EFFECTS OF ADJUVANT HONEY THERAPY ON DISEASE ACTIVITY, LYMPHOCYTE FUNCTION AND LYMPHOCYTE SUBSETS IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

By

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بسم الله الرحمن الرحيم

In the name of Allah, the Beneficent, the Merciful

And your Lord inspired the bee: build homes in mountains and trees, and in (*the hives*) they build for you [68].

Then eat from all the fruits, following the design of your Lord, precisely. From their bellies comes a drink of different colors, wherein there is healing for the people. This should be (*sufficient*) proof for people who reflect [69].

DEDICATION

I would like to dedicate my thesis to my wife, Dr.Shaimaa Khalid, and my mother whose support and encouragement throughout the process of writing this thesis is invaluable.

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LIST OF ABBREVIATION

ACA	Anticardiolipin Antibody
ACR	American College of Rheumatology
AIDS	Acquired immune deficiency syndrome
AIMS	Arthritis Impact Measurement Scale
ANA	Anti-nuclear antibody
APC	Antigen presenting factor
APLs	Antiphospholipid Antibodies
ARA	American Rheumatism Association
AZA	Azathioprine
bcl2	B-cell CLL/lymphoma 2
BILAG	British Isles Lupus Assessment Group
CD4	Cluster of differentiation 4
CPM	Cyclophosphamide
CR1	Complement receptors
CRP	C - reactive protein
DC	Dendritic cells
DHEA	Dehydroepiandrosterone
dRVVT	Dilute Russell viper venom time
dsDNA	Double stranded deoxynucleic antibody
ECLAM	European Consensus Lupus Activity Measure
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunoabsorbent assay
EM	Electron microscopy
ESRD	End stage renal disease
ESR	Erythrocyte sedimentation rate
FDC	Follicular dendritic cells
FTA-ABS	Fluorescent treponemal antibody-absorbed
GC	Germinal centres
GVHD	Graft-versus-host disease
GFR	Glomerular filtration rate
GMB	Glomerular basement membrane
HAQ	Health Assessment Questionnaire
HLA	Human leucocytes antigens
IF	Immunofluorescence
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-2	Interleukin 2
IL-6	Interleukin-6
IL-10	Interleukin-10
KCT	Kaolin clotting time
KFSS	Krupp Fatigue Severity Scale
Kg	Kilogram
LAI	Lupus Activity Index
LCA	Leucocyte common antigen

LH	Luteinizing hormone
LM	Light microscopy
LN	Lupus Nephritis
mAbs	Monoclonal antibodies
MHAQ	Modified Health Assessment Questionnaire
MHA-TP	Microhemagglutination-Treponema pallidum
MHC	Major histocompatibility complex
mls	Mililitres
MMF	Mycophenolate mofetil
NIH	National institute of health
NK	Natural killer
NSAIDs	Non steroidal anti-inflammatory drugs
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
Rnp	Ribonucleoprotein
RPR	Rapid plasma reagin
SCID	Severe combined immunodeficiency
SIS	SLE Index Score
SLAM	SLE Activity Measure
SLE	Systemic lupus erythematosus
SLICC/ACR	Systemic Lupus Erythematosus International Collaborating Clinics/American College of Rheumatology
Sm	Smith
snRNP	Small nuclear ribonucleoprotein
SSc	Systemic sclerosis
TBM	Tingible body macrophages
TGFβ	Transforming growth factor β
Th	T helper
TNF	Tumour necrosis factor
UFEME	Urine for full examination and full examination
UV	Ultraviolet
WHO	World health organization
WT	Weigh

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ABSTRACT

Background: SLE is a prototype autoimmune disease which can affect many tissues and organs of the body. Current treatment with anti-inflammatory and immunosuppressive drugs are able to suppress disease activity but associated with significant short term and long term side effects. Honey has been shown to have anti-bacterial and anti-inflammatory properties. However the role of honey in autoimmune diseases has not been elucidated. We studied the effects of adjuvant honey given together with conventional treatment to assess the effect of honey on disease activity and its immunological effects on lymphocyte function and lymphocyte subsets.

Objectives: To compare the effects of adjuvant honey therapy versus conventional therapy alone on disease activity, to determine the effects of adjuvant honey therapy versus conventional therapy alone on lymphocyte functions and lymphocyte subsets in SLE patients.

Methodology: This was a prospective randomized study. Sixty SLE patients were randomized into two equal groups, group 1 receiving conventional treatment with low dose steroid and cyclophosphamide or azathioprim, group 2 receiving same treatment in addition to honey 20 gm twice a day. Disease activity was measured in all patients every 3 months by SLEDAI score, C3, C4, ANA titre, dsDNA antibodies and CRP. The function of T lymphocyte was determined at baseline and after 6 months by measuring the expression of 3 lymphocyte activation markers (HLADR, CD45RO and CD25) using flow cytometer. Enumeration of lymphocyte subsets (CD3, CD4, CD8, CD19, and

CD16/56) was done by Immunofluorescence staining method using flow cytometer at baseline and after 6 months.

Results: SLEDAI score was significantly decreased in honey group compared to non honey group after 3 and 6 months of follow up (p value < 0.001, 0.023 respectively). C3 and C4 showed a significant increase within honey group (p = 0.002, 0.017 respectively). The level of dsDNA antibodies was found to be decreased significantly within honey group (p = 0.05). The level of CRP was found to be decreased significantly within honey group compared to non honey group after 6 months follow up (p = 0.016). For ANA titre we observed that the mean difference between the groups after 3 months and after 6 months follow up was statistically not significant. For the expression of lymphocyte activation markers there was a significant decrease in the expression of CD8CD45RO and CD8CD25 within honey group compared to non honey group after 6 months follow up (p = 0.04, 0.021 respectively). Regarding lymphocyte subsets there was a significant increase in CD3 and CD16/56 percentage in honey group after 6 months follow up compared with non honey group (p = 0.021, 0.038 respectively). A pattern of increment for CD4 and of decrement for CD8 and CD19 was observed in honey group but it was not statistically significant.

Conclusion: The use of honey therapy as adjuvant to conventional treatment in patients with systemic lupus erythematosus (SLE) was found to be useful in suppressing disease activity and enhancing lymphocyte function and lymphocyte subsets.

ABSTRAK

Latar belakang: SLE merupakan prototaip autoimun yang boleh melibatkan banyak tisu dan organ badan. Rawatan terkini menggunakan dadah anti-radang dan dadah penindas imun berkebolehan untuk menindas aktiviti imun tetapi mempunyai kesan sampingan jangkamasa pendek dan jangkamasa panjang yang signifikan. Kami mengkaji tentang kesan penggunaan madu yang diberikan bersama rawatan konvensional terhadap penyakit dan kesan imun dalam fungsi limfosit dan subset limfosit.

Objektif: Untuk membandingkan kesan diantara terapi tambahan madu dan terapi konvensional terhadap aktiviti penyakit berdasarkan skor indeks aktiviti penyakit (SLEDAI), untuk membandingkan kesan diantara terapi tambahan madu dan terapi konvensional dalam fungsi limfosit, dan untuk membandingkan kesan terapi tambahan madu dan terapi konvensional dalam subset limfosit dikalangan pesakit SLE.

Prosedur: Dalam kajian prospektif secara rawak ini, semua pesakit di perolehi dari Hospital Universiti Sains Malaysia (HUSM), samada mereka yang menghadiri klinik pakar atau mereka yang di masukkan ke wad. Enam puluh pesakit SLE dibahagikan sama rata kepada dua kumpulan secara rawak, kumpulan pertama menerima rawatan secara konvensional dengan dos steroid yang rendah dan cyclophosphamide atau azathioprim, kumpulan kedua menerima rawatan yang sama dengan tambahan madu sebanyak 20 gm dua kali sehari. Aktiviti penyakit diukur untuk semua pesakit setiap 3 bulan menggunakan skor SLEDAI, C3, C4, titer ANA, antibody dsDNA dan CRP. Fungsi T-limfosit ditentukan pada permulaan kajian dan selepas 6 bulan (dua kali) dengan cara mengukur ekspresi 3 penanda pengaktifan limfosit (HLADR, CD45RO dan CD25) menggunakan aliran sitometri. Pengiraan subset limfosit (CD3, CD4, CD45, CD19, dan CD16/56) menggunakan kaedah fenotaip imun dengan penggunaan aliran sitometri pada permulaan kajian dan selepas 6 bulan (dua kali).

Keputusan: Skor indeks aktiviti penyakit (SLEDAI) menunjukkan penurunan yang signifikan dalam kumpulan terapi tambahan madu dibandingkan dengan kumpulan terapi bukan madu selepas 3 dan 6 bulan susulan (p value < 0.001, 0.023 masing-masing).

Komplimen C3 dan C4 menunjukkan ada kenaikan yang signifikan dalam kumpulan terapi madu setelah 6 bulan ($p = 0.002$, 0.017 masing-masing). Paras CRP didapati menurun secara signifikan di dalam kumpulan terapi madu dibandingkan dengan kumpulan bukan madu setelah 6 bulan susulan ($p = 0.016$). Juga didapati paras antibodi dsDNA menurun secara signifikan di dalam kumpulan terapi madu ($p = 0.05$).

Untuk ekspresi penanda pengaktifan limfosit (HLADR, CD45RO dan CD25), terdapat penurunan yang signifikan dalam ekspresi CD8CD45RO dalam kumpulan terapi madu setelah 6 bulan susulan ($p = 0.04$). Terdapat penurunan yang signifikan dalam ekspresi CD8CD25 dalam kumpulan terapi madu setelah 6 bulan susulan ($p = 0.021$).

Berkenaan subset limfosit, didapati bahawa wujud kenaikan yang signifikan untuk peratus CD3 dan CD16/56 dalam kumpulan terapi madu setelah 6 bulan jika dibandingkan dengan kumpulan terapi bukan madu ($p = 0.021$, 0.038 masing masing). Corak kenaikan untuk CD4 dan penurunan untuk CD8 dan CD19 dapat dilihat dalam kumpulan terapi madu tetapi tidak signifikan secara statistik.

Kesimpulan: Penggunaan terapi tambahan madu keatas rawatan konvensional dikalangan pesakit sistemik lupus eritmatosus (SLE) didapati bermanfaat di dalam mengurangkan aktiviti penyakit dan menambahbaik fungsi limfosit dan subset limfosit.

CHAPTER ONE

Introduction

1. Systemic Lupus Erythematosus Overview

Systemic lupus erythematosus is an autoimmune disease that can affect various parts of the body, including the skin, joints, heart, lungs, blood, kidneys and brain. Normally the body's immune system makes proteins called antibodies, to protect the body against viruses, bacteria, and other foreign materials.

In an autoimmune disorder like lupus, the immune system cannot differentiate between foreign substances and its own cells and tissues. The immune system then makes antibodies directed against itself. These antibodies called "auto-antibodies" cause inflammation, pain and damage in various parts of the body.

Systemic lupus erythematosus (SLE) is characterized clinically by protean manifestations, most commonly including arthralgia, arthritis, rash, alopecia, oral ulcers, serositis, leukopenia, central nervous system, and renal involvement (Lahita, 2004). It is an important prototype of systemic autoimmunity and it causes significant disability and premature death in those patients who suffer from it (Lahita, 2004). It is occasional life threatening, often life long, autoimmune disease.

1.1. Epidemiology of SLE

SLE was previously believed to be a rare disease. Since 1960s, there was an increase in the awareness and education about lupus. This has helped many more patients to get an accurate diagnosis making it possible to estimate the number of people with lupus.

The overall prevalence is estimated to be about 1 per 1000. A study from Birmingham, UK, found the prevalence to be 27.7/100,000 in the general population, but nearly 9 times higher in Afro-Caribbean females (Johnson, Gordon *et al.,* 1995). Data from a national health survey in the USA found the self-reported prevalence of SLE (defined as having been given a diagnosis of SLE by a physician) to be 241/100,000 (Simard and Costenbader, 2007). Recognizing that this may well be an over-estimate, combining self-reporting with evidence of a current prescription for anti-malarial, corticosteroids, or other immunosuppressive medications reduced this figure to 53.6/100,000 (Simard and Costenbader, 2007).

Studies carried out in England support the notion that Asians are more predisposed to SLE. For example, Asians of Indian, Pakistani and Bangladeshi origins who resided in Leicester, England, were three times more likely to develop SLE than Caucasian whites (Samanta, Roy *et al.,* 1992).

In Central Australia, a higher prevalence of SLE was found in Aborigines than Caucasian whites (1:1360 versus 1:5170) though the Aborigines were not found to have more aggressive disease (Segasothy and Phillips, 2001).

Population based studies carried out in non-Caucasian countries may provide better evidence for ethnic differences in the prevalence of SLE and that certain non- Caucasian groups are more susceptible to develop the condition than Caucasian whites. Data from China, based on a survey that covered a population of 32 668 carried out in 1984–1985, showed the prevalence of SLE was 70.41 per 100 000 persons overall and 113.33 per 100 000 females according to the 1982 ARA criteria for SLE (Chen, Xu *et al.,* 1987). A study

carried out in Hong Kong, where 95% of its population are ethnic Chinese, showed comparable figures (58.8 per 100 000 persons overall and 104 per 100 000 females) (Mok and Lau, 2003).

Overall, evidence does seem to suggest that SLE is more common in American blacks, Hispanics, Afro- Caribbean's, Native North American Indians and Orientals including Asian Indians and Chinese than in Caucasian populations.

1.1.1. Prognosis and survival

Mortality attributed to SLE in the United States in the 1970s and 1980s has shown an unfavorable outcome for African-American patients (Siegel and Lee, 1973; Lopez-Acuna *et al.,* 1982). The annual age adjusted mortality rate was almost three times higher in black females (17.6 per million person-years) than Caucasian white females (6.0 per million person-years) (Lopez-Acuna *et al.,* 1982). Similarly, the adjusted annual rates of death from SLE between 1968 and 1976 were higher for Asians (Chinese, Japanese and Filipinos) (6.8 per million person-years) than Caucasian whites (2.8 per million person-years) (Kaslow RA, 1982). This was confirmed by other studies in England (Samanta A *et al.,* 1991).

The survival of patients with SLE has improved remarkably in the past few decades and five- and 10- years survival rates are now expected in the range of ~95% and ~90% for most patients. This improved survival, however, is typical only of the Western world whereas in countries such as India (Kumar *et al.,* 1992), and amongst black Caribbean patients (Diadyk *et al.,* 1998), poorer survival continues to be recorded. It is not clear whether this observation is related to differences in ethnic origin alone. Out of interest the

study carried out by Walsh and DeChello (Walsh SJ, DeChello LM, 2001) who attempted to identify demographic and environmental factors that affect the incidence and course of SLE by studying geographical variation in mortality from the disease in the United States. Census data from clusters where there were more or fewer SLE deaths than expected were compared. Higher poverty rates and/or greater concentrations of ethnic Hispanics were found to be associated with increased mortality from SLE. Thus, it seems that both ethnicity and socioeconomic status have an impact on SLE survival.

1.1.2. Prevalence and disease expression in different racial and gender groups in Malaysia

Ethnic Chinese had the highest prevalence of SLE compared to other ethnic groups in Malaysia (Wang *et al.,* 1997). There was a high incidence of renal disease in Chinese and Malays patients, 74% of patients had significant proteinuria and half of these had associated nephrotic syndrome. Indian patients had significantly less incidence of skin manifestation compared to other racial groups. No difference in disease expression was detected between the ethnic Chinese and Indians and between the male and female patients. The overall 5 y and 10 y survival rates were 82% and 70% respectively. Indian patients had the poorest survival rates. Survival rates are similar among the Chinese and Malay patients (Wang *et al.,* 1997).

1.2. Aetiology of SLE

Evidence suggests that SLE has a multi-factorial aetiology. Sex hormones, environmental factors, immunological dysfunction, and genetics have all been hypothesized to be

potential causes of SLE (Balow, Boumpas, *et al.,* 2000). Ninety percent of SLE affected individuals are women. The onset of SLE in women occurs most often during child-bearing years. This data has led the scientists to hypothesize that estrogen levels during child bearing age may be a factor in the development of SLE. Researchers have also noted that flares of SLE have been correlated with ultraviolet light photosensitivity, which may mean that environmental agents have a role in the onset of SLE. The likely environmental triggers include ultraviolet light, drugs, and viruses. These stimuli cause the destruction of cells, and expose their DNA, histones, and other proteins, particularly parts of the cell nucleus (Blatt and Glick, 1999). However, the most interesting theories about the etiology of SLE deal with defects of specific elements of the immune system.

B cells originate from pluripotent haematopoietic stem cells in the bone marrow and undergo several developmental stages before expressing immunoglobulin specific for a single antigen. The specificity of the immunoglobulin is determined by rearrangement of light chain and heavy chain genes. In a healthy individual, immature B cells undergo a "negative selection" process during which B cells that react to self proteins are deleted, inactivated, or undergo further gene arrangement. Immature B cells that recognize multivalent self antigens are eliminated through clonal deletion. Immature B cells that bind to soluble self molecules become anergic. In some cases receptor editing replaces a self-reactive immunoglobulin rearrangement with a successive rearrangement. Theoretically, if the immune system is working properly it should not produce B cells with immunoglobulin that react with self proteins such as DNA. However, individuals with SLE produce antibodies that react with self proteins (Janeway *et al.,* 1999). Researches have hypothesized that individuals with SLE may have a defect in the

negative selection process during B cell development (Bensimon, Chastagner *et al.,* 1994). In SLE, the body's immune system produces antibodies against itself, particularly against proteins in the cell nucleus. SLE is triggered by environmental factors which are unknown (but probably include viruses), in people with certain combinations of genes in their immune system.

All the key components of the immune system are involved in the underlying mechanisms of SLE, and SLE is the prototypical autoimmune disease. The immune system must have a balance (homeostasis) between being sensitive enough to protect against infection and being too sensitive and attacking own proteins (autoimmunity) (Rahman and Isenberg, 2008).

SLE as a chronic inflammatory disease believed to be a type III hypersensitivity response with potential type II involvement, (Hom, Graham *et al.,* 2008).

1.2.1. Genetics

The first mechanism may arise genetically. Research indicates that SLE may have a genetic link. Lupus does run in families, but no single "lupus gene" has yet been identified. Instead, multiple genes appear to influence a person's chance of developing lupus when triggered by environmental factors. The most important genes are located on chromosome 6, where mutations may occur randomly or be inherited. Additionally, people with SLE have an altered RUNX-1 (a protein that is a transcription factor controlling when genes are switched on or off) binding site, which may be either a cause or contributor (or both) to the condition. Altered binding sites for RUNX-1 have also been found in people with psoriasis and rheumatoid arthritis (Crow, 2008).

1.2.2. Environmental triggers

The second mechanism may be due to environmental factors. These factors may not only exacerbate existing lupus conditions, but also trigger the initial onset. They include certain medications (such as some antidepressants and antibiotics), extreme stress, sunlight exposure, hormones, and infections. Some researchers have sought to find a connection between certain infectious agents (viruses and bacteria) but no pathogens can be consistently linked to the disease. UV radiation has been shown to trigger the photosensitive lupus rash, but some evidence also suggests that UV light is capable of altering the structure of the DNA, leading to the creation of autoantibodies (Rahman and Isenberg, 2008).

1.2.3. Drug reactions

Drug-induced lupus erythematosus is a reversible condition that usually occurs in patients being treated for a long-term illness. Drug-induced lupus mimics systemic lupus. However, symptoms of drug-induced lupus generally disappear once a patient is taken off the medication which triggered the episode. There are many medications currently in use that can cause this condition, though the most common drugs are procainamide, hydralazine and quinidine (Rahman and Isenberg, 2008).

1.2.4. Non-SLE forms of lupus

Discoid (cutaneous) lupus is limited to skin symptoms and is diagnosed via biopsy of skin rash on the face, neck or scalp. Often an anti-nuclear antibody (ANA) test for

discoid patients is negative or a low-titre positive. About 1-5% of discoid lupus patients eventually develop SLE (Hughes, 1998).

1.2.5. Clearance deficiency

The exact mechanisms for the development of systemic lupus erythematosus (SLE) are still unclear since the pathogenesis is a multifactorial event. Beside discussed causations, impaired clearance of dying cells is a potential pathway for the development of this systemic autoimmune disease. This includes deficient phagocytic activity, scant serum components in addition to increased apoptosis (Gaipl, Kuhn *et al.,* 2006).

Monocytes isolated from whole blood of SLE patients show reduced expression of CD44 surface molecules involved in the uptake of apoptotic cells. Most of the monocytes and tingible body macrophages (TBM), which are found in the germinal centres of lymph nodes, even show a definitely different morphology in patients with SLE. They are smaller or scarce and die earlier. Serum components like complement factors, CRP and some glycoproteins are furthermore decisively important for an efficiently operating phagocytosis. In SLE patients these components are often missing, diminished or inefficient.

The clearance of early apoptotic cells is an important function in multicellular organisms. It leads to a progression of the apoptosis process and finally to secondary necrosis of the cells, if this ability is disturbed. Necrotic cells release nuclear fragments as potential autoantigens as well as internal danger signals, inducing maturation of dendritic cells (DC), since they have lost their membranes integrity. Increased appearance of apoptotic cells also is simulating inefficient clearance that leads to maturation of DC and also to the

presentation of intracellular antigens of late apoptotic or secondary necrotic cells, via MHC molecules (Munoz *et al.,* 2005).

Autoimmunity possibly results by the extended exposure to nuclear and intracellular autoantigens derived from late apoptotic and secondary necrotic cells. B and T cell tolerance for apoptotic cells is abrogated and the lymphocytes get activated by these autoantigens; inflammation and the production of autoantibodies by plasma cells are initiated (Gaipl *et al.,* 2007).

1.2.6. Accumulation in germinal centres (GC)

In healthy conditions apoptotic lymphocytes are removed in germinal centres by specialized phagocytes, the tingible body macrophages (TBM); that's why no free apoptotic and potential autoantigenic material can be seen. In some patients with SLE accumulation of apoptotic debris can be observed in GC, because of an ineffective clearance of apoptotic cells. In close proximity to TBM, follicular dendritic cells (FDC) are localized in GC, which attach antigen material to their surface and in contrast to bone marrow-derived DC; neither take it up nor present it via MHC molecules. Autoreactive B cells can accidentally emerge during somatic hypermutation and migrate into the GC light zone. Autoreactive B cells, maturated coincidently, normally don't receive survival signals by antigen planted on follicular dendritic cells and perish by apoptosis. In the case of clearance deficiency apoptotic nuclear debris accumulates in the light zone of GC and gets attached to FDC. This serves as a germinal centre survival signal for autoreactive B-cells. After migration into the mantle zone autoreactive B cells require further survival signals from autoreactive helper T cells, which promote the maturation of autoantibody

producing plasma cells and B memory cells. In the presence of autoreactive T cells a chronic autoimmune disease may be the consequence (Gaipl, Kuhn *et al.,* 2006).

1.2.7. Sex hormones

Sex hormones play a part in the pathogenesis of SLE (Clarke *et al.,* 2008). SLE is predominantly a female disease (Cervera R, 2003) and typically has a predilection for women in their child-bearing years (Cervera, Khamashta *et al.,* 2003). First onset of SLE before puberty and after menopause is uncommon (Formiga Moga *et al.,* 1999). In addition, patients with Klinefelter's syndrome, characterized by hypergonadotrophic hypogonadism, are prone to the development of SLE (French and Hughes., 1983). These observations suggest a role for endogenous sex hormones in disease predisposition.

Abnormal oestrogen metabolism has been demonstrated in patients with SLE of both sexes, with an increase in 16α hydroxylation of oestrone, resulting in significantly raised 16α hydroxyestrone concentrations (Lahita, Bradlow *et al.,* 1979). The 16α metabolites are more potent and feminizing oestrogens.

Women with SLE also have low plasma androgens, including testosterone, dihydrotestosterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (Jungers, Nahoul *et al.,* 1982). The concentration of androgens correlates inversely with disease activity (Lahita, Bradlow *et al.,* 1987). Low concentrations of plasma testosterone and raised luteinizing hormone (LH) values (Lahita, Bradlow *et al.,* 1987) have been found in some men with SLE.

Some studies have found that exogenous exposure to estrogen, either through oral contraceptive or oestrogen replacement therapy may increase the incidence of SLE (Sanchez-Guerrero, Karlson *et al.,* 1997).

Taken together, oestrogens may aggravate SLE by prolonging the survival of autoimmune cells, increasing T helper type 2 (Th2) cytokine productions and stimulating B cells to produce autoantibodies. The inhibition of the Th1 response and the enhancement of CD40L expression on lupus T cells may indirectly promote the Th2 response and lead to further B cell hyperactivity (Mok and Lau, 2003).

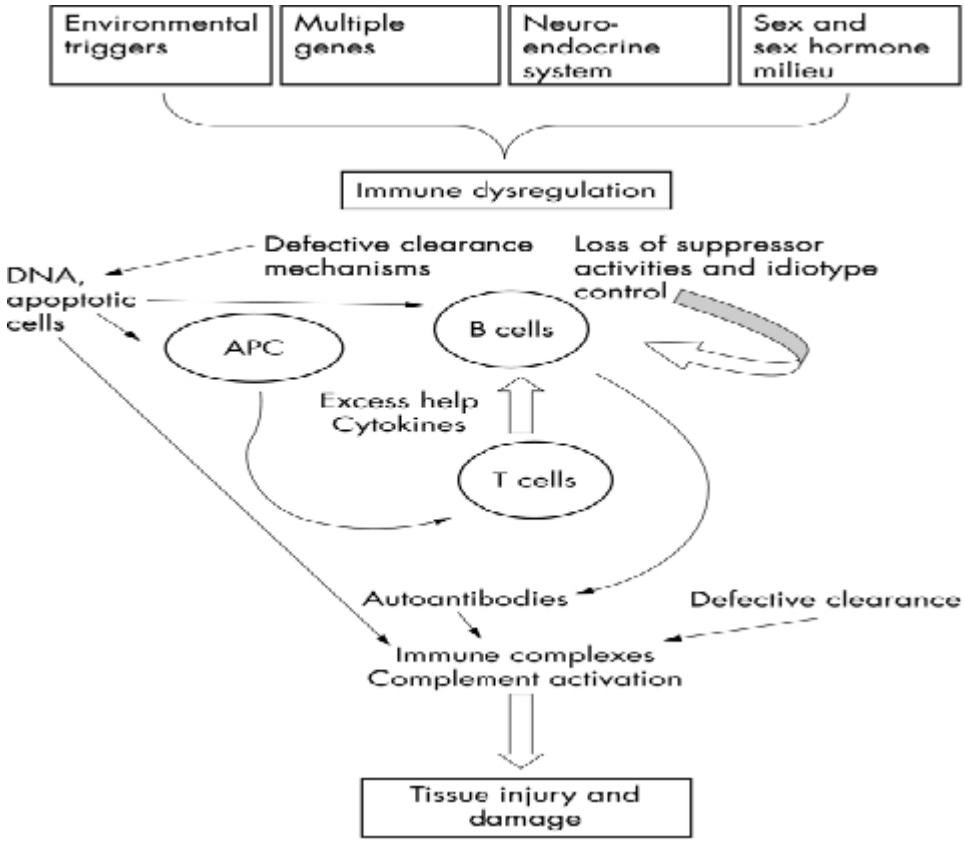

Figure 1.1 Pathogenesis of systemic lupus erythematosus. Adapted from Journal of Clinical Pathology (Mok and Lau, 2003).

1.3. Immunopathology of SLE

The basic pathological features of SLE are that of inflammation and blood vessel abnormalities, which include band or occlusive vasculopathy, vasculitis, and immune complex deposition.

The best characterized organ pathology is in the kidney. By light and immunofluorescence microscopy, renal biopsies in patients with SLE display mesangial cell proliferation, inflammation, basement membrane abnormalities and immune complex deposition, comprising immunoglobulins and complement components. On electron microscopy, these deposits can be visualised in the mesangium and the subendothelial or subepithelial surface of the basement membrane.

Other organ systems affected by SLE usually display non-specific inflammation or vascular abnormalities, although pathological findings are sometimes minimal. Inflammation and necrotising vasculitis can rarely be found. Occlusive vasculopathy is a common histological feature associated with the presence of antiphospholipid antibodies. Atherosclerosis and tissue damage caused by hypertension, corticosteroids, and other drugs can be demonstrated in patients with long standing SLE (Roman, Shanker *et al.,* 2003).

1.3.1. Autoantibodies in SLE

The central immunological disturbance in patients with SLE is autoantibody production. These antibodies are directed at several self molecules found in the nucleus, cytoplasm, and cell surface, in addition to soluble molecules such as IgG and coagulation factors. Antinuclear antibodies are most characteristic and present in more than 95% of patients.

Anti-double stranded DNA (ds-DNA) and anti-Sm antibodies are unique to patients with SLE. In fact, their presence is included in the classification criteria of SLE (Gilboe and Husby, 1999). The Sm antigen is designated as a small nuclear ribonucleoprotein (snRNP) and is composed of a unique set of uridine rich RNA molecules bound to a common group of core proteins and other proteins associated with the RNA molecules. Anti-Sm antibodies react with snRNP core proteins, whereas anti-DNA antibodies bind to a conserved nucleic acid determinant widely present on DNA. Anti-DNA antibody titres frequently vary over time and disease activity but anti-Sm antibody titres are usually constant.

The most remarkable feature of anti-DNA antibodies is their association with glomerulonephritis. Anti-DNA antibodies can be isolated in an enriched form from glomerular eluates of patients with active lupus nephritis and anti-DNA antibodies can induce nephritis in normal and severe combined immunodeficient mice (Vlahakos, Foster *et al.,* 1992, Ehrenstein, Katz et al., 1995) However, the correlation between anti-DNA antibodies and lupus nephritis in not complete because some patients with active nephritis are negative for anti-DNA antibodies, whereas some patients with persistent high titres of anti-DNA may not show renal involvement.

Although an association between certain clinical features of SLE and autoantibodies has been well documented, the pathogenicity of these antibodies has not been adequately studied. The exact immunological mechanisms for injury remain to be elucidated. The pathogenesis of manifestations other than glomerulonephritis is less well understood, although immune complex deposition with activation of complement at relevant sites is a probable mechanism. This is demonstrated by the frequent association of

hypocomplementaemia and signs of vasculitis at the sites of active SLE. Direct antibody mediated damage and cell mediated cytotoxicity on target tissues are other possible mechanisms (Gompertz *et al.,* 1990).

1.3.2. Disturbances of the immune response

SLE is characterized by a myriad of immune system aberrations that involve B cells, T cells, and cells of the monocytic lineage, resulting in polyclonal B cell activation, increased numbers of antibody producing cells, hypergammaglobulinaemia, autoantibody production, and immune complex formation. It appears that excessive and uncontrolled T cell help in the differentiation and activation of autoantibody forming B cells is probably a final common pathway.

The activation of B and T cells requires stimulation by specific antigens. Irritating chemicals such as pristine, bacterial DNA and cell wall phospholipids, and viral antigens can induce anti-DNA antibodies in mice (Hahn, 1998). Moreover, self antigens, such as DNA–protein and RNA–protein complexes may induce autoantibody production (James, Gross *et al.,* 1995). Environmental antigens and self antigens are taken up by professional antigen presenting cells (APCs) or bind to induced antibodies on the surface of B cells. Both professional APCs and B cells process the antigens into peptides and present them to T cells through their surface HLA molecules. The activated T cells in turn stimulate the B cells to produce pathogenic autoantibodies. In addition to contact stimulation, the interaction of B and T cells is facilitated by several cytokines, such as IL-10, and requires accessory molecules such as those of the CD40/CD40L and B7/CD28/CTLA-4 systems to initiate a second signal.

B cell activation is abnormal in patients with SLE. The number of B cells at all stages of activation is increased in the peripheral blood of patients with active SLE (Klinman, Shirai *et al.,* 1991). These B cell abnormalities can precede the development of SLE. Activated lupus B cells have higher intracytoplasmic calcium responses than controls (Liossis, Kovacs *et al.,* 1996). There is also evidence that B cells in patients with SLE are more sensitive to the stimulatory effects of cytokines such as IL-6 than non-SLE B cells (Linker-Israeli, Deans *et al.,* 1991). Moreover, the phenomenon of epitope spreading has been demonstrated in both human and murine SLE (Monneaux and Muller, 2002). Thus, it appears that B cells in patients with SLE are more prone to polyclonal activation by antigens, cytokines and other stimuli.

Abnormalities in T cell function are also evident in patients with SLE. The total number of peripheral blood T cells is usually reduced, probably because of the effects of anti-lymphocyte antibodies (Bakke, Kirkland *et al.,* 1983). There is a skewing of T cell function towards B cell help, leading to enhanced antibody production (Linker-Israeli, Quismorio *et al.,* 1990). Experiments have shown that the early events of T cell activation are defective in patients with SLE compared with controls (Portales-Perez, Gonzalez-Amaro *et al.,* 1997, Fernandez-Gutierrez, de Miguel *et al.,* 1998). Although peripheral lupus T cells are activated, both their capacity for proliferation in response to mitogenic stimulation and IL-2 production are reduced (Horwitz and Garrett, 1977, Alcocer-Varela and Alarcon-Segovia, 1982, Warrington 1988, Linker-Israeli, Bakke *et al.,* 1983). The reasons for the defective Th1 responses in SLE remain speculative. Down regulation by excessive Th2 cytokines, defective interaction between APCs and T cells, the suppressive effects of CD8+ T cells and natural killer (NK) cells, the presence of IL-2

inhibitors, and the down regulation of IL-2 receptors are possible mechanisms (Garcia-Cozar, Molina *et al.,* 1996). Summary of abnormal immune responses in SLE patients are shown in Table 1.1.

1.3.3. Cytokine network in SLE

Cytokine profiles in patients with SLE have been studied extensively. Peripheral blood mononuclear cells (PBMCs) from patients with SLE proliferate less than controls when stimulated with various antigens and mitogens (Horwitz and Garrett, 1977). Supernatants from phytohaemagglutinin (PHA) or autologous mixed lymphocyte reaction stimulated lupus T cells to produce less IL-2 than control T cells. Lupus T cells are less responsive to IL-2 stimulation than normal T cells (Alcocer-Varela and Alarcon-Segovia, 1982; Warrington, 1988; Linker-Israeli, Bakke *et al.,* 1983). However, the expression of IL-2 in freshly prepared SLE PBMCs was increased compared with control PBMCs (Horwitz, Wang *et al.,* 1994). Lupus T cells are capable of producing normal amounts of IL-2 in response to optimal stimulation with PHA combined with phorbol esters or with anti-CD28 antibodies (Garcia-Cozar, Molina *et al.,* 1996; Horwitz, Wang *et al.,* 1994; Horwitz, Gray *et al.,* 1998). As mentioned previously, the impaired in vitro IL-2 production from lupus T cells is probably the result of many factors, one of which is the downregulating effects of certain Th2 cytokines.

The recent discovery of the role played by IL-10 in the pathogenesis of SLE supports this hypothesis. IL-10 is a Th2 cytokine that acts as a potent stimulator of B cell proliferation and differentiation, and thereby a potential mediator of polyclonal B cell activation in SLE. Indeed, studies have shown that spontaneous production of IL-10 from SLE

peripheral blood B cells and monocytes is significantly higher than that of controls (Llorente, Richaud-Patin *et al.,* 1993; Llorente, Richaud-Patin *et al.,* 1994). The expression of IL-10 transcripts is significantly increased in the non-T cell population of PBMCs from patients with SLE compared with controls (Csiszar, Nagy *et al.,* 2000). Moreover, serum IL-10 concentrations are higher in patients with SLE than in controls and are correlated with clinical and serological disease activity and anti-DNA antibody titres (Houssiau, Lefebvre *et al.,* 1995; Park, Lee *et al.,* 1998; Grondal, Gunnarsson *et al.,* 2000). Furthermore, an increased ratio of IL-10 to interferon γ secreting cells in the PBMCs of patients with SLE correlates with disease activity (Hagiwara, Gourley *et al.,* 1996).

The increase in IL-10 production may be a cause of the defective in vitro Th1 response in lupus T cells. This is suggested by a study demonstrating that the addition of blocking antibodies to IL-10 significantly enhanced the proliferative response of lupus PBMCs (Lauwerys, Garot *et al.,* 2000). Conversely, PBMCs from patients with SLE and inactive disease cultured with IL-10 showed a significant augmentation of anti-DNA antibody production (Tyrrell-Price, Lydyard *et al.,* 2001).

IL-12, a heterodimeric cytokine produced by B cells, macrophages, and dendritic cells, promotes cell mediated immune responses but exerts some inhibitory activities on humoral responses (Trinchieri, 1994). IL-12 production was found to be impaired in stimulated PBMCs from patients with SLE compared with matched controls (Horwitz, Gray *et al.,* 1998; Liu and Jones, 1998). The defect in IL-12 production probably lies in the monocytes but not the B cells (Liu, Jones *et al.,* 1999). In contrast, the addition of IL-12 to lupus PBMCs significantly inhibits both spontaneous and IL-10 stimulated

immunoglobulin and anti-DNA antibody production (Houssiau, Mascart-Lemone *et al.,* 1997). Moreover, the production of anti-DNA antibodies by PBMCs from patients with SLE and active disease is inhibited by culturing the cells with IL-12 (Tyrrell-Price, Lydyard *et al.,* 2001). The results from these studies suggest that dysregulation of the IL-10 and IL-12 balance plays a crucial role in the impaired cellular immune responses seen in patients with SLE.

1.3.4. Defective immune regulation

The clearance of immune complexes by phagocytic cells is defective in patients with SLE. This results partly from the reduced numbers of CR1 receptors for complement and functional defects of the receptors on cell surfaces (Mir, Porteu *et al.,* 1988; Kiss, Csipo *et al.,* 1996). Defective clearance may also result from inadequate phagocytosis of IgG2 and IgG3 containing complexes. Allelic polymorphisms of the IgG receptors (FcγR) have recently been described. Some of the polymorphic alleles (FcγRIIA and FcγRIIIA) are associated with lower binding of the Fc portions of IgG2 and IgG3, and hence impaired clearance of immune complexes (Dijstelbloem, Bijl *et al.,* 2000). Indeed, the FcγRIIA and FcγRIIIA genotypes have been associated with susceptibility to SLE and nephritis in certain ethnic groups (Zuniga, Ng *et al.,* 2001). Although consistent results cannot be obtained in patients of different ethnicities, impaired immune complex clearance by phagocytes is an important pathogenetic mechanism in SLE.

In one study also demonstrated that non-inflammatory engulfment phagocytosis of apoptotic cells is impaired in patients with SLE (Herrmann *et al.,* 1998). Persistently

circulating apoptotic waste may serve as an immunogen for the induction of autoreactive lymphocytes and as an antigen for immune complex formation.

The synthesis and secretion of pathogenic autoantibodies in SLE is driven by the interaction of CD4+ and CD8+ helper T cells, and double negative T cells (CD4− CD8−) with B cells (Mohan, Adams *et al.,* 1993). Therefore, cells that normally suppress B cell activation, such as CD8+ suppressor T cells and NK cells are defective in their suppressive activity. It has been shown that CD8+ T cells and NK cells from patients with active SLE are often incapable of down regulating polyclonal immunoglobulin synthesis and autoantibody production (Linker-Israeli, Quismorio *et al.,* 1990). One study reported that CD8+ T suppressor cell function was impaired in patients with active SLE (Filaci, Bacilieri *et al.,* 2001). This impaired suppression of B cells may be one factor that leads to the perpetuation of the disease.

In normal healthy subjects, overproduction of antibodies is prevented by an idiotype network. This network is probably defective in patients with SLE, leading to dysregulation of autoantibody production (Sherer and Shoenfeld Y, 2000).

Table 1.1 Summary of abnormal immune responses in SLE patients.

Hyperactivated B cells • Number of activated B cells producing Ig increased in peripheral blood • B cell abnormalities are present in unaffected family members and may precede SLE development • Lupus B cells are more prone to polyclonal activation by specific antigens • Raised IL-6 and IL-10 concentrations may promote B cell hyperactivity • B cell responses to activating signals are abnormal
Hyperactivated T cells • Number of activated T cells increased in peripheral blood • Abnormal early events of T cell activation • T cell function skewed towards B cell help and Ig production • Lupus T cells produce little IL-2 on stimulation
Abnormal phagocytic functions • Phagocytic cells cannot bind or process immune complexes efficiently • Phagocytosis of apoptotic cells impaired
Abnormal immunoregulation • Defective clearance of immune complexes and apoptotic materials because of qualitative or quantitative defects of early complement proteins (C2, C4, C1q), Fcγ, CR1, and C1q receptors on cell surfaces • Suppressive activity of suppressor T cells and NK cells on activated T and B cell network is inadequate • Idiotypic control of antibody production is dysregulated

1.3.5. Apoptosis and SLE

Apoptosis, or programmed cell death, is a process that leads to the ordered destruction of cells, avoiding the release of intracellular contents into the extracellular microenvironment, where they have a powerful inflammatory effect. Defective apoptosis leading to the prolonged survival of pathogenic lymphocytes was thought to be one disease mechanism for SLE (Suzuki, Ichino *et al.,* 1998). Apoptotic cells are usually cleared by macrophages via a non-inflammatory pathway (Voll RE and I., 1997). Apoptotic and necrotic cells are strong candidates as sources of autoantigens that drive the autoantibody response in autoimmune diseases.

The fast and efficient uptake of dying cells is of main importance to prevent contact of the immune system with intracellular autoantigens (Rovere P, 2000). Defects in the clearance of dying cells may contribute to the etiopathogenesis of systemic lupus erythematosus (SLE). Increased apoptosis or impaired clearance of apoptotic cell material has been implicated in the pathogenesis of human SLE.

Impaired clearance functions for dying cells may explain accumulation of apoptotic cells and subsequently of secondary necrotic cells in various tissues of SLE patients. During cell death by necrosis or apoptosis, autoantigens are cleaved or otherwise modified. These modifications may render cryptic epitopes immune dominant. Dendritic cells may then acquire modified autoantigens, like apoptotic nuclei and chromatin, and consequently start an immune reaction.

Normally, apoptotic cells are swiftly removed by phagocytosis due to surface changes induced by the apoptotic process (Savill J, 2002). This prevents the release of intracellular constituents, including nucleosomes, which are uniquely formed during

apoptosis through cleavage of chromatin by nucleases. However, antibodies against nucleosomes are a hallmark of systemic lupus erythematosus (SLE) (Dieker JW, 2002). Therefore, disturbances in either apoptosis or the phagocytosis of apoptotic cells have been proposed to play a role in the development of autoimmunity, especially in SLE.

The reasons for the defective clearance of apoptotic cells in SLE are not clear. It could be the result of quantitative or qualitative defects of the early complement proteins, such as C2, C4, or C1q (Walport, Davies *et al.,* 1998). The C1q receptors on the surface of phagocytes constitute an extremely important mechanism for the clearance of apoptotic cells (Korb and Ahearn, 1997). Patients or mice with homozygous C1q deficiency develop autoantibodies and a lupus-like syndrome apparently because of the inability to eliminate apoptotic cells effectively, which leads to an increase in the exposure of antigens to the immune system (Walport, Davies *et al.,* 1998; Korb and Ahearn, 1997; Botto, Dell'Agnola *et al.,* 1998).

1.4. Lymphocyte subsets in SLE

Human lymphocytes may be classified in three main populations according to their biological function and their cell surface antigen expression: T lymphocytes, B lymphocytes and natural killer cells (NK). T lymphocytes (CD3+), the precursors of which originate in the bone marrow and then migrate and mature in the thymus, can be subdivided as well in functionally different populations. The most clearly defined of these are helper/inducer T cells (CD3+CD4+) and suppressor/cytotoxic T cells (CD3+CD8+). T cells produce no antibodies and are the mediators of cell immunity. Helper/inducer lymphocytes are a subset of T lymphocytes (CD3 +) that are CD4 + cells.

CD3 + CD4 + counts are used to characterize and monitor some forms of immunodeficiency and autoimmune diseases.

Suppressor/cytotoxic lymphocytes are a subset of T lymphocytes (CD3 +) that are CD8 + cells. Suppressor/cytotoxic lymphocyte values lie outside the normal reference range in some autoimmune diseases, and in certain immune reactions such as acute graft-versus-host disease (GVHD) and transplant rejection. The CD8 + subset is elevated in many patients with either congenital or acquired immune deficiencies.

CD19 is the broadest lineage- specific surface marker for B cells and it is present on the surface of virtually all B lymphocytes, including early B progenitor cells. CD19 expression is maintained in B- lineage cells that have undergone neoplastic transformation. B lymphocytes (CD19+) are the producers of antibodies; they mediate humoral immunity particularly effective against toxins, whole bacteria, and free viruses.

The immunological status of patients can be determined by assessing the level of CD16-positive natural killer (NK) cells, and a simultaneous detection of other NK- cell markers and enumeration of other lymphocyte subpopulations. NK cells (CD3-CD16+CD56+) mediate cytotoxicity against certain tumors and virus-infected cells. NK-mediated cytotoxicity does not require class I or class II major histocompatibility complex (MHC) molecules to be present on the target cell. CD56 is the prototypic marker of human NK cells, and it is also expressed by a subset of CD4- positive and CD8- positive T cells in peripheral blood (Paglieroni and Holland, 1994).

1.5. Lymphocyte activation markers in SLE

Activation and proliferation of immunocompetent cells is the basic immunologic response to alloantigens (Corradin *et al.,* 1977), which could be evaluated by the

expression on T cells of CD25, HLA-DR molecules, and by the loss of the high molecular mass isoform of the CD45 molecule (CD45RA) and the expression of the low molecular mass isoform CD45RO (De Francesco *et al.,* 1996) and thus termed ‘‘activation antigens.’’

CD25 are naturally occurring immunoregulatory T cells originate in the thymus, constitutively express CD25 and comprise 5-10% of murine and approximately 2% of human peripheral CD4+ T cells (Shevach, 2002, Baecher-Allan *et al.*, 2001). Within the human CD4+ subset only cells coexpressing high levels of CD25 appear to have potent regulatory function (Baecher-Allan *et al.*, 2001, Wing *et al.*, 2002). The precise role of this distinct T cell subset in human autoimmunity is as yet undefined.

The primary function of HLA-DR is to present peptide antigens, potentially foreign in origin, to the immune system for the purpose of eliciting or suppressing T-(helper)-cell responses that eventually lead to the production of antibodies against the same peptide antigen.

CD45RO are memory T cells generated during a primary response appear to survive for long periods and are therefore available to provide immediate protection as well as to assist in the provision of rapid and effective responses if and when a relevant Ag is re-encountered (Goldrath and Bevan, 1999).

Such activation antigens are easily evidenced by immunofluorescence assay and flow cytometric analysis using monoclonal antibodies (mAbs) as specific reagents. By two- and three colour immunofluorescent staining it is also possible to identify, within bulk lymphocyte cultures, the T-cell subpopulations involved in the activation process.

Activation of lymphocytes is a complex, yet finely regulated cascade of events that