The link between *Proteus mirabilis*, environmental factors and autoantibodies in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a relatively common and potentially disabling immune-mediated inflammatory systemic disease, predominantly affecting women and characterised by multiple small joint arthritis. Extensive data supports the roles of genetic, environmental and microbial factors in the triggering and development of this disease. Proteus mirabilis is considered as the main microbial culprit in the causation of RA. The evidence for the role of these microbes in RA and their links with commonly associated autoantibodies such as rheumatoid factors and anti-citrullinated peptide antibodies have been elucidated together with their relations with some of the non-microbial environmental factors which have been implicated in the aetiopathogenesis of RA. The most likely mechanism in the development of RA is "molecular mimicry" where Proteus antigens were found to share homologous sequences, which cross-react with certain self-antigens present in synovial tissues. This could raise possibilities for implementing a new therapeutic strategy in the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory and potentially disabling arthritic disease affecting between 1–2 millions of people worldwide. It usually occurs in middle-aged women, and presents with arthritis involving several peripheral small joints with a symmetrical distribution as well as associated extra-articular manifestations (1).

A general consensus exists among clinical and biological scientists, which supports the notion that RA is an autoimmune disorder resulting from a complex interaction between genes, environmental factors and the immune system (2). There is also a possibility for an inter-relation between the triggering microbe and these disease-inducing elements that are involved in the development of RA.

Genetics of RA and the role of HLA genes

During the last few years, new genetic loci have been identified to be associated with a susceptibility risk to develop RA. A series of genome-wide association studies and meta-analyses have led to discovery of over 100 loci for RA (3). Among these genes, however, certain major histocompatibility complex alleles provide the strongest association with RA. HLA-Dw4 gene, which was originally discovered in the mid 1970s by workers in Dallas, was found to be more frequently associated with RA (4). Later with the use of more sensitive techniques various HLA-DRB1 alleles have been found to be associated with high risk of RA development among many ethnic populations (5). Some of these alleles such as HLA-DR4 subtypes (DRB1*0401, *0404 and *0405), HLA-DR1 (DRB1*0101), and HLA-DR6 (DRB1*1402) were found to be more associated with the RA risk (Fig. 1), whilst other HLA-DR4 subtypes (DRB1*0402 and *0403) did not have such an association (6). This discrepancy in the association of HLA-DRB1 genes with RA was found to be due to the presence of a conserved hexameric amino acid sequence in the third hypervariable regions of all RA-associated HLA-DRB1 alleles involving positions 69-74 and consisting of glutamic acid, glutamine, lysine or arginine, arginine, alanine and alanine "EQK/RRAA", which has led to the "shared epitope" (SE) hypothesis (7). In a later study, HLA-DR10 (DRB1*1001) genes were

also found to be significantly associated with the increased risk of RA among the Spanish population, giving a new "ERRRAA" sequence to the SE with an arginine substitution at the 70th position of that region (8). It has also been reported that more than 90% of RA patients possess one or more of these SE-containing RA-associated HLA-DRB1 (HLA+SE) alleles (9), regardless of the ethnic backgrounds.

Based on these results it seems that each of these genetic loci have an important role in the aetiopathogenesis of RA. However, considerably low disease concordance rates among monozygotic twin pairs with RA (10) emphasises the importance of an environmental, mainly microbial, factor to have a potentially triggering role in this disease.

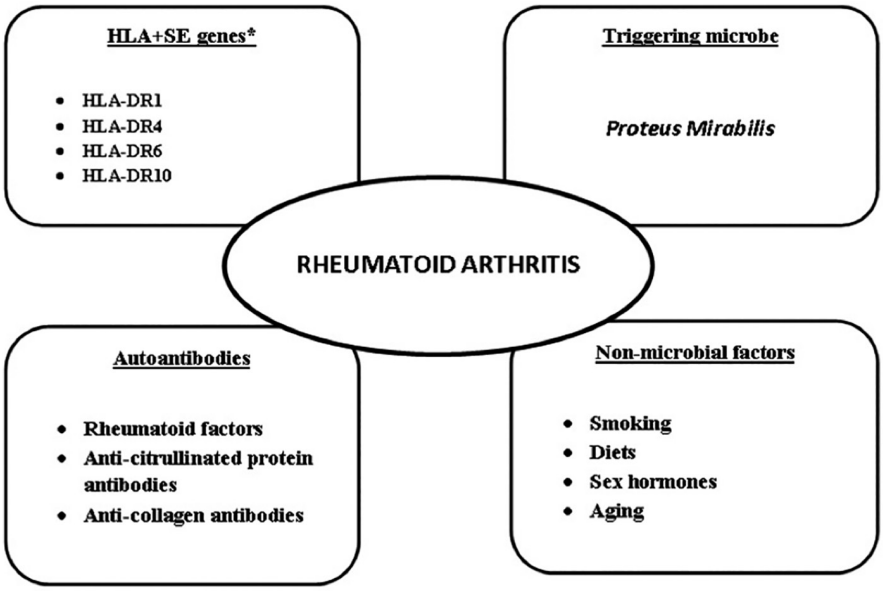

Fig. 1. Prominent factors affecting the development of rheumatoid arthritis (*HLA+SE: shared epitope-containing RA-associated HLA genes).

The link between *Proteus* and other environmental factors in RA

The potential role of non-microbial environmental factors in the aetiopathogenesis of RA has been extensively studied. Among these, smoking, dietary and hormonal factors have been more frequently investigated (Fig. 1). Whether each one of these factors acts solely or as a promoter of infections in RA susceptible individuals is still unclear.

Smoking has been implicated as one of the most important extrinsic risk factors which might have a potential role in the development of RA (11). A possible link between smokers and *Proteus* urinary tract infections in patients with RA has been previously reviewed (12). In a study from Norway patients with RA have been found to respond significantly to specific dietary measures such as fasting and a one-year vegetarian diet, and the serum samples from these patients were shown to have had progressively dropping levels of anti-*Proteus* antibodies throughout the one-year dietary regime (13).

It is also well documented that RA is more likely to exacerbate after pregnancy and during puerperium (14) where the incidence of urinary tract infections were found to be higher. This might possibly be attributable to the anatomical structure of the female reproductive system or due to hormonal changes. In a recent study, although pregnant patients with RA had not shown considerable changes in the disease course, the majority of these patients, however, were found to have significant flares and exacerbations in disease activity during the postpartum periods (15).

The relation between microbial infections and humoral autoimmunity in RA

Infectious agents have been the most well studied environmental factors suggested to be involved in the triggering and development of autoimmune diseases. The best example of a relation between infection and immune-mediated pathology is acute rheumatic fever, which occurs following exposure of susceptible hosts to *Streptococcus pyogenes* usually presenting with acute tonsillitis (16).

One of the fundamental features of RA is its association with antibodies against several self-antigens, most commonly involving antibodies against IgG heavy chains, termed rheumatoid factors (RF), and those against citrullinated proteins, namely anti-citrullinated protein/peptide antibodies (ACPA) as well as antibodies against collagens (17). Although these autoantibodies, especially RFs and ACPAs have been linked to the disease severity, and possibly in the pathological process of RA, their exact roles in the aetiopathogenesis of this disease have not been yet fully clarified.

Longitudinal studies have shown that autoimmunity features may begin many years before the clinical manifestations of RA (18) designating that they are not considered as essentially inciting factors in RA. In a recent study it has been shown that single nucleotide polymorphisms driving levels of N-glycosylation have no association with RA susceptibility indicating that there is no preliminary direct relation between RFs and the development of RA (19). These findings could implicate that RFs are produced or triggered by infections, as they have been also reported to occur in patients with subacute bacterial endocarditis and other infections (20). Furthermore, in a collaborative study carried out by two groups from USA and Canada, early RF-positive RA patients had significantly higher levels of *Proteus* antibodies when compared to RF-negative RA patients as well as patients with other rheumatic diseases (21).

Antibodies to citrullinated proteins have also been shown to bind to cross-reactive antigens present in many environmental factors including microbes and dietary substances (22). The citrullinated proteins recognised by ACPA antibodies are produced as the result of deimination of an arginine residue into citrulline by the effect of protein

arginine deiminase (PAD) enzyme (23), which was found to be highly abundant and more active within the synovial joints of patients with RA (24). It is pertinent also to note that each of the "ESRRAL" and "IRRET" amino acid sequences which are present in the haemolysin and urease enzymes of *Proteus* respectively as well as the cross-reactive sequences present in HLA-DRB1 alleles and type XI collagens, all contain a doublet of positively charged arginines and they could be acted by PAD enzymes from granulocytes to form cyclical citrullinated proteins (CCP). Whether the polyclonal response of anti-CCP antibodies found in RA patients is involved with arginine-containing amino acid sequence awaits further examination (25).

The link between collagen autoantibodies and RA has been investigated mainly in animals. A widely accepted model for RA is collagen-induced arthritis, which is elicited by immunising genetically susceptible mouse strains with native type II collagens (26). Transgenic mice lacking endogenous class II molecules but expressing human HLA genes such as RA-associated, HLA-DRB1*0401, were found to be prone to develop collagen-induced arthritis whilst those with RA-resistant, HLA-DRB1*0402 genes, were not showing signs of arthritis (27). It has been shown also that IgG anti-collagen II antibody levels were increased significantly in patients with RA when compared to healthy subjects or to patients with osteoarthritis and that their levels were correlated with high concentrations of disease activity markers such as erythrocyte sedimentation rate, C-reactive protein (CRP), tumour necrosis factor-α and interleukin-6 (28).

The link between collagen type XI and RA, however, has been studied on the molecular and cross-reactivity levels only, where *Proteus* urease enzymes were found to possess "IRRET" sequence which stereo-chemically resembles "LRREI" amino acid motif present in the collagens type XI, which are abundant within hyaline cartilage of the synovial joints. Moreover, sera from active RA patients showed haemolytic activities against sheep red blood cells coated with "LRREI" synthetic peptides indicating that RA patients have anti-urease antibodies which show active cytotoxic immune responses to collagen fibres (29).

The role of *Proteus mirabilis* in RA

Proteus mirabilis is a Gram-negative bacterium most commonly found in the urinary tract system especially the kidneys. It is considered as one of the common cause of cystitis, pyelonephritis or asymptomatic bacteriuria especially in females (30).

Although various infectious species including bacteria, viruses and fungi have been claimed to have potential roles in the causation and development of RA, *Proteus mirabilis*, however, has been considered as the most likely culprit based on the following evidence:

A) Immunological and molecular links between Proteus and RA

An extensive amount of data exists which supports the role of *P. mirabilis* in the aetiopathogenesis of RA. Many studies were carried out by various independent groups from different parts of the world and involved molecular, immunological and microbiological investigations on many antigenic profiles of *Proteus* in patients with RA.

1. The first evidence of a link between *Proteus* microbe and RA was reported more than four decades ago where it has been shown that among a panel of 30 microbial agents tested, the mean geometric titres of antibodies were raised only against *Proteus* OXK and herpes virus hominis microbes in 22 newly diagnosed RA patients when compared to 22 control subjects (31).

2. Using Coomb's agglutination method, serum samples from rabbits immunised with HLA-DR4 positive lymphocytes were found to react mainly to *Proteus* but not to eight other tested microbes (32). Similarly, HLA-DR4 positive allogeneic tissue typing sera were found to bind to *P. mirabilis* more significantly than to *E. coli* bacteria (33).

3. Molecular similarity was observed between the "EQ/KRRAA" shared epitope sequence present in RA-associated HLA-DRB1 molecules and the "ESRRAL" amino acid peptides from haemolysins of *Proteus* (34). Molecular homology was also found to exist between the "IRRET" amino acid sequence from *Proteus* urease enzymes and the "LRREI" motif present in collagen type XI (35). Serum samples with specific reactivities to synthetic peptides containing "EQ/KRRAA" sequence were found to bind more significantly to those containing "ESRRAL" sequence of the *Proteus* haemolysin molecules. Similarly, anti-ESRRAL serum was found to react to the "EQ/KRRAA" containing peptides. Moreover, anti-ESRRAL peptide antibodies were found to bind to mouse fibroblasts expressing RA-associated HLA-DRB1 alleles but not to cells carrying other HLA molecules not associated with RA (36).

4. An *in vitro* study showed cytotoxic activities between IgG antibodies from RA patients against HLA-DR4-peptides as shown by increased haemolysis for sheep red blood cells coated with HLA-DRB1*0404 peptides containing the "EQRRAA" sequences when compared to sera from patients with ankylosing spondylitis and healthy subjects (29).

5. Antibodies against various antigens from *P. mirabilis* were found to be significantly elevated among RA patients from many different countries throughout the world (37). These antibodies have been shown to be specific to *Proteus* only as there were no such elevations in antibodies against 27 other microbes included in these studies (38). Furthermore, a group from Poland has also found that antibodies against other *Proteus* urease antigens were significantly higher in RA patients than in healthy controls (39). More recently, a group from Greece has shown that Greek RA patients have more significantly elevated class-specific antibodies against urease and haemolysin cross-reactive and non cross-reactive synthetic peptide antigens from *Proteus mirabilis* and that positive correlations were shown to occur between erythrocyte sedimentation rate and IgM anti-urease F antigens as well as between CRP and IgM anti-urease C antigens (40). So far RA patients from various populations in 17 different countries have shown elevated levels of anti-*Pro-*

teus antibodies when compared to corresponding controls (Table I) (41).

B) Microbiological link between Proteus urinary tract infections and RA

Previous reports have shown that the incidence of urinary tract infections is increased in patients with RA (42, 43). These observations, however, could not be confirmed by others (44). This discrepancy in the epidemiological link between urinary tract infections and RA could be partly explained by existing subclinical or occult infections which are merely characterised by *Proteus* bacteriuria in patients with RA, and this has been confirmed by two independent studies from London and Dundee.

In a controlled study of a group of patients with RA from London, *P. mirabilis* microbes were isolated from the urine of 63% female and 50% male patients and these results were found to be significant in comparison to the female (32%) or male (11%) healthy controls. However, the frequency of the isolation of *Proteus* from urine of women and men patients without RA (osteoarthritis, fibromyalgia, psoriasis, gout, and systemic lupus erythematosus) was 35% and 7% respectively, which were similar to those obtained from healthy controls (45). Furthermore, a positive correlation was found to exist between high anti-*Proteus* antibody levels in sera of RA patients and the number of colony-forming units in the urinary specimens obtained from these patients (46). In another study carried out by a group from Dundee, UK, a significantly increased isolation rate of *Proteus* microbes from the urine of 33% RA patients were detected when compared to those of only 4% of the gender-matched healthy individuals (47). It should be noted that women are more likely to have recurrent urinary tract infections which could explain why RA is more prevalent in females.

Table I. Geographical locations of RA patients having significantly elevated levels of anti-*Proteus* antibodies. (Modified from Ebringer and Rashid, 2014 – ref. 41).

	Year of study	Country	Location
1	1985	ENGLAND	London
	1988		Winchester
	1992		Newcastle
	1995		Stevenage
	1996		Stevenage
	1997		Winchester
	2007		Stevenage
2	1988	IRELAND	Dublin
3	1994		
	1995	FRANCE	Toulouse
			Brest
4	1995	SCOTLAND	Dundee
5	1995	BERMUDA	Hamilton
6	1995	NORWAY	Oslo
7	1997	JAPAN	Otsu
8	1997	INDIA	Chandigarh
9	1998	NETHERLANDS	Amsterdam
10	1998	TAIWAN	Taichung
11	1999	SPAIN	Barcelona
12	2000	RUSSIA	Moscow
13	2004	FINLAND	Helsinki
14	2005	U.S.A.	Bethesda & Philadelphia
15	2005	CANADA	Montreal
16	2012	POLAND	Kielce
17	2016	GREECE	Athens

Molecular mimicry mechanism and the pathogenesis of RA

Molecular mimicry between antigenic profiles of infectious agents and the normal human host cell proteins possibly represents a most likely mechanism responsible for the autoimmunity in RA (48). Various medical conditions such as rheumatic fever, RA, ankylosing spondylitis, Guillain-Barré syndrome, dengue haemorrhagic fever, primary idiopathic thrombocytopenic purpura and multiple sclerosis could possibly develop as the results of the molecular similarities and cross-reactivity reactions existing between invading microbes and self-antigens that lead to productions of microbial antibodies which could bind to the cross-reactive self-antigens in the targeted tissues (Table II).

Molecular similarities exist between antigenic epitopes present in the urease and haemolysin enzymes of *Proteus* microbes and the homologous antigens present in collagens and the RA-associated HLA+SE alleles within the targeted tissues in the joints and other parts of the body. As a small percentage of individuals carrying HLA+SE genes are prone to develop RA, we suggest that only those people who are exposed to repeated attacks of asymptomatic *Proteus* urinary infections are liable to produce high levels of anti-*Proteus* antibodies with the resultant inductions of autoantibodies against cross-reactive self-antigens (Fig. 2). In a previous collaborative study involving Spain, Norway and England, it has been shown that high anti-*Proteus* antibody levels were detectable mainly in patients with active RA as indicated by rises in the disease activity markers such as CRP and a positive correlation was also found to exist between the levels of anti-*Proteus* antibodies and the concentration of CRP in these patients (49).

Failure to detect microbial components at the main pathological sites, mainly joints (50), supports the suggestion that the immune system in RA patients could be triggered by microbes residing at extra-articular mucosal sites such as the urinary tract.

The concept of RA development can be summarised as follows: when individuals with high genetic risk factors such as those carrying HLA+SE alleles, are exposed to asymptomatic *Proteus* urinary tract infections, productions of auto-antibodies such as ACPA, RF, anti-HLA-DRB and anti-collagen antibodies are induced as the result of molecular mimicry or cross-reactivity between *Proteus* antigens and self-antigens (29). The induction of inflammations and pathological lesions consequently results from the productions and effects of complements, cytokines, free radicals as well as other components of the inflammatory mediators which lead to

Table II. Medical conditions showing the links between microbial infections and humoral autoimmunity via molecular mimicry.

Disease	Causative microbe	Microbial antigens	Self-antigens	Targeted tissues	References
Rheumatic fever	*Streptococcus pyogenes*	N-acetyle-β-D-glucosamine G1cNAc and lysoganglioside	Cardiac myosins Dopamine receptors	Heart valves Basal ganglia	(16) (59)
Rheumatoid arthritis	*Proteus mirabilis*	Haemolysin Urease	HLA-DRB1 genes Collagens XI	Joints and other organs Joints	(34) (35)
Ankylosing spondylitis	*Klebsiella pneumoniae*	Nitrogenase reductase Pullulanase-A Pullulanase-D	HLA-B27 genes HLA-B27 genes Collagens I, III & IV	Joints and other organs Joints and other organs Joints	(60) (61) (61)
GBS	*Campylobacter jejuni*	Lipopolysaccharide Gangliosides	Neuronal Gangliosides	Peripheral nerve axons	(62)
DHF	Dengue virus	Non-structural protein-1	Plasminogens	Coagulations and fibrinolysis system	(63)
PITP	*Helicobacter pylori*	Cytotoxin-associated gene-A	Glycoproteins	Platelets	(64)
Multiple sclerosis	*Acinetobacter*	4-CMLD 3-OACT-A	MBP MOG	Nerve myelin sheath Nerve myelin sheath	(65) (65)

GBS: Guillain-Barré syndrome; PITP: Primary idiopathic thrombocytic purpura; DHF: Dengue haemorrhagic fever; 4-CMLD: 4-carboxy-muconolactone decarboxylase; 3-OACT-A: 3-oxoadipate CoA-transferase; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein.

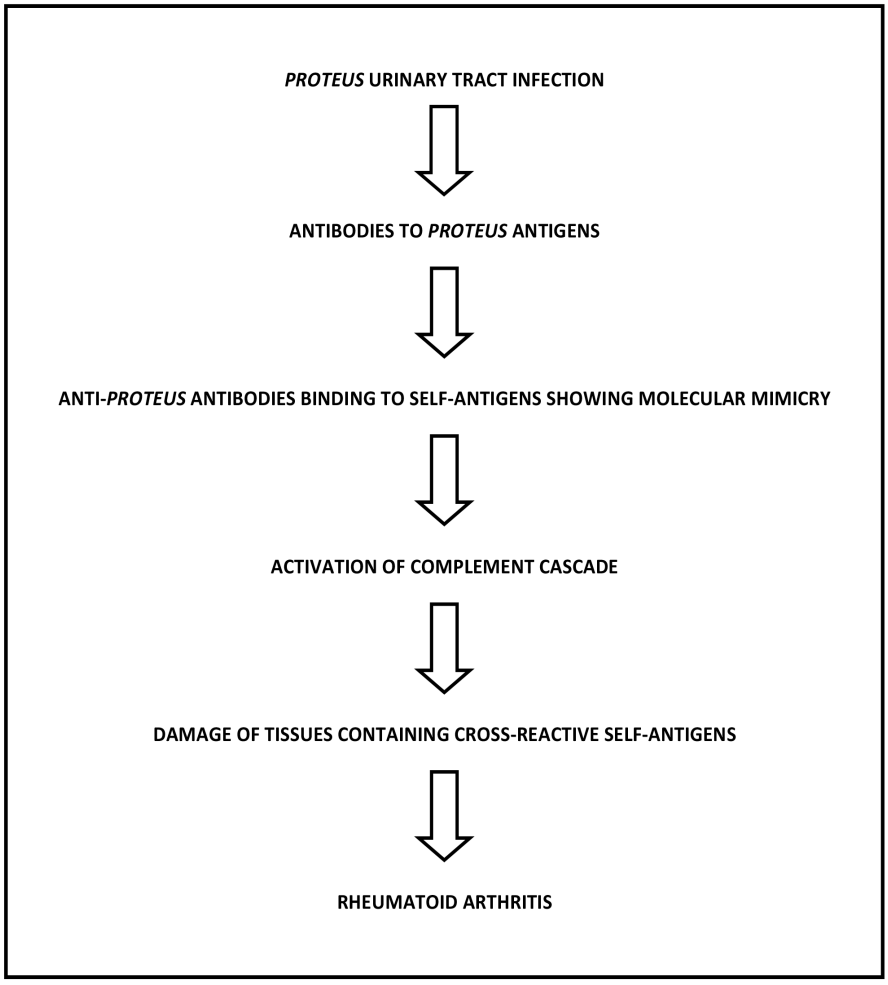

Fig. 2. Proposed pathogenetic process of rheumatoid arthritis.

recruitments and aggregations of immune and phagocytic cells at the sites of inflammations. In a recent study it has been shown that there was evidence of local proliferation but not cellular influx of fibroblasts responsible for the synovial hyperplasia in collagen antibody-induced arthritis, the mice model of RA, indicating that the main pathological process in RA starts at the site of pathological inflammations, namely the joints (51).

A new therapeutic strategy involving the use of anti-*Proteus* measures in RA

The nature of the disease having chronic, progressive and incapacitating effects that shorten survival rates in patients with RA, promotes the use of some aggressive therapeutic measures especially in the early period of the active disease, which include disease modifying anti-rheumatic drugs and biological agents (52). These measures, however, are not curative and even not devoid of deleterious side effects. Therefore, a proposal for the use of other therapeutic measures that stop or prevent exposures of susceptible individuals to excessive *Proteus* urinary tract infections via the use of antibiotics. For example, in a study curried out

by a group from Omaha, it was found that early seropositive RA patients had better response to treatment with doxycycline plus methotrexate versus methotrexate alone based on the disease activity responses (53) , and this result was supported by other groups (54, 55), dietary manipulations, such as the use of vegetarian diets (13, 56) and high fluid intakes (57), as well as cranberry juice (58) can be included together with the currently used anti-rheumatic treatments.

Conclusions

Based on the results of many independent studies it can be concluded that repeated asymptomatic urinary tract infections by *P. mirabilis* could be considered as the main triggering factor in the development of RA via pathological mechanism of molecular mimicry or cross-reactivity. The association of certain environmental factors such as smoking, hormones and diets in RA has been shown to have possible links with *Proteus* microbes. Antibodies to various *Proteus* antigens have been found to be elevated among RA patients from many different countries worldwide and these antibodies had shown cross-reactivity and cytotoxicity features with RA-associated HLA-DRB alleles and collagens found in the articular tissues. Furthermore, the relatively constant associations of certain autoantibodies such as RFs and ACPAs with RA have been linked to *Proteus* microbes and all these might be involved in the aetiopathogenetic process and development of RA. These extensive results provide a new approach to the treatment of this disease by combining the reduction of the *Proteus* microbial load together with the currently used biologicals.

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