories. In the majority of cases, periods of disease activity are interspersed with intervals of spontaneous remission, the duration of the remission periods lasting between a few months and several years. This disease
pattern offers the most favourable prognosis. The second, fairly common, course, is characterized by a progressive and unremitting synovitis, interrupted by sporadic flares typified by severe joint pains and effusions, marked inflammation, fever, general malaise, anaemia, and debilitation. A third, far less frequent course, is one of very aggressive and rapid, polyarticular synovitis, accompanied by very high RF titers, nodules, and extensive extra-articular organ involvement.

1/1/1/4 DIAGNOSTIC CRITERIA FOR RA

Because of the inherent difficulties in making an accurate diagnosis of RA, the American Rheumatism Association (ARA) formulated a list of eleven diagnostic criteria. Differential diagnosis is dependent on the number of criteria satisfied (Kellgren et al [eds], 1963). However, this system is currently in desuetude, and has been largely superseded by another set of criteria, which were advanced at the third International Symposium of Population Studies of the Rheumatic Diseases, in New York in 1966 (Bennett & Burch, 1967). These criteria, being more restrictive, exclude mild, non-deforming disease, sero-negative arthritis, and cases in which only one or two joints are affected. Both sets of criteria are listed below:-
ARA CRITERIA

1. Morning stiffness
2. Pain on motion or tenderness in at least one joint
3. Swelling of one joint representing soft tissue or fluid
4. Swelling of at least one other joint (soft tissue or fluid) with an interval free of symptoms no longer than 3 months
5. Symmetrical joint swelling (simultaneous involvement of the same joint, right and left)
6. Subcutaneous nodules over bony prominences, extensor surfaces, or near joints
7. Typical roentgenographic changes which must include demineralization in periarticular bone as an index of inflammation; degenerative changes do not exclude diagnosis of RA
8. Positive test for RF in serum
9. SF; a poor mucin clot formation on adding SF to dilute acetic acid
10. Synovial histopathology consistent with RA, viz:
   (a) Marked villous hypertrophy
   (b) Proliferation of synovial cells
   (c) Lymphocyte/plasma cell infiltrate in subsynovium
   (d) Fibrin deposition within or upon microvilli
11. Characteristic histopathology of RNs biopsied from any site

DIAGNOSIS

Classic RA- 7 criteria needed
Definite RA- 5 criteria needed
Probable RA- 3 criteria needed
To meet criteria 1-5, symptoms or signs must be present for at least 6 weeks.

NEW YORK CRITERIA
RA is present if criteria 1 and 2 plus either 3 or 4 are met:

1. History of an episode of 3 painful limb joints. Each group of joints (e.g., proximal phalangeal joint) is counted as one joint, scoring on each side separately
2. Swelling, limitation of motion, subluxation and/or ankylosis of three limb joints. Necessary inclusions: (a) at least one hand, wrist or foot; (b) symmetry of one joint pair. Exclusions: (a) distal interphalangeal joints; (b) fifth proximal interphalangeal joints; (c) first metatarsophalangeal joint; (d) hips
3. Radiographic changes (erosions)
4. Serum positive for RFs

1/1/1/5 BIOCHEMICAL/IMMUNOLOGICAL MARKERS OF RA DISEASE ACTIVITY

Diagnosis of RA, and its response to treatment, are aided by standard laboratory tests monitoring biochemical and immunological changes during disease activity. Essentially, these are indicators of systemic inflammation, and are therefore not absolutely specific
for RA. The acute phase response to the synovial insult is characterized by numerous pro-inflammatory metabolic alterations, including increased synthesis of various hormones, accelerated protein catabolism, and gluconeogenesis. Certain liver-derived protein inflammatory mediators such as fibrinogen, are mobilized. Serum levels of C-reactive protein (CRP), evaluated by electrophoresis, are routinely measured during the course of RA, since they often exceed 1,000-fold normal levels during active disease. There are several other acute phase proteins amenable to electrophoretic analysis. These include ceruloplasmin, α₁-antitrypsin, haptoglobin, and serum amyloid A. Due to elevated fibrinogen levels, both the erythrocyte sedimentation rate (ESR) and plasma viscosity (PV) are raised during acute inflammation. Measurement of the ESR is the most frequently performed laboratory test used in monitoring the course of RA, since the experimental technique is relatively simple and reliable. Decreased serum levels of iron, as evaluated by haemoglobin concentrations, are indicative of disease activity. The absolute number of circulating platelets is markedly raised during inflammation.

Immune hyperactivity is another feature of the acute phase. The total white cell count is often elevated, due to an increase in circulating PMNLs. Whilst the overall number of peripheral blood lymphocytes (PBL), and the relative proportions of T/B-cell subsets, are not generally altered in acute RA, IgM, (and sometimes IgG and IgA), titers, as assessed by serum protein
electrophoresis, are commonly elevated, since they are present as RFs. RFs are antibodies directed against antigenic determinants on IgG Fc fragments. It is now well established that RA patients with positive titers of RF (seropositive RA), are prone to more aggressive disease than are their seronegative counterparts. The presence of IgM-RF in the form of immune complexes, is strongly correlated with severe and debilitating disease symptoms. Hence, quantitation of serum IgM-RF levels is often useful in the evaluation of disease severity. There is a neoteric suggestion that serum complexes containing IgA, appear to presage erosive arthritis (Westedt et al, 1986). Serum and synovial levels of certain complement components, particularly C3 and C4, are generally assayed. Whilst serum levels are seldom depressed, synovial fluid analysis is of greater diagnostic value, in that reduction in complement correlates well with the extent of synovitis. Circulating immune complexes, as measured using latex reagents, are perhaps more indicative of vasculitis, rather than inflammation, in RA.

1/1/1/6 DRUG TREATMENT IN RA

1/1/1/6/1 FIRST LINE AGENTS

Non-steroidal anti-inflammatory drugs with analgesic (and sometimes anti-pyretic) properties (NSAIDs), are initially indicated in RA. A wide range of NSAIDs of varying potency are currently in use. Although
structurally heterogeneous, their universal mode of action is to block PG biosynthesis, by inhibition of the enzyme, cyclo-oxygenase, which converts arachidonic acid to precursor cyclic endoperoxides. It is often necessary for the individual to attempt several NSAIDs, before a suitable choice can be made. In former decades, aspirin and other salicylate preparations were initially chosen. Nowadays, however, salicylates are infrequently used, in view of their well-acknowledged adverse effects on the gastro-intestinal tract.

More commonly prescribed are the phenylpropionic acid derivatives such as ibuprofen, which is alleged to have fewer side effects than most other NSAIDs. Alternative drugs within this group are: naproxen, fenbufen, fenoprofen, flurbiprofen, ketoprofen and tiaprofenic acid. Naproxen is often a first choice agent, since it combines good efficacy with minimal adverse effects. The remaining compounds tend to be less well tolerated, particularly in the gut. The phenylacetic acid derivatives, diclofenac (voltarol) and etodolac, are similar in action to naproxen. Amongst the pyrazolones, phenylbutazone is a powerful anti-inflammatory agent, but is now restricted to hospitalized ankylosing spondylitis patients, because of occasional serious side effects such as aplastic anaemia; azapropazone (rheumox) is chemically related to phenylbutazone, and is similar in efficacy to naproxen, but is disadvantageous in producing a high incidence of rashes. The oxicams, e.g., piroxicam, are fairly effective and prolonged in their action, but are somewhat less well
tolerated than naproxen. Indomethacin is particularly potent, but is associated with a considerable number of side effects; the other indole-related compounds, sulindac (clinoril), and tolmetin (tolectin), appear to compare favourably with ibuprofen and naproxen. Thus, there is a wide range of NSAIDs available, although it must be asserted that a number of newly synthesized agents have been withdrawn from the market, either by the manufacturers, or by the Committee of Safety of Medicines, during the last few years, because of potentially dangerous adverse effects.

1/1/1/6/2 SECOND LINE AGENTS

Whenever NSAIDs fail to control symptoms of RA, and there is the likelihood of progressive joint damage or extra-articular disease, second line drugs are warranted. These are more radical in their mechanism of action, having disease modifying, or remission inducing, properties. Because their beneficial effects are not apparent for weeks or months, they are sometimes termed "slow-acting anti-rheumatic drugs" (SAARDs). Side effect profiles are quantitatively greater than those of first line drugs, and their beneficial effects are generally experienced after several weeks of therapy. Because of these strictures, treatment with second line agents is undertaken by a physician with special knowledge and experience of their use.

Gold (Au I) salts, d-penicillamine (d-PEN, distamine),
and more recently, sulfasalazine, are the most common second line drugs in rheumatological practice. Gold (i.e. sodium aurothiomalate or sodium aurothioglucose) is initially administered by deep intra-muscular injections once weekly, for about three months, whence an effective body concentration is reached. Thereafter, a maintenance dose is delivered until remission of disease symptoms is obtained. An oral gold preparation, auranofin (triethylphosphine gold), has recently been developed, and is presently under evaluation. Gold salts bind avidly to plasma albumin, and rapidly diffuse into the inflamed synovium. Their precise mechanism(s) of action are unclear, although they appear to inhibit the migration of mononuclear cells to the vicinity of inflammation. Gold salts may also stabilize lysosomal membranes, thereby restricting the release of destructive pro-inflammatory products. Adverse effects such as dermatitis, stomatitis, blood disorders, and renal and hepatic damage, occur in about 33% of patients treated, which restricts the usage of gold in RA. Furthermore, the possible occurrence of renal impairment, necessitates close supervision of the patient, and regular monitoring of urine for protein.

d-PEN is a suitable alternative to gold in a therapeutic regimen for RA. Like gold, it possesses immunomodulatory properties, notably interference with the complement cascade, and suppression of immunoglobulin production. It is also a chelator of certain metal ions including gold (Au I), thereby contra-indicating any possibility of combination therapy. Undesirable effects of
d-PEN account for its discontinuation in about one third of patients treated. Gastrointestinal disorders, allergic reactions, and impairment of taste and blood clotting, are characteristic adverse conditions. The potential for renal damage to develop after several months of therapy, entails close supervision, and frequent analysis of blood and urine.

Apart from SASP (discussed separately in section 1/3), other second line agents include antimalarials and immunosuppressants. Antimalarials of the chloroquine group are occasionally employed in RA. Their chief disadvantage is that of ocular toxicity; they are otherwise fairly weak second line agents. Amongst immunomodulator drugs, azathioprine (imuran) is of similar therapeutic value as gold and penicillamine, although bone marrow suppression may occur. Cyclophosphamide and chlorambucil are more potent than azathioprine, but are more toxic. Further limitations are imposed upon immunosuppressants, particularly amongst younger patients, due to their carriage of carcinogenic and teratogenic, risks, the magnitude of which are uncertain.

1/1/1/6/3 "THIRD LINE" AGENTS- ADRENAL STEROIDS

Usage of systemic adrenal steroids in RA is reserved for cases which have failed to respond to, or tolerate, several second line agents. Occasionally, a corticosteroid may be combined with a SAARD at the inception of therapy, to relieve inflammation during the interim period. The
anti-inflammatory effects of corticosteroids are mediated by inhibition of the enzyme, phospholipase A₂, which releases cellular arachidonic acid. Thus, corticosteroids, like NSAIDs, block PG synthesis, but additionally suppress the lipoxygenase pathway, by which LTs are formed. This is the basis for the very high efficacy of adrenal steroids in gratuitous inflammatory states such as RA, and for their potentially dangerous non-specific suppression of protective inflammation, should an infection arise. Intra-articular injections of corticosteroids such as prednisolone and hydrocortisone, are sometimes administered with extreme astringency, to RA patients, when inflammation of a particular joint is inordinately severe.

1/1/2 OVERVIEW OF THE SPONDYLOARTHROPATHIES

The spondyloarthropathies form a composite group of seronegative arthritides, which share certain features. The entire group consists of six distinct diseases, viz:

(a) ankylosing spondylitis (AS)
(b) Reiter’s syndrome (RS)
(c) psoriatic arthritis (PsA)
(d) reactive arthritis associated with enteropathic infection, initiated by yersiniae, shigella, salmonella and campylobacter organisms (ReA)
(e) arthritis associated with IBD
(f) Whipple’s disease
ANKYLOSING SPONDYLITIS (AS)

AS is the prototype of the spondyloarthopathies. Cardinal features of AS include inflammation at the junction of bone and ligament (enthesopathy), sacroiliitis, spondylitis, inflammatory ocular disease, and asymmetric arthritis predominantly involving the proximal synovial joints such as the shoulders, hips, and knees. About 1% of Caucasians are afflicted; the ratio of males to females is roughly equal when sacroiliitis is present, but with spondylitis, it is approximately 3:1. Age of AS onset is generally during the second and third decades, and there is a strong familial prevalence. AS is very highly correlated with the presence of the HLA-B27 haplotype. About 90% of Caucasian AS subjects are B27 positive, compared with 8% within the general population. Tissue typing would therefore seem invaluable, should diagnostic difficulties arise.

The most frequent clinical manifestations of AS are mid- and low-back pain and stiffness, thoracic cage pain, enthesopathic pain at various sites, and arthritic signs such as early morning stiffness, and joint swelling. Synovitis associated with AS is, however, non-progressive, and thus rarely causes joint deformity and functional loss, prototypical of untreated RA. Uveitis occurs in about 25% of patients, and is characterized by pain, rubor, and abnormal light sensitivity (photophobia). AS chiefly affects the joints of the axial skeleton, causing
inflammation, which in turn elicits fibrosis. The ensuing pathological course is one of calcification, followed by ligamentous ossification, and vertebral fusion by bony bridging of the joint margins. The spine becomes irreversibly immobilized, giving the radiographic appearance of the "bamboo spine". Treatment is based on a regimen of physical exercises, the purpose of which, is to minimize the development of disabling spinal rigidity. The use of NSAIDs is also indicated.

1/1/2/2 REITER'S SYNDROME (RS)

RS is characterized by urethritis, conjunctivitis, mucocutaneous lesions, and arthritis. The age of disease onset is nearly always during the third or fourth decade, and the male:female ratio is 10-15:1. RS is frequently classified as a reactive (infectious) arthritis, since it occurs after venereal exposure, or dysentery. The venereal type is more prevalent in the USA, and in approximately 33% of cases, is associated with Chlamydia trachomatis induced urethritis. The post-dysenteric disease, consequent upon gastrointestinal infection with Shigella organisms, predominates in Europe, Africa, and the Far, and Middle, East. Like AS, the serotype HLA-B27 is present in about 75% of Caucasians with RS.

Clinical symptoms of RS appear between 1 and 4 weeks following dysentery or venereal exposure. Urethritis, characterized by urethral discharge, frequency of urination, and a burning sensation, usually develops at an
early stage. Conjunctivitis presents as a mild inflammation with irritation and burning, and is generally self-limiting, lasting up to 48 hours. In some cases, however, acute iridocyclitis occurs, which may cause ocular damage if untreated. Mucocutaneous lesions, usually painless, are generally confined to the oropharynx, penis, and surfaces of the feet. Arthritis is often abrupt in its onset, and generally affects the knees and ankles, although joint distribution is non-specific. Swelling, pain and effusions are cardinal symptoms. Enthesopathy, as envisaged in AS, is also a notable feature. Other clinical symptoms of RS include fever, malaise and weight loss.

The prognosis of RS is variable. The majority of patients fail to succumb to further episodes, whilst about one third experience recurrences or sustained disease activity. In 15% of cases, AS complicates the progression of RS. Although there are no specific diagnostic tests, detection of serum antibodies to Shigella organisms, may prove fruitful. Cultures of a urethral discharge occasionally yield evidence of Chlamydia infection. During active disease, elevated numbers of circulating leukocytes, and a rise in ESR, are frequently noted. Treatment of RS depends on the severity of symptoms. Extensive skin lesions might warrant the use of a topical corticosteroid. The arthritis generally responds to NSAIDs combined with physical therapy. Enthesopathy may require local corticosteroid injections.
PsA is a systemic disorder which occurs in 5-7% of people afflicted with psoriasis. Clinical symptoms of the arthritic component are very similar to those of RA, viz: fatiguability, early morning stiffness, pain, swelling, and limitation of joint motion. Occasionally there is weight loss, fever, and general malaise. The severity and extent of psoriasis do not correlate with arthritis activity. AS, with or without sacroiliitis, is seen in 20-33% of PsA subjects.

The arthritis falls into three categories. Patients with Group I synovitis, initially present with an asymmetric, oligo- or poly-, arthritis which is non-specific, but the distal and proximal interphalangeal joints of both the hands and feet, are especially involved. However, the arthritis can become symmetrical over a period of time. Digital deformities ("sausage" digits) commonly arise as a result of both interphalangeal involvement, and attendant flexor tendon sheath effusions. Onychodystrophy occurs in about two thirds of PsA subjects with asymmetric arthritis. The prognosis is favourable for the majority of Group I patients, especially if their joint involvement remains asymmetric, although about 25% develop a destructive, aggressive disease similar to untreated RA. Group II patients initially present with a symmetrical synovitis. About 50% of cases eventuate in destructive, deforming disease. Group III comprises cases of spondyloarthritis, in which AS, spondylitis, or
sacroiliitis co-exist with psoriasis. Amongst this group, peripheral arthritis does not necessarily occur.

Diagnosis is aided by radiography, which may detect osteoporosis and bone resorption, as well as ankylosis, if present. There are no specific laboratory findings, although levels of acute phase reactants such as CRP, and immunological markers, e.g., circulating immune complexes, are raised. Tissue typing is useful, since the B27 antigen is present in one third of PsA sufferers; B27 incidence is approximately 60% amongst those patients with spondyloarthritis. There is also evidence associating HLA-B17, Bw38, Bw39, and DRw4 antigens with PsA.

Treatment in milder cases of PsA consists of a regimen of bed rest, physical exercises, and NSAIDs. In more severe disease, oral or intra-articular corticosteroids are occasionally employed. SAARDs such as gold and hydroxychloroquine have been attempted with a reasonable degree of success. In very severe psoriasis or PsA, the folate antagonist, methotrexate, has been used.

1/1/2/4 WHIPPLE’S DISEASE

Whipple's disease is a very rare systemic disorder, whose cardinal symptoms are: weight loss, fever, skin pigmentation, abdominal pain, lymphadenopathy, and peripheral arthritis. The disease, which was first recognized in 1907, is characterized by malabsorption in the small intestine, with deposition of glycoproteins within macrophages located in the lamina propria.
Later studies have shown that 68% of patients with this condition, exhibit signs of polyarthritis, although the time which elapses between the onset of arthritic symptoms, and other disease features, can exceed five years. Although the arthritis is usually confined to the knees and elbows, it can encompass the wrists and metacarpophalangeal joints, and is non-deforming. In this respect, there is similitude with IBD-associated arthritis. Moreover, there is some evidence for the increased frequency of the HLA-B27 serotype in this disorder.

It would seem that Whipple's disease is really a reactive arthritis, since the glycoprotein deposits are of bacterial origin, although no organism has yet been identified. Finally, it must be stressed that appropriate antibiotic therapy results in amelioration.

1/1/3 OVERVIEW OF INFLAMMATORY BOWEL DISEASE

IBD includes both idiopathic ulcerative colitis (UC), and Crohn's disease ([CD], sometimes referred to as regional enteritis). IBD is a chronic disorder of unknown aetiology, which afflicts men and women equally. Its prevalence is world-wide, although generally more common among Caucasians. The incidence of UC is somewhat greater than that of Crohn's disease. Clinical manifestations of IBD most frequently occur between the ages of 15 to 30 years, although the disease may begin at any age. Despite a familial tendency, no genetic markers have yet been
associated with IBD. Like RA, the pathology of IBD suggests a fundamental immunological anomaly.

1/1/3/1 PATHOLOGY OF IBD

UC is characterized by an acute inflammatory lesion in which rectal involvement occurs in more than 95% of cases. This lesion extends throughout the colon, occasionally as far as the terminal ileum. It is continuous, and is confined to the mucosal and submucosal intestinal epithelial layers. The epithelium becomes infiltrated with neutrophils, and the presence of superficial ulcerations is pathologically typical. Occasional deep ulcers are seen, with development of microabscesses in the vicinity of the crypts. Very severe inflammation causes marked colonic distention, an emergency condition known as "toxic megacolon". Chronic UC is characterized by gradual deposition of connective tissue, and the epithelial cells may eventually become neoplastic.

CD differs from UC, in that it is not exclusively colonic. Rectal involvement is also less frequent. About two thirds of patients exhibit ileitis. The associated inflammation may be discontinuous; inflammatory lesions are interspersed with regions of normal bowel ("skip" lesions). Inflammation is transmural, extending from the luminal, to the serosal, surface, with frequent mesenteric involvement. Adherence of inflamed bowel loops either to each other, or to other organs, may cause luminal obstruction. Deep ulcerations and thickening of the bowel
ensue, together with an infiltrate comprising neutrophils, T- and B-lymphocytes, and macrophages.

1/1/3/2 CLINICAL ASPECTS OF IBD

Clinical manifestations of acute UC include diarrhoea, fever, rectal bleeding, abdominal pain, and weight loss, the severity of symptoms depending on the degree of inflammation. Although most UC cases are mild or moderate, about 10-15% of patients experience a rapid, abrupt disease onset, requiring immediate hospitalization to prevent complications such as colonic perforation, which may develop as a result of toxic megacolon. In the majority of UC patients, the clinical course is one of acute disease episodes alternating with remission periods of up to several years duration.

Symptoms of CD depend mainly upon the location of the inflammatory lesion. Signs of ileal involvement include colicky pain, moderate diarrhoea, and occasionally, palpable masses due to adherence of inflamed bowel loops. Rectal bleeding is very rare, although occult blood loss is not infrequent. When the colon is involved, diarrhoea is a more prominent feature.

Both CD and UC are associated with a miscellany of extra-intestinal manifestations. Of particular interest are the occurrences of peripheral arthritis and ankylosing spondylitis. IBD patients develop a synovitis affecting the larger peripheral joints such as the knees and ankles (enteropathic arthritis). Its onset may precede clinical
symptoms of IBD, although it generally concurs with the development of intestinal symptoms. The clinical course of synovitis usually reflects that of the primary disease; flares of arthritis coincide with acute exacerbations, and remission periods tend to concur with steady-state disease activity. Enteropathic arthritis does not eventuate in erosive, deforming, articular disease.

AS complicates 5-10% of IBD cases, whilst sacroiliitis is present in up to 17% of patients, and often occurs in the absence of spondylitis. The incidence of both sacroiliitis and AS in IBD, is strongly associated with the HLA-B27 serotype. Clinical features of IBD-associated AS closely resemble those seen in the idiopathic disease.
1/2 AETIOLOGICAL THEORIES OF RA

1/2/1 REQUIREMENTS FOR AN AETIOLOGICAL MECHANISM

The aetiopathogenesis of RA remains obscure and perplexing despite much painstaking research over several decades. That it is mediated by the immune system is now unquestionable. Although RA is generally classified as an autoimmune disorder, in that disease severity closely correlates with RF titers (Waaler, 1940; Rose et al, 1948), its status vis a vis autoimmunity is disputable. In a true autoimmune disease, whether organ specific (e.g autoimmune thyroiditis), or systemic (e.g systemic lupus erythematosus [SLE]), the relevant interaction of autoantibody with its target, is itself the underlying cause of disease symptoms (Strakosch et al, 1982; Gershwin & Steinberg, 1974). In the case of RA, there is no firm evidence to suggest an aetiopathogenic role for RFs. Nevertheless, an appreciation of their nature, and a proposal for their origin in RA, seem warranted. Further bewilderment arises upon consideration of two fundamental questions regarding the immunopathology of RA, viz: (a) the selectivity of the joint for the inflammatory response, and (b) the initiation and perpetuation of this process. Like RF production, these two points require elucidation, in order to formulate a genuine aetiological mechanism.

1/2/2 THE POSSIBLE ROLE OF RF IN THE PATHOGENESIS OF RA

The term "rheumatoid factor" is perhaps somewhat
misleading, since RF production is not unique to RA. RF titers have been detected in various infectious diseases such as leprosy (Cathcart et al, 1961), and in other arthritides such as SLE (Pope et al, 1981), and Sjogren's syndrome (Dunne et al, 1979). Vaccination of healthy people with tetanus toxoid, has been shown to increase the numbers of precursor RF-secreting cells in the peripheral blood (Welch et al, 1983). Thus, RF production appears to be a normal, adaptive immune response. However, RF secretion is both quantitatively greater in RA, and qualitatively different in belonging chiefly to the IgM class, and exhibiting more cross-reactivity with animal IgG (Pope et al, 1981).

RF synthesis is evoked by three distinct types of stimuli, viz: (a) immune complexes, (b) exogenous antigens bearing cross-reactive determinants to human IgG, and (c) polyclonal B-cell activators. Induction of RF by circulating immune complexes, has been demonstrated in sub-acute bacterial endocarditis patients (Carson et al, 1978), and in normal humans injected with tetanus toxoid (Welch et al, 1983). However, an aetiological concept for RA, based entirely on immune complex formation in response to a hypothetical antigen, appears implausible for several reasons. Firstly, RFs are present in the sera of only 70-90% of RA cases (Vaughan, 1972). Secondly, the time of RF appearance may vary between several years preceding clinical disease onset (Aho et al, 1985), and long after RA diagnosis (Vaughan, 1972; Jones et al, 1986). Thirdly, rheumatoid synovial membranes are predominantly infiltrated by T-cells, rather than B-lymphocytes and plasma cells.
(Ziff, 1974; van Boxel & Paget, 1975). Also, T-cell lymphokines have been detected in RA synovial fluids (Smith, 1980; Fontana et al, 1982). These data might indicate a major pathogenic role for cell mediated immunity, rather than RF production, in RA.

Much evidence has accrued, to support the notion that RF production can be stimulated by exogenous antigens, cross-reactive with human IgG (Hannestad, 1969; Cunliffe & Cox, 1980). The existence of IgM RF molecules, reactive with both nuclear antigen, and autologous IgG, within a subset of polyclonal RF, in RA subjects, has been reported (Hannestad & Johannessen, 1976; Johnson, 1979). The fact that these dual specificities are confined to a small fraction of the total RF, suggests that these antigens are not the sole cause of RF production.

Induction of RF generation, by polyclonal B-cell activators such as pokeweed mitogen (PWM) and Epstein-Barr virus (EBV), has been demonstrated in both normal (Fong et al, 1981), and rheumatoid, PBL (Tsoukas et al, 1980; Olsen et al, 1982). Whereas PWM induces T-cell dependent, IgM RF secretion by mature B-cells, EBV activates precursor B-cell clones, without T-cell co-operation (Fong et al, 1983). It was proposed that RFs, as seen in RA, comprise a family of closely related molecules, derived by mutational events from germ-line gene segments, which specify a primordial RF important to the species. Essentially, successive transitions in B-cell status would serve to increase RF diversification (Carson et al, 1981). Consistent with this hypothesis, is the observation that RF in RA, is chiefly manufactured by the more mature B-cells (Pasquali et al,
1981). Moreover, there are favourable arguments for the survival value of RF. Its complement fixing capacity facilitates lysis and killing of cells bearing surface IgG antibodies (Elson et al, 1983). Protection is also conferred against circulating immune complexes in chronic infections, since binding of RF to these aggregates, increases their size, thereby permitting their clearance by reticuloendothelial cells (van Snick et al, 1978). It is also conceivable that RFs can clear tissues of idiotype-anti-idiotype complexes, thus acting as a component of normal immunoregulation (Morgan et al, 1979).

In the 1970s, autoantibodies were reputed to arise from unbridled B-cell activity, consequent upon impaired non-specific T-cell mediated suppression (Fudenberg, 1971; Cantor et al, 1978). However, in both normal humans and RA patients, RF production is believed to be subject to classical regulation by T-helper/suppressor populations (Koopman, 1981). No solid evidence of T-cell dysfunction, or of reduction in absolute numbers of T-lymphocytes, in RA, has been claimed to date, thereby repudiating the notion that an imbalance between help and suppression, is responsible for the elevated RF levels in RA. Jerne (1974) hypothesized that antibody production is controlled via a network of idiotypic and anti-idiotypic antibodies. This concept has inevitably been applied to RF production, and is supported by substantial evidence (Bonagura et al, 1982; Koopman et al, 1983a) Human anti-idiotypic antibodies have been shown to inhibit B-cell release (Bona & Fauci, 1980; Mudawar et al, 1980), and to exert direct suppression of RF synthesis (Koopman et al, 1983a). The control of RF
production by idiotypic/anti-idiotypic mechanisms would therefore seem an attractive theory.

1/2/3 PROPERTIES OF JOINTS

There are three unique characteristics of joints which segregate them as ideal organs for the localization and perpetuation of inflammation. Firstly, being structurally designed for movement, joints are subject to a substantial degree of generalized wear and tear, besides an increase in blood flow. Secondly, the joint space is lined by mesenchyme rather than epithelial tissue, and there is an absence of basement membrane. These structural features permit the formation of neovasculature, with consequent increase in blood flow to the subsynovial tissues. In addition, the joint space is a low pressure cavity, providing little resistance to the influx of low molecular weight inflammatory components. Thirdly, there is a notable absence of blood vessels in cartilage. This feature permits the sequestration of immune complexes within the collagenous network, without any clearance facility.

1/2/4 EXOGENOUS AGENTS ASSOCIATED WITH RA

1/2/4/1 OVERVIEW

Broadly speaking, there have been two general approaches regarding the aetiology of RA. Several researchers have concentrated on the synovial acquisition of a novel antigen, while others have focused their attention on the
possibility of a primary genetic abnormality, culminating in an immunoregulatory dysfunction. The former hypothesis will be considered first. Experimentally demonstrated agents which have been implicated in the initiation of an inflammatory arthritis, both in man, and in animal models, can be classified as exogenous or endogenous. Exogenous agents encompass a wide range of intact bacteria, viruses and mycoplasmata, all three of which have been extensively studied. Endogenous candidates include collagen and peptidoglycans (PGLYs).

The earliest research into RA was based on the rationale that a specific exogenous agent, able to initiate an inflammatory arthritis, which in many respects resembles RA, may provoke an immune response in a vulnerable host, with resultant RA. In the 1930s, it was believed that RA (often confused with rheumatic fever) was associated with a streptococcal infection (Cecil et al, 1931; Nicholls & Stainsby, 1931). Although the distinction between RA and rheumatic fever was later clarified (Green, 1942; Copeman, 1964), research continued to be directed towards isolating intact bacteria from human rheumatoid joints (Bauer et al, 1951; Person & Sharp, 1976). Attention was shifted to diphtheroids as aetiologic agents, when these were detected in synovial tissues (Stewart et al, 1969). Isolation of these organisms was relinquished, when it was discovered that they form part of the normal skin flora, and therefore were probably culture contaminants. Around the same period, Mycoplasma fermentans was implicated in RA, since it was shown that membranes of this organism could inhibit the migration of RA leukocytes, but not those of
osteoarthritis patients and normal people, in culture (Williams et al, 1970). Furthermore, *M. fermentans* was claimed to be present in RA synovial fluids (Williams, 1967). However, the methods used were technically flawed, because the culture medium contained horse serum, which proved antigenic. Using more sophisticated techniques, it has been convincingly demonstrated that elevated titers of antibodies to *Proteus mirabilis*, a normal commensal in the gut, were present in RA sera (Ebringer et al, 1985; Ebringer et al, 1988), although there is not as yet, any concrete evidence for the existence of this organism in rheumatoid joints.

During more recent years, it has perhaps been realized that one of the greatest drawbacks to the "acquired intact organism" approach, lies in the difficulty in providing a satisfactory explanation as to the perpetuation of the inflammatory process in RA. Research has become increasingly directed towards isolating bacterial and viral, debris or secreted products, from rheumatoid tissues, in the hope that such particles play a role in RA. The advent of bacterial PGLYs and stress proteins, as potential aetiologic agents, has exemplified this approach.

1/2/4/2 ANIMAL MODELS OF BACTERIAL AND MYCOPLASMATA-INDUCED ARTHRITIS

Excessive levels of atypical *Clostridium perfringens* have been observed in faecal cultures from rheumatoid patients. In vivo, these organisms were shown to migrate as far as the upper jejunum, whereas, in normal individuals,
they were confined to the colon. 67% of RA patients exhibited this abnormal intestinal flora, as compared with only 0.9% of healthy controls (Olhagen & Mansson, 1974). This work was extrapolated to a study in pigs. These animals were fed a protein-rich diet, which indirectly led to increased clostridial growth, concurrent with the development of a rheumatoid-like arthritis (Mansson et al, 1971).

_Erysipelothrix rhusiopathiae_ is now recognized as a major cause of infectious arthritis in pigs, and is the most commonly isolated organism from suilline arthritic joints (Ward, 1922). The disease may either present as an acute form, having a high mortality if untreated, or as a chronic infection characterized by urticaria and erythematous plaques. The accompanying arthritis is sometimes unremitting; the synovial pathology very closely resembles that found in human RA, to the extent of synovial thickening, villus formation, and a lymphoid infiltrate (Drew, 1972). Serum levels of "RF-like molecules" have also been reported (Timoney, 1971). Interestingly, Erysipelothrix arthritis can be sustained even in the absence of the live organism (Timoney, 1971).

Mycoplasma have been isolated from rheumatoid synovial tissues (Taylor-Robinson & Taylor, 1976; Cassell & Cole, 1981), although there are conflicting reports (Person et al, 1973a; Person et al, 1973b). These discrepancies may be explicable by difficulties in cultivation of mycoplasmas, particularly since they are believed to form very close associations with mammalian host lymphoid cells (Cassell et al, 1978). It has been revealed that they
adsorb host cell surface antigens onto their own surfaces (Wise et al., 1978). By this means, it is possible that mycoplasmas can mimic the host cell, thereby evading the host's immune defences, and permitting their continuity throughout the rheumatoid process. *Mycoplasma hyorhinis*-induced arthritis has been demonstrated using porcine models (Ross, 1973; Decker & Barden, 1976). Interestingly, the organism can be readily isolated from the arthritic joints only in the early disease stages (Barden & Decker, 1971; Decker & Barden, 1976), possibly inferring that alterations in surface structures, occur with progression of disease activity.

1/2/4/3 BACTERIAL STRESS PROTEINS

Recently, a possible role for mycobacterial stress proteins in the genesis of RA, has been claimed (Holoshitz et al., 1986). It was formerly demonstrated that the onset of an adjuvant arthritis (AA), could be evoked in genetically susceptible rats, 14 days after intradermal injection of whole, heat-killed, *Mycobacterium tuberculosis* (Mt) in mineral oil containing an emulsifying agent. AA is characterized by an inflammatory pansynovitis with pathological sequelae very similar to those of untreated RA (Pearson, 1956). AA was later shown to be transferable via lymphocytes from donor to syngeneic recipient rats (Pearson & Wood, 1964).

These findings were reproduced and expanded upon, in that a specific T-cell clone (denoted A2b), able to induce AA by transfer to irradiated rats, was isolated from an AA
immunized animal (Holoshitz et al, 1983). The A2b clone was subsequently shown to recognize both an Mt epitope, and a self-antigen of cartilage proteoglycan. Molecular cloning of the Mt epitope revealed that it resided within a 65kD Mt stress protein (van Eden et al, 1985). It was further demonstrated that RA patients exerted specific T-cell reactivity to the Mt epitope (Holoshitz et al, 1986). Significantly, synovial fluid lymphocytes from RA subjects of disease duration less than one year, exhibited high proliferative responses to the Mt 65kD protein, yet their peripheral blood lymphocytes (PBL) were almost totally refractory (Res et al, 1988). Furthermore, PBL of patients of disease duration between 1-10 years, were shown to exert high specific responsiveness to the Mt epitope (Holoshitz et al, 1986). These data led to the proposal that RA may be caused by synovial lymphocyte clones, cross-reactive with the Mt 65kD protein, and with self articular cartilage (Holoshitz et al, 1986). Propagation of these T-cell clones would presumably autoimmune the host against its own cartilage tissue.

The fact that the Mt 65kD protein is a stress or "heat shock" protein, is noteworthy. These molecules are synthesized by bacteria, when grown at high temperatures (Young et al, 1987). Stress proteins have been highly conserved throughout evolution, and there appears to be an extraordinarily high degree of homology between those of prokaryotic, and eukaryotic, organisms (Thole et al, 1988). It has been postulated that bacteria undergoing phagocytosis, are "under stress". Stress proteins may be synthesized under these conditions during RA, and presented
to the appropriate synovial T-cells (Young et al, 1987). This hypothesis, if valid, could realistically explain the perpetuation of synovial inflammation. Clearance of bacteria such as Mt from the synovium, by phagocytic cells, could serve to amplify the production of stress proteins. It is conceivable that the T-cell epitope, present on the Mt 65kD protein, may be common to several bacterial species. This might explain why AA is not exclusive to mycobacteria (Flax & Waksman, 1963; Paronetto, 1970). Thus, it is possible that T-cell clones in pre-rheumatoid joints, may be expanded in response to multifarious bacterial species. The above findings are very encouraging, though further investigations employing stress proteins from other bacterial sources, are merited.

1/2/4/4 VIRUSES

There has been much interest in the possibility of a viral aetiology for RA. It was originally claimed that particles of identical morphology to the elementary bodies of known viruses, were present in rheumatoid tissues, but not in those of control subjects (Eagles et al, 1937). However, since the viral nature of these particles was not verified, only sporadic investigations of this nature have been published (Clarke, 1958; Utz et al, 1959). Interest in a viral aetiology for RA was revived, when it was shown that the rubella virus (RV), was unable to infect rheumatoid synovial fibroblasts, and replicate therein (Grayzel & Beck, 1970), despite the fact that RV infection was known to be accompanied by a mild polyarthritis, of
short duration (Johnson & Hall, 1958; Ogra & Herd, 1971). The implication was that an unknown, intrinsic virus caused this interference (Grayzel & Beck, 1970). Recently, parvovirus-like agents (designated RA-1) have been isolated from rheumatoid synovial tissues (Simpson et al., 1984). It was shown that polyclonal antibodies against RA-1, detected reactive antigen in synovial cells from RA, but not osteoarthritis, patients (Simpson et al., 1984).

The realization that slow-acting viruses can play a role in producing tissue pathology, characteristic of chronic diseases hitherto believed to arise from degenerative processes, e.g., kuru (Gajdusek & Gibbs, 1972; Gajdusek, 1977), has been applied to RA. Consequent searches for virus-like particles, in this particular context, in rheumatoid tissues, have been forthcoming (Warren et al., 1969).

Type C oncornoviruses have the ability to become incorporated into mammalian host cells, where they manufacture viral proteins which may be expressed on host cell surfaces, thus altering the cells' antigenic determinants from "self" to "non-self" (August & Strand, 1977). How this process is accomplished at the molecular level is not yet clear, but this type of antigenic modification is especially intriguing, from the viewpoint of autoimmune disease production. Indeed, these viral events have been studied with reference to SLE (Strand & August, 1974). The quest for similarly acquired novel antigens on rheumatoid cell surfaces, might prove fruitful.

EBV was once reputed to play a major role in the pathogenesis of RA. It was initially observed that RA sera
reacted with an antigen extracted from the EBV-carrying cell line, denoted Wil-2 (Alspaugh & Tan, 1976). This antigen (named RA-associated nuclear antigen, RANA) was specifically located in human B-lymphocytes transformed by EBV, suggesting a link between prior exposure to EBV, and susceptibility to RA (Alspaugh et al, 1978). It was subsequently shown that rheumatoid patients had a higher frequency of anti-RANA antibodies, than had normal people (Catalano et al, 1979; Venables et al, 1981). However, conflicting results have also been published (Silverman & Schumacher, 1981). Also in the context of EBV, there have been reports that rheumatoid subjects failed to regulate polyclonal B-cell activation in response to EBV, with consequent elevation in anti-EBV antibodies (Alspaugh et al, 1981; Depper et al, 1981). This abnormality may be due to a deficiency in gamma interferon synthesis by rheumatoid T-suppressor cells (Hasler et al, 1983), although it has also been shown that this defect was rectified, by removal of macrophages from the cell system (Lai et al, 1977). It would seem likely that this dysfunction is a secondary feature of RA.

Several viral methodologies have been applied to RA. Rheumatoid patients have been serologically tested for specific anti-viral antibodies (Chandler et al, 1971), and for virally induced enzymes such as DNA/RNA polymerases (Spruance et al, 1975). Sequencing of DNA and RNA, synthesized by rheumatoid synovial cells, has been performed, in order to establish regions of homology with viral nucleic acids (Person et al, 1973b). Although this type of approach has not yet produced any definitive
evidence for a viral role in RA, the possibility of a hitherto unknown virus being causative to RA, cannot be entirely discounted.

1/2/5 ENDOGENOUS AGENTS IN RA

1/2/5/1 BACTERIAL PEPTIDOGLYCANS

Endogenous agents implicated in the aetiopathogenesis of RA, include peptidoglycans (PGLYs) and collagen, both of which are integral structural components of articular cartilage. PGLYs are essential constituents of all bacterial cell walls. Throughout the entire spectrum of bacterial species, there is extraordinarily little divergence in their chemical structure (Ghuysen, 1968; Schleifer & Kandler, 1972). Most of the studies involving PGLYs in RA, have utilized the gram-positive cocci (Kohashi et al, 1976; Cromartie et al, 1977).

It has been demonstrated that rabbits immunized with streptococci, were able to produce RF, cross-reactive with a PGLY epitope, and with the Fc region of autologous IgG (Bokisch et al, 1973). More recently, it was reported that BALB/c mice, upon immunization with RF preparations derived from seropositive RA patients, could raise antibodies against *Streptococcus pyogenes* cell-wall PGLYs. It appeared that an idiotypic complementarity existed, between RF and the anti-PGLY antibodies elicited (Johnson et al, 1985). There is evidence that a chronic inflammatory arthritis could be induced experimentally in rats, by intraperitoneal injection of a sterile, aqueous suspension containing
streptococcal cell wall fragments (Cromartie et al, 1977). It is conceivable that bacterial, and joint cartilage, PGLYs may associate. This alteration in matrix components might culminate in autoimmunity to cartilage, and hence RA. However, further studies are merited, to discern whether bacterial PGLYs are truly arthritogenic.

1/2/5/2 COLLAGEN

There has been considerable interest in collagen as an aetiological agent in RA, following the development of a rat model of collagen arthritis (CA). Upon intradermal injection with emulsified, native type II collagen, 40% of rats treated, were shown to develop a polyarthritis, with pathological sequelae resembling AA (Trentham et al, 1977; Trentham, 1982). CA was elicited only when intact type II collagen was injected, and was associated with an immune response to type II collagen, the degree of which was much more pronounced in the arthritic rats (Trentham et al, 1978a). CA was transferable via spleen, and lymph node, cells of immunized donor rats, to syngeneic, non-immunized recipients (Trentham et al, 1978b). It was later demonstrated that the factor responsible for disease transfer, consisted primarily of IgG anti-type II collagen antibodies, which bound to autologous type II collagen of articular cartilage (Stuart et al, 1982).

Evidence of collagen autoimmunity in RA, was initially provided by the detection of serum antibodies to type I collagen (Steffen & Timpl, 1963). Other workers noted antibodies to types II, III, IV, and V, collagens, both in
RA sera (Adriopoulos et al., 1976; Michaeli & Fudenberg, 1974), and synovial fluids (Menzel et al., 1978). A proportion of the synovial fluid antibodies exist as immune complexes (Menzel et al., 1976). Cell mediated immunity was also reported in RA. Approximately 75% of RA subjects secreted a lymphokine, leukocyte inhibition factor (LIF), upon lymphocyte stimulation *in vitro* with native, types II and III, collagens (Trentham et al., 1978c). Another lymphokine, lymphocyte-derived monocyte chemotactic factor for monocytes (LCDF), was also shown to be produced by lymphocytes likewise stimulated, but not by normal control cells (Stuart et al., 1980).

Immune reactivity to denatured collagen (gelatin) has been shown to exceed that towards native collagen (Stuart et al., 1980). It might therefore be inferred that collagen autoimmunity is a secondary phenomenon in RA, being consequential to collagen denaturation during the inflammatory process.

Antibodies to type II collagen have been noted in other arthritides, e.g., PsA (Trentham et al., 1981) and scleroderma (Mackel et al., 1982), in chronic liver disease (Menzel et al., 1980), and in some normal subjects (Solinger et al., 1981), suggesting that collagen autoimmunity is not exclusive to RA. Neoteric evidence has indicated a genetic basis for anti-collagen antibodies (Solinger et al., 1981). The immune response to denatured collagen, both in RA patients and in controls, as measured by LIF production, was shown to be strongly associated with the histocompatibility marker, HLA-DR4 (Solinger & Stobo, 1981). These researchers further demonstrated that the
immunogenicity of the collagen macromolecule, lay in its unique amino acid sequences, rather than in antigenic determinants, created by spatial arrangements within its secondary or tertiary structure (Solinger et al, 1981). It was also shown that HLA-DR4 negative, collagen non-responders, possessed specific suppressor T-cells which inhibited collagen responsiveness (Solinger et al, 1981). In summary, autoimmunity to collagen is probably a secondary phenomenon in RA, being primarily dependent on the presence of the HLA-DR4 marker. The significance of the HLA-antigens vis a vis RA, is discussed in the following section.

1/2/6 GENETIC THEORIES OF RA

1/2/6/1 ARGUMENTS IN FAVOUR OF A GENETIC THEORY

The "acquired antigen" hypothesis for the aetiology of RA, might adequately explain the raised RF titers in the disease, and even the sustenance of inflammation. However, none of the aforementioned mechanisms offer a satisfactory explanation as to why only a minority of individuals are predisposed to RA; nor do they explain the inter-patient variation in disease severity. Furthermore, they leave unanswered, the perplexing question as to why an apparently healthy person should spontaneously develop the disease, in the absence of any concurrent illness. Finally, they discount its somewhat obvious familial trend. It appears that any environmental stimulus of potential arthritogenicity, per se, can only partially explain the
genesis of RA. The most likely possibility is that the co-action of genetic and environmental factors, determines whether, and when, an individual succumbs to RA.

A survey of over 600 RA cases, employing clinical and radiological criteria, revealed that the prevalence of seropositive RA was about twice the expected rate, among family members (Lawrence, 1967). Further evidence in support of a familial trend, was yielded by the finding of greater concordance in monozygotic twins, when compared with sex-matched, dizygotic counterparts (Lawrence, 1970). A lack of concordance was found between monozygotic, seronegative twins, thereby implicitly repudiating the possibility of shared environmental factors as being causative of seropositive RA (Lawrence, 1970). These data are suggestive of a true genetic distinction between seropositive, and seronegative, RA, an inference which was later corroborated by immunogenetic studies, vide infra.

1/2/6/2 HLA ANTIGENS AND RA

Like other autoimmune disorders such as Graves' disease and Goodpasture's syndrome, there exists a significant association between seropositive RA, and the presence or absence of specific HLA class II antigens (Svejgaard et al, 1983; Jaraquemada et al, 1986). In the case of seropositive RA, the Ia antigen, HLA-DR4, has been clearly established, with a relative risk of 4.2 (McMichael et al, 1977; Panayi et al, 1978; Thomsen et al, 1979). This correlation is not as strong as that between the HLA-B27 serotype and ankylosing spondylitis (relative risk 87.4) (Svejgaard et
The serologically determined antigen, HLA-DR4, is closely associated with the lymphocyte-defined determinant, HLA-Dw4, and is present in approximately 35-70% of seropositive RA cases (McMichael et al., 1977). However, the frequency of HLA-DR4 does not differ significantly between seronegative patients and controls (Stastny, 1980), nor between male and female RA subjects (Jaraquemada et al., 1986). Additionally, a positive correlation between the DR4 antigen and serum RF levels, was demonstrated (Stastny, 1980), implying that DR4 may influence the degree of immune responsiveness to autologous IgG. As previously discussed, RF production in RA, might be governed by mutational events. Alternatively, HLA-DR4, or one or more closely linked genes, codes directly for antigens to autologous IgG Fc receptors. The precise time of phenotypic expression (RA onset) may be environmentally dependent.

The major histocompatibility complex (MHC), which encodes "Ia-like" (class II) antigens such as DR4, in man, is comparable with the murine Ia (H-2) antigen system, which is concerned with immunoregulation (Demant, 1973; Zinkernagel & Doherty, 1977). The concept of linkage disequilibrium (i.e., the non-random occurrence of two or more distinct alleles at each of two particular genetic loci on a single chromosome), has provoked the formulation of four prospective immune mechanisms, each having aetiological potential for RA (Winchester, 1981). HLA-DR4 linked genes may code for antigen-specific hyper-responsiveness, such as autoimmunity to collagen or IgG. Secondly, representation of antigenic
hypo-responsiveness, may render a host incapable of mounting a particular immune response to an exogenous antigen. Another scenario is that of a generalized immunoregulatory dysfunction, represented by a prolongation of virtually all immune responses. The underlying defect could be made manifest by excessive T-helper cell function, or by a decline in non-specific T-cell suppression. Lastly, a situation of abnormal cellular differentiation within the synovium, is envisioned. For instance, synoviocytes or macrophages, bearing surface Ia antigens, may become hyper-activated by non-specific stimuli. Consequential recruitment of large numbers of T- and B-lymphocytes, could in turn, generate the type of synovial lesion present in the early stages of RA (Winchester, 1981). Further human MHC loci (viz: HLA-DP and HLA-DQ), as well as HLA-DR, which are also comparable with the murine I region loci, have been fairly recently discovered (Duquesnoy et al, 1979; Shaw et al, 1980). In addition, between the HLA-A,B,C and DR loci, present on the short arm of the human sixth chromosome, are four intervening loci which code for important complement components. The inclusion of the latter together with the HLA loci, is termed the "extended HLA haplotype". A number of these haplotypes, known to be associated with DR-related diseases, contain genes encoding functionally inadequate, or absent, proteins (Svejgaard et al, 1983). Individuals bearing certain extended haplotypes, are less capable of clearing circulating immune complexes, and also exhibit lowered phagocyte function (Svejgaard et al, 1983). Genetic studies using HLA gene probes and endonuclease restriction
enzymes, have enabled restriction fragment length polymorphisms to be characterized within the HLA system (reviewed by: Reeves, 1987). Further research in this field, utilizing molecular biological methodologies, will hopefully, illuminate some of the underlying immunogenetic mechanisms concerned with RA.

1/2/6/3 CLONAL DIVERSIFICATION AND RA

Prior to the discovery of the HLA system, and its acknowledged role in autoimmunity, research into autoimmune disorders was strongly influenced by Burnet's "clonal diversification" theory. This postulated that certain individuals are predisposed to specific autoimmune diseases, because they possess "preforbidden" clones, potentially able to diversify upon sequential somatic mutational events, thereby culminating in autoimmunity. Presumably, normal individuals are afforded protection, by the absence of such clones (Burnet, 1959). Preforbidden clones would evidently express antigen receptors which are within a few mutational events from autoreactivity. In RA, a situation might be envisaged, in which distinct "preforbidden" B-cell precursors undergo somatic mutations, the outcome being RF generation. Numerous environmental mutagens would serve to increase the occurrence of specific mutations, merely by triggering "preforbidden" clonal replication. Such a mechanism could realistically explain the increased frequency of RA with age. Since immunoglobulins are coded for by V-genes present on chromosomes 2, 14 and 22 in man (reviewed by: Male et el,
1987), there need not be any incongruity between the possible implications of Burnet's hypothesis, and the role of HLA antigens in the initiation of autoimmune diseases. Jerne's network proposal, vide supra, that clonal deletion is mediated by anti-idiotypic reactions, extends the potential for variability in the immune response repertoire, throughout life. However, the extent to which the above concepts are pertinent to the aetiology of RA, is conjectural.

1/2/7 ADDITIONAL AETIOLOGICAL THEORIES FOR RA

1/2/7/1 IMPAIRED GALACTOSYL-TRANSFERASE ACTIVITY

It was recently demonstrated that the activity of the enzyme, galactosyl-transferase (GT), was diminished in rheumatoid, as contrasted with, age and sex-matched, control B-cells (Axford et al, 1987). GT catalyzes the sequential addition of galactose molecules to the oligosaccharide chains, in the Cy2 region of IgG. Significantly, the Cy2 domains reside within the Fc portion (Axford et al, 1987). It is highly probable that decreased galactosylation alters the Fc structure, resulting in autoimmunity, with consequent RF production. Since human gene probes for the enzyme are presently operative (Appert et al, 1986; Humphreys-Beher et al, 1986), molecular investigations should be forthcoming. At this stage, it is only possible to speculate that predisposing genes are responsible for the dysfunction of B-cell GT, in RA.
Over fifty years ago, it was suggested that a negative correlation existed between schizophrenia and RA. This proposal was based on the observation that there was "not a single arthritic" amongst 2,200 hospitalized psychotics (Nissen & Spencer, 1936). Three years later, this finding was endorsed in an autopsy examination of 3,000 psychotics (Gregg, 1939). Two later studies, using current diagnostic criteria for both schizophrenia and RA, confirmed the earlier observations (Mellsop et al, 1974; Mohamed et al, 1982). Several interpretations have emerged to explain these remarkable results. It was initially suggested that the placid lifestyle of hospitalized schizophrenics, protected against articular trauma (Gregg, 1939). A later study of patients suffering from joint damage following injury, revealed a slight increase in susceptibility to RA, symptoms of which were shown to occur long after the trauma had healed (Julkinen et al, 1974). A more probable explanation, is that minimal psychophysiological stress, in conjunction with genetic components, are responsible for this very low incidence of RA, amongst schizophrenics. A positive association between the histocompatibility markers, HLA-A1 and B8, and severe schizophrenia, has been reported (McGuffin et al, 1981). It is particularly intriguing that both antigens have been shown to be negatively correlated with RA (McDermott et al, 1986; Ollier et al, 1986).

Biochemical abnormalities linking the two diseases, have been noted. Whereas an elevation of prostaglandin levels,
especially those of the E series, is representative of RA, schizophrenia is associated with PG deficiency, causing neurotransmitter defects (Mathe et al, 1980). It has also been shown that serum levels of beta-endorphins, were lowered in RA (Denko et al, 1982), the converse applying to schizophrenics. (Brambilla et al, 1984). Evidently, further studies, clarifying the roles of these bio-molecules in rheumatoid disease, are required, before consideration of their possible implications in the pathogenesis of RA.

1/2/7/3 GUT INVOLVEMENT IN RA

There is a multiplicity of evidence for an association between the gut and inflammatory joint diseases. It is now well established that inflammatory gastrointestinal disorders such as Crohn's disease and ulcerative colitis, are frequently accompanied by a peripheral arthritis (McEwan et al, 1982). Reciprocally, certain seronegative arthritides such as ankylosing spondylitis, Reiter's disease, and reactive arthritis, are associated with enteric bacteria (reviewed by: Isdale & Wright, 1989). Extensive small bowel, resection or by-pass surgery, can precede a peripheral arthritis (Fernandez-Herlihy, 1977). As pre-mentioned, serum antibody titers to the gut commensal, Proteus mirabilis, are raised in active RA (Ebringer et al, 1985; Ebringer et al, 1988). Serum IgA RF levels correlate with RA disease activity (March et al, 1987), as do IgA immune complexes (Westedt et al, 1986), implicating the gut in RA, since lamina propria lymphocytes from inflamed intestines, secrete increased amounts of IgA.
Further evidence for an association between RA and the gut, is provided by the observation that the polymeric, rather than the monomeric, form of IgA, in IgA-RF, predominates in RA synovial fluids (Elkon et al, 1982). Also, IgA-RF has been detected in synovial membrane plasma cells, although synovial RF is chiefly of the IgG and IgM classes (Koopman et al, 1983b).

Based on this knowledge, a study monitoring the distribution of re-injected, radio-labelled leukocytes, in 26 active RA subjects receiving NSAIDs, revealed abnormal localization of radiation, in the gut of 12 patients (Segal et al, 1986). These results were interpreted as indicative of a possible gastrointestinal lesion, through which foreign antigens could enter the circulation. Formation of immune complexes, and their subsequent interaction with synovial macrophages, may initiate RA (Segal et al, 1986).

However, it has also been claimed that RA therapy with NSAIDs, induced bowel inflammation, with concomitant, increased mucosal permeability (Bjarnason et al, 1984; Bjarnason et al, 1987). In the aforementioned study (Segal), 10 additional patients afflicted by other arthritides, and taking NSAIDs, were included. None of these exhibited abnormal leukocyte scans. Gut abnormality was, however, noted in two RA subjects receiving no drug treatment. These findings taken in toto led to the suggestion that NSAIDs may only serve to enhance leukocyte accumulation in the rheumatoid gut (Segal et al, 1986). Further, strictly controlled, studies are required to clarify this ambiguity.

It has also been postulated that an abnormality in...
migratory behaviour, is exhibited by lymphocytes operating within the secretory immune system (Sheldon, 1987; Sheldon, 1988). This is termed the "iteropathy" concept (iter = journey) (Sheldon, 1987). It is argued that gut-associated lymphoid tissue and synovial membrane are related, inasmuch that high endothelial venules (HEVs), the sites at which itinerant lymphocytes emerge, are present in both tissues (Freemont, 1987). The "iteropathy" concept proposes that intestinal epithelial lymphocytes may, in RA, exert a pathological tendency to home to synovial membrane sites (via HEVs), rather than to their native gut mucosal tissues (Sheldon, 1988). It is possible that these cells may encounter, in the gut, an antigen cross-reactive with a self epitope present in the joint. Such an antigen (e.g., a bacterial cell wall component autoreactive with articular cartilage) would render certain lymphocyte clones iteropathic. These activated clones would home to the synovium, expanding therein, and initiating inflammation. Although the underlying mechanism(s) by which intestinal epithelial lymphocytes become iteropathic, require clarification, several of the pre-mentioned endogenous and exogenous agents might act secondarily, provided that the initial immunization occurs within the gut. Indeed, the repertoire of exogenous agents could be extended to include those implicated in the gut-associated arthritides. Moreover, this hypothesis can explain the occurrence of both seropositive and seronegative RA, since there are no restrictions imposed upon the immunizing antigen. Mechanistic studies of lymphocyte trafficking are warranted.
CONCLUSIONS

The aetiopathogenesis of RA is yet enigmatic. The mere fact that such a wide vista of proposed agents, and suggested mechanisms, has arisen over 60 years, may imply that the aetiology of this disease is, indeed, multifactorial. It appears likely that a genetic predisposition plus an environmental trigger, are prerequisites for RA susceptibility. The sufficiency of any one mechanism *per se* is very slim. Our present knowledge and understanding of immunoregulation are limited, though expansile. With the advancement of cytological and immunogenetic techniques, it is anticipated that further concepts regarding the control of immune responsiveness, will be forthcoming during the next decade.
SASP is a compound comprising 5-amino salicylic acid (5-ASA) azo linked to sulfapyridine (SP). Its full chemical nomenclature is: 4 Pyridyl-(2) aminosulfonyl-3-carboxy-4'-hydroxybenzol.

SASP was originally formulated in Sweden by Dr Nana Svartz, during the late 1930s, as a prospective anti-rheumatic drug. The underlying rationale was to combine a sulfonamide with an analgesic salicylate, having a strong affinity for connective tissue, since it was believed at the time, that RA had an infective aetiology, and that joint inflammation was potentially responsive to antibiotics (Svartz, 1942). A subsequent clinical trial, in which over 400 RA patients were treated with SASP, was undertaken between 1940 and 1946. This proved largely successful, in that 63% of cases improved clinically (Svartz, 1948). As a result of these findings, Svartz recommended that SASP treatment should be continued indefinitely ("treatment for a brief period, e.g., one month, is useless in chronic polyarthritis"), starting with a dose of 6g daily, and reducing this to a maintenance dose of 1.5g/day, upon clinical amelioration. Symptoms of SASP toxicity arose in several patients, shortly after the inception of therapy; she advised re-commencement with very low doses of SASP (less than...
1g/day), with incremental increases up to 6g daily, over a 10-14 day period (Svartz, 1948).

In an attempt to repeat Svartz's original work in Britain, an open trial involving 60 hospitalized, active RA patients, wherein 20 received SASP, a further 20, gold injections, and the remainder acted as controls, was conducted (Sinclair & Duthie, 1948). The results were unfavourable, in that, although there was an initial clinical improvement in all 3 groups, at final follow-up, clinical deterioration was notable in about 50% of cases, and there was shown to be no significant difference in disease status between SASP, or gold, treated patients, and controls. The explanation for these negative findings, lay in a failure to adhere to Svartz's instructions regarding duration of SASP therapy, i.e: SASP was given for a mean duration of only 60 days. Furthermore, when toxicity to SASP occurred, the regimen was to re-introduce the drug at the maximum dose of 6g/day, thereby accounting for its early withdrawal in several cases (Sinclair & Duthie, 1948). Unfortunately, the latter study tended to discourage further consideration of SASP in RA. Apart from two studies (Kaczynski & Sokolowski, 1950; Kuzell & Gardner, 1950), it was not until some 20 years later, that interest in SASP as a potential anti-rheumatic drug, was revived, by McConkey and co-workers, in Birmingham.

Their previous experience with dapsone, a sulphone then commonly used in the management of RA, revealed that this agent possessed limited therapeutic value (McConkey et al, 1976). This led McConkey to consider alternative
drugs, one of which was SASP. The reasons for this choice were: (a) SASP was believed to exert immunomodulatory effects in UC; (b) the SP moiety was chemically related to dapsone; and (c) SP was known to be effective in treating dermatitis herpetiformis, a condition in which deposition of immune complexes may play a major pathogenetic role (McConkey, 1986). An initial, open trial was conducted, in which 32 active RA patients received a maintenance dose of 2g SASP daily (McConkey et al, 1978). 25 individuals were able to tolerate the drug for longer than one month; the majority of these patients improved clinically, and their ESR, and C-reactive protein levels, fell substantially. It was inferred that SASP exerted second line effects in RA. However, the incidence of adverse effects was fairly high (7/32 patients). Gastrointestinal symptoms, and headaches, were predominant. Two patients developed megaloblastic anaemia, ascribed in one case to folate deficiency, and to vitamin B12 deficiency, in the second. There was also one instance of neutropenia, which resolved upon SASP withdrawal.

This work was extended to a larger open study, in which 74 RA patients were given enteric-coated SASP, at a maintenance dose of 2g/day. Clinical improvement was noted in 49 cases, and only 7 patients withdrew due to adverse effects (McConkey et al, 1980). A more sophisticated and exacting trial was undertaken in Leeds, in which 15 patients fulfilling a number of criteria for active RA, were initially treated with NSAIDs alone, and then prescribed SASP. Various clinical and biochemical indices of RA were monitored for up to 6 months of SASP therapy.
(Bird et al., 1982). This system had already been developed for comparison of the efficacy of various compounds in RA, viz: gold (sodium aurothiomalate), d-PEN, hydroxychloroquine, aspirin, and alclofenac. Results obtained with SASP compared favourably with, if not quite as well as, gold, d-PEN, and hydroxychloroquine. SASP was, however, far more effective than the first line drugs, aspirin and alclofenac, thus confirming its second line action in RA.

The next, and seemingly logical, step, was to perform a direct comparison of SASP with an established second line agent. A two-centre, controlled and blinded trial held at Leeds and Birmingham, was accordingly performed (Neumann et al., 1983). 63 outpatients with active RA were initially treated with a NSAID alone for 4 weeks, after which they were randomly allocated for SASP, or d-PEN, treatment (31:32 per group respectively). SASP was initially administered at a dose of 0.5g/day, with stepwise increments up to a maintenance dose of 2g daily. d-PEN was started at 125mg/day, and gradually increased to a maintenance dose of 500mg daily. All patients were clinically monitored using standard tests (i.e., global clinical score, morning stiffness, grip strength, Ritchie index, and pain score), and their ESR and serum CRP, and certain haematological indices including circulating platelets and leukocytes, were assessed at 4-weekly intervals, up to 16 weeks. 8 and 12, SASP and d-PEN treated subjects, respectively, withdrew due to adverse effects. Of the remainder, significant clinical improvement, and reduction of ESR, CRP, and leukocyte and
platelet counts were notable in both groups, thus indicating that SASP, like d-PEN, radically interfered with the pathological progression of RA. A series of similar, comparative clinical trials, have followed (Carroll et al, 1982; Pullar et al, 1983; Farr et al, 1984), and have stimulated much interest in the pharmacology, and mode of action of, SASP in RA.

Furthermore, there is mounting evidence to suggest that SASP exerts ameliorative effects in the seronegative spondyloarthropathies. A double blind trial, in which 37 HLA-B27 positive patients with definite AS, according to ARA criteria, received either SASP or placebo, for 3 months, revealed a marked reduction of inflammatory activity, in parallel with clinical improvement, among those treated with SASP (Feltelius & Hallgren, 1986). The findings of a concurrent, double blind, controlled trial, which differed inasmuch that it was restricted to active AS patients without peripheral arthritis, suggested that SASP acted in a disease remitting capacity (Dougados et al, 1986). The latter data were interpreted as implicative of a local antimicrobial action in the gastrointestinal tract (Dougados et al, 1986), since it has been reported that microbial carriage within the gut, caused acute exacerbations of AS (Ebringer, 1983). Beneficial effects of SASP have been demonstrated in patients with enteric ReA (Mislants et al, 1986; Trnavsky et al, 1988), and with PsA (Farr et al, 1990).
1/3/1/2 DEVELOPMENT OF SASP IN IBD

Fortuitously, in her earliest studies, Svartz tested the drug on several UC patients with accompanying peripheral arthritis, obtaining substantial symptomatic control (Svartz, 1942). Due to the Second World War, SASP did not reach America or Britain until the 1950s. Two early American studies of SASP in UC (Morrison, 1953; Moertal & Bargen, 1959), yielded favourable results in 73% and 64%, of 60 and 183, subjects, respectively. The first comparative trials of SASP in UC, in Britain, soon followed (Watkinson, 1961; Truelove et al, 1962). The incidence of disease remission or improvement, was, however, slightly lower (about 50%) in both these studies. Subsequent controlled trials of SASP versus placebo, in patients experiencing active UC, have revealed the efficacy of SASP, in terminating such disease attacks or exacerbations, in 65-80% of cases (Lennard-Jones et al, 1960; Baron et al, 1962; Dick et al, 1964). Later, controlled trials of inactive UC were undertaken, in which a maintenance dosage of SASP (2g daily) was given to UC patients in remission. The findings indicated that SASP was highly effective in preventing further relapses, in a significant proportion of cases (Misiewicz et al, 1965; Dissanayake & Truelove, 1973). Long-term treatment with SASP is essential, since it was shown in a similar study, that SASP treatment lasting only 1-3 months, was no more efficacious than placebo, in preventing UC relapses (Riis et al, 1973).

The cumulative data from the afore-cited trials have
established the therapeutic value of SASP in UC. Controlled trials of the efficacy of SASP in Crohn's disease, have been fraught with difficulties, due to the intrinsic complexities, especially regarding the location of the inflammatory site, in this disease. A concerted attempt was made, by independent groups of American and European physicians, to conduct such a trial. SASP and placebo groups were matched for CD disease severity, and site of inflammation (Singleton et al, 1979; Summers et al, 1979). From 151 patients with active CD, 74 were allocated for SASP treatment (4-6g/day); 77 served as placebo controls. Disease assessment was carried out after 4 months; 38% of SASP treated cases exhibited signs of disease improvement, compared with 26% among controls. It was noted that patients whose disease involved the colon, responded somewhat better to SASP, than those whose disease was confined to the small bowel. Clinical amelioration was confirmed in subsequent trials (Anthonisen et al, 1974; van Hees et al, 1981; Malchow et al, 1984), although the prophylactic benefit of SASP in small bowel CD, remains disputable.

1/3/2 PHARMACOKINETICS OF SASP

Several studies have elucidated the fate of SASP, after oral ingestion (Das & Dubin, 1976; Das et al, 1979; Peppercorn & Goldman, 1972; Schroder & Campbell, 1972). 10-20% of ingested SASP is absorbed mainly intact, in the ileum, and is returned unchanged via the hepatobiliary system. Hence, blood levels of the parent molecule are
very low (Das et al, 1979). 80-90% of ingested SASP is delivered to the colon, where it is reduced by the enzyme, azo reductase, present in colonic bacteria, yielding SP and 5-ASA (Azad Khan et al, 1983). It has been shown that inhibition of the growth of colonic flora by ampicillin, eventuated in less cleavage of the azo bond (Day & Houston, 1981). Liberated SP is absorbed from the colon, and metabolized in the liver by acetylation or hydroxylation, and conjugation with glucuronic acid, prior to urinary excretion (Peppercorn & Goldman, 1973). Serum SP levels are variable, being partly dependent upon the rate of acetylation. Acetylator phenotypes are bivariate ("slow" and "fast" acetylators) (Das et al, 1973a). 5-ASA is very poorly absorbed; it accumulates in the colon where it is acetylated, and is eliminated in the faeces (Das & Dubin, 1976; Peppercorn & Goldman, 1973; Schroder & Campbell, 1972). A gradient is created by the SASP, SP, and 5-ASA moieties in the colon. SASP predominates at the ileo-caecal junction, whilst very high levels of 5-ASA are attained in the rectum. Steady-state concentrations (μg/ml) of SASP, SP, and 5-ASA, acquired at the ileo-coecal valve and sigmoid colon, respectively, during continuous intake of 4g SASP daily, are: SASP- 5,300 and 100; SP- 700 and 1,200; and 5-ASA (free and acetylated)-400 and 7,400 (reviewed by: Hoult, 1986). Approximate, mean steady-state serum concentrations of SASP, SP and its metabolites, and 5-ASA and its derivatives, respectively, are: 12, 30, and <1μg/ml (reviewed by: Hoult, 1986). SASP, like 5-ASA, has a very high affinity for proteins, and is 98% bound to plasma proteins, whereas plasma SP is 45%
protein bound (Farr et al, 1985).

### 1/3/3 MECHANISM OF ACTION OF SASP IN IBD, AND RA

#### 1/3/3/1 EVIDENCE FOR THE ROLE OF 5-ASA IN COLONIC IBD

Since the establishment of SASP in IBD has preceded its development in RA, the majority of mechanistic studies of SASP and its constituents have necessarily pertained to IBD, with particular relevance to colonic disease. Determination of the therapeutic component in UC was pioneered by Azad Khan et al, (1977), who performed a controlled, blinded clinical trial, in which 3 groups of active UC participants, received retention enemas of either SASP, SP or 5-ASA for 14 days. Based on an analysis of histological changes in rectal mucosa biopsy samples, it was concluded that the 5-ASA moiety was therapeutically active, SP merely serving as a carrier, ensuring that the 5-ASA was liberated in the colon. This finding has been corroborated by a later clinical trial, in which it was shown that effective control of UC, could be obtained by a regimen of therapy with an oral preparation of 5-ASA (Dew et al, 1982).

A prophylactic role for 5-ASA in UC, is perhaps what might be expected, in view of the very high levels attained locally in the colon, and excreted in the faeces. Also in support of a local action, is the high affinity of both SASP and 5-ASA for connective tissue, as shown in an early autoradiographic study in mice (Hanngren et al, 1963). The fact that SASP is much less effective in the
management of Crohn's disease restricted to the small bowel, lends extra support for the role of the 5-ASA component, rather than the intact SASP molecule, in colonic IBD.

1/3/3/2 EVIDENCE THAT SP IS THE ACTIVE COMPONENT IN RA?

Whereas the active moiety has long since been established in IBD, this is not the case in RA. However, a clinical trial wherein two groups of 30 RA patients were treated for 24 weeks with SP or 5-ASA, revealed that SP exerted a similar second line action as did SASP. 5-ASA was shown to have only a weak first line effect. The doses given were the molar equivalents of 3g SASP daily (Pullar et al, 1985a). These findings were reproduced in a very similar study (Taggart et al, 1986). It was thus propounded that SP was equally effective as the parent molecule, and that it exhibited an anti-microbial action in the small bowel, granted that the onset and sustenance of RA were induced by a micro-organism such as clostridium (Pullar et al, 1985b). Detection of SASP and SP in the synovial fluids of RA subjects receiving oral SASP, has been claimed (Farr et al, 1985). These findings have led to the speculation that SP depresses systemic activity in RA, whilst 5-ASA, with its high affinity for connective tissue, acts locally on synovial inflammation (Farr et al, 1985).
EFFECTS OF SASP, SP, AND 5-ASA ON ARACHIDONATE METABOLISM

There is abundant evidence for excessive production of PGs (Donowitz, 1985; Hoult & Moore, 1980; Hampton & Hawkey, 1984) and LTs (Lauritsen et al, 1986; Peskar et al, 1985; Sharon & Stenson, 1984) by inflamed colonic mucosal tissues. This is also the case in rheumatoid synovial tissues, as previously described. Some of the functions of the major arachidonate metabolites are listed below (modification of: Hoult, 1986):-

PRO-INFLAMMATORY ACTIONS

1. Vasodilatation of small blood vessels, erythema: PGE₂, PGI₁
2. Raised vascular permeability, oedema: PGE₂, PGI₁, LTC₄, LTD₄
3. Potentiation of pain-causing mediators: PGE₂, PGI₁
4. Leukocyte chemotaxis: LTB₄
5. Activation of respiratory burst in PMNLs: LTB₄
6. Stimulation of bone resorption: PGE₂
7. Toxic cellular effects of oxidants released by PG cyclo-oxygenase-hydroperoxidase enzyme

ANTI-INFLAMMATORY ACTIONS

1. Feedback suppression of lymphocyte activation: PGE₂
2. Reduced splenocyte antibody production in
tumour-bearing animals: PGE₂
3. Reduced secretion from inflammatory cells: PGE₂ (high doses)
4. Feedback inhibition of fibroblast proliferation and collagen synthesis: PGE₂

It was initially demonstrated that SASP was capable of suppressing PG biosynthesis (Collier et al, 1976; Gould et al, 1981), although it was later shown that both SASP and 5-ASA were very weak inhibitors of cyclo-oxygenase mediated, conversion of arachidonic acid to eicosenoids (Hoult & Moore, 1980), in contrast to potent NSAIDs such as indomethacin and flurbiprofen (Hoult & Moore, 1980; Rampton & Hawkey, 1984). Paradoxically, the latter NSAIDs are not only ineffective in the treatment of active UC, but may actually precipitate relapses in quiescent UC patients (Hoult & Moore, 1980; Rampton & Hawkey, 1984). The reasons for this incongruity are unclear; possibly the effects of vital cytoprotective vasodilator PGs are partially or totally lost.

It has been shown that, whereas high doses of 5-ASA (5mM/l) inhibited PG synthesis, low-dose 5-ASA (500μM/l) actually stimulated PG formation in colonic fragments (Hoult & Page, 1981). This biphasic property is shared by the synthetic phenolic compounds, designated NDGA, and BW 755c (Hoult & Page, 1981). At low concentrations, these molecules are capable of acting as free radical scavengers, thereby preventing the self-destruction of the cyclo-oxygenase enzyme, a process which occurs during PG synthesis, and which is probably caused by intermediate
hydroperoxy-PGs (Hoult & Moore, 1980). At high concentrations, 5-ASA, NDGA and BW 755c are believed to exert a scavenging effect upon activated oxygen species required for arachidonate oxidation, thereby depriving cyclo-oxygenase of its substrate (Hoult, 1986).

There is substantial evidence suggesting that SASP, but neither 5-ASA, SP, nor NSAIDs, is a potent suppressor (active at up to 100μM/1) of human, and rabbit, colonic PG 15-hydroxydehydrogenase (PGDH), the enzyme which catalyzes the initial step in PG degradation (Hoult & Moore, 1980; Hillier et al, 1982; Kolassa et al, 1985). This finding led to the postulate that the prophylactic benefit of SASP in UC, may be mediated by an increase in colonic cytoprotective PGs (Hoult & Moore, 1978). Opposing this theory, is the fact that SASP therapy in UC, was associated with a fall, rather than an elevation, in PG secretion, as determined by in vivo rectal dialysis (Lauritsen et al, 1984). Furthermore, enhanced cytoprotection by PGs, arising as a result of PGDH inhibition by SASP, is difficult to equate with convincing evidence indicating that the 5-ASA component played a therapeutic role in UC.

SASP and 5-ASA also differ from aspirin-like drugs in their capacity to inhibit lipoxygenase-catalyzed LT synthesis from arachidonic acid (Dreyling et al, 1986; Nielsen et al, 1987; Sircar et al, 1983). In a recent study using human PMNLs, 50% inhibition of LTB₄ generation was attained at SASP and 5-ASA concentrations of 4-5mM/1, but acetyl 5-ASA was ineffective (Nielsen et al, 1987).
Since PMNLs predominate in acute inflammatory conditions such as UC, it has been hypothesized that the anti-inflammatory manifestations of SASP and/or its metabolites, are principally due to inhibition of PMNL activation. Activated neutrophils release lysosomal enzymes (Weissmann et al., 1971) and reactive oxygen radicals (McCord & Fridovich, 1978), both of which species are capable of tissue damage and abnormal immune responses (McCord & Fridovich, 1978). It has been reported that intact SASP, but not its metabolites, blocked neutrophil chemotaxis in vitro (Molin & Stendahl, 1979; Rhodes et al., 1981). This might simply reflect suppression of LTB₄ generation, particularly because similar concentrations of SASP were needed to produce half-maximal inhibition of both chemotaxis (Rhodes et al., 1981) and LTB₄ production (Nielsen et al., 1987).

SASP has been shown to inhibit the binding of the synthetic tripeptide, N-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) to a specific receptor on PMNLs, thus reducing PMNL activation, as assessed by chemotaxis and superoxide generation. 5-ASA was also suppressive, though to a lesser degree (Stenson et al., 1984). SASP, SP and 5-ASA have been shown to inhibit both superoxide production and lysosomal enzyme release, in response to serum-coated zymosan and immune complexes (Molin & Stendahl, 1979; Neal et al., 1987). Using either a cell-free xanthine-xanthine oxidase system, or a zymosan stimulated PMNL system, it
was demonstrated that SASP, SP, and 5-ASA, at steady-state physiological concentrations, were each able to suppress superoxide, hydrogen peroxide, or hydroxyl radical, production (Miyachi et al, 1987). In a very recent study, micromolar doses of SASP and SP were shown to inhibit neutrophil superoxide production, elicited either by FMLP, or by the calcium ionophore, A 23187. It was demonstrated that the inhibitory effect was due to interference with intracellular calcium (Ca++) mobilization, since an increase in cytosolic Ca++ was required for neutrophil activation by FMLP and A 23187 (Kanerud et al, 1990).

1/3/6 FURTHER PHARMACOLOGICAL EFFECTS OF SASP

1/3/6/1 ANTI-BACTERIAL EFFECTS OF SASP, SP AND 5-ASA

Due to the sulfonamide component of SASP, and perhaps because clostridial species have been associated with both IBD (Bolton et al, 1981) and RA (Olhagen & Mannson, 1968), several studies of the possible anti-bacterial action of SASP and its components, have been undertaken. It was reported that SASP treatment of patients with proctocolitis or Crohn's disease, resulted in a diminution in the numbers of clostridia, enterobacteria, and non-sporing anaerobes (West et al, 1974). A neoteric study indicated that in vitro growth of Clostridium difficile could be inhibited by SASP, SP and 5-ASA (Sandberg-Gertzen et al, 1985). RA patients, after treatment with SASP, were shown to exhibit a reduction in faecal levels of
C. perfringens, whilst no change in clostridial numbers was observed in similar patients, following d-PEN therapy (Neumann et al, 1987).

1/3/6/2 EFFECTS OF SASP ON FOLATE METABOLISM

IBD is associated with folate deficiency, as evidenced by low serum folate levels. This is partly due to impaired jejunal absorption of folates (Franklin & Rosenberg, 1973). SASP therapy has been shown to cause further malabsorption in colitis patients (Franklin & Rosenberg, 1973; Halsted et al, 1981). It has been reported that SASP, but not its metabolites, was a competitive inhibitor of jejunal brush border folate conjugase, an enzyme that hydrolyzes polyglutamyl folate to its monoglutamate form, prior to intestinal transport (Reisenauer & Halsted, 1981). Furthermore, it has been demonstrated that SASP competitively inhibited three folate-dependent enzymes, dihydrofolate reductase, serine transhydroxymethylase, and methylenetetrahydrofolate reductase, derived from rat liver homogenates, at concentrations similar to those required for blocking intestinal folate transport. SP and 5-ASA were ineffective in this system (Selhub et al, 1978).

1/3/6/3 EFFECTS OF SASP ON IRON METABOLISM

It has been shown that concurrent administration of SASP and ferrous sulfate to healthy volunteers, resulted in decreased serum iron levels, but no overall
interference with iron metabolism. Complementary in vitro studies indicated that the addition of ferrous sulfate, to SASP dissolved in sodium hydroxide, induced the formation of an insoluble precipitate (Das & Eastwood, 1973). This might suggest that SASP forms insoluble complexes with ferrous ions (Fe++) in the gut, thereby impeding the absorption of iron.

1/3/7 SASP ANALOGUES

In IBD, the function of the SP group is to act as a carrier molecule for 5-ASA, delaying its release until SASP undergoes azo reductive cleavage in the colon. Based on strong evidence that circulating SP is responsible for the majority of side effects associated with SASP therapy, such as dose-related anorexia and nausea, skin rashes, blood dyscrasias (Das et al, 1973b; van Hees, 1979), and reversible infertility in men (Toovey et al, 1981), SASP analogues in which 5-ASA was azo-linked to a seemingly harmless carrier molecule, have been developed. The most extensively studied compounds include olsalazine (Dipentum), which comprises two azo-linked 5-ASA molecules (Sandberg-Gertzen et al, 1983a, 1983b), ipsalazide (5-ASA azo-coupled with 4-amino benzoyl glycine), and balsalazide (5-ASA 4-amino B-alanine) (Chen et al, 1983).

Studies with oral olsalazine disodium have revealed that the molecule is poorly absorbed in the small bowel (Sandberg-Gertzen et al, 1983a) and colon (Sandberg-Gertzen et al, 1983b). Like SASP, olsalazine
undergoes enterohepatic circulation (van Hogezaend et al, 1985). Steady-state serum levels attained, are low, although its serum half-life is longer than that of SASP (Willoughby et al, 1982; van Hogezaend et al, 1985). Since this is not due to a decrease in clearance rate, it has been proposed that olsalazine and SASP were distributed differently in the body (van Hogezaend et al, 1985). Olsalazine, like SASP, has been shown to decrease colonic PGE₂ levels, in UC patients and normal controls (Lauritsen et al, 1984). One major clinical trial of UC patients who had previously been unable to tolerate SASP, demonstrated that olsalazine was well tolerated in 80% of these cases. Male infertility was notably absent. In the same study, olsalazine was shown to be significantly efficacious in preventing relapses (Sandberg-Gertzen et al, 1986).

Preliminary studies of balsalazide and ipsalazide in rodents, indicated that both drugs were non-toxic, and that peak serum levels of both carrier molecules, in healthy volunteers, following a single drug dose of 2g, did not exceed 3µg/ml (Chan et al, 1983). A recent, comparative double-blind study of UC patients undergoing maintenance therapy with balsalazide or SASP, showed that balsalazide was equally effective as SASP, and gave rise to fewer side-effects (McIntyre et al, 1988). None of these compounds has been tested in RA, presumably because the SP group is widely accepted as the relevant therapeutic agent in this disease.
Studies of SASP and its metabolites on lymphocyte function, have been reported sporadically, and there is as yet, no definitive evidence to suggest that SASP acts in an immunosuppressive capacity, in either IBD or RA. In the 1970s, much emphasis was placed on the pathogenetic concept of reduced suppressor T-cell activity, vis a vis IBD (Hodgson et al, 1978; Sachar et al, 1976). Several in vivo and in vitro IBD studies were undertaken, in which quantitative changes in a variety of circulating lymphoid subsets, were compared before, and after, SASP therapy (Rubinstein et al, 1978; Thayer et al, 1979; Holdstock et al, 1982). The results of such studies are inconclusive as to whether SASP and its components exert immunosuppressive properties. The work of Rubinstein et al, (1978) indicated that active IBD patients with UC or idiopathic proctitis, exhibited increased levels of circulating complement receptor positive cells (CRLs) and activated monocytes, whilst circulating T-cell percentages and mitogen responses, were lowered. Reversal towards normality was noted following successful SASP therapy. However, no such effects were detected, by in vitro incubation of patients' cells with SASP and its metabolites, at physiological concentrations (Das et al, 1973c). A similar study involving 12 and 6, UC and CD patients, respectively, revealed no changes in the absolute numbers and percentages
of circulating T cells, CRLs, and Ig-bearing B-lymphocytes, upon SASP treatment. Concomitant responses to skin test antigens did not differ significantly during SASP therapy. Antibody-dependent cellular cytotoxicity (ADCC) activity, in vitro, was shown not to be inhibited by SASP or its components, even at supra-physiological doses (Thayer et al, 1979). In a study of CD patients taking SASP, it was reported that the activity of Con A-induced suppressor T-cells, remained unaltered by SASP, both in vivo, and in vitro, using 100μg/ml SASP, SP, or 5-ASA (Holdstock et al, 1982).

One possible reason for these largely negative findings, is that PBL, rather than mucosal lymphocytes from the vicinity of inflammation, were tested. By using an enzymatic procedure to isolate lymphocytes from colonic biopsy specimens, of colitis patients, and normal controls, it was demonstrated that SASP therapy exerted a profound normalization of initially reduced T-cell percentages, increased B-cell percentages, and initially elevated, total mononuclear cells per gram of colonic mucosal tissue (Miyazaki et al, 1985). In support of an immunosuppressive role for SASP, it was reported that skin grafts were prolonged in mice receiving the drug, and that symptoms of experimental allergic encephalitis, were significantly abated in guinea pigs treated with SASP (Campbell, 1973). SASP has also been shown to inhibit PHA-induced ADCC in vitro, albeit at concentrations far in excess of those found in the sera of SASP-treated patients (Holm & Perlmann, 1988). It has been postulated that the immunosuppressive effects of SASP were exerted by
anti-folate actions on lymphocytes (Baum et al., 1981). The latter showed that SASP inhibited tetrahydrofolate-dependent, conversion of glycine to serine, in cultured rat lymphocytes; 50% inhibition occurred at 0.1mM/l (40μg/ml). SP and 5-ASA were ineffective in this system (Baum et al., 1981).

1/4/2 EVIDENCE FOR LYMPHOCYTE ABNORMALITIES IN RA

There is universal agreement that large numbers of T-lymphocytes, besides B-cells, are present in chronic rheumatoid synovial fluids (Traycoff et al., 1976; Froland et al., 1973) and tissues (van Boxel & Paget, 1975; Klareskog et al., 1982). It is also widely acknowledged that the numbers of Ig-secreting B-cells, in synovial fluids and tissues, are abnormally high in RA (Al-Balaghi et al., 1984; Petersen et al., 1984). Immunofluorescence studies of RA synovial sections have revealed the presence of, primarily IgM and IgG, RF-producing, B-lymphocytes (Munthe & Natwig, 1972). IgM RF-secreting cells have also been demonstrated in synovial membrane eluates (Vaughan et al., 1976). There is strong evidence for the presence of circulating, activated B-lymphocytes, as demonstrated by a significant increase in the ratio of surface IgM+/IgD+ B-cells in very severe cases of RA (Youinou et al., 1984), and by spontaneous DNA synthesis (Al-Balaghi et al., 1984). It has been reported that absolute numbers of circulating activated B-cells, correlated directly with severity of disease (Bell & Pinto, 1984). This led to the inference that, when RA symptoms are chiefly articular, active Ig
synthesis is confined to cells within the synovium. Conversely, extra-articular disease manifestations were associated with the subsistence of Ig-secreting, activated cells, in the peripheral blood (Bell & Pinto, 1984).

The responses of synovial T-lymphocytes in active RA, to mitogenic stimuli, have been extensively studied. These cells exhibited subnormal responses to PHA, Con A, and PWM, in contrast to rheumatoid PBL (Peterson et al., 1982; Peterson et al., 1983; Abrahamsen et al., 1978). It is now believed that this refractoriness was caused by a localized increase in T-cell activation, as defined by expression of HLA-DR antigens (Burmester et al., 1981; Klareskog et al., 1982). Activated synovial T-cells, predominantly of the CD8 phenotype, have been shown to express IL-2 receptors, and to be capable of spontaneous replication (Burmester et al., 1984; Kontinnen et al., 1985; Lemm & Warnatz, 1986). It is unclear whether circulating T-lymphocytes are refractory to mitogenic stimulation in RA. Normal responsiveness to PHA has been reported by several investigators (Dunne et al., 1982; Slavin & Strober, 1981), although depressed PHA and Con A responses, have also been noted in RA patients with erosive disease (Silverman et al., 1976). It has been reported that the presence of activated T-cells, expressing increased HLA-DR antigens and IL-2 receptors, was much less apparent in the peripheral blood of RA patients (Burmester et al., 1984; Lemm & Warnatz, 1986).

The precise mechanisms which propagate continuous T-cell activation within the rheumatoid synovium, are at present, unclarified. It seems likely that secreted cytokines play a major role. IL-1, a macrophage derived cytokine, has been
shown both to induce IL-2 receptors on cloned T-cell lines (Kaye et al, 1984), and to promote IL-2 production by T-cells in vitro (Smith et al, 1980). In support of these claims, substantial levels of IL-1 have been detected in synovial fluids of RA subjects (Wood et al, 1983; Nouri et al, 1984). A similar role for IL-2 is another possibility, since considerable IL-2-like activity has been demonstrated in RA synovial fluids in vivo (Lemm & Warnatz, 1986; Wilkins et al, 1983). Cultures of Con A stimulated, synovial fluid lymphocytes from active RA patients, were shown to secrete enhanced quantities of IL-2 (Ruschen et al, 1988).

Increased production of PGE₂ by RA synovial macrophages, is well established (Sturge et al, 1978). PGE₂ and other macrophage derived molecules, e.g peroxides, of rheumatoid synovial provenance, have been shown to exert potent suppression of local T-cell activation (Klareskog et al, 1985; Metzger et al, 1980). It might therefore be anticipated, that these mediators exert a down-regulatory effect on RA synovial T-lymphocyte activation, although there is no decisive evidence for this.

Alterations in the composition, and molecular organization, of cellular membranes, and an overall loss in their fluidity, is a cardinal feature of several disease states, such as leukaemia (Inbar et al, 1977) and diabetes (Baba et al, 1979). Using a fluorescence polarization technique to measure cell membrane fluidity in vitro, it was demonstrated that PBL membranes from patients with active RA, were more viscous than those from normal persons (Beccerica et al, 1988). This led to the proposal that
rheumatoid synovial lymphocytes were exposed to free radicals and reactive oxygen species, known to induce peroxidation of polyunsaturated fatty acids of membrane phospholipids (Beccerica et al, 1988).

1/4/3 STUDIES OF SASP PERTAINING TO RA

Neoteric studies suggest that the ameliorative effects of SASP and/or its components, in RA, and perhaps in the spondyloarthropathies, might be mediated by immunomodulatory effects on lymphocyte function. Using a mouse model of collagen arthritis, it was shown that subcutaneous administration of either SASP or SP to DBA/1 mice, impeded the development of arthritis. Contiguous in vitro experiments indicated a profound depression of proliferative responses, mounted by rat collagen II-specific, murine T-cells, when cultured with syngeneic accessory cells, plus SASP at micromolar concentrations (Klareskog et al, 1987). It was concluded that SASP could exert a direct inhibition of class II dependent T-cell activation (Klareskog et al, 1987).

It has been reported in a recent study, that several immunological aberrations in RA patients, reverted to normal after 12 weeks of successful SASP therapy (Symmons et al, 1988). Although no changes in lymphocyte subset numbers were noted, IgM and RF titers, and total numbers of circulating activated lymphocytes, fell significantly. There was also shown to be a marked normalization in PBL responsiveness to Con A, yet transferrin receptor (TfR) expression was unchanged (Symmons et al, 1988). The latter
finding may be of rheumatological significance. SASP is known to chelate ferrous ions (Das & Eastwood, 1973), which are deposited in large quantities in rheumatoid synovia (Muirden, 1966), where they almost certainly play a role in generating free radicals (Blake et al, 1981). There is also evidence to suggest that TfR expression is iron dependent (Bridges & Cudkowicz, 1984). In order to account for the prolongation of TfRα on circulating PBL, despite successful SASP therapy, it was proposed that the drug may exert its therapeutic action in RA, by chelating ferrous ions in the synovial milieu (Symmons et al, 1988).

A contemporaneous study revealed that SASP, but not its metabolites, inhibited mitogen induced proliferation of normal, and rheumatoid, PBL, at high doses (0.25mM/l). Synthesis of IgM RF in vitro was also shown to be suppressed by SASP at physiological concentrations, and experiments using purified lymphocyte subpopulations, indicated a major inhibitory effect of SASP on B-lymphocyte activity (Comer & Jasin, 1988). In this study, very little immunosuppression was attributed to SP and 5-ASA (Comer & Jasin, 1988). Very recent work has indicated that intact SASP exerted a profound inhibition of murine T-cell dependent antibody responses to sheep red blood cells, at concentrations as low as 0.2mM/l; SP and 5-ASA were ineffective. This suppressive effect was shown to be accompanied by a dose-dependent depression of IL-2 production (Fujiwara et al, 1990).
At the time of commencement of this work (1985), very little was known about the effects of SASP and its metabolites, in RA. Since there was abundant evidence indicating that lymphocytes, especially T-cells, played a major role in the progression of inflammatory synovitis, it was proposed that one means by which SASP exerted its second line effects in RA, was by lymphocyte modulation. Furthermore, since activated lymphocytes were known to prevail in rheumatoid synovia, it seemed expedient to study the effects of SASP and its constituents, on lymphocyte proliferation in response to standard mitogens.

Preliminary experiments established that SASP, but neither SP, nor 5-ASA, was able to induce a profound suppression of healthy human, and murine, lymphocyte proliferation, elicited by T- and B-cell mitogens. Essentially, two model systems of SASP-induced inhibition of mitogenesis, were developed for the purposes of this study, viz: (a) PHA induced proliferation of human PBL, and (b) Con A induced proliferation of murine (and porcine) splenocytes. The Con A mouse model served as a basis for analyzing the inhibition due to SASP, and for providing clues to its pharmacological action. Experiments were designed, firstly to confirm with reasonable confidence, that the observed suppression was, indeed, a biological phenomenon, with possible relevance to RA. For instance, it was necessary to eliminate the possibility that the inhibitory effect seen, was simply due to non-specific cytotoxicity, or interference with the binding of mitogen
to its cell surface receptor site. Secondly, experimental variables, viz: cell numbers, and concentrations of mitogens and sera, all of which factors might have influenced the degree of SASP-induced suppression, were studied. Thirdly, an attempt was made, to probe likely mechanisms by which SASP exerted its inhibitory action in vitro. It was of interest to discover whether SASP operated primarily on lymphocytes, or on accessory cells. The possibility that the suppression due to SASP, could be abrogated by addition of IL-2 to SASP-containing cultures, was investigated. Two other plausible modes of action of SASP, i.e: folate antagonism, and induction or activation of, suppressive PGs, were also studied. Finally, the mouse model was utilized for comparison of the relative potency of various 5-ASA azo derivatives and isomers. The purpose of these studies was to obtain clues to the biochemically active portion, and mode of action of, SASP.

The model of SASP-induced suppression of human PBL proliferation by PHA, was utilized in a longitudinal, collaborative study of RA patients undergoing therapy with SASP or d-PEN. It was clearly expedient to compare the suppressibility of patients' lymphocytes by SASP (and SP) in vitro, at the inception, and after 12 weeks, of, SASP, and d-PEN, treatment. Alterations in RA lymphocyte susceptibility to SASP, were assessed in relation to individual clinical responses to treatment, and to changes in various immunological and biochemical markers of disease activity.
2 MATERIALS AND METHODS

Superscript numerals after a methodological description identify another person as having performed the preceding work described. His/her name and contribution are elsewhere acknowledged.

2/1 MATERIALS

2/1/1 PATIENTS AND HUMAN CONTROLS

45 seropositive patients fulfilling the ARA criteria for definite RA, were recruited into the study. All had active disease with the following: (a) morning stiffness > 1 hour; (b) Ritchie index > 15; (c) PV > 1.85 cp (upper limit of normal = 1.72 cp); (d) CRP > 2mg/dl (upper limit of normal = 1mg/dl); (e) at least 3 clinically inflamed joints. None had received second line drugs within the preceding 3 months, or had ever been on steroids or other immunosuppressive agents. 35 patients were treated with SASP (2g daily); 10 received d-PEN (maintenance dose 500mg/day). All patients received NSAIDS concurrently, the dose of which was unaltered throughout the period of study.

14 patients were clinically assessed at 0 and 12 weeks of SASP therapy. At each visit, articular index, grip strength, morning stiffness, and visual analogue pain score, were noted. Total numbers of circulating lymphocytes, PV, serum CRP, and plasma levels of IgG, IgM, IgA, C3, and C4, were monitored. Lymphocyte subsets were
enumerated using a fluorescence activated cell sorter (FACS), which detected fluorochrome-labelled monoclonal antibodies directed against CD3, CD4, CD8, CD24, and DR, antigens, as well as kappa and lambda light chains. Serum levels of SASP, SP, 5-ASA, acetyl-SP, and N-acetyl 5-ASA, were assayed using high pressure liquid chromatography (HPLC).

The remaining 31 (21, SASP; 10, d-PEN) patients were clinically assessed at 0 and 12 weeks of therapy. They were categorized as "responders" if, after 12 weeks of treatment, at least 4 of the following criteria were fulfilled: (a) morning stiffness < 15 mins; (b) Ritchie index < 5; (c) PV < 1.72; (d) CRP < 1mg/dl; (e) no joint pain by history. Concurrently, their lymphocytes were used for PHA-induced proliferation assays, in the presence of 200 and 300μg/ml SASP; PBL from 16 of the 21 SASP-treated patients, were cultured with PHA plus 1mg/ml SP. By this means, it was possible to assess the extent of RA lymphocyte suppressibility by SASP (and SP) in vitro, at 0 and 12 weeks of second line therapy, and to compare this between clinical responders and non-responders.

Peripheral blood mononuclear cells (PBMC) were also obtained from 6 healthy laboratory personnel, of age range 20-45 years. Cells were cultured with PHA in the presence of SASP, SP and 5-ASA.

2/1/2 ANIMALS

Mice used in all studies were CBA/Ca females, aged 8-15 weeks, specific pathogen free upon arrival (OLAC). They
were healthily maintained in a warm atmosphere, under conditions of minimal stress. All mice were allowed at least one week to acclimatize to their environment, before sacrifice. Fresh porcine blood was collected from Parker's Abattoir, Freemen's Common Road, Leicester.

2/1/3 REAGENTS

2/1/3/1 MEDIA

2/1/3/1/1 PREPARATIVE MEDIA

RPMI 1640 (Gibco, 042-02511-H) was buffered with either 25mM NaHCO₃, or 1M HEPES, for preparing mouse splenocytes, and human PBL, respectively. It was supplemented with 100 IU penicillin/ml and 100μg/ml streptomycin, the pH being adjusted to 7.4 with 4M NaOH. Mouse peritoneal macrophages were prepared in medium as for human PBL, with the addition of 38μg/ml sodium heparin.

2/1/3/1/2 CELL CULTURE MEDIA

All lymphocytes were cultured in Iscoves' modified Dulbecco’s medium (Flow, 12-359-54), supplemented with antibiotics as above, plus 200mM l-glutamine. Mouse macrophages were maintained in RPMI 1640 buffered with HEPES, as described above, with the addition of 200mM l-glutamine.
MACROPHAGE WASH AND PEROXIDE ASSAY MEDIUM

Hanks' balanced salts solution ([HBSS], Sigma, H-1387) was supplemented with 1M HEPES and 25mM NaHCO₃ (HBSS/HEPES).

SERA

Foetal calf serum ([FCS], Sera Lab, batch 501113) was stored in 3ml aliquots at -20°C. In all experiments, frozen aliquots were thawed out within a few hours before the initiation of cell cultures. Erythrocyte-free, autologous human serum was prepared from a clotted fraction of whole venous blood, donated by patients or volunteers, for separation of their PBL. It was heated at 56°C in a water bath for 30 minutes, before use.

DRUGS

SASP (S-0883), SP (S-6252), 5-ASA (A-3021), 4-ASA (A-3380), and 3-ASA (A-9786), were bought from Sigma, UK. Di-sodium salts of olsalazine, and of its isomers, viz: (a) azo di-4-amino salicylic acid (two azo linked molecules of 4-ASA, denoted 4ASA-N=N-4ASA), and (b) 5-ASA azo 4-ASA, a hybrid of 4-ASA and 5-ASA (denoted 4-ASA-N=N-5-ASA), were kindly donated by Pharmacia, Sweden, as were 5-ASA azo 4-amino benzoic acid (sodium salt, denoted 5-ASA-N=N-4-ABA), and 5-ASA azo benzene sulfonic acid (sodium salt, denoted 5-ASA-N=N-BSA). Balsalazide and ipsalazide (di-sodium salts) were gifts from Biorex
Laboratories Ltd, UK. Sodium indomethacin was generously supplied by Merck, Sharp & Dohme Laboratories, UK. Misoprostol, a synthetic PGE1 analogue, was kindly provided by Searle, USA.

SASP was dissolved in autologous human serum at 2mg/ml, or in FCS at 1mg/ml, for human, and murine, cell cultures respectively. Experiments which required free SASP, entailed dissolving it in ICM; SP was dissolved in ICM at 200µg/ml for mouse cell cultures, and at 2mg/ml for human PBMNC cultures. Misoprostol was dissolved in absolute ethanol, to give a stock concentration of 10mg/ml. Aliquots were stored at -70°C. Immediately prior to use, frozen aliquots were thawed out, and diluted tenfold sequentially in phosphate buffered saline, pH 7.4, (PBS, Sigma, P-4417); corresponding ethanol controls were prepared in PBS. All other drugs were readily dissolved in ICM. Stock solutions were always freshly prepared, and sterilized by filtration through a 0.2µm millipore membrane.

2/1/3/4 MITOGENS

Con A (Sigma, C-2010), PHA (Sigma, L-9132), PWM (Sigma, L-9379), and LPS (Difco, E.coli, 0111:B4), were dissolved in ICM, and millipore filtered. In all cases, the optimal mitogen concentration, both for human, and mouse, lymphocyte cultures, was first determined. Mitogens were stored in 1ml aliquots at -20°C, at 20x the optimal concentration used in cell cultures, viz: Con A at 50µg/ml; PHA at 200 and 300µg/ml for murine and human cells respectively; PWM at 500µg/ml; LPS at 50µg/ml. Fluorescein
isothiocyanate-labelled Con A (FITC-Con A, Sigma, C-7642) was dissolved in PBS, diluted to stock concentrations of 300, 400, and 500 μg/ml, and stored at -20°C prior to use. All stock solutions once opened, were not re-frozen, since repeated freezing and thawing can potentially alter mitogenic activity (Sigma, personal communication).

2/1/4 ADDITIONAL REAGENTS FOR MACROPHAGE PEROXIDE ASSAY

(i) p-HYDROXYPHENYLACETIC ACID ([pOHPAA], Sigma, H-4377) at 7.4 mg/ml in HBSS/HEPES.

(ii) HORSE RADISH PEROXIDASE ([HRP], type I, Sigma, P-8125) at 10U/ml in HBSS/HEPES. The assay mixture comprised: pOHPAA: HRP: HBSS/HEPES, in the ratio 2:1:15 respectively.

(iii) PHORBOL MYRISTATE ACETATE ([PMA], Sigma, P-8139) at 10 μg/ml in HBSS/HEPES.

(iv) 0.2M BORATE BUFFER (pH 10.4) was prepared from boric acid. The pH was adjusted using 1M NaOH.

2/1/5 ELISA REAGENTS

(i) ELISA COATING BUFFER at pH 9.6, was prepared by dissolving 1.59g Na₂CO₃, 2.93g NaHCO₃, and 0.2g NaN₃ per litre of distilled water.

(ii) ELISA SERUM DILUENT at pH 7.3, was prepared by adding 9g NaCl, 1g bovine serum albumin ([BSA], Sigma A-9647),
0.2g NaN₃, 0.5ml Tween 20, and 0.2g MgCl₂·6H₂O, per litre of distilled water.

(iii) ELISA SUBSTRATE DILUENT at pH 9.8, was prepared by dissolving 97ml diethanolamine, 0.1g MgCl₂·6H₂O, and 0.2g NaN₃ in 800ml distilled water. The pH was adjusted to 9.8 with 1M HCl, and the volume made up to 1 litre.

(iv) ELISA SALINE WASH comprised 9g NaCl plus 0.5ml Tween 20 per litre distilled water.

2/1/6 MISCELLANEOUS REAGENTS

(i) [METHYL-³H]-THYMIDINE, radioactive concentration 37MBq/ml, specific activity 185GBq/mmol (³H TdR), (Amersham International, TRA 120), was diluted 1 in 33 with sterile "Nanopure" water. 2ml aliquots were stored at 4°C. Batches were used within 6 months of the date of radiochemical analysis quoted by the manufacturers.

(ii) HUMAN RECOMBINANT IL-2 (Sigma, T-3267) was dissolved in PBS to a concentration of 1,000U/ml, aliquotted in 1ml volumes, and stored at -70°C. Samples were thawed out immediately before addition to cell cultures, and were not re-frozen.

(iii) 2% PARAFORMALDEHYDE SOLUTION (PFA) was prepared by dissolving 0.2g paraformaldehyde powder (Sigma, P-6148) in 10ml "Nanopure" water, and heating to 70°C in a water bath, with gentle stirring. 0.5ml of 1mM CaCl₂·2H₂O was added to
the cooled solution, which was then filtered through Whatman No 1 filter paper, and stored at 4°C prior to use.

(iv) DEXTRAN/HEPARIN MIXTURE (DHM) was made up by dissolving 0.9g NaCl, 3g d-glucose, 3g dextran (MW 500K, Sigma, D-5251), and 5,000 units of sodium heparin in 100ml "Nanopure" water. The mixture was filtered through a millipore membrane, aliquotted in suitable quantities, and stored at -20°C.

(v) ACRIDINE ORANGE/ETHIDIUM BROMIDE MIXTURE (AO/EB) comprised 0.1mg of each reagent dissolved in 100ml PBS.

(vi) CARBONYL IRON (Sigma, C-3518), used for macrophage separation, was prepared on the day prior to the experiment. 3.5g iron powder was suspended in absolute ethanol by vigorous vortexing, and thence stored at room temperature (RT) until required. Before use, the ethanol was drawn off by pipette, and the powder washed extensively with sterile PBS, in 7ml of which it was finally resuspended.

2/2 METHODS

2/2/1 PREPARATION OF MOUSE SPLENOCYTES

Mice were sacrificed by cervical dislocation, and the spleens rapidly, and aseptically, excised; fatty tissue was carefully trimmed away. Spleens were transferred to a sterile, stainless steel, coffee strainer, rinsed briefly
with preparative medium, and gently teased using the barrel of a sterile 2ml syringe. Cells were washed twice through the mesh; the suspension was aspirated and discharged several times using a sterile 10ml pipette, and transferred to a sterile conical tube. This was allowed to stand on ice for 10 mins to enable debris to sediment. The cell suspension was centrifuged at 1,000 RPM for 5 mins at 4°C, and the supernatant discarded. The cell pellet was re-washed; cells were suspended in ICM, and enumerated using a Coulter counter (Coulter Electronics). They were adjusted to the desired concentration in ICM.

2/2/2 PREPARATION OF HUMAN (AND PORCINE) PBMC

50ml of human venous blood or whole porcine blood were mixed with 12ml DHM, in order to sediment erythrocytes. The white cell fraction was washed and resuspended in preparative medium. Aliquots were layered onto lymphocyte separation medium (Ficoll/sodium metrizoate mixture, Flow, 16-922-54), and centrifuged at 400 x g m/s² for 30 mins, at 18°C. Mononuclear cells were obtained from the interface layer, washed once, and resuspended in 1ml ICM. Cells were enumerated, and adjusted to a final concentration of 2 x 10⁶/ml.

2/2/3 CELL CULTURES

2/2/3/1 MURINE SPLENOCYTES

Quintuplicate cultures were set up in cylindrical wells
of sterile, 96-well, microtiter plates (Nunc, 1-67008). Each well received a total volume of 200μl, which included:

(i) 10% FCS with or without dissolved SASP
(ii) mitogen, viz: PHA at 5μg/ml; Con A at 2.5μg/ml; PWM at 25μg/ml; LPS at 2.5μg/ml
(iii) other drug(s)
(iv) 10^6 cells (PHA and Con A cultures); 1.2 x 10^6 cells (PWM and LPS cultures)
(v) IL-2 at 25, 50 and 100U/ml (when appropriate)

These concentrations, and this sequential order of addition of ingredients, were adhered to, unless otherwise stated.

Plates were incubated in a cleaned, humidified, modular incubator chamber, at 37°C in an atmosphere of 5% CO₂/95% air. After 48 hours (24 hours in the case of LPS cultures), 0.02 MBq of ³H TdR were added. Cultures were harvested 24 hours later, using a semi-automated cell harvester, by deposition of cells onto filter paper discs (Skatron, Northumbria Biologicals). Filters were dried overnight at 37°C in air, and transferred to scintillation vials, to which were added 3ml of xylene-based scintillation fluid (Optiphase-X, LKB, Plc). Cellular incorporation of ³H TdR was measured using a scintillation counter (Packard, Minaxi); raw data were expressed as counts per minute (CPM).
Quintuplicate cultures were set up in hemispherical wells of sterile, 96-well, microtiter plates (Titertek, Flow, 76-013-05). The total volume per culture was always 200μl, and comprised:
(i) 20% heated homologous serum with or without dissolved SASP
(ii) SP or 5-ASA if required
(iii) 10μl stock PHA at 300μg/ml (i.e. 15μg/ml per well)
(iv) 3 x 10⁶ cells

In all experiments, ingredients were added in this particular sequence. Cells were then cultured as described for murine splenocytes. Concurrently obtained autologous serum was employed, because lymphocyte behaviour in vitro was assumed to reflect more closely, than in vivo, if the supporting serum was identical.

2/2/4 MEASUREMENT OF LYMPHOCYTE VIABILITY

Cell viability was measured at the termination of culture, by differential staining with AO/EB. Briefly: one part AO/EB was mixed with one part cell suspension, and layered onto a haemocytometer grid. A total of over 250 cells was counted using fluorescence microscopy at x300 magnification. Live cells appeared green, dead cells orange. Duplicate counts were performed on each sample, and the mean percentage viability quoted.
For these experiments, cells were initially cultured in cylindrical, sterile Linbro tissue culture plate wells (Flow, 76-053-05). Since the surface area of the wells was approximately 11.7 times greater than that of the microtiter wells, volumes of FCS, Con A, and cell suspension were adjusted by this factor. Several cultures were incubated in the presence of 0 or 70μg/ml serum-bound SASP for 24 hours, under the conditions previously stated. After this period, cells were gently scraped from the well surfaces using a plastic pipette tip, pooled, and washed twice in PBS. They were resuspended in ICM, counted, and adjusted to the desired concentration. Viabilities of washed cells were determined, and invariably exceeded 90%.

Cells were re-cultured in septuplicate in microtiter plate wells for a further 48 hours, with or without Con A, and in the presence or absence of 70μg/ml of SASP. Cultures were tritiated after 24 hours of re-incubation, and harvested 24 hours later. As a control for the washing and scraping procedure, several cell cultures set up with or without SASP, were scraped but not washed, whilst others received no scraping. Linbro well plates were reincubated under the same conditions as the washed cells, and tritiated 24 hours later using 0.234MBq ³H TdR. After 72 hours of incubation, their cells were scraped and transferred in aliquots to microtiter wells, in which they were harvested.
2/2/6 MEASUREMENT OF RELATIVE Ig IN PWM SUPERNATANTS

2/2/6/1 PREPARATION OF PWM SUPERNATANTS

Preliminary experiments showed that maximal Ig production by PWM stimulated mouse splenocytes, is achieved after 96 hours of culture (data not shown). Thus, cells were incubated with or without PWM in the presence of SASP, SP, or 5-ASA at various doses up to 100µg/ml. After 96 hours, micro-titer well plates were centrifuged in specially designed holders (Cooke Instruments Ltd, UK) at 900 RPM for 5 mins. 150µl of cell supernatant from each of quintuplicate cultures were pooled together and stored in sterile vials at -70°C.

2/2/6/2 PREPARATION OF ALKALINE PHOSPHATASE CONJUGATE

Dialysis tubing was pre-boiled in 2% NaHCO₃ plus 1mM ethylene-diamine-tetra-acetic acid (EDTA), rinsed thoroughly in distilled water, boiled for a further 10 minutes in the latter, and allowed to cool. It was stored at 4°C submersed in sterile distilled water, until required. Immediately before use, it was thoroughly washed inside and out with sterile distilled water.

1.4ml rabbit anti-mouse total Ig (Dako, Z259), dissolved in 1ml PBS, was mixed with 5mg alkaline phosphatase (Sigma, P-5521) at RT for 10 mins. The mixture was dialyzed against PBS for 18 hours at 4°C, with two changes of buffer. 25% glutaraldehyde was then added to the dialysis
sac, giving a final concentration of 0.2%. This was incubated for 90 mins at RT, and further dialyzed against PBS overnight, with two changes of buffer. The sac was then transferred to a sterile flask containing 0.05M Tris buffer plus 1mM MgCl$_2$.6H$_2$O at pH 8, and dialyzed overnight at 4°C with two changes of buffer. The enzyme conjugate was transferred from the sac to a sterile vial, to which were added 1% BSA plus 0.02% NaN$_3$. It was stored at 4°C.

2/2/83 ELISA ASSAY PROCEDURE

ELISA micro-titer plate wells (Titertek, Flow, 78-594-01) were coated with 200µl rabbit anti-mouse total Ig (as above), diluted 1 in 1,000 in coating buffer, pH 9.6. Plates were covered with blotting paper dampened with distilled water, and incubated overnight in a covered tray at RT. Wells were subsequently washed (x4) with ELISA saline wash, using a washing device (Immuno-wash-12, Nunc, Gibco). PWM, and control, supernates were diluted 1 in 20 in ELISA diluent buffer, pH 7.3. 200µl volumes were added to quadruplicate coated wells. In addition, 200µl of positive and negative control sera (CBA/Ca mouse serum and FCS respectively, both diluted 1 in 2 in diluent buffer), were likewise dispensed. Plates were incubated for 2 hours at RT, and washed as described above.

The prepared enzyme conjugate was diluted 1 in 1,000 in diluent buffer, and 200µl added to all wells except the substrate controls. Plates were incubated for a further 3 hours at RT. Alkaline phosphatase substrate tablets (each consisting of 5mg of p-nitrophenyl disodium phosphate plus...
filler, Sigma, 104-105) were dissolved in diethanolamine buffer, pH 9.8, at 1mg/ml. After washing the plates, 200μl substrate were added to all wells except the conjugate controls. After sufficient coloration had developed, the reaction was terminated by rapid addition of 50μl of 3M NaOH, per well. Optical density (OD) was measured at 405nm, using an ELISA reader (Dynatech, MR 600).

2/2/7 MACROPHAGE DEPLETION STUDIES

PBMNC were prepared from 60ml of venous blood, obtained from one healthy volunteer (myself), and adjusted to 20 x 10⁶/ml in sterile PBS. Aliquots containing 12 x 10⁶ cells were dispensed into two polypropylene tubes, and the volume per tube was made up to 5ml with PBS plus 10% FCS. The remaining cells were X-irradiated with 1,500 rads (150 Gy). 12μl of freshly prepared carbonyl iron were added to one tube; the other served as a control. Both tubes were incubated in a water bath at 37°C for 15 mins, with occasional gentle shaking, and were then held on a magnet for 10 mins at RT, to allow the iron to sediment. Cell suspensions were carefully aspirated using a sterile Pasteur pipette, washed twice in PBS, suspended in ICM, and enumerated. At this stage, a small aliquot of each sample was subjected to FACS analysis, using monoclonal antibodies directed at surface markers on macrophages and lymphocytes (CD14 & CD45 respectively), in order to evaluate the degree of macrophage depletion. 6

Control cells were adjusted to 2 x 10⁶/ml. Since carbonyl iron treatment caused a decrease in total cell
concentration, with a corresponding increase in the ratio of lymphocytes to macrophages, the appropriate number of filler cells (i.e. irradiated PBMNC) was added to the test cells to compensate for this factor, before adjustment to $2 \times 10^6$/ml. Thereafter, cells were cultured in the presence or absence of autologous serum-bound SASP at 200, and 300$\mu$g/ml, and PHA, as described.

2/2/8 MEASUREMENT OF PEROXIDE PRODUCTION BY ACTIVATED MACROPHAGES

2/2/8/1 MACROPHAGE CULTURE

Mice were sacrificed by cervical dislocation, and erythrocyte free macrophages were obtained aseptically, by thoroughly washing and aspirating the peritoneal cavity with 2ml macrophage preparation medium. Cells were enumerated, and adjusted to $10^6$/ml in the same medium. 0.5ml aliquots containing $5 \times 10^6$ cells were dispensed into sterile tissue culture plate wells (Linbro, Flow). Plates were then incubated at 37°C, in a humidified atmosphere of 5% CO$_2$ for 60 mins, to enable sufficient cells to adhere to the well surfaces. Non-adherent cells were removed by washing with HBSS/HEPES. Finally, 0.45ml of macrophage maintenance medium was added to all cultures, which also received 10% FCS with or without 100$\mu$g/ml SASP. Macrophage monolayers were grown for 72 hours at 37°C, in a humidified atmosphere containing 5% CO$_2$ in air. At 24 and 48 hour intervals, cultures were washed twice with HBSS/HEPES. 0.45ml maintenance medium plus 10% FCS were added to all
cultures. FCS, containing dissolved SASP, was re-added to those cultures which had already received SASP; the concentration of SASP remained constant at 100μg/ml, throughout the incubation period.

2/2/87 PREPARATION OF HYDROGEN PEROXIDE STANDARDS

Immediately prior to determining \( \text{H}_2\text{O}_2 \) production by macrophage cultures at 72 hours, graded volumes of up to 100μl of 200μM stock \( \text{H}_2\text{O}_2 \), were added in duplicate to 4ml polystyrene tubes (Sarstedt, 55.478). The total volume of each standard was adjusted to 100μl with HBSS/HEPES. 0.9ml assay mixture (i.e 4-OHPAA and HRP in HBSS/HEPES) were then added to all samples. These were incubated at 37°C for 60 mins. The reaction was terminated by the addition of 1ml of ice cold borate buffer, pH 10.4, per tube. The reactants were allowed to equilibrate to RT.

After 10 mins, fluorescence was measured using a fluorimeter (Perkin Elmer 1000M), fitted with a micro-flow cell (volume 50μl). Excitation and emission wavelengths were set to 314nm and 414nm respectively. Samples were transferred to the micro-flow cell by means of a sampling pump, with timed peristaltic action (Ilacon Ltd, Gilbert House, River walk, Tonbridge, Kent). The instrument was set to zero using borate buffer alone.

After 72 hours of incubation, macrophage monolayer-containing wells were washed thrice with HBSS/HEPES. Macrophages subsequently received 100μl PMA followed by 0.9ml assay mixture. They were thereafter incubated and assayed as described above; fluorescence was
assessed upon direct transfer of the liquid from the culture wells to the micro-flow cell via the peristaltic pump.

2/2/9 FITC-CON A BINDING STUDIES

Porcine PBL were preferred to mouse splenocytes for studying the possibility that SASP interferes with Con A-lymphocyte binding. Preliminary experiments had shown that the dose of FITC-Con A required for optimal fluorescence, corresponds well with that needed for maximal stimulation of pig PBL, but not mouse splenocytes. It was previously shown that maximal Con A stimulation of porcine PBL, occurs at a concentration of $10^6$ cells per culture. 50% suppression was obtained with 100μg/ml SASP; 90-100% suppression occurred at 200μg/ml (data not shown).

Pig PBL were prepared as for human cells, counted and suspended in ICM at the desired concentration. Cultures were set up in cylindrical micro-titer well plates, in a total volume of 160μl, which comprised:

(i) FITC-Con A at 15, 20 and 25μg/ml
(ii) Free SASP at 100, 200 and 400μg/ml
(iii) $10^6$ cells

Cultures were incubated at 37°C for 30 mins. 160μl of 2% PFA were subsequently added to all wells. Cells were fixed for 15 mins at RT. Plates were then centrifuged at 900 RPM for 5 mins, and the majority of cultures were washed twice with PBS. Cells were resuspended in 30μl of 50% glycerol, and viewed by fluorescence microscopy, using a 50x water immersion objective. In order to control for the removal of
SASP prior to examination of cells, a few cultures remained unwashed; cells were viewed directly, after 15 mins fixation with PFA.

2/3 TREATMENT OF EXPERIMENTAL RESULTS

2/3/1 TREATMENT AND PRESENTATION OF DATA

For each experiment involving mitogen induced lymphocyte proliferation, means and standard deviations (SD) of replicate CPM values were calculated for cultures containing mitogen alone (control CPM), mitogen plus specified doses of drug(s) (test CPM), and for those devoid of both mitogen and drug(s) (background CPM). It was often more convenient to express test CPM as a percentage of control CPM. The terms "percentage stimulation" and "percentage suppression" were calculated using the formulae:-

\[
\text{% stimulation} = \frac{\text{CPM (test)} - \text{CPM (background)}}{\text{CPM (control)} - \text{CPM (background)}} \times 100
\]

Percentage suppression = 100 - % stimulation

Background CPM were, unless stated, very low in comparison with control CPM (< 3,000 CPM for murine Con A cultures; < 1,000 CPM for human PHA cultures). The term "stimulation index (SI)" is also used in this context, being calculated by the formula:-
SI = \frac{CPM (control)}{CPM (background)}

Due to inter-experimental variation between control CPM values, data (mean ± SD) from one of two or more reproducible experiments, are usually presented for murine Con A experiments.

The effect of SASP, SP, and 5-ASA, upon relative Ig synthesis by PWM-treated lymphocytes (as assayed by ELISA), was calculated by the formula:

\[
\frac{OD [DRUG (x) + PWM] - OD [DRUG (x) NO PWM]}{OD [DRUG (0) + PWM] - OD [DRUG (0) NO PWM]} \times 100
\]

where \( x \) = drug concentration in supernatant; OD at 405nm

2/3/2 STATISTICAL ANALYSIS

2/3/2/1 RA STUDIES

In the first group of 14 patients, multiple correlations using Spearman's and Pearson's coefficients, were sought between the clinical and laboratory indices of disease activity, and proportions and absolute numbers of lymphocyte subsets, at 0 and 12 weeks of SASP therapy.* Comparisons of individual variables at 0 and 12 weeks were performed using Wilcoxon's Matched Pairs Signed Rank Test.

In the second group of 21 (SASP) patients, the extent of SASP (and SP) mediated suppression of PHA responses of clinical responders and non-responders, was compared at 0
and 12 weeks using Wilcoxon's Signed Rank Test. Responses within patients at 0 and 12 weeks were compared by Wilcoxon's Sum Test. In the third group of 10 (d-PEN) patients, SASP induced suppression was compared at 0 and 12 weeks by means of the Signed Rank Test. However, the material was insufficient to permit comparisons between d-PEN responders and non-responders.

2/3/2 MURINE EXPERIMENTS

Computerized regression analysis, based on the method of least squares, was performed on data points relating: % of control CPM, in the presence of fixed doses of SASP, to control CPM values. Regression analysis was performed by direct computation on data relating: % of control CPM, in the presence of a fixed dose of SASP, to cell number per culture. In all other murine experiments, statistical comparisons, where apposite, were made between test, and control, raw data, using Wilcoxon's Ranked Sum Test.
## Table 1

Control and Background CPM, and Stimulation Indices (SI) for Murine Experiments Performed for Con A, PHA, PWM and LPS

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Control CPM Mean ± SD</th>
<th>Bkgd CPM Mean ± SD</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON A</td>
<td>124,952 ± 5,845</td>
<td>2,660 ± 235</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>131,750 ± 3,641</td>
<td>2,236 ± 398</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td>87,315 ± 4,178</td>
<td>1,651 ± 100</td>
<td>52.9</td>
</tr>
<tr>
<td>PHA</td>
<td>28,655 ± 3,375</td>
<td>908 ± 54</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>48,865 ± 2,519</td>
<td>3,168 ± 418</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>28,207 ± 1,722</td>
<td>1,056 ± 168</td>
<td>26.7</td>
</tr>
<tr>
<td>PWM</td>
<td>51,852 ± 2,116</td>
<td>16,264 ± 2,562</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>38,010 ± 2,837</td>
<td>3,703 ± 336</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>8,986 ± 369</td>
<td>430 ± 60</td>
<td>20.9</td>
</tr>
<tr>
<td>LPS</td>
<td>84,002 ± 2,003</td>
<td>20,274 ± 741</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>87,040 ± 3,785</td>
<td>19,290 ± 679</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>91,660 ± 1,956</td>
<td>15,958 ± 604</td>
<td>5.7</td>
</tr>
</tbody>
</table>
% CONTROL CPM

FIGURE 1(a) PHA

[DRUG] (micrograms/ml)

5-ASA  SP  SASP
Figure 1(c) PWM
TABLE 2(a)

VIABILITIES OF CELLS CULTURED WITH CON A AND SASP

<table>
<thead>
<tr>
<th>SASP (μg/ml)</th>
<th>VIABILITY (%)</th>
<th>CPM ± SD</th>
<th>% CONTROL CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95.4 ± 0.3</td>
<td>141,177 ± 4,671</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>93.8 ± 1.2</td>
<td>129,507 ± 5,282</td>
<td>91.7</td>
</tr>
<tr>
<td>50</td>
<td>92.2 ± 2.5</td>
<td>87,555 ± 6,001</td>
<td>62.0</td>
</tr>
<tr>
<td>75</td>
<td>94.4 ± 0.1</td>
<td>41,950 ± 3,933</td>
<td>29.7</td>
</tr>
<tr>
<td>100</td>
<td>94.3 ± 0.7</td>
<td>4,175 ± 1,196</td>
<td>3.0</td>
</tr>
</tbody>
</table>

TABLE 2(b)

VIABILITIES OF CELLS CULTURED WITH SASP, BUT NOT CON A

<table>
<thead>
<tr>
<th>SASP (μg/ml)</th>
<th>VIABILITY (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.8 ± 1.0</td>
</tr>
<tr>
<td>25</td>
<td>90.1 ± 1.2</td>
</tr>
<tr>
<td>50</td>
<td>80.0 ± 1.8</td>
</tr>
<tr>
<td>75</td>
<td>79.5 ± 0.7</td>
</tr>
<tr>
<td>100</td>
<td>72.4 ± 4.4</td>
</tr>
<tr>
<td>TIME OF ADDING CON A TO CELLS (MINS)</td>
<td>SASP (µg/ml)</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>-60</td>
<td>70</td>
</tr>
<tr>
<td>-60</td>
<td>0</td>
</tr>
<tr>
<td>-30</td>
<td>70</td>
</tr>
<tr>
<td>-30</td>
<td>0</td>
</tr>
<tr>
<td>-15</td>
<td>70</td>
</tr>
<tr>
<td>-15</td>
<td>0</td>
</tr>
<tr>
<td>-5</td>
<td>70</td>
</tr>
<tr>
<td>-5</td>
<td>0</td>
</tr>
<tr>
<td>-1</td>
<td>70</td>
</tr>
<tr>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>+1</td>
<td>70</td>
</tr>
<tr>
<td>+1</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 4

**DRUG**

SASP 100 µg/ml

SP

5-ASA

5-ASA 38 µg/ml

SP 63 µg/ml

% SUPPRESSION

96

10

-1

8
**TABLE 4**

EFFECT OF INCUBATING CELLS FOR 24 HOURS WITH CON A IN THE PRESENCE OR ABSENCE OF SASP @ 70μg/ml, WASHING, AND RE-ADDING CON A WITH OR WITHOUT 70μg/ml SASP FOR THE REMAINDER OF CULTURE

<table>
<thead>
<tr>
<th>Treatment at 0-24 (Hrs)</th>
<th>24</th>
<th>24-72</th>
<th>CPM ± SD</th>
<th>% Cont CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 NO SASP WASH CON A</td>
<td></td>
<td></td>
<td>176,220 ± 9,756</td>
<td>100.0</td>
</tr>
<tr>
<td>2 SASP WASH CON A</td>
<td></td>
<td></td>
<td>185,029 ± 8,473</td>
<td>105.0</td>
</tr>
<tr>
<td>3 NO SASP WASH NONE</td>
<td></td>
<td></td>
<td>110,452 ± 6,689</td>
<td>62.7</td>
</tr>
<tr>
<td>4 SASP WASH NONE</td>
<td></td>
<td></td>
<td>114,756 ± 4,671</td>
<td>65.1</td>
</tr>
<tr>
<td>5 NO SASP WASH SASP</td>
<td></td>
<td></td>
<td>152,624 ± 5,430</td>
<td>86.6</td>
</tr>
<tr>
<td>6 SASP WASH SASP</td>
<td></td>
<td></td>
<td>143,717 ± 6,914</td>
<td>81.6</td>
</tr>
<tr>
<td>7 NO SASP WASH SASP/CON A</td>
<td></td>
<td>128,715 ± 3,997</td>
<td>73.0</td>
<td></td>
</tr>
<tr>
<td>8 SASP WASH SASP/CON A</td>
<td></td>
<td>141,411 ± 7,999</td>
<td>80.2</td>
<td></td>
</tr>
<tr>
<td>9 NO SASP NONE NONE</td>
<td></td>
<td></td>
<td>120,309 ± 10,335</td>
<td>100.0</td>
</tr>
<tr>
<td>10 SASP NONE NONE</td>
<td></td>
<td></td>
<td>66,770 ± 5,568</td>
<td>55.5</td>
</tr>
<tr>
<td>11 NO SASP SCRAPE NONE</td>
<td></td>
<td></td>
<td>92,522 ± 4,803</td>
<td>100.0</td>
</tr>
<tr>
<td>12 SASP SCRAPE NONE</td>
<td></td>
<td></td>
<td>73,620 ± 5,972</td>
<td>79.6</td>
</tr>
</tbody>
</table>
% CONTROL CPM

\[ y = 67.921 + 0.14668x \]

\[ R^2 = 0.767 \]

FIGURE 5(a) SASP @ 25ug/ml (n=21)
% CONTROL CPM

FIGURE 5(b) SASP @ 50ug/ml (n=19)

\[ y = 36.148 + 0.20711x \]

\[ R^2 = 0.604 \]
FIGURE 7(a)

\[ y = -59.083 + 10.750x \]
\[ R^2 = 0.994 \]
### TABLE 5

**EFFECT OF MACROPHAGE DEPLETION ON SASP-INDUCED SUPPRESSION OF HUMAN PBL PROLIFERATION, IN RESPONSE TO PHA**

<table>
<thead>
<tr>
<th>SASP (µg/ml)</th>
<th>CARBONYL IRON</th>
<th>CPM ± SD</th>
<th>% CONTROL CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>93,841 ± 2,447</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>-</td>
<td>95,207 ± 5,197</td>
<td>101.8</td>
</tr>
<tr>
<td>300</td>
<td>-</td>
<td>51,347 ± 5,353</td>
<td>54.7</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>61,814 ± 4,011</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>+</td>
<td>55,664 ± 1,942</td>
<td>90.1</td>
</tr>
<tr>
<td>300</td>
<td>+</td>
<td>25,737 ± 1,122</td>
<td>41.6</td>
</tr>
</tbody>
</table>
Figure 11

OD (414nm)

\[ y = 6.0611 + 5.4255x \]

\[ R^2 = 1.000 \]
% CONTROL CPM

[Diagram showing a graph with % CONTROL CPM on the y-axis and [MISOPROSTOL] (micrograms/ml) on the x-axis. The graph plots a curve that rises steeply as the x-axis values increase.]
<table>
<thead>
<tr>
<th>MP (μg/ml)</th>
<th>SASP (μg/ml)</th>
<th>CPM ± SD</th>
<th>% CONTROL CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>109,416 ± 6,175</td>
<td>100.0</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
<td>97,967 ± 5,317</td>
<td>89.5</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>66,025 ± 1,803</td>
<td>60.3</td>
</tr>
<tr>
<td>0</td>
<td>60</td>
<td>55,385 ± 7,685</td>
<td>50.6</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>89,415 ± 4,722</td>
<td>81.7</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>77,010 ± 6,746</td>
<td>70.4</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>56,193 ± 6,832</td>
<td>51.4</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>38,350 ± 2,758</td>
<td>35.0</td>
</tr>
</tbody>
</table>
### TABLE 7

**EFFECT OF LOW DOSES OF INDOMETHACIN (IM) ON SASP INDUCED SUPPRESSION OF CON A STIMULATION**

<table>
<thead>
<tr>
<th>IM (µg/ml)</th>
<th>SASP (µg/ml)</th>
<th>CPM ± SD</th>
<th>% CONTROL CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>132,834 ± 13,610</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>67,044 ± 6,482 (C)</td>
<td>50.5</td>
</tr>
<tr>
<td>0.2</td>
<td>50</td>
<td>66,662 ± 6,964 NS</td>
<td>50.2</td>
</tr>
<tr>
<td>0.4</td>
<td>50</td>
<td>79,070 ± 5,337 *</td>
<td>59.5</td>
</tr>
<tr>
<td>0.6</td>
<td>50</td>
<td>83,098 ± 6,949 *</td>
<td>62.6</td>
</tr>
<tr>
<td>0.8</td>
<td>50</td>
<td>78,473 ± 5,233 *</td>
<td>59.1</td>
</tr>
</tbody>
</table>

* p < 0.01; + p = 0.01; data are compared with IM control containing SASP (C); NS = non-significant (p > 0.05). IM within this dose range had no effect on CPM of cultures lacking SASP (see text).
TABLE 8
EFFECT OF ADDITION OF IL-2 TO MURINE SPLENOCYTE CULTURES
STIMULATED BY CON A, CONTAINING 100 µg/ml SASP

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN ± SD</td>
</tr>
<tr>
<td>IL-2</td>
<td>6,686 ± 879</td>
</tr>
<tr>
<td>NONE</td>
<td>3,005 ± 150</td>
</tr>
<tr>
<td>IL-2 + SASP</td>
<td>373 ± 100</td>
</tr>
<tr>
<td>SASP</td>
<td>166 ± 78</td>
</tr>
<tr>
<td>CON A</td>
<td>176,431 ± 8,070</td>
</tr>
<tr>
<td>CON A + SASP</td>
<td>113,881 ± 4,846</td>
</tr>
<tr>
<td>CON A + IL-2</td>
<td>171,554 ± 7,357</td>
</tr>
<tr>
<td>CON A + IL-2 + SASP</td>
<td>123,954 ± 6,194</td>
</tr>
</tbody>
</table>
### TABLE 9

COMPARATIVE SUPPRESSION OF CON A-INDUCED MURINE SPLENOCYTE PROLIFERATION BY VARIOUS DRUGS TESTED

<table>
<thead>
<tr>
<th>DRUG</th>
<th>MW</th>
<th>S50% (µg/ml)</th>
<th>S50% (µM/l)</th>
<th>S50% DRUG 50% SASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SASP</td>
<td>401</td>
<td>55</td>
<td>0.14</td>
<td>1.0</td>
</tr>
<tr>
<td>OLSALAZINE. 2Na</td>
<td>346</td>
<td>235</td>
<td>0.68</td>
<td>4.8</td>
</tr>
<tr>
<td>4-ASA-N=4-ASA.2Na</td>
<td>346</td>
<td>500</td>
<td>1.45</td>
<td>10.4</td>
</tr>
<tr>
<td>4-ASA-N=5-ASA.2Na</td>
<td>346</td>
<td>425</td>
<td>1.23</td>
<td>8.8</td>
</tr>
<tr>
<td>BALSAZIDE. 2Na.H₂O</td>
<td>433</td>
<td>600</td>
<td>1.39</td>
<td>9.9</td>
</tr>
<tr>
<td>IPSALAZIDE. 2Na.H₂O</td>
<td>419</td>
<td>535</td>
<td>1.28</td>
<td>9.1</td>
</tr>
<tr>
<td>5-ASA-N=4-ABA</td>
<td>308</td>
<td>950</td>
<td>3.08</td>
<td>22.0</td>
</tr>
<tr>
<td>5-ASA-N=5-BSA</td>
<td>344</td>
<td>&gt;1000</td>
<td>&gt;2.91</td>
<td>&gt;20.8</td>
</tr>
<tr>
<td>SP</td>
<td>252</td>
<td>&gt;252</td>
<td>&gt;1.00</td>
<td>&gt;7.1</td>
</tr>
<tr>
<td>5-ASA</td>
<td>152</td>
<td>&gt;1000</td>
<td>&gt;6.58</td>
<td>&gt;47.0</td>
</tr>
<tr>
<td>4-ASA</td>
<td>152</td>
<td>&gt;1000</td>
<td>&gt;6.58</td>
<td>&gt;47.0</td>
</tr>
<tr>
<td>3-ASA</td>
<td>152</td>
<td>&gt;1000</td>
<td>&gt;6.58</td>
<td>&gt;47.0</td>
</tr>
</tbody>
</table>

S50% denotes the concentration of drug required to induce 50% suppression of control CPM. S50% values were estimated from Figs. 14-16; in the case of drugs which failed to suppress by 50%, the symbol > (greater than: the highest concentration tested) is used. Relative suppression values of drug, i.e., as compared with S50% SASP, were calculated from S50% concentrations as expressed in µM/l (column 3).
### TABLE 10

CLINICAL AND LABORATORY VARIABLES (EXPRESSED AS MEANS +/- STANDARD ERRORS [SEM]) IN 14 RA PATIENTS AT 0 AND 12 WEEKS OF SASP THERAPY

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>WEEK 0</th>
<th>WEEK 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Index</td>
<td>16.4 (1.8)</td>
<td>7.4 (1.5)**</td>
</tr>
<tr>
<td>Grip strength (R+L)</td>
<td>226.0 (21.0)</td>
<td>294.0 (28.0)**</td>
</tr>
<tr>
<td>Pain score (VAS mm)</td>
<td>48.0 (5.0)</td>
<td>25.0 (7.0)**</td>
</tr>
<tr>
<td>Morning stiffness (mins)</td>
<td>270.0 (18.0)</td>
<td>132.0 (36.0)**</td>
</tr>
<tr>
<td>Haemoglobin (gm/dl)</td>
<td>13.2 (0.5)</td>
<td>13.1 (0.4)</td>
</tr>
<tr>
<td>Platelets (x 10^9/l)</td>
<td>400.0 (27.0)</td>
<td>387.0 (25.0)</td>
</tr>
<tr>
<td>Plasma viscosity (cp)</td>
<td>1.92 (0.05)</td>
<td>1.84 (0.05)*</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>4.30 (0.6)</td>
<td>1.60 (0.4)**</td>
</tr>
<tr>
<td>IgG (gm/l)</td>
<td>13.7 (1.0)</td>
<td>13.8 (1.2)</td>
</tr>
<tr>
<td>IgA</td>
<td>3.2 (0.4)</td>
<td>3.0 (0.5)</td>
</tr>
<tr>
<td>IgM</td>
<td>1.3 (0.2)</td>
<td>1.3 (0.2)</td>
</tr>
<tr>
<td>C3 (mg%)</td>
<td>138.5 (8.4)</td>
<td>137.0 (11.2)</td>
</tr>
<tr>
<td>C4 (mg%)</td>
<td>24.0 (2.8)</td>
<td>24.4 (1.8)</td>
</tr>
<tr>
<td>Total lymphocytes (x 10^9/l)</td>
<td>2.0 (0.17)</td>
<td>1.99 (0.2)</td>
</tr>
<tr>
<td>% CD3+</td>
<td>73.3 (2.1)</td>
<td>70.8 (1.6)</td>
</tr>
<tr>
<td>% CD4+</td>
<td>48.2 (3.4)</td>
<td>45.1 (2.6)</td>
</tr>
<tr>
<td>% CD8+</td>
<td>11.6 (1.7)</td>
<td>10.1 (1.1)</td>
</tr>
<tr>
<td>% DR+</td>
<td>18.5 (1.5)</td>
<td>17.6 (1.2)</td>
</tr>
</tbody>
</table>

** p <0.01; * p <0.05; values compared at 0 and 12 weeks by Wilcoxon's Matched Pairs Signed Rank Test.

Clinical responders were those patients who fulfilled at least 4 of the following criteria at 12 weeks of SASP therapy:

- (a) morning stiffness <15 mins
- (b) Ritchie (articular) index <5
- (c) PV <1.72
- (d) CRP <1mg/dl
- (e) pain score = nil
FIGURE 17. CLINICAL RESPONSES TO SAPS
% SUPPRESSION

FIGURE 18. CLINICAL NON-RESPONDERS TO SASP

[SASP] (micrograms/ml)

200

NS

300

NS

0 WEEKS

12 WEEKS
### TABLE 11
CLINICAL VARIABLES (MEANS +/- SEM) IN 21 RA PATIENTS AT 0 AND 12 WEEKS OF SASP THERAPY

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>WEEK 0</th>
<th>WEEK 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular index</td>
<td>26.0 (1.5)</td>
<td>8.0 (2.2)*</td>
</tr>
<tr>
<td>Grip strength (mm Hg) (R+L)</td>
<td>142.0 (14.4)</td>
<td>206.0 (12.0)</td>
</tr>
<tr>
<td>Pain score (VAS mm)</td>
<td>65.0 (5.5)</td>
<td>20.0 (3.9)*</td>
</tr>
<tr>
<td>Morning stiffness (mins)</td>
<td>132.0 (9.2)</td>
<td>36.0 (7.6)*</td>
</tr>
<tr>
<td>Haemoglobin (gm/dl)</td>
<td>11.4 (0.4)</td>
<td>11.5 (0.4)</td>
</tr>
<tr>
<td>Plasma viscosity (cp)</td>
<td>2.04 (0.04)</td>
<td>1.81 (0.04)*</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>4.90 (0.2)</td>
<td>2.1 (0.3)*</td>
</tr>
</tbody>
</table>

* p < 0.05 by Wilcoxon's Signed Rank Test.
A CPM ($10^4$)

-10

-10

+10

+20

+30

+40

+50

+60

+70

+80

+90

t₀   t₁₂

NON-RESPONDERS
(n=4)

RESPONDERS
(n=12)
FIGURE 21

CPM (x 10^9)
240
220
200
180
160
140
120
100
80
60
40
20
0

NON-RESPONDERS
(n=7)

RESPONDERS
(n=14)
Δ CPM (x 10^3)
+10
+20
+30
+40
+50
+60
+70
+80
+90
+100
+110
+120
+130

FIGURE 22

NON-RESPONDERS
(n=3)

RESPONDERS
(n=7)
3 RESULTS

3.1 EFFECT OF SASP, SP AND 5-ASA ON MITOGENESIS

Figures 1(a)-1(d) illustrate the effects of SASP, SP, and 5-ASA, at concentrations of up to 100μg/ml, on mouse splenocyte proliferation, in response to optimal doses of T-, and B-cell, mitogens. In all cases, SASP exhibited a potent, dose-dependent, suppressive effect, discernable at 25μg/ml, and reaching approximately 90% suppression at 100μg/ml. Generally, SP exerted a mild degree of inhibition, but this was never statistically significant for any mitogen. 5-ASA tended to increase the level of proliferation to around 20% above control values, in Con A cultures (p < 0.01 at 50μg/ml 5-ASA and above). Table 1 indicates the mean, control and background CPM values, and SIs, of triplicate experiments for each mitogen. Although the background CPM appeared rather high in the case of LPS, and in one PWM experiment, the variation between CPM of replicate cultures seemed sufficiently low to justify the SIs quoted.

Figure 1(e) demonstrates the effect of 0-400μg/ml SASP and its moieties, on healthy human PBL proliferation in response to PHA. Data points were derived from the mean, % control CPM values for 6 volunteers. (Control CPM ranged from 27,000 to 186,000; background CPM were less than 1,000 in all cases). Evidently, SASP was profoundly suppressive, causing a particularly sharp decline in CPM, at concentrations between 200 and 400μg/ml. The action of SP
was negligible, whilst 5-ASA induced a slight, but statistically non-significant, elevation in CPM, as for murine splenocytes. Figure 2 is a comparison of the dose range required, for SASP-mediated inhibition of PHA responsiveness, for both species (data are taken from Figs.1(a) and 1(e)). Human PBL appeared somewhat more resistant to SASP than did mouse spleen cells; 50% suppression occurred at approximately 300-350μg/ml and 25-50μg/ml, for human and murine cells respectively.

**Fig.3** illustrates the effect of SASP and its moieties, on relative Ig production (as assayed by ELISA) by mouse splenocytes stimulated with PWM for 96 hours. Clearly, SASP elicited a profound, dose-dependent, suppression of Ig production (OD), which correlated well with its inhibition of PWM-induced lympho-proliferation. The effect of SP upon relative Ig synthesis, was negligible. Again, it is arguable whether low dose 5-ASA enhanced the response: although the figure suggested a 20% rise above control levels, this finding was refuted statistically (i.e., p > 0.05).

### 3/2 VERIFICATION OF SASP INDUCED SUPPRESSION

Having established that SASP, but not its main metabolites, could suppress mitogen induced lymphocyte proliferation, albeit at supra-physiological doses, the next logical step was to perform a series of experiments, to determine whether this inhibition could potentially reflect a true biological effect, due to the intact SASP molecule. All these investigations were carried out using a
single experimental model, that is: Con A induced mouse splenocyte proliferation.

Fig. 4 illustrates the requirement for the parent SASP, to achieve virtually total suppression, at 100\(\mu\)g/ml (0.25mM/l); co-culture with 0.25mM/l SP plus 0.25mM/l 5-ASA resulted in only 10% suppression. Similar experiments were undertaken, using 0.5 and 1mM/l, SP and 5-ASA. In both cases, the degree of suppression did not differ significantly from the values given in the figure (data not shown). Hence, it would appear that the suppressive action of SASP, could not be ascribed to a concerted effect of SP and 5-ASA.

Tables 2(a) and 2(b) show the effect of graded doses of SASP on cell viability, measured after 72 hours incubation with or without Con A. No appreciable increase in cytotoxicity was observed among Con A stimulated cultures, set up in the presence of up to 100\(\mu\)g/ml SASP (see Table 2(a)). However, incubation of non-stimulated cultures with incremental doses of SASP, resulted in a small, but steady, decline in viability, about 16% cytotoxicity occurring at 75\(\mu\)g/ml SASP (Table 2(b)).

The possibility that SASP (dissolved in FCS) inactivated the Con A molecule, when the two were present simultaneously, before addition of cell suspension to the culture wells, was considered. Experiments were performed in which FCS, with or without 70\(\mu\)g/ml dissolved SASP, was added at least 60 minutes before delivery of cell suspension. The same wells then received Con A at various times before, and 1 minute after, addition of cells. The results of a typical experiment are outlined in Table 3.
Clearly, the co-presence of SASP and Con A, for any length of time not exceeding 60 minutes, did not alter the degree of suppression by SASP. These findings were highly reproducible, and it was indeed shown in similar experiments, that even when SASP and Con A were co-present for as long as 3 hours prior to cell culture, there was no appreciable difference in the degree of suppression. Therefore, it appears highly improbable that SASP binds to the Con A molecule in such a manner as to render the mitogen inactive.

3/3 ANALYSIS OF SASP INDUCED SUPPRESSION

Having demonstrated that the suppressive action of SASP, was unlikely to be a consequence of cytotoxicity, mitogen inactivation, or an additive effect of its metabolites, SP and 5-ASA, various analytical studies were performed using the murine Con A model, to gain further insight into the mode of action of this drug.

3/3/1 VARIABLES INFLUENCING THE EXTENT OF SASP INDUCED SUPPRESSION

It was of interest to discover whether the degree of SASP mediated suppression, could be influenced by alterations in the experimental conditions. The effects of variation in: (a) the dose of Con A, (b) the number of cells, and (c) the concentration of FCS, were therefore studied.
Experiments were carried out, wherein cultures containing 0, 25 and 50μg/ml SASP, were stimulated by sub-optimal Con A concentrations. By this means, it was possible to obtain a range of sub-maximal levels of stimulation.

Figures 5(a) and 5(b) are scatter plots illustrating the relationship between: % control CPM in the presence of two fixed doses of SASP, and control CPM values. Linear regression lines are fitted, and their corresponding equations, and product-moment correlation coefficients (R^2) with two degrees of freedom, displayed. In both cases, a linear trend was apparent (p < 0.01 at 25 and 50μg/ml SASP).

Another series of experiments employed cultures containing variable concentrations of SASP, not exceeding 50μg/ml, plus optimal (2.5μg/ml), or sub-optimal (0.225μg/ml), Con A. The rationale was to determine whether SASP, at physiological concentrations, could suppress mitogenesis under these conditions. Fig.6 portrays the results of a single experiment. SASP exerted no significant degree of suppression of optimally stimulated cells (control CPM = 242,984), whereas cultures treated with sub-optimal Con A (control CPM = 56,768), exhibited a small, though statistically non-significant (p > 0.05), degree of inhibition with 15μg/ml SASP. Significant suppression (p <0.01) was achieved at SASP concentrations in excess of 15μg/ml, under these conditions.
3/3/1/2 EFFECT OF VARIATION IN CELL NUMBERS

The effect of variation of cell numbers, upon optimally stimulated Con A cultures containing 50μg/ml SASP, was investigated (see Figs. 7(a) & 7(b)). Fig. 7(a) indicates a linear relationship (best-fit line) between % control CPM attained in the presence of 50μg/ml SASP, and cell number (data from one highly reproducible experiment). Linearity is apparent (p < 0.01, since R^2 = 0.994 for n = 9) for cell numbers between 7 x 10^5 and 1.5 x 10^6 per culture; the regression equation (of y on x) is:

\[ y = 10.75x - 59.083 \]

where \( y \) = % control CPM; \( x \) = cell no \( 10^6 \)

Theoretically, from the above equation, total suppression is achieved when the number of cells present is approximately 5.5 x 10^6 per well.

Fig. 7(b) complements Fig. 7(a), in that it illustrates the relationship between CPM and cell number, in the presence of 50μg/ml SASP, and shows that the CPM of control cultures steadily declined as the number of cells was increased above 10^6 per well. Notably, the "SASP" curve tends to plateau, as the control curve descends.

3/3/1/3 EFFECT OF SERUM CONCENTRATION

For these studies, SASP was initially dissolved in either FCS or ICM, and added to Con A cultures, all of
which already contained 10% FCS. Replicate experiments showed no substantial difference between the degree of suppression obtained, at any dose of SASP within the range: 25-100μg/ml, whether the drug was dissolved in medium or FCS. Hence, the effect of adding 0 or 50μg/ml SASP (dissolved in ICM), to Con A cultures containing up to 25% FCS, was studied. Fig. 8(a) shows the results of a prototypical experiment. The relationship between: CPM and % FCS per culture is displayed. At low serum concentrations (0, 5 and 10%), a marked degree of SASP induced suppression was apparent (p <0.01 in all 3 cases). At FCS levels of, and above, 15%, the effect of SASP was abrogated. It should be mentioned that the background CPM were consistently low (<3,000 CPM), even at the highest FCS levels used.

Additionally, the effect of a constant dose of SASP upon Con A cultures set up with 0 or 10% FCS, was studied. It was found that: % SASP-mediated suppression of cultures devoid of FCS was linearly related to the control CPM values, as was demonstrated for cultures containing 10% FCS (see Figs. 5(a) and 5(b)). Thus, a valid comparison of the degree of suppression by SASP, between cultures containing 0 and 10% FCS, could only be made when the control CPM values were equal. Experiments were therefore carried out, utilizing sub-optimal concentrations of Con A. One such experiment, in which % control CPM obtained in the presence of 50μg/ml SASP, is plotted against control CPM values, is illustrated in Fig. 8(b). Within the range of CPM obtained, it is clear that cultures containing FCS were indubitably less susceptible to SASP, than were those lacking FCS.
3/3/2 TEMPORAL EFFECTS OF SASP

The effect of graded doses of SASP, when added at 0, 4, 24, and 46 hours, after the outset of cell culture with Con A, was studied. Fig. 9 illustrates the findings of one such experiment. It can be seen that, when SASP was added at the inception of culture, there was a marked, dose-dependent decline in % control CPM, reaching total suppression at 100µg/ml SASP. However, addition of SASP at 4 hours after the initiation of culture, resulted in significantly less suppression (p < 0.01 for all doses of SASP). A difference of 40% control CPM (60% suppression), was apparent at 50µg/ml SASP (p < 0.01). No significant difference in SASP-induced suppression was observed when SASP was added at 24, as compared with 4, hours. Addition of SASP as late as 46 hours, elicited a lesser, but significant, reduction in % control CPM, at concentrations exceeding 50µg/ml, when compared with control cultures (p < 0.01). This reduction was significantly less than that of cultures which received SASP at 0, 4, and 24 hours (p < 0.01).

3/4 FURTHER ANALYSIS OF SASP INDUCED SUPPRESSION

3/4/1 EFFECT OF SASP ON LYMPHOCYTE BINDING OF CON A

Since SASP was known to possess a high affinity for proteins, it was postulated that the drug bound to extrinsic, or intrinsic, cell surface (glyco)proteins, in such a manner as to sterically hinder the binding of the
Con A molecule. This hypothesis was tested, by incubating porcine PBMNC briefly with FITC-Con A and SASP, before fixation, and examination under fluorescence microscopy. At all concentrations of FITC-Con A used (i.e.: 15, 20 and 25µg/ml), bright fluorescence was obtained. All control, and SASP-treated, cells were shown to exhibit full or partial surface ring staining, and in about 25% of the cell population viewed, polarized fluorescence (capping) was visible. In some cases, shedding of caps was clearly observed. These features were notable, even at the highest dose of SASP employed (400µg/ml), known to cause total suppression of pig PBL proliferation in response to Con A (data not shown). Moreover, no difference was detected in SASP treated cells which remained unwashed throughout.

3/4/2 DETERMINATION OF REVERSIBILITY OF SASP INDUCED SUPPRESSION

In order to determine whether the suppression of Con A-induced lymphocyte proliferation by SASP, could be reversed, mouse splenocytes were cultured for 24 hours with Con A, and 0 or 70µg/ml SASP. Cells were then washed thoroughly, and re-cultured for a further 48 hours, with either: (a) Con A, (b) SASP at 70µg/ml, (c) Con A plus 70µg/ml SASP, or (d) neither. The findings from one of 3 replicate experiments are displayed in Table 4. It can be seen that the CPM value obtained, when Con A cultures without SASP, were set up for 24 hours, washed, and then re-cultured with mitogen alone, was 175,220 (No.1). This value is regarded as being the standard, representing 100%
stimulation (zero suppression). The table indicates that Con A cultures which contained SASP for the first 24 hours of incubation, and which were subsequently washed and re-cultured with Con A alone, were refractory to the suppressive action of SASP (No.2).

There was no significant deviation in the CPM obtained, between pairs of cultures (3 & 4; 5 & 6; 7 & 8) which differed inasmuch that SASP was present or absent for the first 24 hours, irrespective of their treatment during the last 48 hours of incubation, provided that the cells were washed at 24 hours. Cultures 9 & 10 were controls for the washing procedure, and indicated a considerable reduction in CPM, upon exposure to SASP for 72 hours, as would be predicted; pairs 11 and 12 represent cultures which were scraped but not washed, after 24 hours. These data, taken in toto, suggest that the suppressive effect of SASP could be reversed, by washing the drug free from SASP-treated cells, after as long as 24 hours of culture.

CPM values of cultures 7 & 8 (incubated with SASP plus Con A, after washing at 24 hours), were significantly lower than those of controls (p <0.05), implying that SASP exerted some suppression of re-stimulated cultures. The CPM of cultures 3 and 4 appeared to be inordinately low. However, this finding was not reproduced in similar experiments.

3/4/3 EFFECT OF SASP ON ACCESSORY CELLS

In order to determine whether SASP influenced accessory cell function, human PBMNC were partially depleted of
macrophages by carbonyl iron treatment, and cultured with PHA, in the presence of 0, 200 and 300μg/ml SASP. The extent of macrophage depletion was evaluated by FACS analysis. Figure 10 is a copy of the FACS printouts, for PBMNC treated with and without carbonyl iron. The figure indicates (in red print): % macrophages present in carbonyl iron-treated, and control, cell suspensions. These values were 26.1% and 37.2% respectively. This represented an overall macrophage depletion of:

\[
\frac{37.2 - 26.1 \times 100}{37.2} = 29.8\%
\]

Each box indicates "X-Y" dot displays, generated by the 6 possible permutations, of 4 parameters used in discriminating between macrophages (red dots) and lymphocytes (blue dots). The parameters employed were: (a) FL-1 = fluoresceinated anti-lymphocyte marker, CD45; (b) FL-2 = fluoresceinated anti-monocyte marker, CD14; (c) VOL = cell volume; (d) SSC = 90° light scatter (side scatter).

Table 5 shows the CPM obtained, when test and control cells were cultured with PHA and SASP. The effect of partial depletion of accessory cells, was to reduce the total control CPM obtained, from 93,841, to 61,814 (i.e., by approximately one third). This reduction in CPM paralleled the extent of accessory cell depletion (29.8%). Although there appeared to be a slight increase in susceptibility to SASP, at both 200 and 300μg/ml, in carbonyl iron-treated cultures, as compared with untreated cells, this difference was not statistically significant.
In conjunction with cell depletion studies, the effect of continuous culture of mouse peritoneal macrophages with 100μg/ml SASP, upon peroxide generation, was investigated. The results of a single peroxide standard curve, generated immediately prior to the determination of peroxide production, by 72 hour, SASP-treated and control, cultures (one of two similar experiments), are illustrated in Fig.11. Evidently, there was a linear relationship between: absorbance (OD) at 414nm, and nM of hydrogen peroxide present in standard volumes. Data points are the means of duplicate OD values (maximum deviation between pairs = 0.4). OD readings (mean & SD, n=6) for macrophages cultured with 0 and 100μg/ml SASP for 72 hours, were: 14.7 ± 1.5, and 16.3 ± 2.1, respectively, which approximated to 1.6, and 1.9nM hydrogen peroxide produced. There was found to be no significant difference between these values (p > 0.05). Similar results were obtained in a subsequent, confirmatory experiment.

3/4/4 EFFECT OF SASP ON PROSTAGLANDIN (PG) ACTIVITY

The possible interference of SASP with PG activity, was first studied directly, using the murine Con A system. The effect of the synthetic PGE1 methyl ester analogue, misoprostol (MP), upon lymphocyte transformation, was initially tested over a wide dose range. The findings of a single experiment are depicted in Fig.12. It can be seen that a profound, dose-dependent decrease in CPM occurred at MP concentrations above 0.05μg/ml. The effect of co-culture with MP plus moderate doses of SASP, was subsequently
investigated; the data from one experiment are presented in Table 6. In this case, MP at 5μg/ml elicited a reduction in CPM of 20,000 (18.3% suppression). Co-addition of SASP at 20, 40 or 60μg/ml with a fixed dose of MP (5μg/ml), neither reversed, nor enhanced, the inhibition caused by the latter. These results were subsequently reproduced.

An attempt was made, to discern whether SASP induced suppression could be reversed by the PG inhibitor, indomethacin (IM). Initially, the effect of IM on lymphocyte transformation, was studied. At concentrations below 2μg/ml, no significant difference in CPM was found, when compared with control values. IM at higher doses was inhibitory (data not shown). The effect of low doses of IM plus 50μg/ml SASP, was investigated. Fig. 13 illustrates the results of a single experiment. CPM obtained with SASP alone, were about 42% of control values. A small rise in CPM (not greater than 7%), was detected at all doses of IM up to 1μg/ml, although this was not statistically significant at any particular dose.

In order to discover whether this rise in CPM could be increased, the dose of IM was varied between 0.2 and 0.8μg/ml, whilst that of SASP remained constant (50μg/ml). Data from one experiment are displayed in Table 7. Cultures containing SASP alone exhibited 67,044 CPM. The effect of co-addition of IM at 0.4, 0.6 and 0.8μg/ml, was to elevate the degree of stimulation by approximately 11,000-16,000 CPM. Although small, this rise was statistically significant for these three doses of IM (p < 0.01). This finding was confirmed in replicate experiments. The effect of SP was also tested in this system; unlike SASP,
co-addition of SP (at 50, 100 & 200μg/ml) and IM, failed to produce any significant deviation from control CPM (data not shown).

3/4/5 EFFECT OF ADDITION OF FOLATE TO SASP CULTURES

The possibility that SASP-mediated suppression could be reversed by addition of folic acid, was studied. Con A cultures were set up with 50 and 100μg/ml SASP, in the presence of varying doses of folate, not exceeding 50μg/ml (higher doses were suppressive). Even at the highest folate concentrations used, there was found to be no reversal of the inhibitory effect of SASP (data not shown).

3/4/6 EFFECT OF SASP ON IL-2 PRODUCTION

The postulate that SASP might mediate its suppression by down-regulation of IL-2 production, was considered. Hence, it was intriguing to discover whether the suppressive effect of SASP on lymphocyte proliferation by Con A, could be reversed by the concomitant addition of IL-2 to SASP-containing cultures. A series of experiments was carried out, in which cells were co-cultured with SASP, at a range of concentrations not exceeding 200μg/ml, plus IL-2 at doses of 25, 50 and 100U/ml. The data from a characteristic experiment are shown in Table 8. In this case, the concentration of SASP was 100μg/ml; that of IL-2 was 25U/ml. All CPM values of necessary controls are tabulated. It can be seen that IL-2 alone caused an increase in background CPM, which was totally suppressed by
SASP. There was no significant reversal of the suppressive effect of SASP on Con A cultures. The extent of SASP-mediated inhibition was fairly large in this instance. However, even at lower doses of SASP, which induced far less suppression, addition of IL-2 proved ineffective (data not shown). Moreover, no abrogation of the SASP effect was achieved, when higher concentrations of IL-2 were employed.

3/5 UTILIZATION OF SASP-MEDIATED SUPPRESSION

3/5/1 EFFECTS OF RELATED COMPOUNDS ON LYMPHOCYTE PROLIFERATION

3/5/1.1 EFFECTS OF 5-ASA-AZO DERIVATIVES

The effects of various azo-linked compounds of 5-ASA were studied, utilizing the Con A murine model of lymphocyte transformation. The results are illustrated in Fig.14 and Table 9. In the figure, data points represent mean ± SD of % control CPM, obtained in replicate experiments. For convenience, drug concentrations are expressed in μg/ml, rather than mM/l. This seems justifiable, since the logarithmic values of the molecular weights (MW) of the 6 analogues, fall within the narrow range of 2.45 and 2.60 (MW of all 5-ASA azo compounds tested are presented in the table). It is clear from the figure, that SASP exhibited far greater potency than any of the other derivatives. Olsalazine, though less suppressive than SASP, elicited a very steep decline in
CPM, at a dose range between 100 and 400μg/ml. Both balsalazide and ipsalazide were significantly less inhibitory than olsalazine (p <0.01 at concentrations exceeding 200μg/ml), despite a relatively large degree of inter-experimental variation. The compounds 5-ASA-N=N-BSA, and 5-ASA-N=N-4-ABA, were even weaker inhibitors of Con A-induced lymphocyte proliferation. Both these analogues, besides balsalazide and ipsalazide, actually caused a slight enhancement in CPM, at lower levels, although this rise was not statistically significant for any individual compound.

3/5/1/2 COMPARATIVE EFFECTS OF OLSALAZINE ISOMERS

The effects of olsalazine, and of its isomers, 4-ASA-N=N-5-ASA, and 4-ASA-N=N-4-ASA, were studied using the mouse Con A model. Fig.15 depicts the findings of replicate experiments. The data for olsalazine are as already shown (see Fig.14). Clearly, olsalazine exerted a greater suppressive action than either of its isomers (p <0.01 for all doses exceeding 200μg/ml); the inhibitory capacities of the two 4-ASA-azo derivatives were comparable with those of balsalazide and ipsalazide. Interestingly, 4-ASA-N=N-4-ASA appeared somewhat less potent than the hybrid, 4-ASA-N=N-5-ASA, at, and above, 600μg/ml (p <0.05 at 600, 800 & 1,000μg/ml).

3/5/1/3 EFFECTS OF 5-ASA, 4-ASA AND 3-ASA

In conjunction with these studies, the actions of 5-ASA...
and its isomers, 4-ASA, and 3-ASA, were investigated using the same model system. The comparative effects of these compounds are shown in Fig. 16. A moderate increase in CPM above control values, was apparent for all three isomers, at the lower dose range employed (p < 0.01 at 400 & 600 µg/ml for all three drugs); at higher concentrations, there was a steady decline in CPM. From the figure, 5-ASA would appear to effect a slightly greater rise in CPM, as compared with its isomers, but this tendency failed to reach statistical significance, since the degree of inter-experimental variation was considerable. The data for 4-ASA and 5-ASA, taken in conjunction with Fig. 15, would also imply that the azo linkage was essential for the suppressive effects of olsalazine and its 4-ASA-N=N-4-ASA isomer, as was the case with SASP (see Fig. 4), since, by analogy, the carrier molecules, 4-ASA and 5-ASA, per se, exerted no intrinsic suppression.

3/5/2 SASP THERAPY IN RA: QUALITATIVE CHANGES IN LYMPHOCYTES AND CORRELATION WITH CLINICAL RESPONSE

Table 10 shows the results of clinical and immunological variables in 14 patients, immediately before, and after 12 weeks of, SASP therapy. There were significant improvements in articular index, grip strength, pain score, morning stiffness, PV, and CRP. No significant changes were observed in complement, or Ig, levels. Similarly, the total numbers and proportions of lymphocyte subsets remained unchanged upon SASP treatment.

Serum levels of SASP and its metabolites at 12 weeks
(μg/ml; mean ± SE) were as follows: SASP, 7.85 ± 1.6; SP, 14.3 ± 3.5; acetyl SP, 10.7 ± 2.4; 5-ASA, 0.3 ± 0.04; N-acetyl 5-ASA, 0.8 ± 0.4. There was found to be no correlation between these levels, and any immunological values measured; nor did these levels correlate with patients' individual clinical responses to SASP therapy (see page 81).

In the second group of 21 SASP-treated patients, there were statistically significant improvements in articular index, pain score, morning stiffness, PV and CRP (see Table 11). Figs. 17 and 18 illustrate the effects of SASP, on PHA-induced proliferation of PBL obtained from this group of patients, at 0 and 12 weeks of SASP therapy. Clinical responders (n=14) and non-responders (n=7) were compared. An overall reduction of SASP-induced suppressibility, in responders and non-responders, occurred after 12 weeks. The mean suppression achieved with 200μg/ml SASP, for the entire group (n=21) at 0 and 12 weeks, was 19% and 11.1% respectively; at 300μg/ml SASP, it was 62.6% and 53.7% respectively (data not shown). These values were statistically non-significant at either dose. Within the responder group alone, however, there was a significant decline in SASP-induced suppressibility of these patients' PBL proliferative responses to PHA, after 12 weeks of SASP therapy. This decrease was highly significant at 200 and 300μg/ml SASP (p < 0.005 for both doses, see Fig.17). By contrast, the non-responder group failed to exhibit this trend (see Fig.18).

Fig. 19 gives a comparative breakdown of the suppression at 0 and 12 weeks, in terms of CPM (Δ CPM, i.e: CPM of
control cultures minus CPM obtained in the presence of 200μg/ml SASP), for PBL of individual patients. The figure shows that, within the responder group, there was a decrease in PBL susceptibility to SASP, in 12/14 patients, after 12 weeks of therapy. Amongst non-responders, 3/7 patients exhibited this trend. It is also notable that, in general, non-responders' cells were initially less suppressible than those of responders, although this observation did not quite achieve statistical significance at the 5% level.

Fig. 20 illustrates the effect of 1mg/ml SP, upon lymphocyte responses to PHA, expressed as Δ CPM, for patients at 0 and 12 weeks of SASP treatment. Due to insufficiency of cells, the number of patients' PBL studied was 16/21 (n=12, n=4, for responders and non-responders respectively). A decrease in Δ CPM (suppressibility) at 12 weeks, was apparent in 6/12 responders, and 3/4 non-responders. These findings were statistically non-significant, inasmuch that there was no trend towards a decrease in suppressibility, of PHA-induced proliferation of PBL, amongst responders, after 12 weeks of SASP therapy. The low sample number of non-responders precluded statistical analysis of this group, although there was a strong tendency towards a decrease in suppression (by approximately 30,000 & 45,000 CPM), of 2 patients' PBL, after 12 weeks. None of the non-responders' cells, within the "SASP" group, exhibited a fall in suppressibility of this magnitude (see Fig.19). This observation would suggest that SP was dissimilar to SASP in this respect.

Fig. 21 indicates the CPM values for PHA-stimulated,
control cultures of individual responders and non-responders, at 0 and 12 weeks of therapy. There were clearly no characteristic differences in the mean control CPM, for PBL of patients within either group, before and after treatment with SASP, suggesting that the quantity of SASP-induced suppression, was independent of the degree of lymphocyte proliferation induced by PHA.

The question arose, as to whether the correlation between increased PBL robustness towards SASP in vitro, and successful SASP therapy, resulted from RA disease remittance, or stemmed from an effect specifically attributable to the action of SASP in vivo. In an attempt to answer this query, a similar study was accordingly performed, utilizing PBL from 10 RA patients receiving another second line agent, viz: d-PEN. Fig.22 is a plot of Δ CPM (suppression) of PHA-stimulated PBL (0 & 12 weeks) from clinical responders (n=7) and non-responders (n=3) to d-PEN, when cultured with 300µg/ml SASP. Although the sample material was rather small, the figure would appear to outline a general trend towards a decline in SASP-induced suppression upon d-PEN treatment, irrespective of responder status. However, a statistical comparison of Δ CPM (n=10), at 0 and 12 weeks of therapy, marginally failed to reach significance (p > 0.05). This was also true for the responder group alone (n=7). The small sample of non-responders precluded inter-group comparison, although there would appear to be a trend towards decreased suppressibility, with treatment, in 3/3 non-responders to d-PEN.
4 DISCUSSION

4/1 OVERVIEW OF RESULTS

There is decisive evidence that SASP, at concentrations up to 100μg/ml (0.25mM/l), exerted a profound, dose-related inhibition of lymphocyte proliferation, in response to optimal concentrations of PHA, Con A, PWM, and LPS, in mouse splenocyte cultures. SP, however, was shown to be only mildly suppressive, at doses of 75μg/ml or greater, whilst the effect of 5-ASA at this concentration range, was to elevate slightly, the degree of proliferation. Furthermore, relative Ig production by PWM-stimulated mouse spleen cells, was dose-dependently reduced when SASP was included in cultures; equivalent concentrations of SP and 5-ASA were ineffective. Thus, both T- and B-lymphocytes are subject to the effect of SASP.

It was also demonstrated that SASP exerted a similar suppressive influence on human PBL proliferation, in response to PHA. A comparison of the effect of SASP on human, and murine, PHA responses, revealed that mouse splenocytes appeared to be approximately 4-10 times more susceptible, than were human PBL, to this drug.

Using a defined mouse model system of Con A-dependent lymphocyte proliferation, it was shown that SASP-mediated suppression could not be ascribed to a cytotoxic effect, since, after 72 hours of culture with Con A and maximal doses of SASP, no appreciable difference in cell viability was observed, between test and control cultures. However, there was a small, but notable, decline in percentage
viability, amongst cells exposed to SASP in the absence of mitogen, which may imply that Con A affords a degree of cyto-protection against SASP. It was also shown that the suppressive action of SASP, was inconsequential to a concerted effect of its metabolites, SP and 5-ASA; the SASP molecule in its entirety was responsible for inducing suppression. The possibility that SASP mediated its effect by inactivating the Con A molecule, could also be excluded with reasonable certainty. It can therefore be confidently stated, that the in vitro suppression attributed to SASP, is non-artefactual, and may therefore be germane to the in vivo situation in RA.

Under optimal culture conditions, supra-physiological doses of SASP were required for inhibition of mitogenesis. However, the degree of suppression of Con A-induced mouse splenocyte proliferation, was influenced by subtle changes in culture conditions, notably serum levels, Con A concentration, and cell numbers; in fact, it was possible to nullify the inhibitory effect of SASP, by the inclusion of excessive doses of FCS, or large numbers of cells, in Con A cultures. Furthermore, there was consistent evidence suggesting that the suppression attributed to SASP, could be totally averted, by washing the drug free from Con A cultures, after as lengthy an interval as 24 hours of incubation.

Temporal studies using the murine Con A model indicated that SASP had to be present at the outset of culture, in order to exert its full suppressive capacity, and that it was highly probable, that the drug acted at a fairly early stage of the lymphocyte activation process. Studies using
fluoresceinated Con A almost certainly invalidated the concept that SASP may have bound to the cell surface, in such a manner as to hinder the appropriate interaction between the Con A molecule and its cellular receptor. Both SASP-treated, and control, cells, exhibited typical ring staining and capping, the extent of which were unaltered either qualitatively or quantitatively, even when very high doses of SASP were tested.

It was shown that partial removal of macrophages from human PBMNC, prior to culture with mitogen and SASP, resulted in no further suppression. Contiguous experiments indicated that culture of mouse peritoneal macrophages, with 100μg/ml SASP for 72 hours, did not impede their ability to generate peroxide, upon stimulation with phorbol ester. These data would seem to imply that SASP operates primarily on lymphocytes, rather than on accessory cells.

Prospective studies of plausible mechanisms, whereby SASP mediated its suppression of lymphocyte activation in vitro, revealed that the inhibitory effects of the drug, could not be overcome by the addition of folate or IL-2, to SASP-treated Con A cultures. Furthermore, it was shown that SASP appeared not to act as a PGE₁ antagonist; nor did it exert a synergistic effect with PGE₁, since murine splenocytes, when co-cultured with suppressive doses of the PGE₁ analogue, misoprostol, plus modest doses of SASP, resulted in a decrease in CPM, consistent with that expected if the two agents were acting independently. Interestingly, the NSAID, indomethacin, albeit within a narrow concentration range, was able to accomplish a minor degree of reversal, of SASP-induced suppression.
The murine Con A model served as a basis for studying the relative suppressibility of various 5-ASA-related compounds. There was clear evidence that none of the 5-ASA azo-linked derivatives tested, was as potently suppressive as SASP. In molecular terms, olsalazine was consistently found to be about fivefold less potent than SASP. Both ipsalazine and balsalazine, which differ chemically by a single methylene group (MW = 14), were equi-suppressive, but significantly less so than olsalazine. The 5-ASA derivatives azo-linked to benzene sulfonic acid, and to 4-amino benzoic acid (i.e.: 5-ASA-N=N-BSA and 5-ASA-N=N-4-ABA respectively), induced equal suppression, and were the weakest inhibitors of lymphocyte proliferation, amongst the group.

Comparison of the relative suppressibility of olsalazine (5-ASA-N=N-5-ASA) with its isomers, 4-ASA-N=N-4-ASA and 4-ASA-N=N-5-ASA, yielded intriguing results. It was demonstrated that both of the 4-ASA-containing compounds elicited markedly less suppression, than did olsalazine. The effect of inclusion of either of the separate moieties, 5-ASA, or 4-ASA, at concentrations below 0.5mg/ml, in Con A cultures, was to increase the degree of proliferation above control values; at higher levels of these agents, suppression occurred. Similar findings were observed with 3-ASA. These data substantiate the requirement for the intact, azo-linked molecule, in order to induce maximal suppression.

A collaborative study of RA patients undergoing SASP therapy, revealed that, after 12 weeks of treatment, a beneficial effect occurred in most patients, as envisaged
by changes in clinical, and laboratory, indices of inflammation. Serum levels of SASP, SP (free and acetylated) and 5-ASA (free and acetylated) were similar to those quoted in several publications (Das et al, 1973c; Pullar et al, 1985a; Schroder & Campbell, 1972), and were much lower than levels required to suppress PHA-induced PBL transformation \textit{in vitro}. Furthermore, there was a dissociation between serum levels of SASP and its metabolites, and clinical efficacy, a finding which is endorsed by the work of others (Das et al, 1973c; Pullar et al, 1985a). No significant alterations in absolute lymphocyte count, or numbers of cells bearing CD3, CD4, CD8, CD24, or DR, antigens, were noted at 12 weeks. Likewise, there was no effect on the numbers of cells bearing kappa or lambda light chains.

Amongst those patients who improved clinically, a significant reduction in SASP-induced (but not SP-induced), suppressibility of their PBL responses to PHA \textit{in vitro}, was apparent at 12 weeks, as compared with that observed immediately prior to treatment. This trend was notably absent, amongst RA patients whose disease failed to ameliorate upon SASP therapy. Although not statistically significant, non-responders' PBL, as contrasted with PBL of responders, were seemingly less sensitive to SASP-mediated suppression, prior to treatment. There was found not to be an inverse relationship, between control CPM values and suppressibility attained, as might be anticipated by analogy with the murine Con A model. Hence, these findings are interpreted as representing a qualitative lymphocyte change, due either to reduced disease activity, or mediated
specifically by SASP \textit{in vivo}.

In this latter context, an attempt was made, to study the effect of similar doses of SASP on PHA-induced proliferation of PBL obtained from RA subjects, immediately before, and after 12 weeks of, d-PEN treatment. Although the sample material was small, the effect of therapy appeared to produce a trend towards decreased PBL suppressibility, in the majority of patients studied (7/10), irrespective of clinical responsiveness (in fact, a time related diminution in susceptibility to SASP, occurred in 3/3 non-responders' cells). However, this trend was found to fall marginally short of statistical significance at the 5\% level. Comparison of the degree of suppression attained at 0 and 12 weeks of d-PEN therapy, amongst the responder group alone, clearly indicated no significant change.

4/2 DISCUSSION OF IN VITRO FINDINGS

4/2/1 DISCUSSION OF SASP-INDUCED SUPPRESSION

The initial results, demonstrating SASP-induced suppression of mitogen responses, have generally been substantiated by the work of others. SASP-mediated inhibition of lymphocyte proliferation \textit{in vitro}, was first reported by Ali et al, (1982), who investigated the effects of this drug and its moieties on PHA responses, mounted by guinea pig lymph node cells and human PBL. This group obtained total inhibition of PHA-induced, tritiated thymidine incorporation, for both cell types, at 100\mu g/ml
SASP; a modicum of suppression of normal human PBL responsiveness, was claimed at physiological levels of SASP. They were unable to demonstrate suppression with SP and 5-ASA at a similar dose range (Ali et al, 1982). The findings reported herein are largely in accordance with theirs, although in this study, human PBL were rather less susceptible to SASP.

A recent publication, confirming several aspects of this work, indicated that significant inhibition of tritiated thymidine incorporation, by normal human PBL in response to Con A, PHA and PWM, could be elicited by SASP at a minimal concentration of 100μg/ml, without cellular toxicity. SP at the same concentration, suppressed the response to PHA, by up to 15% (Comer & Jasin, 1988). These workers also reported that PWM-induced, IgG, and IgM, synthesis, was dose-dependently reduced by SASP, maximal inhibition occurring at 100μg/ml. SP and 5-ASA were ineffective when added separately, and in concert (Comer & Jasin, 1988). The work of Fujiwara et al, (1990), previously alluded to, supports the results herein, inasmuch that: (a) the concentration of SASP required to effectively suppress murine splenocyte responses to T-lymphocyte dependent antigens (0.2mM/l), and (b) the specificity for the intact molecule as distinct from its components, in order to attain suppression, were both in agreement.

These studies intimated that SASP inhibited both T- and B-cell responses to mitogens. In support of a B-cell effect, it has been shown in our laboratory, that SASP depressed antibody formation in response to bacterial
Antigens. Relative Ig production by mouse splenocytes, when incubated for 4-7 days with heat-killed preparations of *Salmonella hadar* and *Klebsiella pneumoniae*, was strongly inhibited by the continuous presence of 50μg/ml SASP in culture; SP was found to exhibit a minor degree of inhibition (unpublished data).

The afore-mentioned studies have all indicated that SP and 5-ASA played only a minor role, if any, vis à vis depression of lymphocyte activation by mitogens and antigens. However, it has been claimed that SP, 5-hydroxy SP and 5-ASA, but not the parent SASP, suppressed rheumatoid PBL responses to Con A (Symmons et al, 1988). Furthermore, PHA responses of PBL from RA patients were significantly lowered by 5-ASA alone. PWM-induced transformation of RA, and normal control, PBL, was shown to be inhibited by SP and 5-ASA, whereas SASP and N-acetyl SP only suppressed PWM responses mounted by healthy control PBL. 5-ASA was found to be substantially toxic to non-activated normal control PBL, but not to PBL from active RA subjects. (Symmons et al, 1988). These findings, where relevant, would appear grossly discordant with those reported herein. However, it should be noted that the drug doses used by these researchers, were intended to approximate to the uppermost therapeutic serum levels attained (i.e, SASP @ 50μg/ml; SP @ 100μg/ml; 5-ASA @ 2μg/ml), and that the extent of suppression appeared not to exceed 50%, for any of the drugs tested (Symmons et al, 1988). The present results indicated that a small proportion of resting cells were killed by low doses of SASP (25μg/ml), although Con A-treated lymphocytes were
resilient to SASP levels as high as 100μg/ml. Viabilities of quiescent cells, cultured with 5-ASA, were not tested. However, CPM values of PWM-treated cultures were slightly enhanced by low 5-ASA concentrations.

In the context of suppression of mitogen-induced responses by the metabolites of SASP, it was suggested in one publication, that SP was the active portion of the SASP molecule, since equimolar concentrations of either SASP or SP, effectively inhibited the cytotoxic action of PHA-treated, normal PBL upon Chang (human liver) cells (Holm & Perlmann, 1968). In this system, complete suppression was obtained after 24 hours of incubation with 1mM/l drug (i.e., SASP @ 400μg/ml; SP @ 250μg/ml). Inhibition of cell-mediated cytotoxicity was correlated with lymphocyte killing by SASP or SP, whilst the Chang cells were resistant to both drugs under these conditions (Holm & Perlmann, 1968). Viabilities of PHA-treated, normal and rheumatoid, PBL, exposed to SASP concentrations of up to 1mM/l, were not systematically determined in the present study. By comparison with the murine data, conclusively indicating that SASP-induced suppression was inconsequential to cytotoxicity, it would appear highly improbable, that SASP-induced cell death was selectively manifested in different species. Furthermore, the studies performed by Holm and Perlmann (1968) necessitated the presence of allogeneic cells in PHA cultures, thereby generating cytotoxic T-cells (predominantly of the CD8+ phenotype), from a pool of non-lytic precursor cells. It is known that CD8-bearing lymphocytes are especially sensitive to free radical activity (Allan et al, 1986). It
would not, therefore, be inconceivable, that T-cells of the CD8+ lineage were selectively killed by the high doses of SASP and SP, employed by these researchers.

Human PBL, as opposed to murine splenocytes, were markedly more resilient to the suppressive effect of SASP on PHA responsiveness. These findings should be viewed cautiously, before concluding that they reflect an inter-species variation in lymphocyte behaviour, since there were essential differences between the methodologies employed, for the two cell types. Chiefly, mouse splenocytes were subjected to mechanical traumata (shear forces) during their preparation. This factor may have been at least partly responsible for rendering murine cells more susceptible to the suppressive action of SASP.

It was shown in this study, that slight alterations in experimental conditions, viz: mitogen concentrations, serum levels, and cell numbers, were able to influence the degree of SASP-induced suppression of the murine Con A response. There have been no reports of similar investigations. A prospective attempt to obtain suppression by SASP, at therapeutic levels, proved successful under conditions of sub-optimal stimulation. The existence of an inverse linear relationship, between the degree of cellular incorporation of tritiated thymidine, and suppression elicited by 25μg/ml SASP, could suggest that, under conditions of lowered cellular proliferation, SASP-induced suppression is attainable at physiological concentrations. Whether this phenomenon can be achieved with the metabolites of SASP, is conjectural, since studies of the effects of sub-optimal mitogen concentrations, were restricted to SASP. Con A
stimulated mouse splenocyte cultures containing low cell numbers, or being devoid of FCS, were also subject to a greater degree of suppression at 50μg/ml SASP. If SASP concentrations approximating to mean therapeutic levels, were to be similarly studied, it would not be unrealistic to envisage the occurrence of significant SASP-induced suppression.

It may be argued that stimulation with sub-optimal Con A concentrations, reduces the number of lymphocytes entering S phase, as would be the case when the initial cell number is low. If so, this would imply that the number of proliferating cells, determines the extent of SASP-mediated suppression. However, it should be noted that, when supra-optimal cell numbers were present in culture, a corresponding linear decrease in suppression occurred, despite a concomitant decline in tritiated thymidine incorporation. The latter feature might be indicative of rising concentrations of suppressor factors secreted, as the number of cells was increased.

The capacity of excess FCS to confer cyto-protection against the suppressive effect of SASP, seems very unlikely to be due simply to an enhancement of lymphocyte proliferation, since: (a) background CPM were consistently low, even when FCS concentrations reached 25%, (b) there was a steady reduction in control CPM, with increased serum levels, and (c) at equal control CPM values (presumably suggesting equivalent numbers of cells undergoing proliferation), the extent of SASP-induced suppression was significantly greater, in Con A cultures lacking FCS, as compared with those containing 10% FCS. Thus, the ability
of high FCS concentrations to abrogate SASP-mediated suppression efficiently, would therefore implicate an effect attributable to the serum. By increasing the FCS concentration, the quantity of protein per culture is accordingly raised. Excessive protein may effectually saturate, or avidly bind, SASP molecules, thereby serving to prevent the SASP from cellular contact.

Attempts were made to probe the suppressive effect of SASP further. Although the mode(s) of action of the drug in vitro, was/were not elucidated, several plausible mechanisms were excluded, albeit with varying degrees of certitude. It was shown that the addition of folic acid, even at high concentrations, failed to reverse SASP-induced suppression, suggesting that SASP does not act as a folate antagonist in this system. However, it might appear that addition of the tetrahydrofolate derivative (THF), would be more expedient, since it has been demonstrated that THF partially reversed the suppressive action of SASP, on THF-dependent metabolic pathways, in intact lymphocytes (Baum et al., 1981).

Regarding a possible anti-PG role for SASP, it was shown that the drug has no antagonistic effect on the PGE₁ analogue, misoprostol. A minor reversal of SASP-mediated (but not SP-mediated) suppression, was observed with low doses of indomethacin, which might suggest that SASP exerts a slight up-regulatory effect on other PGs, which are both synthesized by activated lymphocytes, and are inhibitory to their proliferation. These include PGs of the A and F series, in addition to PGE₂ (Goodwin et al., 1977).

Using two methodologies, it would appear that SASP does
not influence macrophage function. Although the macrophage depletion studies may be open to criticism, on the grounds that the extent of depletion was only partial, it must be stated that preliminary experiments indicated that, mitogen-induced lymphocyte proliferation fell sharply upon extensive accessory cell depletion, thereby imposing considerable limitations on this methodology. However, several similar experiments with suitable controls, were performed, in which mouse splenocyte populations were more exhaustively depleted of macrophages, and cultured with Con A, plus doses of SASP not exceeding 100μg/ml. Results indicated no substantial difference in suppressibility by SASP, between control, and carbonyl-iron treated, cells, although it was impossible to assess accurately the efficacy of macrophage depletion, by FACS analysis, due to the lack of availability of fluoresceinated anti-mouse monoclonal antibodies.

One aspect of macrophage function, i.e., peroxide production, was unimpaired by continuous and prolonged, prior exposure to 100μg/ml SASP. This would imply that macrophage viability is retained in the presence of SASP. Taking the data from both sets of experiments into account, it would seem unlikely, but certainly not impossible, that SASP exerts its suppressive action via macrophages, perhaps by down-regulating IL-1 production. Very recently, it was reported that mouse thymocyte proliferation, upon co-stimulation with PHA plus supernatants derived from, human monocytes pre-incubated with or without SASP plus E.coli endotoxin, was reduced when SASP was present in the pre-incubation mixture. The observed inhibition was
interpreted as being caused by a depressive effect, mainly upon IL-1 activity, and possibly on other co-stimulatory factors such as IL-6 (Remvig & Andersen, 1990). No suppression was obtained with equimolar SP, 5-ASA or N-acetyl 5-ASA. Nevertheless, it must be stressed, that the dose of SASP required for 50% suppression using this assay, was inordinately high (2mM/l; 800μg/ml), although there was no apparent toxicity to the monocytes, a finding which would appear compatible with the results reported herein, regarding peroxide generation.

Interference with the binding of Con A to its cell surface receptor, can almost certainly be excluded as a prospective mechanism of action of SASP. Furthermore, cap formation, ensuant upon the redistribution of Con A receptors on the cell surface, by lateral movement in the plane of the cell membrane, was uninhibited by SASP. This finding would tend to suggest that SASP does not mediate its suppression at the cytoskeletal level, since sequential cell surface events, following Con A binding, and culminating in cap formation, are acknowledged to be subject to control by microtubular proteins (Unanue & Karnovsky, 1974; Schechter, 1980). The fact that the suppressive effect of SASP could be completely abolished, by washing the drug free from the cells, after 24 hours, would tend to substantiate these results.

Temporal studies excluded the possibility that SASP exerted a pronounced inhibition of the cellular uptake of tritiated thymidine, since the addition of SASP after 46 hours of culture (2 hours prior to tritiation), resulted in comparatively less suppression, than when SASP was added to
cultures at earlier times. The most interesting aspect of these studies, however, was the finding of equivalent suppression, when the drug was added at 4 or 24 hours, following the initiation of culture. The fact that the amount of suppression elicited, when SASP was added at 4 or 24 hours, was considerably less than that evoked when SASP was present from the start, could suggest that SASP inhibits a cellular event, which begins during the first 4 hours or so, of lymphocyte stimulation with Con A. Mechanisms underlying the early stages of T-cell activation by mitogens, have been partially delineated. Interaction of mitogen with its cell membrane receptor, induces an influx of Ca++ (Mills et al, 1985), with consequent activation of protein kinase C, and resultant IL-2 synthesis and secretion (Depper et al, 1984). IL-2 receptors (IL-2Rs) are first detected within 4-6 hours of lymphocyte stimulation with PHA (Depper et al, 1984); optimal expression of IL-2Rs is dependent on the presence of exogenous IL-2 (Depper et al, 1985). The findings reported herein, however, revealed that the addition of IL-2 to SASP-containing, Con A treated cultures, did not reverse the suppressive effect of SASP to any extent, irrespective of the dose of IL-2 added, and the degree of cell proliferation achieved. This result conflicts with that of Fujiwara et al, (1990), who demonstrated that the inhibition of T-cell mediated antibody responses by SASP, was accompanied by a depression of IL-2 production.
Several prospective modes of action of SASP were investigated using the murine Con A model, some of which have been eliminated as realistic possibilities. At this stage, hypothetical mechanisms, based on present knowledge of the cellular events underlying lymphocyte activation pathways, combined with deductions drawn from these investigations, require formulation. Since this study has revealed that similar concentrations of SASP, were inhibitory to mitogen-induced, T- and B-lymphocyte proliferation, a tenable mode of action for SASP in vitro, should encompass both cell types.

An important clue is furnished, by the finding of total restoration of lymphocyte proliferation, upon washing the drug free from cultures, after 24 hours of incubation. Reversal of SASP-mediated suppression of PHA-induced RNA synthesis, by removal of the drug, has been demonstrated by others, albeit after only 3 hours incubation (Holm & Perlmann, 1968). The interpretation placed upon the present results, is that SASP does not arrest the lymphocytes in the G1 phase of the cell cycle. It seems unlikely that the drug acts as a metabolic inhibitor, in the sequence of events which constitute lymphocyte activation by Con A, since SASP-treated cells were committed to DNA synthesis upon removal of the drug; thus, it is postulated that the lymphocytes are successfully driven into G1, but are unable to progress towards S phase, because SASP (hypothetically) inhibits an essential activation signal, by which the transition from G1 to S phase is mediated.
There is now an accumulation of evidence, suggesting that IL-2 plays a significant role in mediating T-lymphocyte proliferation (Depper et al, 1983; Depper et al, 1984). The capacity of IL-2 to drive activated T-cells into S phase, is dependent on three crucial factors, viz: (a) the concentration of exogenous IL-2, (b) the density of IL-2Rs per cell, and (c) the period of time available for the exogenous IL-2 to interact with the cells. Thus, the control of proliferation by IL-2, is subject to a critical, cumulative number of IL-2Rs being acquired by individual cells. There is evidence indicating that peak levels of IL-2Rs per cell, are realized at 48-72 hours after PHA activation (Depper et al, 1984). Furthermore, the expression of IL-2Rs is a process which depends on de novo RNA and protein synthesis (Depper et al, 1984). Blockade of human IL-2Rs, by means of the murine monoclonal antibody, anti-Tac, has been shown to prevent lymphocyte entry into S phase (Depper et al, 1983). It is proposed that one feasible, general mechanism by which SASP mediates its suppression in vitro, is via interference with the ability of IL-2 molecules to effectively interact with IL-2Rs.

This hypothesis is consistent with several features of SASP-induced suppression, which arose from this work. Firstly, it could explain the failure to overcome the inhibitory effect of SASP, by addition of exogenous IL-2, even at very high doses. Secondly, it furnishes an adequate explanation of the washing experiments and kinetic studies. Thirdly, it is compatible with the inability of SASP to affect accessory cell function, e.g., by down-regulating IL-1 production, since, if this were the case, the addition
of IL-2 would be expected to effect at least a partial reversal of SASP-mediated suppression. Fourthly, it could offer a satisfactory explanation of the fact that, the addition of SASP at either 4, or 24, hours after the outset of culture, resulted in equivalent suppression. Finally, this postulate would appear consistent with the finding of equi-suppression of T- and B-cell responses by SASP, since fairly recent evidence suggests that IL-2 receptors can be expressed on B-lymphocytes (Robb et al, 1984), and that B-cells can respond to exogenous IL-2 (secreted by T-cells), by increased Ig secretion (Waldmann et al, 1984).

The precise nature by which SASP could interfere with the binding of IL-2 to IL-2Rs, can only be speculated upon. The IL-2R consists of a 55kD single glycopeptide, which traverses the cell membrane, and includes a short cytoplasmic segment (Leonard et al, 1984). It is possible that SASP binds to a critical region of the extra-cellular peptide component of the IL-2R. The high affinity of SASP for proteins, is known to be associated with the para(4)-aminophenolic structure of the 5-ASA moiety (Cirstea et al, 1983). 4-aminophenol has been shown to form spontaneous protein conjugates (Cirstea et al, 1980). Thus, the 5-ASA moiety could serve to anchor the SASP molecule to a crucial binding site, present on the IL-2R, leaving the SP portion free to react accordingly, the net result being interference with the binding of IL-2 to its receptor.

The general hypothesis formulated, could also incorporate the possibility that SASP blocks the expression of IL-2Rs, perhaps by impeding the necessary de novo
protein/RNA synthesis. Another proposition is that SASP binds to exogenous IL-2, thereby inducing a conformational change, which frustrates its recognition by the IL-2R. However, if this is the case, it is conceivable that the presence of high concentrations of IL-2 in SASP-containing cultures, would to some extent overcome the inhibitory effect.

Another plausible, but perhaps less likely, general mechanism, by which SASP exerts its in vitro inhibition, is by blockage of the appropriate interaction between transferrin molecules and their receptors (TfRs). Following PHA activation of T-cells, TfRs are induced after expression of IL-2Rs, immediately prior to S phase (Neckers & Cossman, 1983). Blockade of TfR expression is known to prevent mitogen-activated T-cells from undergoing entry into S phase (Neckers & Cossman, 1983). Studies by Mendelsohn et al. (1983), have demonstrated that an anti-human TfR monoclonal antibody, which specifically blocks the binding of transferrin, also suppresses human lymphocyte proliferation in response to PHA. This inhibition was shown to be reversible, by removal of the antibody, after up to 48 hours of exposure (Mendelsohn et al., 1983). It is conceivable that SASP exhibits a similar, direct action on TfRs, in the murine Con A system employed herein. However, a possible difficulty arises from the fact that TfR expression, and therefore lymphocyte proliferation, can be enhanced by the addition of IL-2, to Con A cultures (Pelosi-Testa et al., 1988).
Thus, two essential modes of action have been propounded, to explain the phenomenon of SASP-mediated suppression in vitro. Both mechanisms could entail the binding of SASP to cell membrane-associated proteins, since SASP displays high-affinity protein binding at alkaline pH (Cirstea et al, 1983). The relative quantity of suppression elicited by the various 5-ASA azo derivatives tested, might reflect differences in binding affinity, granted that these agents share a common receptor site. Although SASP was by far the most suppressive, it is interesting to note that olsalazine (5-ASA-N=N-5-ASA) was more potent than the remaining four compounds, and only five times less effective than SASP. Moreover, olsalazine was significantly more suppressive than its isomer 4-ASA-N=N-4-ASA. Possession of the 5-ASA (para-aminophenol) component, as distinct from the 4-ASA (ortho-aminophenol) group, could serve to increase the protein binding affinity of the relevant compound, thereby elevating its suppressive potency. This could explain why olsalazine (comprising two 5-ASA moieties) is considerably more potent than its 4-ASA-containing isomers. The differential suppression induced by the various 5-ASA azo derivatives, might reflect differences in the protein binding affinities of the carrier molecules. The fact that the compound 5-ASA-N=N-BSA, in which the carrier is benzene sulfonic acid (essentially SASP without the amino-pyridine group), was the weakest suppressant among the compounds tested, emphasizes the requirement for the pyridine ring, linked to
the sulfonamide component, i.e., the entire SP moiety, for obtaining potent suppression characteristic of SASP. However, with the exception of SASP, all the remaining 5-ASA azo compounds tested, possess only two aromatic ring structures. It might prove worthwhile to investigate the effects on lymphocyte proliferation, of 5-ASA azo-linked derivatives which more closely resemble SASP. The SP residue comprises a basic component (the pyridine ring), attached to a hydrophobic moiety (the sulfonamide ring). It may appear that the presence of a base, in conjunction with a hydrophobic residue, is a fundamental requirement for the (hypothetical) effect of SASP, assuming that the drug binds to its apposite site, via the 5-ASA constituent.

Under cell-free conditions, SASP has been shown to exert competitive inhibition, with respect to the substrate, dihydrofolate, for the active site of the enzyme, dihydrofolate reductase (Selhub et al, 1978). This folate receptor site possesses an ionizable region, a hydrophobic region, and a weakly acidic region (Blakley, 1969); it has been argued that SASP can bind to the folate receptor, since (a) the 5-ASA moiety is potentially interactive with the ionizable region, (b) the sulfonated benzene component can be recognized by the hydrophobic region, and (c) the basic nitrogen of the pyridine ring, can become neutralized by interaction with the weakly acidic region (Selhub et al, 1978). It is conceivable that SASP (at least initially) interacts in a similar manner, with cell membrane-associated structures.

5-ASA, and its isomers, 4-ASA and 3-ASA, displayed an enhancement of lymphocyte proliferation, at the lower dose
range employed, and were suppressive at higher concentrations. Moreover, the dose-response curves were similar for all three isomers. These data would suggest that the increased proliferation observed, was not due to the formation of immunogenic complexes with serum proteins, as might be anticipated with 5-ASA, in view of its para-aminophenolic structure (Cirstea et al, 1983). A more likely explanation of these findings, is that PG synthesis is depressed, perhaps by excessive scavenging of intermediate hydroperoxy-PGs needed for arachidonate oxidation, as pre-mentioned (Hoult, 1986).

The decline in CPM, seen at concentrations exceeding 600μg/ml (> 4mM/l), might be caused by a reduction in the rate of α-(methylamino)-isobutyric acid uptake, by the "A" transport system, a cellular event which is induced by mitogen, and which is known to be inhibited by various NSAIDs, including salicylates (Seng & Bayer, 1986). It has also been demonstrated that indomethacin, which at low concentrations, enhances PHA induced lymphocyte proliferation, is inhibitory at higher levels (Seng et al, 1987). Such inhibition was shown to be due to arrested cell growth in G1; this phenomenon was interpreted as being secondary to the inhibition of amino acid transport (Bayer et al, 1979). It must be stressed that, in the murine Con A system used in the present study, both increased proliferation and suppression were induced by 5-ASA, at concentrations very far in excess of physiological levels.
4/3 DISCUSSION OF RA STUDIES

The fact that, after 12 weeks of treatment with SASP, the absolute numbers of, PBL, and various lymphoid sub-populations, remained constant, would suggest that SASP did not exert any quantitative effects on circulating lymphocytes, in RA. These findings are accordant with those of others, who also noted a significant decline in IgM and RF, titers, as well as a reduction in circulating low density lymphocytes, and a trend towards decreased refractoriness of PBL to Con A, upon SASP therapy (Symmons et al, 1988). By utilizing SASP-induced suppression of PBL proliferation, in response to Con A in vitro, our work indicated that SASP appeared to influence lymphocyte behaviour, in parallel with clinical responsiveness. Interestingly, the degree of PHA-induced lymphocyte proliferation, did not vary significantly with therapy, and was not substantially different, between responders and non-responders. This observation is difficult to equate with the findings of Symmons et al, (1988). It is notable, however, that in a series of unpublished studies, in which RA patients’ PBL responses to various mitogens, were recorded prior to, and at subsequent times after, the initiation of SASP therapy, Con A-induced responses were consistently lower than those elicited by PHA.

Although SASP therapy influenced clinical responders’ lymphocyte susceptibility to the drug in vitro, the finding that no such effect was observed with SP, would implicate the parent molecule alone as a modulator of this particular aspect of lymphocyte behaviour, despite the fact that
serum SP levels (even after excluding acetyl-SP levels), observed in patients undergoing SASP therapy, were considerably higher than those of SASP. The question as to whether the reduction of in vitro suppressibility, ensuant upon SASP therapy, was specifically mediated by SASP, or was the resultant of clinical amelioration, wrought by other means, remains unresolved. In either case, a hypothetical explanation of this phenomenon is warranted.

Increased membrane viscosity of circulating lymphocytes, as previously referred to, characterizes active RA. Membrane damage is associated with lipid peroxidation, since increased levels of free radical oxidation products, have been detected in rheumatoid, sera and synovial fluids (Lunec et al, 1981). It has been shown that membrane fluidity plays an important role in regulating cellular functions, such as the conformation of membrane-bound enzymes (Lenaz et al, 1983), diffusion (Edidin, 1974), and the degree of receptor protein exposure (Borochov & Shinitzky, 1976). It is therefore postulated that: the damage inflicted upon lymphocyte membranes, as occurs in active RA, renders affected cells more liable to SASP-induced suppressibility. SASP, SP and 5-ASA are all known to act as free radical scavengers in vitro, at therapeutic concentrations (Miyachi et al, 1987). SASP also chelates ferrous ions, thereby indirectly helping to reduce serum and synovial levels of reactive oxygen species, since Fe++ catalyzes the production of the (extremely toxic), hydroxyl radical, from hydrogen peroxide. Hence, the temporal effect of therapy with SASP, could permit restoration of lymphocyte membrane function. Thus,
lymphocyte susceptibility would revert to normal, as was apparent in the case of clinical responders. It is not easy to interpret the inability of non-responders' PBL, to exhibit this feature. However, it appeared from the study, that PBL from non-responders, were generally less suppressible than those of responders, before SASP treatment. On the basis of the hypothesis formulated, it might seem that lymphocyte membranes of non-responders to SASP, are initially less damaged. It should also be mentioned, that patients' PBL cultures were set up with autologous serum, simultaneously obtained. Hence, the extent of SASP-induced suppressibility, could, at least partly, reflect levels of reactive oxygen radicals present in the supporting sera.

Lymphocytes of patients treated with d-PEN, generally became less sensitive to SASP \textit{in vitro}, after 12 weeks, irrespective of clinical amelioration. d-PEN (3,3-dimethyl cysteine), by virtue of its sulfhydryl (thiol) group, is able to chelate various metal ions, especially copper (I) (Rudge & Perrett, 1988). Like iron, raised levels of free copper, have been detected in rheumatoid synovial fluids and sera (Milanino et al, 1985). d-PEN may exercise constraints on free radical activity \textit{in vivo}, by formation of copper complexes. It might therefore be anticipated, that successful RA treatment with d-PEN, as with SASP, would produce a likeness in circulating lymphocyte behaviour.
The results presented herein afford possible clues indicating: (a) the relevant therapeutic moiety, (b) the site, and (c) the mechanism of action, of SASP on rheumatoid lymphocytes in vivo. There is irrefragable evidence to suggest that, under optimal culture conditions, the suppression observed is mediated almost entirely by the parent compound. In molecular terms, SASP is at least thirty-fold more potent than either component, SP or 5-ASA. In respect of SP, there is an apparent dichotomy between these findings, and the acknowledged, but erroneous, belief that SP is necessarily the "active therapeutic ingredient of the SASP molecule in RA". The work of Pullar et al. (1985b), already referred to, initially made this claim, since RA patients responded equally well to similar doses of either SASP or SP. This deduction is open to criticism on the grounds that: ingested SASP remains intact until it undergoes reductive cleavage in the colon, whereas oral intake of SP creates a different situation, in that it is readily absorbed from the ileum. Thus, serum and synovial levels of SP attained, would be considerably higher than those achieved, upon administration of the parent SASP. It is illogical to disregard the possibility of an effect mediated by the entire SASP molecule.

This work did ascribe a minor degree of suppression to
SP, which might be expected, since PHA responses have been shown to be depressed by sulphonamides such as dapsone (Beiguelman & Pisani, 1974). The important point is that, even if SP is therapeutically equi-potent as SASP, in RA, it is less well-tolerated than the parent drug. Although this work demonstrated that the SASP molecule in its entirety, is, in all probability, the "active constituent", it must be stressed that all the 5-ASA azo derivatives lacking the SP group, caused less suppression than SASP. This suggests that the possession of the SP moiety, is a paramount requisite for inducing the potent immunosuppression characteristic of SASP.

Likewise, the 5-ASA moiety is essential for the immunosuppressive role played by the intact SASP molecule, although it is hard to envisage the occurrence of substantial anti-PG activity in RA, in view of the relatively low 5-ASA levels acquired in rheumatoid sera and synovial fluids. It would seem more likely that the 5-ASA component helps to anchor the SASP molecule to connective tissue in the synovial milieu, where anti-inflammatory effects may be mediated by free radical scavenging.

It was proposed that SASP exerts its suppression of lymphocyte proliferation in vitro, essentially by interference with either IL-2Rs or TfRs. Both hypotheses are germane to the in vivo scenario of RA, because of the preponderance of circulating activated lymphocytes, of both T- and B-cell lineage, during the the course of disease. Levels of these cells are known to fall, upon successful treatment with other major second line agents, such as d-PEN and gold (Alexander et al, 1984; Pardo & Levinson,
1983; Gul et al, 1984), besides SASP. It has also been argued that all the recognized SAARDS are potentially able to block the drive towards IL-2 production \textit{in vivo} (Bacon and Salmon, 1986). d-PEN complexed with copper (Cu++) has been shown to impede human T-helper cell function (Lipsky & Ziff, 1980). The anti-malarials, chloroquine and hydroxychloroquine, have both been reported to inhibit IL-1 production by macrophages, by interference with lysosomal functions (Mackenzie, 1983). Sodium aurothiomalate dissociates \textit{in vivo}, forming elemental gold, which is phagocytozed by macrophages, and selectively concentrated in the inflamed synovium (Vernon-Roberts et al, 1976). Perhaps most significantly, cyclosporin-A, which specifically interferes with IL-2 generation, by inhibiting its transcription by messenger RNA (Kasaian & Biron, 1990), is efficacious in RA. Upon withdrawal of cyclosporin-A, rheumatoid disease is known to flare rapidly (reviewed by: Bacon & Salmon, 1986).

Thus, by analogy with other SAARDS and immunosuppressive drugs, based on \textit{in vivo} and \textit{in vitro} data, there are realistic grounds for supposing that SASP modulates rheumatoid activated lymphocytes. Granted that SASP acts selectively on activated T- and B-cells, by blockage of IL-2Rs or possibly TfRs, thereby inhibiting lympho-proliferation, the question arises as to the site at which it exerts this particular immunosuppressive effect. One (superficially) problematic aspect of these (and to some extent other workers') studies, is that the levels of SASP required to inhibit lymphocyte proliferation, in response to optimal concentrations of mitogens, \textit{in vitro},
are very much greater than those attained in rheumatoid sera and synovial fluids. However, there are three factors which may help to surmount this disparity. Firstly, although the concentrations of PBL employed in culture with PHA, approximated to those attained in the blood-stream, it must be emphasized that mitogens are *polyclonal activators*, thereby maximizing the proportion of potentially mitotic lymphocytes, present in micro-cultures. In RA, the percentage of circulating activated lymphocytes (i.e., those capable of proliferation), must necessarily be considerably lower, per unit volume. If SASP binds to receptor sites chiefly expressed on activated cells, it is evident that lower drug concentrations are required, when the proportion of potentially mitotic cells is reduced, as was demonstrated *in vitro*. Secondly, activated lymphocytes in RA are subject to membrane damage, due to their encounter with reactive oxygen radicals present in synovial fluids and sera. As previously discussed, traumatized cells might become more susceptible to the effects of SASP (and perhaps SP). Lastly, cultured lymphocytes are densely packed and relatively static. Both these features could serve to restrict their contact with SASP. Cellular aggregation is also facilitated in the presence of lectins such as PHA. Contrastingly, circulating lymphocytes are continuously in a state of flux. Hence, it is not inconceivable that SASP (and probably SP) can exert suppression of lymphocyte proliferation systemically. In fact, a systemic action may be favoured, since SASP binds avidly to connective tissue in the synovium, where its immunomodulatory role may be diverted towards free radical
scavenging. Likewise, the predominance of PMNLs at the site of inflammation, could also serve to detract SASP molecules. Furthermore, although there is an abundance of activated lymphocytes in the synovial milieu, these cells are probably in very close contact, and therefore may be protected from the effects of SASP.

It may, perhaps, be argued that the lack of correlation between serum levels of SASP and individual clinical responsiveness to SASP treatment in RA, as was reported by ourselves, vitiates the argument in favour of a systemic action for SASP. It seems, in my present opinion, a trifle simplistic and presumptuous, to anticipate such a direct relationship, for several reasons. Firstly, it must be stated that all patients received the same dose of SASP (2g/day), irrespective of weight, the magnitude of which was inevitably subject to variation. Secondly, the results of this study pertain to only one possible mechanism of action of SASP (i.e., a down-regulatory effect on activated lymphocyte proliferation). Several alternative mechanisms of action of SASP in vivo have been reviewed here, and it would seem that this drug exerts manifold effects, all of which may be therapeutically relevant, and subject to inter-patient variation. Thirdly, the procedure for monitoring serum drug levels (HPLC) entailed measuring only free, and protein-bound, SASP, as recognized by its intact azo bond. By definition, lymphocyte bound, or internalized, SASP (e.g., SASP located within phagocytozed immune complexes) is necessarily excluded from serum. It was proposed, from the in vitro studies demonstrating that SASP mediated suppression could be overcome by the addition
of high serum concentrations, that SASP molecules are
effectually "saturated" or "masked" in the presence of
excess protein. This may have rheumatological importance,
since active RA is associated with increased circulating
acute phase proteins, and high molecular weight immune
complexes. Thus, it is feasible that SASP may be trapped
within serum proteins, and remain incognito. For these, and
probably other reasons, it might appear that serum levels
of SASP, as measured by HPLC, are not necessarily an
accurate reflection of those attained in the blood-stream.

Indomethacin was shown to effect a minor degree of
reversal, of SASP-mediated suppression in vitro. It is
noteworthy that the concentration range of IM required
(0.4-0.8µg/ml), can be achieved in vivo, following oral
intake of low doses (up to 50mg) of IM. (Swingle & Kvam,
1982). This might have possible clinical implications,
should RA patients be treated with SASP and IM
concurrently. Whether this effect occurs with other NSAIDs,
is speculative.

Finally, regarding its site of action, mention must be
made, of the very high levels of free SASP, which prevail
in the small intestine, following oral administration of
the drug. In view of this observation, it has been
postulated that SASP exerts a local immunosuppressive
influence, on gut-associated lymphoid tissue (GALT)
(Sheldon, 1988). Favouring this argument, it has been
demonstrated using a mouse model, that serum antibody
titers to orally administered Cholera toxin, an immunogen
which acts solely within the gut, are significantly lowered
in animals receiving SASP in their feed, as compared with
placebo-treated mice (Sheldon & Pell, in press). The proposition that SASP operates at this site, must pre-suppose the occurrence of an immunizing event within GALT, which eventuates in arthritis. It may indeed, transpire, that the efficacy of SASP in the reactive arthropathies, reflects inhibition of the expansion of potentially arthritogenic lymphoid clones, by the mechanisms already proposed. However, in the case of RA, gut involvement remains unresolved, and evidently, much research within this hitherto (perhaps undeservedly) neglected area, is necessary, before a true assessment of the site(s) of action of SASP in rheumatoid disease, can be made.

4/4/2 POTENTIAL FOR SASP ANALOGUES IN RA THERAPY?

The present study has established the requisition for the integrity of the SASP molecule, besides the need for both the 5-ASA and SP groups, for its effectiveness in vitro. Based on the assumption that all the 5-ASA azo derivatives tested, suppress lymphocyte proliferation by a common mechanism which is germane to RA, there is clearly very little, if any, potential for most of these compounds as suitable alternatives to SASP, in the management of RA. Olsalazine is a possible exception, being only fivefold less potent than SASP, and advantageous in possessing a considerably longer serum half-life (van Hogezand et al, 1982).

Development of suitable SASP analogues for RA therapy, must ideally entail circumventing the problem of
undesirably high serum levels of SP, as well as increasing the bioavailability of the active compound. In these respects, it is interesting to note that methylsulfasalazine (MeSASP), which differs from SASP, in possessing a methyl group at position 3 of the pyridine ring, has been tested in a preliminary trial of 21 RA patients, with a reasonable degree of success (Astbury et al, 1990). By virtue of the additional methyl group, lipid solubility is increased. Absorption of the parent compound is thereby facilitated, as indicated by an elevation, and corresponding reduction, in, plasma MeSASP and MeSP levels, respectively (Astbury et al, 1990). The effect of MeSASP upon human PBL proliferation elicited by PHA, was studied in our laboratory, and although the results were not conclusive, it appeared that MeSASP was a somewhat less potent suppressant than SASP (unpublished data).

A possible alternative method of increasing the bioavailability of the parent analogue, is by modification of the SASP molecule, in order to prevent its scission by intestinal bacterial enzymes. One possible means of accomplishing this, is by replacing the azo bond with two methylene groups, thus affording a more inert structure. However, this possibility remains speculative, and until the precise mode of action of SASP at the atomic level, is elucidated, the practicality of this type of approach is conjectural.
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APPENDIX: PUBLICATIONS WHICH INCLUDE WORK COVERED HERE

