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MOLECULAR GENETIC ANALYSIS OF THE C1-INHIBITOR GENE
IN HEREDITARY ANGIO-OEDEMA

ALLAN RUSSELL McPHADEN

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Medicine at the University of Glasgow

The research described in this thesis was carried out at the Beatson Institute for Cancer Research, Garscube Estate, Bearsden, Glasgow

September 1990

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<td>-------------</td>
</tr>
<tr>
<td>A</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Base pair</td>
</tr>
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<tr>
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<td>Deoxyribonuclease</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Guanine</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
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</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SSC</td>
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</tr>
<tr>
<td>T</td>
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</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-hydroxymethyl propane 1:3 diol</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
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The development of recombinant DNA technology has enabled the molecular genetic basis of a number of inherited diseases to be established. This thesis describes the application of this technology to the study of hereditary Cl-inhibitor deficiency or hereditary angio-oedema (HAE). In addition, the findings of a clinical survey of HAE in Scotland are presented.

Hereditary angio-oedema is an autosomal dominant disease in which there is either a quantitative (Type I HAE) or functional (Type II HAE) deficiency of Cl-inhibitor, this protein being an important inhibitor of complement system activation and an inhibitor of other serine protease dependent plasma mediator systems. In Type I HAE, which constitutes approximately 85% of cases, functionally normal Cl-inhibitor is present in the serum but at low levels. This contrasts with Type II HAE in which there are normal or sometimes elevated levels of serum Cl-inhibitor as measured by immunoassay, though most of this is in the form of a functionally inactive molecule. Clinically Type I and Type II disease cannot be distinguished since both present with recurrent attacks of subcutaneous or submucous oedema.

In this study restriction fragment length polymorphism (RFLP) analysis of the Cl-inhibitor gene was carried out on genomic DNA isolated from 25 normal, unrelated individuals and on genomic DNA samples from 12 Type I HAE kindred and two Type II HAE kindred. A total of 38 restriction endonucleases were used to digest each DNA sample followed by agarose gel electrophoresis and Southern blotting of each sample onto nylon hybridisation membranes. Each digested DNA sample was then probed with a radiolabelled exon 2-8 Cl-inhibitor
cDNA which represents the full protein coding sequence of the Cl-inhibitor gene.

Four of the 12 Type I HAE kindred were shown to have unique disease-specific RFLPs affecting one allele of the Cl-inhibitor gene. Localisation studies clearly demonstrated that the gene mutations responsible for each RFLP affected exon 4, the 3' exon/intron boundary of exon 6, exon 7 and exon 8 of the Cl-inhibitor gene. Family studies showed that each mutation co-segregated with the disease. Mutations affecting exon 6 and exon 8 had not been reported previously in Type I HAE. Both these mutations were small, possibly point mutations and it is highly likely that the mutation at the extreme 3' boundary of exon 6 results in loss of the donor splice site for excision of the sixth intron during RNA processing. The effect of the small exon 8 mutation is less certain. Nucleotide sequence analysis of both these mutated areas is currently being undertaken. This should help to establish their likely effects. The remaining two mutations, which were shown to be a complete exon 4 deletion and a complete exon 7 deletion, had recently been documented in Type I HAE by other investigators. No RFLPs were identified in the two Type II HAE kindred.

Detection of a disease-specific RFLP in 33% of the Type I HAE kindred tested represents a significantly higher detection rate than other published studies in which 16% was the previous maximum. This increase appears to reflect the greater number of restriction enzymes employed since no other methodological differences were apparent. These results suggest that Type I HAE is likely to be due to a multiplicity of gene mutations as is seen in other genetic
diseases including β-thalassaemia, haemophilia A and haemophilia B.

Cl-inhibitor gene RFLPs were generated by the restriction enzymes Kpn I and Hgi AI in the normal population. The Kpn I RFLP had not been reported previously and was shown to be due to a mutation that lay approximately 10kb downstream of the Cl-inhibitor gene. The Hgi AI RFLP had already been observed in normal individuals from North America and arose due to a point mutation within the Cl-inhibitor gene itself. Both RFLPs fulfill criteria which are likely to make them useful as indirect genetic markers of the mutant Cl-inhibitor allele in those HAE families who lack a disease-specific RFLP.

The clinical survey of HAE in Scotland identified a total of 46 patients which represents a disease incidence of approximately one patient per 110,000 of the population. This is the first documented attempt to calculate disease incidence in Scotland. It became evident from the survey that despite the development of more effective treatment regimes in recent years, significant disease-associated morbidity and mortality still existed within the relatively young patient population. This observation provided ample justification for studying the molecular genetics of HAE since an understanding of this aspect of the disease is central to future disease prevention in the form of prenatal diagnosis.
INTRODUCTION

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1.1 Cl-inhibitor deficiency

Hereditary Cl-inhibitor deficiency or hereditary angio-oedema (HAE) is an autosomal dominant disease caused by either a quantitative (Type I HAE) or functional (Type II HAE) deficiency of Cl-inhibitor, an important inhibitor of activation of the complement system of proteins and of other serine protease dependent plasma mediator systems. Although not a common disease its clinical presentation is often characteristic and before the advent of modern drug therapy it was responsible for the deaths of many patients in their early adult years. In addition to the hereditary forms of the disease, acquired Cl-inhibitor deficiency is now a well recognised entity. The autoimmune aetiology of this acquired deficiency state will be described.

It is appropriate to commence this introduction with a concise review of the complement system in order to ensure that the Cl-inhibitor protein is placed in context. Thereafter a detailed description of Cl-inhibitor structure and function will be undertaken, an understanding of which is central to an appreciation of the pathogenesis of Cl-inhibitor deficiency states.

1.2 The complement system

The complement system comprises a group of at least 20 distinct plasma proteins which have a key role to play in host defence against infection and in helping to mediate inflammation (1). These proteins can be divided into four main groups, namely the classical pathway components, the alternative
pathway components, the terminal components and the control proteins (Table 1). Today the interactions that occur between the different components during complement system activation are fairly well defined although the fine details of some of these molecular interactions still remain unresolved.

Complement activation occurs in a cascade fashion similar to that seen in the coagulation and kinin mediator systems. At several points in the activation sequence an inactive precursor molecule or zymogen is activated with the acquisition of proteolytic activity which is specific for its substrate. This substrate is in turn activated ready to carry out its role in the cascade. One of the main consequences of such a cascade system is that stepwise amplification occurs at each activation step since the newly generated proteolytic enzyme is able to activate a number of substrate molecules. For this reason tight control of the cascade is maintained by a number of mechanisms, one of which is the presence of plasma and cell membrane regulatory proteins. Cl-inhibitor is one of the plasma proteins which regulates complement activation. The complement system can be activated by either the classical or alternative pathways. Classical pathway activation usually follows binding of antigen to antibody, whereas alternative pathway activation may occur independently of antibody. The alternative pathway is continuously undergoing low-grade turnover only becoming fully activated in the presence of a suitable activating substance. Phylogenetically the alternative pathway probably represents a more ancient activation pathway which is independent of the specific immune response, namely antibody. For this reason it is able to
<table>
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<tr>
<th>COMPONENT</th>
<th>SERUM CONC. (µg/ml)</th>
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<td><strong>CLASSICAL PATHWAY</strong></td>
<td></td>
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</tr>
<tr>
<td>Clq</td>
<td>75</td>
<td>18(6x3)</td>
</tr>
<tr>
<td>Clr</td>
<td>100</td>
<td>1</td>
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<td>Cls</td>
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<td>430</td>
<td>3</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>150</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
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<tr>
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</tr>
<tr>
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<tr>
<td><strong>CONTROL PROTEINS</strong></td>
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</tr>
<tr>
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<tr>
<td>C4 BINDING PROTEIN (C4bp)</td>
<td>250</td>
<td>6-8</td>
</tr>
<tr>
<td>I (C3b/C4b INACTIVATOR)</td>
<td>50</td>
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</tr>
<tr>
<td>H (β1H GLOBULIN)</td>
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<td>1</td>
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<tr>
<td>S PROTEIN (vitronectin)</td>
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</tr>
<tr>
<td>CARBOXYPEPTIDASE N</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>
respond very quickly to invasion by micro-organisms.

Activation of either pathway results in target-cell bound enzymes that cleave and activate C3 and C5 (C3 convertase and C5 convertase respectively). Subsequent activation of the multimolecular membrane attack complex (MAC) from the terminal complement components C5-9 can then occur. The MAC can act to lyse the target cell or bacteria. In addition, a number of biologically active peptides, not necessary for perpetuating the cascade, are released during the activation sequence. A number of these peptides are active as inflammatory mediators, the relevance of which will become apparent later during the discussion of the pathogenesis of the clinical manifestations of Cl-inhibitor deficiency.

1.2.1 Classical pathway

The classical pathway consists of six components Cl, C4, C2, Cl-inhibitor, C4 binding protein (C4bp) and Factor I (I) which are necessary for the formation and regulation of the classical pathway C3 convertase, C4b2a, and the C5 convertase, C4b2a3b (Figure 1). The initial activation step in vivo is the interaction of Cl with antigen-antibody complexes which contain IgM or IgG antibody (2). Cl itself is a calcium dependent macromolecule comprising three glycoproteins Clq, Clr and Cls as two reversibly interacting subunits Clq and the tetramer Cls-Clr-Clr-Clr (Clr₂Cls₂) (3). It is the Clq moiety which interacts with the immunoglobulin Fc region. Multivalent attachment is required for Clq activation meaning that one cell surface bound molecule of IgM is sufficient for activation whereas two adjacent molecules of IgG are needed (4). Immune complex
FIGURE 1
Activation cascade of the complement system of proteins. For clarity the control proteins have not been included.
interaction with Clq is not the only mechanism of classical pathway activation. A number of other substances such as the lipid A moiety of endotoxin, mitochondrial membranes, certain gram negative bacteria and complexes of C-reactive protein with pneumococcal polysaccharide can also produce Cl activation (5). The relative importance of such activation in vivo is unclear. In addition, Cl activation may occur in the absence of an activator. This process of spontaneous autoactivation is rather inefficient and appears to be prevented by Cl-inhibitor in normal individuals (6).

The Clq subcomponent of Cl binds to an activator and so leads to a conformational change in Clq which in turn allows conversion of theClr and Cls subcomponents to their active forms as serine proteases (7). This conversion involves autocatalytic cleavage of native Clr to active Clr which acts to produce a single proteolytic cleavage in native Cls to generate activated Cls (8,9). Activated Cls, as part of the Cl macromolecule, can then carry out proteolytic cleavage and activation of C4 and C2. C4 is cleaved into C4b and C4a with the small peptide C4a being released as one of the anaphylatoxins (10). Newly generated C4b has two important biological activities. Firstly, it is able to bind covalently to immune complexes or other acceptor molecules on activating surfaces through the formation of an ester or amide bond between C4b and the acceptor molecule. This bond forming ability is extremely labile and is dependent upon the presence in native C4 of an internal thiolester group which becomes accessible on activation (11). Only around 10% of C4 which is converted to C4b will bind in this manner to an activating surface. The remaining 90% will react with the
hydroxyl group of water to form C4bi which is unable to participate in further complement activation. Secondly, newly generated C4b possesses a stable magnesium ion dependent binding site for C2 or, more specifically, the C2b fragment of C2 (12). C2 can therefore become bound to C4b to form the proconvertase C4bC2 which is acted upon by activated Cls cleaving C2 into C2a and C2b (13). Thereafter C2a becomes bound to C4b and it is this C2a fragment which possesses the enzymatic site of the classical pathway C3 convertase, C4b2a (14,15). This C3 convertase is inherently unstable with C2a rapidly decaying off the complex although it can be reconstituted with fresh cleavage of C2 by activated Cl (12). This instability helps prevent the generation of excessive classical pathway C3 convertase activity.

Rigid control of classical pathway activation is maintained by Cl-inhibitor, C4bp and I. A more detailed discussion of the role of Cl-inhibitor will be presented later, however, in simple terms it inhibits activated Clr and Cls. C4bp and I control the availability of C4b and so control assembly of the classical pathway C3 convertase, C4b2a. I achieves this by degrading C4b to C4c and C4d in the presence of its co-factor C4bp which, as its name suggests, binds to C4b (16). Spontaneous decay/dissociation of C4b2a appears to be enhanced by C4bp (17).

1.2.2 Alternative pathway

Six components, namely C3, factor B (B), factor D (D), factor P (P), factor H (H) and factor I (I) are involved in the initiation, generation and maintenance of the alternative pathway C3.
and C5 convertases, C3bBbP and C3bBbC3b respectively. The activation mechanisms are complex and are summarised in Figure 1.

Initiation occurs through low-grade hydrolysis of fluid-phase C3 to give C3(H₂O) (18). In the presence of magnesium ions C3(H₂O) can bind B which is then cleaved by D which is normally present in plasma in its activated form. Ba is released leaving C3(H₂O)Bb as the initial C3 convertase (19). This low efficiency fluid-phase convertase cleaves C3 into C3a, an anaphylatoxin, and metastable C3b, the latter being deposited on and covalently binding to the surfaces of surrounding particles (20). The C3b-dependent positive feedback or amplification loop can now operate (Figure 1) since each newly generated C3b molecule has the potential to form the more efficient C3 convertase, C3bBb, in the presence of B, D and magnesium ions. This can occur both in the fluid-phase and on the surface of nearby particles. Factor P has a stabilising effect on C3bBb since it retards the decay of Bb from C3bBb and hence prolongs the half-life of the alternative pathway C3 convertase (21). Control of alternative pathway amplification is carried out by H and I both in the fluid-phase and on non-activating surfaces. I, in the presence of H as a co-factor, degrades C3b to iC3b which can no longer bind B to generate the C3 convertase (16). In addition, H alone acts to both restrict the formation of, and to accelerate the rate of decay of C3bBb and C3bBbP (22). It should be mentioned at this point that certain widely distributed cell-associated proteins serve a regulatory role in the complement system. Decay acceleration factor (DAF), membrane co-factor protein (MCP) and complement receptor type 1 (CR1) are thought to be important (23). DAF prevents
alternative pathway C3 convertase assembly and mediates convertase
dissociation, an effect which is also observed for the classical
pathway C3 convertase. MCP has co-factor activity for I mediated
degradation of C3b to iC3b. CR1 has both DAF and MCP activity.

Overall it is thought that prevention of C3b deposition on
host cells by the actions of these regulatory proteins stops damage
occurring to host tissues during episodes of complement
activation (23). This tight regulation of alternative pathway
activation is valid for fluid-phase and non-activating surfaces.
However, substances which activate the alternative pathway do so
through the fact that their surfaces provide a sanctuary for the C3b
generated by C3(H₂O)Bb. This surface-bound C3b is protected from the
control proteins H and I but it is still able to bind B and P to
form C3bBbP (24,25). Alternative pathway activators therefore allow
a shift from low-grade fluid-phase activation to efficient
solid-phase turnover. Such activators often have surfaces which are
low in sialic acid (26,27), the net effect of which is to reduce the
number of regulatory protein binding sites on C3b without affecting
factor B binding sites (28). In the future it is likely that
substances other than sialic acid will be found to be important in
distinguishing activating surfaces from non-activators.

1.2.3 Terminal sequence

The terminal sequence is the final common pathway that leads
to completion of the complement activation cascade irrespective of
whether initial activation occurred via the classical or alternative
pathways (Figure 1). One molecule of C5, C6, C7 and C8, plus up to
18 C9 molecules constitute the terminal sequence proteins whose highly ordered interaction results in the formation of the membrane attack complex (MAC), C5b-9. When assembled, this macromolecular complex has a hydrophobic outer layer which allows insertion into biological membranes. A hydrophilic core creates a transmembrane channel through which water and ions can pass. Insertion of sufficient numbers of MACs into a target-cell membrane can lead to the membrane becoming "leaky" with resulting osmotic lysis and cell death. The terminal sequence therefore represents a mechanism by which the complement system can mediate cell injury or can lyse susceptible bacteria.

Activation of the terminal sequence follows formation of C5 convertase activity. This is dependent upon covalent binding of C3b adjacent to cell surface or activating surface bound classical and alternative pathway C3 convertases, C4b2a and C3bBb respectively (29). C3b covalently binds to the C4b component of C4b2a whereas it binds to the C3b moiety of C3bBb. Each of the C5 convertases is therefore designated C4b2a3b and C3bBbC3b. This newly bound C3b acts as a binding site for native C5 and renders it susceptible to cleavage into C5a and C5b by the C2a or Bb moiety of each convertase. In the classical pathway C5 convertase, C4b2a3b, C4b is also involved in the C5 binding site. Each C3 convertase therefore generates the C3b necessary to convert its specificity to a C5 convertase.

Assembly of the membrane attack complex follows C5 cleavage into C5a and C5b. C5a, the third anaphylatoxin, is released leaving C5b which has a metastable binding site for C6. Bimolecular C5b6
remains loosely bound to C3b on the target-cell surface until interaction with C7 occurs. At this time the C5b67 complex undergoes a hydrophilic-amphiphilic transition and inserts itself into lipid membranes in its immediate vicinity (30). The lipid membranes may belong to nucleated cells, erythrocytes, bacteria or viruses and the insertion event commits MAC assembly to a specific site. The inserted C5b67 now acts as a membrane-bound C8 "receptor" by binding C8 and the C5b-8 complex penetrates deeper into the hydrophobic bilayer creating a 30Å diameter transmembrane channel (31). Subsequent binding of C9 occurs and as increasing numbers of C9 molecules are incorporated (C9 polymerisation) the channel structure increases its size up to a 100Å diameter pore in the membrane (32). Significant water and ion fluxes can then occur with possible lysis of the target (33). Interestingly calcium ion flux induced by MAC formation in nucleated cells can produce cell activation rather than cytolysis.

Not surprisingly inhibitors of MAC channel formation exist as a defence measure against bystander host cell lysis at sites of complement activation. Plasma S protein and very low density lipoprotein (VLDL) inhibit the binding of C5b-7 complexes to membranes (34). In addition, S protein prevents C9 polymerisation (35). A number of widely distributed membrane associated proteins including C8 binding protein, 20 kilodalton homologous restriction factor or CD59 also exist and act to inhibit MAC mediated pore formation (36,37,38). Their importance is demonstrated by the fact that deficiency states can lead to inappropriate host cell lysis as occurs in paroxysmal nocturnal
haemoglobinuria (39).

From the foregoing description it is apparent that tight control of the complement activation cascade is maintained at many points, such is the potential biological potency of the system once activated. It is not surprising that defective functioning of one of these control measures, such as occurs in Cl-inhibitor deficiency, has important and potentially detrimental consequences for the host as will be described later. First it is appropriate to briefly outline the biological activities of the complement system to allow a better appreciation of its areas of involvement.

1.2.4 Biological activities of the complement system

A number of biological activities important to host defence are mediated, at least in part, through the complement system. It is now known to be involved in mediating cytolysis (33), increasing vascular permeability (40), promoting vasodilatation (41), inducing oedema formation (42,43), increasing neutrophil adhesiveness (44,45), stimulating inflammatory cell chemotaxis (46,47), mediating opsonisation and enhancing phagocytosis (48,49,50,51), inducing release of toxic oxygen species and lysosomal enzymes from phagocytic cells (52,53,54,55), controlling the handling of immune complexes (56), stimulating leukocytosis (57) and modulating lymphocyte functions (58). The complement components and peptides thought to mediate each process are shown in Table 2. Vasodilatation, increased vascular permeability and oedema formation are of particular relevance in Cl-inhibitor deficiency states. Their possible mediation by complement system cleavage products will be
<table>
<thead>
<tr>
<th>BIOLOGICAL ACTIVITY</th>
<th>COMPONENT(S) INVOLVED</th>
</tr>
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<tbody>
<tr>
<td>Cytolysis</td>
<td>MAC (C5b-9)</td>
</tr>
<tr>
<td>Increased vascular permeability,</td>
<td></td>
</tr>
<tr>
<td>vasodilatation and oedema formation.</td>
<td></td>
</tr>
<tr>
<td>Increased neutrophil adhesiveness</td>
<td>C5a, C5a des arg</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>C5a, C5a des arg, C3a (weak), C4a (very weak)</td>
</tr>
<tr>
<td>Opsonisation and phagocytosis</td>
<td>C3b, iC3b, C4b</td>
</tr>
<tr>
<td>Release of toxic oxygen species and lysosomal enzymes</td>
<td>C5a, C5a des arg, C3b, C3e</td>
</tr>
<tr>
<td>Handling of immune complexes</td>
<td>C3b</td>
</tr>
<tr>
<td>Leukocytosis</td>
<td>C3e, C3d-k</td>
</tr>
<tr>
<td>Modulation of lymphocyte functions</td>
<td>C4a, C3a, C3d-k, C5a, C5a des arg</td>
</tr>
</tbody>
</table>
described later. The vital role played by the complement system in ensuring the biological integrity and indeed survival of an organism is amply demonstrated by complement deficiency states other than hereditary Cl-inhibitor deficiency. Examples of these deficiency states and their major associated diseases (59) are listed in Table 3. Development of these diseases is not surprising when the list of biological activities is considered.

1.3 Cl-inhibitor

1.3.1 Discovery and physico-chemical characterisation

During the 1950s human serum was found to contain a heat-sensitive substance that inhibited the enzymatic activity of activated Cl (60). This substance was called Cl-esterase-inhibitor and was first isolated by Pensky et al in 1961 (61). Independently, but around the same time, Schultze et al described a protein they named alpha-2-neuramino-glycoprotein whose function initially was unknown (62). Subsequent work demonstrated its identity with Cl-esterase-inhibitor and thereafter the two names were used interchangeably (63). Today the name Cl-inhibitor is preferred.

Characterisation of Cl-inhibitor has shown it to be one of the most heavily glycosylated proteins in human plasma with carbohydrate accounting for approximately 35% of its 104-105,000 dalton molecular weight (64). Electrophoretically it is an alpha-2-globulin and structurally it consists of a single polypeptide chain (65). Electron microscopy suggests a two domain structure comprising a
<table>
<thead>
<tr>
<th>DEFICIENT COMPONENT</th>
<th>DISEASE</th>
</tr>
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<tbody>
<tr>
<td>Clq</td>
<td>Immune complex disease including systemic lupus erythematosus (SLE)</td>
</tr>
<tr>
<td>Clr</td>
<td>Immune complex disease including SLE and glomerulonephritis</td>
</tr>
<tr>
<td>Cls</td>
<td>Immune complex disease including SLE</td>
</tr>
<tr>
<td>C2</td>
<td>Immune complex disease including SLE or an SLE-like illness</td>
</tr>
<tr>
<td>C4</td>
<td>Immune complex disease including an SLE-like illness</td>
</tr>
<tr>
<td>C3</td>
<td>Recurrent bacterial infections especially by encapsulated organisms;</td>
</tr>
<tr>
<td></td>
<td>immune complex disease including SLE</td>
</tr>
<tr>
<td>Factor P</td>
<td>Fulminant bacterial infections, particularly Neisserial infections</td>
</tr>
<tr>
<td>Factor D</td>
<td>Recurrent bacterial respiratory tract infections</td>
</tr>
<tr>
<td>Factor I</td>
<td>Recurrent bacterial infections including respiratory tract infections</td>
</tr>
<tr>
<td></td>
<td>and meningitis</td>
</tr>
<tr>
<td>Membrane attack</td>
<td>Recurrent systemic meningococcal or gonococcal bacteraemia</td>
</tr>
<tr>
<td>components C5, C6, C7, C8 or C9</td>
<td></td>
</tr>
<tr>
<td>Factor H</td>
<td>Recurrent meningococcal infections</td>
</tr>
</tbody>
</table>
33nm long, 2nm diameter rod-like domain and a terminal 4nm diameter globular head domain. This structure is entirely compatible with the known low sedimentation coefficient of 3.67S (66).

The carbohydrate composition and pattern of attachment to the polypeptide chain are interesting. There appear to be 20 oligosaccharide units attached to the amino-acid backbone, 17 of these units being attached to the N-terminal region of the polypeptide chain (67). Both O-linked and N-linked glycosylation is present, the former initially being suggested by a relatively high content of N-acetylgalactosamine amongst the carbohydrate moieties and by the relatively high serine/threonine content of the protein. The function of the glycosylation is unclear. Neither the N-linked nor the O-linked oligosaccharide units appear to be necessary for the functional activity of Cl-inhibitor against activated Cls (68,69). This finding is probably not surprising when it is realised that the reactive site of Cl-inhibitor is located at its C-terminal end, well away from the highly glycosylated N-terminus. The possibility that heavy glycosylation was necessary for efficient secretion of the protein after synthesis has been considered, however, experimental evidence derived from the study of Hep G2 cells did not support this idea (70). Since mononuclear phagocytes, fibroblasts and umbilical vein endothelial cells also synthesise Cl-inhibitor (71,72,73) a role for glycosylation in these cell types should also be sought. The activity of deglycosylated Cl-inhibitor against other target proteases has not been investigated.

The entire 478 amino-acid sequence of Cl-inhibitor is now
known along with the sequence of its 12 amino-acid N-terminal signal peptide (67). The reactive site or active centre of the Cl-inhibitor molecule lies close to the carboxy-terminal end as described by Salvesen et al (74). They localised the site by carrying out amino-acid sequence analysis of the four kilodalton carboxy-terminal peptide released from Cl-inhibitor during complex formation with activated Cls. This analysis indicated that the reactive site was centred round the arginine residue at position 444. This was termed the PI residue of the reactive site. The residue adjacent to this arginine on the carboxy-terminal side was threonine 445 and this was called the PI' residue. Based on these initial observations subsequent work has now established the overall mechanism of interaction of Cl-inhibitor with target proteases. The reactive site contains a substrate sequence or bait sequence which is recognised by the substrate binding site of the target protease (75). Guided by the bait sequence the target protease complexes with Cl-inhibitor, recognises the PI arginine residue then cleaves the acyl bond between it and the PI' residue so creating the four kilodalton peptide sequenced by Salvesen. Creation of this peptide also explains the appearance of the new N-terminal threonine residue described by Nilsson (76). Cl-inhibitor is now covalently bound through its reactive site PI residue to the target protease and since Cl-inhibitor has only one reactive site it forms equimolar complexes with its target proteases. Furthermore, the covalent nature of the bond formed between the inhibitor and the protease explains the resistance of the enzyme-inhibitor complex to powerful denaturants (77,78). Protease inhibition is achieved because access
of substrate to the protease is prevented by Cl-inhibitor which is covalently bound to the protease.

It is now universally accepted that Cl-inhibitor can be assigned to the serpin (serine protease inhibitor) group of proteins by virtue both of the properties just described and because of sequence homology with other members of the serpin family. Included in the serpin family are alpha-1-antitrypsin, alpha-1-antichymotrypsin, antithrombin III, mouse contrapsin, heparin cofactor II and endothelial cell plasminogen activator inhibitor (79,80). All of these are plasma protease inhibitors except the last one, which is a cell-associated inhibitor. On the basis of sequence homology angiotensinogen, chicken ovalbumin and barley protein Z are also included in the serpin family despite having no known serine protease inhibitory activity (79,81). The degree of sequence homology which exists between Cl-inhibitor and other serpins is approximately 20-27%, a percentage homology similar to that seen between many other serpin family members (67). The areas of homology between Cl-inhibitor and the other serpins are scattered over 80% of the length of the polypeptide chain although most lie between amino-acid residue 120 and the carboxy-terminus. Discrete stretches of strong homology are interspersed with less homologous stretches. Based on these data it is felt that the genes coding for the serpins, including the Cl-inhibitor gene, are likely to have arisen from a common ancestral gene.

The exact tertiary structure of Cl-inhibitor has not been fully characterised. However, comparison with fellow serpin alpha-1-antitrypsin, whose structure is known, shows similarities
particularly in certain key areas (82). A good example is the β-sheet that forms a large planar surface of serpin molecules including Cl-inhibitor. One strand of this β-sheet is thought to bend back on itself to form a highly stressed loop over the planar surface so connecting the active centre to the carboxy-terminal segment of the molecule. The hinge region at the base of this loop is highly conserved amongst the serpins, including Cl-inhibitor, because maintenance of this stressed loop appears to be critical for serpin function (67). By comparison, those sequences which are least conserved often appear to map to areas on the surface of the molecule away from the stressed loop. It is argued that it is these surface differences that help to define overall protease specificity, absolute specificity being decided by the active centre bait sequence and PI residue. Recent work relating conformational stability of the serpins to alterations in structure during complex formation with their target proteases has shed light on a few previously unexplained observations in serpin biochemistry. As just described, α₁-antitrypsin, α₁-antichymotrypsin, Cl-inhibitor and antithrombin III have been shown to have a stressed loop structure containing the PI residue. On complexing with the target protease the acyl bond between the PI and PI' residues is cleaved thereby inducing a large conformational change in the serpin which results in "wide" separation of the newly created amino-terminus and carboxy-terminus. The overall effect is to relax the stressed loop so increasing the conformational stability of the molecule (83). This seems to explain why resynthesis of the cleaved PI-PI' peptide bond does not occur in vivo with recovery of a
"reactivated" inhibitor. Furthermore, this transition from a strained intact state to a relaxed modified conformation may explain why new antigenic determinants (neoantigens) are exposed on the surface of Cl-inhibitor and alpha-1-antitrypsin following their interaction with a target protease (84,85). It is suggested that exposure of these neoantigens may be important in the recognition of serpin/protease complexes by cell surface receptors involved in their clearance from the circulation (86). This process, which does not seem to be specific for a particular serpin/protease complex, may be dependent upon exposure of a shared serpin sequence which in the native molecule is hidden in the interior but which is externalised ready for use after the inhibitor complexes with its target protease.

In summary, Cl-inhibitor is a protein whose functional activity can be disrupted by small, critically placed alterations in its structure. This fact will be evident when the Cl-inhibitor gene mutations responsible for the dysfunctional Cl-inhibitor molecules that characterise Type II HAE are described.

1.3.2 Biological roles of Cl-inhibitor

Cl-inhibitor is capable of inactivating a number of plasma serine proteases including activated Clr and Cls, plasmin, kallikrein and coagulation factors XIa and XIIa plus the enzymatically active fragments derived from XIIa, XIIf. It is therefore potentially involved in regulating a number of the proteolytic processes which are central to complement activation, coagulation, fibrinolysis and inflammation. The involvement of
Cl-inhibitor in each of these major biological effector systems will be described.

Cl-inhibitor inactivates enzymatically active Clr and Cls, the initial enzymes of the classical pathway of complement activation (87,88,89,90,91). The interaction is through the reactive site of each molecule and it is a typical serpin interaction. Cl-inhibitor is the most important circulating inhibitor of activated Clr and Cls and, until recently, was thought to be the only serpin with significant inhibitory activity against activated Clr and Cls (92). Interestingly, however, a protease inhibitor produced by fibroblasts called protease Nexin I has been shown to have activity against activated Cls (93). Nexin I, now shown to be a serpin family member, has marked structural similarity to Cl-inhibitor over the area of its reactive site, so helping to explain its activity against activated Cls. It has been suggested that because Nexin I is produced by fibroblasts and is at a very low concentration in the plasma, its role is more likely to be one of mediating protease control extravascularly. By comparison, Cl-inhibitor is abundant in the plasma as are its main target proteases and so it is better suited to an intravascular inhibitory role. Nexin I also resembles Cl-inhibitor in that it has demonstrable inhibitory activity against coagulation factor XIIa and kallikrein.

The rate of interaction of Cl-inhibitor with activated Clr and Cls is not identical in that activated Clr reacts more slowly with Cl-inhibitor compared with activated Cls (94). In addition, the rate of interaction with activated Cls is less affected by alterations in temperature, ionic strength or calcium concentration (94,95). The
The exact physiological significance of these observations is not certain.

The foregoing description was based on observations made on the interactions between Cl-inhibitor, activated Clr and Cls as isolated components. In vivo, however, Clr and Cls are found as part of the calcium dependent macromolecular Cl complex along with Clq. It is therefore essential to examine the effects of Cl-inhibitor on macromolecular Cl to ensure that all its biologically relevant actions are appreciated. At this point a more detailed description of the subunit structure of macromolecular Cl is required. This will facilitate an understanding of the proposed interactions between Cl-inhibitor and Cl that are now thought to be central to controlling the two main types of Cl activation, namely immune complex induced activation and spontaneous autoactivation.

Macromolecular Cl comprises a complex of Clq with the tetramer Cls-Clr-Clr-Cl (Clr_s Cls_s). Clr and Cls are now known to have at least two main domains, an interactive and a catalytic domain, each of which is divided into subdomains (96). The interactive subdomains are thought to be responsible for at least three interactive functions - 1) calcium binding; 2) mediating Clr-Cls interaction as part of tetramer formation (Cls-Cls-Cls-Cls); 3) mediating interaction between the Cls-Cls-Cls-Cls tetramer and Clq. The catalytic subdomains or γ-B domains also appear to have at least three functions - 1) mediating the serine protease activity of Clr and Cls; 2) substrate recognition; 3) in the case of Clr mediating monomer-monomer interaction as part of tetramer formation (Cls-Cls-Cls-Cls). It is this Clr monomer-monomer interaction to
form a Clr dimer (-Clr-Clr-) that is now thought to be central to Cl autoactivation (97). Since this interaction is mediated by a θ-B region interaction, binding of Cl-inhibitor to this same region, as will be described presently, could be important for the control of autoactivation.

In summary, there appear to be distinct subdomains in Clr and Cls which mediate tetramer formation, Clq binding, serine protease activity and Cl autoactivation. Cl activation and Cl-inhibitor interaction with the serine protease and autoactivation related domains will now be described.

Two forms of macromolecular Cl activation appear to exist. Firstly, there is the process of rapid, highly efficient activation in the presence of a known activator such as an immune complex. Secondly, there is spontaneous Cl autoactivation which is a slower, less efficient process. The role that Cl-inhibitor plays in modulating the first form of Cl activation is well established. In this situation it covalently interacts with the already activated serine protease domains of Clr and Cls within macromolecular Cl (89). It does not act to prevent Cl activation (98). This covalent interaction is accompanied by dissociation of the activated macromolecular Cl into Clq, which remains bound to the immune complex, and Cl-inhibitor-Clr-Clss complexes (99). For this release reaction to occur the Cl-inhibitor interaction with Clr appears more important than the Cls interaction (90). Measurement of these Cl-inhibitor-Clr-Clss complexes in plasma and other biological fluids has been used by some investigators to quantify classical complement pathway activation (100,101).
Controversy arises when the mechanisms responsible for Cl autoactivation and its control by Cl-inhibitor are considered. The concept of autoactivation is generally accepted but the fine details of the molecular mechanisms involved are unclear. Two main schools of thought exist. On one side there is evidence to support the idea that spontaneous autoactivation of Cl is a rather slow process brought about by thermal motion acting on macromolecular Cl to generate activated Clr (102). The role of Cl-inhibitor is to rapidly and covalently complex with the small amounts of activated Clr generated and so inhibit its enzymatic activity. Spontaneous autoactivation of Cl in this model is therefore kept under tight control in normal individuals by a classical serpin interaction. The alternative theory suggests that the key to spontaneous autoactivation of Cl is the self activating ability of Clr. When present as Clr₂ in macromolecular Cl (ClqClr₂Cls₂), Clr₂ is said to be able to self activate so leading to the generation of activated Cls (97,103). It is suggested that in addition to covalent binding of Cl-inhibitor to already activated Clr, as described in the previous paragraph, there is a second form of interaction which specifically prevents Clr₂ self activation and so prevents Cl autoactivation. This interaction is dependent upon non-covalent binding of Cl-inhibitor to the β-β region of Clr at a point that appears not to involve the Clr serine protease subdomain (104). The net result is prevention of spontaneous autoactivation of Cl, although in this role Cl-inhibitor is not acting as a serpin. It is possible that the heavily glycosylated amino-terminus, an area which is not necessary for its serpin activity, is required for this
interaction. Weak specific binding of Cl-inhibitor to native Cl had been observed previously (105) and had been reported as being mediated by a domain on Cl-inhibitor which was distinct from its active centre. This may represent a similar interaction to the one which is responsible for inhibition of Cl autoactivation. Further work is required to define these interactions fully.

Recently Okada and Utsumi have described another possible \( \beta-B \) region interaction. When an immune complex binds to the Clq subcomponent of native Cl the CH3 domain of the IgG in the immune complex appears to compete with Cl-inhibitor for access to the \( \beta-B \) domains of the Clr dimer present in the same macromolecular Cl unit (106). Prevention of Cl-inhibitor binding to the \( \beta-B \) region allows the Clr dimer to be activated following the immune complex/Clq interaction.

The relevance of the foregoing discussion becomes apparent when clinical Cl-inhibitor deficiency is considered. A characteristic finding is excessive activation of the classical pathway of complement in the absence of known Cl activators. In this situation the importance of uncontrolled spontaneous Cl autoactivation as a result of the Cl-inhibitor deficiency state becomes obvious. It is also relevant that certain small immune complexes which are normally incapable of inducing classical pathway activation can do so in experimental systems in which Cl-inhibitor levels are low (107). Lowering of the threshold for complement activation by these complexes seems to have occurred. Should in vivo correlates for this exist, this could help to explain the apparent lack of detectable, accepted classical pathway activators in
Cl-inhibitor deficiency states. Clearly much still has to be learned about Cl activation and its control both in normal and pathological situations.

1.3.3 Cl-inhibitor involvement in the coagulation, fibrinolytic and kallikrein systems

Cl-inhibitor inactivates a range of serine proteases other than activated Clr andCls. The proteases involved are coagulation factors XIa, XIIa and XIIf, plasmin and kallikrein (108,109,110,111). In vivo the inhibition of factors XIIa, XIIf and kallikrein appears to be of biological significance. Cl-inhibitor seems to inactivate about 90% of XIIa and XIIf generated in vivo with this percentage falling to 40%-50% for kallikrein (112,113,114,115). Alpha-2-macroglobulin inhibits most of the remaining 50%-60% of kallikrein. By comparison, Cl-inhibitor appears to make a very small contribution to the in vivo inactivation of factor XIa and plasmin. Most of the former is inactivated by alpha-1-antitrypsin whereas alpha-2-antiplasmin and alpha-2-macroglobulin inhibit the vast majority of plasmin generated in vivo (116,117). Cl-inhibitor inactivates all these proteases in a manner similar to that for activated Clr and Cls. It is possible that some of these proteases could produce classical pathway activation in Cl-inhibitor deficiency states. Plasmin is, however, the only protease for which there is definite experimental evidence of an ability to activate Cl (118).

In summary, since evidence exists for activation of both the coagulation and kinin systems in Cl-inhibitor deficiency states, it
is possible that such activation may contribute to the clinical and biochemical manifestations of the disease.

Overall the foregoing description has been an attempt to highlight those areas of Cl-inhibitor structure and function which are felt to be relevant to a discussion of disease induced by Cl-inhibitor deficiency. The remainder of this introduction will now focus on Cl-inhibitor deficiency with particular emphasis being placed on the hereditary forms of the disease. Included will be a description of clinical aspects of hereditary Cl-inhibitor deficiency followed by the molecular pathology and molecular genetics of the disease.

1.4 Hereditary Cl-inhibitor deficiency

1.4.1 Historical aspects

The first description of this disorder was written by Quincke in 1882 but it was Osler in 1888 who first produced a detailed account of the disease (119,120). A later description of the disorder as it affected five generations of one family placed it in a hereditary disease category (121). The name hereditary angioneurotic oedema was widely used for the disease as a result of the observation that emotional stress could precipitate symptomatic attacks (122). Today hereditary Cl-inhibitor deficiency or hereditary angio-oedema (HAE) is preferred. Donaldson and Evans (123) are credited with first identifying that a deficiency of
plasma Cl-inhibitor was the cause of HAE although in the year prior to their description Landerman et al (124) noted a decrease in the serum levels of an inhibitor of kallikrein in patients with HAE. Hindsight shows that Landerman was describing a deficiency of Cl-inhibitor.

1.4.2 Clinical features of HAE

Such was the thoroughness of his report that nearly all the major clinical characteristics of HAE were described by Osler in 1888 (120). The disease is characterised by recurrent attacks of acute, circumscribed, non-inflammatory subcutaneous and submucosal oedema (125). Anatomically the face, limb extremities, genitalia, upper respiratory tract and gastro-intestinal tract are affected most commonly by the oedema which is classically non-pitting, non-pruritic and is not accompanied by marked erythema or increased temperature in the affected area. The symptoms experienced by a patient during an attack are dependent on the main sites affected. The subcutaneous oedema, which can be readily observed, usually starts from a single site and spreads outwards to a variable degree until, in severe cases, it may affect a whole limb or the entire face. A feeling of tightness or tingling may precede the appearance of the oedema by a few hours but subsequently the usual time course followed is an increase in the degree and extent of oedema for up to 48 hours then gradual reduction over the next 48 or 72 hours. A similar time course is followed by oedema at other anatomical sites. Children in particular can develop erythematous mottling or, less commonly, an appearance similar to erythema marginatum or erythema
multiforme either with or without typical attacks of subcutaneous oedema. It seems that the skin manifestations of HAE are more common in patients before puberty, involvement of other sites, with the possible exception of the gastro-intestinal system, being seen more frequently thereafter.

Involvement of the upper respiratory tract, particularly laryngeal oedema, represents the most life-threatening and often the most acutely distressing clinical manifestation of HAE. Death by asphyxiation due to laryngeal oedema is the only recorded fatal complication of the disease (126).

Gastro-intestinal involvement can occur anywhere between mouth and anal canal producing a wide range of symptoms. Abdominal pain represents by far the most common symptom, the pain typically being an acute cramping or colicky pain superimposed on a persistent dull ache (127). In addition, anorexia, nausea, vomiting, abdominal distension and watery diarrhoea are all well recognised symptoms. Abdominal symptoms may occur without any sign of skin involvement and may even precede the onset of cutaneous signs by a number of years. Abdominal pain may lead to unnecessary surgery with the true diagnosis only becoming apparent later. Compared with involvement of the preceding systems, oedema of the genito-urinary system, pulmonary oedema and even localised cerebral oedema are extremely rare events.

Symptomatic attacks of angio-oedema leading to diagnosis often commence during childhood, usually before the onset of puberty (128). Exceptions do exist in that symptoms and signs have been recorded as commencing during infancy and even as late as the
sixth decade of life in a few individuals. However, for most patients with a long history of the disease, the fifth and sixth decades herald a welcome reduction in both the severity of symptoms and the frequency of attacks. A number of years often elapse between the appearance of symptoms and definitive diagnosis due to a combination of factors including mild symptomatology, mild or no initial cutaneous involvement and a high medical threshold for diagnosis. Even a sudden asphyxial death may be recorded in a family without the diagnosis being made.

An important consideration in HAE is the nature of the trigger which precipitates each acute attack. An identifiable trigger is noted by approximately 50% of patients with the commonest trigger being tissue trauma to either a limb or to the oropharynx (126,128,129). Tooth extraction and tonsillectomy are particularly notorious for inducing laryngeal swelling. Included in a long list of other precipitating factors are infection, emotional stress, prolonged pressure or vibration, over-exposure to sunlight, menstruation, early pregnancy and the oestrogen containing oral contraceptive pill (130). Interestingly the second and third trimesters of pregnancy often induce a reversal of the increased frequency of attacks seen in early pregnancy. The basis for this appears to be the absolute increase in the amount of circulating Cl-inhibitor that occurs later in pregnancy due to increased blood volume, although the serum Cl-inhibitor concentration is decreased.
1.4.3 Abnormalities in the complement, coagulation and fibrinolytic systems in HAE

An abnormal complement profile is seen in HAE. Cl-inhibitor deficiency allows excessive Cl activation and subsequent consumption of its substrates C4 and C2 with resulting diminished serum concentrations of both proteins (130). C4 levels are commonly low even during periods of disease remission. During periods of active disease C4 frequently falls to very low levels and activated Cl can be detected in the serum. Since C4 and C2 consumption is in the fluid-phase little C3 convertase activity is generated and C3 levels remain within the normal range although an increased rate of C3 turnover has been observed (131). As already described one of the central problems in the pathogenesis of HAE is the nature of the mechanisms responsible for Cl activation. It is likely that in most circumstances immune complex induced activation is not important. Uncontrolled autoactivation of Cl requires serious consideration in HAE. At 37°C HAE plasma shows spontaneous activation of the complement system at a rate which is inversely proportional to the Cl-inhibitor level (132). In addition, normal plasma partially depleted of Cl-inhibitor shows significant spontaneous Cl autoactivation when the Cl-inhibitor concentration is around 25% of normal, a concentration which is close to that found in the plasma of many HAE patients. It seems that increased autoactivation of Cl in situations of Cl-inhibitor deficiency is a very significant finding.

A further source of activated Cl in HAE may be as a result of interactions between components of the coagulation and fibrinolytic
cascades and Cl. Cl-inhibitor is thought to be physiologically important for the inactivation of activated factor XII (XIIa and XIIf) and kallikrein. Since it is known that activated factor XII and kallikrein are able to generate plasmin from plasminogen and that plasmin can activate Cl, the amount of activated Cl generated from such a source is likely to increase in a Cl-inhibitor deficiency state (118,133,134,135,136). The demonstration of kallikrein and activated factor XII in HAE plasma lends support to this theory (137,138). Furthermore, tissue trauma, which commonly induces attacks of angio-oedema in HAE patients, is known to result in factor XII activation (139). Overall, therefore, evidence exists to support the involvement of the coagulation, fibrinolytic and kallikrein systems in generating activated Cl. Also of relevance is the recent description of a C2-like plasma protein which can be cleaved by kallikrein to generate small peptides which may be involved in the mediation of the physical signs and symptoms of HAE. This protein is described in Section 1.4.4.

1.4.4 Mediation of symptoms in HAE

Characterisation of the mediator or mediators which induce the tissue oedema in HAE has raised much controversy and recently has come full circle. The histopathological changes seen in affected tissues from patients with HAE consist of marked interstitial oedema and venular dilatation. No significant inflammatory cell infiltrate is present. The main conclusion drawn from these observations was that conventional inflammatory mediators were unlikely to be responsible for the tissue changes. Initial lines of investigation
identified and partially purified a small, heat-stable kinin-like peptide from the plasma of HAE patients (140). This peptide had the ability to increase vascular permeability when injected into guinea pig skin as well as being able to induce smooth muscle contraction and was therefore suspected of being bradykinin. Further investigation however showed that compared with bradykinin it differed in size, electrophoretic mobility, isoelectric point and trypsin susceptibility (141). Furthermore, the biological effects of bradykinin following subcutaneous injection include pain and erythematous swelling, features not seen in patients with HAE (125). Attention now focussed on the complement system itself as the source of the peptide for the following reasons. Firstly, C4 and C2 appeared necessary for peptide generation in that its appearance in HAE serum could be inhibited by antibodies against C4 and C2 (142). Secondly, intradermal injection of activated Cls into normal individuals induced local, pain-free, non-pruritic swelling whose macroscopic and microscopic appearance closely resembled the angio-oedema seen in HAE patients (143). Thirdly, the active enzymatic site of Cls was required for generating the peptide. Fourthly, C2 deficient individuals failed to respond to intradermal injection of activated Cls whereas a C3 deficient patient responded normally (144,145,146). These results were confirmed using an animal model in which C2 deficient guinea pigs did not respond to intradermal injection of activated Cls but responsiveness developed after partial restoration of their C2 levels with purified C2 (147). These data suggest that C2 is necessary for peptide generation but it may not be the source of the peptide. Evidence which suggested that the
A kinin-like peptide was derived from C2 came from a study in which activated Cls, C2 and plasmin were incubated together (148). This resulted in a small peptide which increased vascular permeability and produced smooth muscle contraction being released from the carboxy-terminus of C2b. The observations of Curd et al (149), Fields et al (150) and Smith and Kerr (151), however, did not support the generation of kinin activity from C2. For example, Smith and Kerr (151) incubated large amounts of C2 with trypsin, activated Cls, plasmin and kallikrein but failed to show any kinin generation. Overall, these groups of workers were more in favour of bradykinin being the mediator of tissue oedema in HAE. It was argued that Cl-inhibitor deficiency allowed kallikrein to act unhindered on high molecular weight kininogen to produce bradykinin and so tissue swelling. Recently, however, Rosen et al have demonstrated generation of a small set of peptides from the carboxy-terminus of C2b using activated Cls and plasmin in combination (152). They used these peptides along with a 25 amino-acid long synthetic peptide corresponding to the carboxy-terminus of C2b to cause rat smooth muscle contraction and to increase human vascular permeability with oedema formation following their intradermal injection. Finally, the most recent potential source of a mediator of tissue changes in HAE is the newly described C2-like plasma sialoglycoprotein sgp 120 (153). It has been shown that kallikrein can cleave this protein to generate a small peptide which causes capillary leakage and tissue oedema in guinea pigs (154). Cl-inhibitor has no direct inhibitory effect on this active peptide, however, it does inhibit kallikrein which is necessary for generation of the peptide from sgp 120. This
possibly represents an important pathogenetic interaction between the complement system and the kinin generating/contact activation system in HAE. Further work is required to establish the importance of sgp 120 in HAE.

In summary, although the pathogenesis of HAE is still not understood current evidence indicates that the complement, coagulation, fibrinolytic and kinin systems may all be contributing either directly or indirectly to producing the tissue changes seen during acute attacks of the disease. This complexity of interactions reflects the diverse actions of Cl-inhibitor. Sgp 120 may be shown to be of great importance.

1.4.5 Acquired Cl-inhibitor deficiency

This form of Cl-inhibitor deficiency is less common than HAE (155,156). It is important to be aware of the diagnosis since the clinical presentation is usually identical to HAE. The complement profile is also very similar in that serum Cl-inhibitor, C2 and C4 levels are low with C3 levels being normal. Unlike HAE, however, serum Cl levels are often very low due to Cl consumption. The aetiology of acquired Cl-inhibitor deficiency is interesting. There appear to be two mechanisms which can give rise to the disease, both of which are auto-antibody mediated. Firstly, in those cases where the disease is associated with a malignant B cell lymphoproliferative disorder an auto-anti-idiotype antibody is produced (155). This antibody is directed against the monoclonal immunoglobulin expressed on the surface of the malignant B cells and leads to idio type/anti-idio type immune complex formation, Cl
activation and Cl-inhibitor consumption. Secondly, there is a group of patients where no B cell malignancy is present but auto-antibodies are found (156). These antibodies are directed against Cl-inhibitor itself and act to inhibit its function by binding to the inhibitor and possibly affecting its structure. Furthermore, binding of the auto-antibody to Cl-inhibitor renders the inhibitor more susceptible to proteolytic cleavage and so inactivation by its normal substrates including activated Clr, activated Cls and plasmin. The net effect of both mechanisms is to produce Cl-inhibitor deficiency.

1.4.6 Treatment of HAE

It was only after the biochemical basis of HAE was established that treatment regimes became less empirical and more targeted. Today treatment can be categorised under two main headings: 1) prophylaxis and 2) treatment of an acute attack (130). 1) Prophylaxis. This can be divided into long term and short term prophylaxis both of which are achieved by the same therapeutic agents. Short term prophylaxis is reserved for patients who normally experience minimal disease activity and so do not require long term prophylaxis. Nevertheless these patients may develop acute attacks of laryngeal oedema when they undergo a surgical procedure, particularly dental extraction or operations requiring general anaesthesia with endotracheal intubation.

The two main groups of agents used in prophylaxis are attenuated androgenic steroids and anti-fibrinolytic drugs. It was Spaulding in 1960 who first demonstrated the efficacy of oral
methyltestosterone in preventing attacks of HAE (157). This drug was used initially due to the misconception that it might be effective because of its anti-histaminic properties. Anti-histamines are however ineffective in HAE despite the presence of histaminuria (158). Methyltestosterone was effective because it increased the circulating levels of Cl-inhibitor and relieved the deficiency state (159). The major problem with methyltestosterone and some of its derivatives was their marked masculinising side-effects (130). It was Gelfand et al who demonstrated the beneficial effect of danazol in HAE in 1976 (160). Danazol is a derivative of ethinyltestosterone with weak androgenic properties but is effective at preventing attacks of angio-oedema by increasing circulating levels of Cl-inhibitor. The benefits of methyltestosterone were seen but there was a marked reduction in the incidence of the undesirable side-effects. 17-alpha-alkylation of the steroid is necessary for activity in HAE since non 17-alpha-alkylated steroids have no therapeutic effect (161). Today a second attenuated androgen, stanazolol, is also available for the treatment of HAE (162). After starting treatment with danazol or stanazolol, Cl-inhibitor levels start to rise within a few days, a maximal effect being seen after 7-14 days although Cl-inhibitor levels rarely enter the normal range (163). Interestingly clinical improvement is not dependent upon achieving maximal Cl-inhibitor levels (164). Indeed, a beneficial effect is often apparent before any measurable increase in serum Cl-inhibitor levels is seen.

Anti-fibrinolytic agents comprise the second group of drugs used prophylactically in HAE. In general they are used less
frequently today than the attenuated androgenic steroids with some individuals showing a relatively poor response to them. Fortunately such individuals often respond well to steroids. The two agents that can be used are epsilon-aminocaproic acid (EACA) and its analogue tranexamic acid which is more potent than EACA in treating HAE (165,166,167). Their mechanism of action is thought to be twofold. Firstly, Cl activation appears to be reduced due to drug-induced inhibition of plasmin which can then no longer activate Cl. Secondly, at higher concentrations EACA seems to be directly inhibitory to Cl activation (130). Today EACA is not used in HAE because the high dosages required put patients at risk of developing drug-induced myopathy.

2) Treatment of acute attacks. The mainstay of treatment in this situation is Cl-inhibitor replacement therapy either as Cl-inhibitor concentrate or as fresh frozen plasma (168). When available, Cl-inhibitor concentrate is to be preferred, however it is expensive, it can be difficult to obtain and there is the danger of it containing the human immunodeficiency virus or hepatitis B virus. Securing the airway if threatened by laryngeal oedema and symptomatic relief such as analgesia are other important aspects of treating acute attacks (130).

In summary, current treatment regimes are more effective at controlling HAE in many patients. It is now less common for an asphyxial death to be encountered in HAE compared with a generation before when approximately one third of all patients died of the disease in their early adult years. It should be remembered, however, that modern treatment is not without its problems and no
cure is achieved. The only hope of cure lies in an analysis of the molecular pathology and molecular genetics of the disease.

1.4.7 Molecular pathology of HAE

HAE is an autosomal dominant disease which can be divided into two types (169). Type I HAE, which comprises approximately 85% of cases, is characterised by reduced levels of circulating Cl-inhibitor, the levels during remission ranging from 5% to 30% of normal in most cases. The Cl-inhibitor protein that is present is functionally normal. This contrasts with Type II HAE in which there is normal or elevated antigenic levels of circulating Cl-inhibitor. However, the majority of this Cl-inhibitor is dysfunctional with only small amounts of normal Cl-inhibitor being present. Clinically the two disease types are indistinguishable. A consequence of the normal serum levels of Cl-inhibitor in Type II HAE is that antigenic screening for Cl-inhibitor deficiency will often fail to detect Type II disease. Assessment of Cl-inhibitor functional activity is required to make this diagnosis (170).

A level of functionally normal Cl-inhibitor of 30% or below in Type I and Type II HAE is an interesting finding considering that both forms of the disease have an autosomal dominant mode of inheritance. With this mode of inheritance HAE patients must be heterozygous for the gene defect and so a normal gene must be present. In this situation the expectation would be that under the influence of the normal gene circulating Cl-inhibitor levels should be close to 50% of normal. In Type I HAE it was initially proposed that a defect in Cl-inhibitor gene regulation rather than an
intrinsic structural gene abnormality would explain these findings. By comparison, in Type II HAE, since both a normal and a mutant Cl-inhibitor protein were present, it was more likely that a structural gene defect was responsible for this form of the disease. It is now accepted that the reason for the reduction below 50% in circulating normal Cl-inhibitor levels in both forms of the disease is consumption of normal Cl-inhibitor through its interaction with susceptible proteases (171,172). The single normal Cl-inhibitor allele is not able to sustain a production rate sufficient to maintain circulating Cl-inhibitor levels at 50%. Analysis of the catabolic rate of Cl-inhibitor in HAE patients is consistent with this concept (173). Further supportive evidence was derived from in vitro studies of Cl-inhibitor synthesis that had been carried out using mononuclear phagocytes and fibroblasts isolated from patients with Type I HAE (72,174). Cl-inhibitor synthesis rates as assessed by intracellular and extracellular Cl-inhibitor concentrations and Cl-inhibitor mRNA abundancies in these studies were found to be 40% to 50% of normal.

In Type II HAE many dysfunctional Cl-inhibitor proteins have been studied. In the investigations carried out by Donaldson and her colleagues the dysfunctional protein from eight different kindred appeared to show heterogeneity in both structure and function of the mutant proteins when their electrophoretic properties and their inhibitory capabilities were analysed (175). When the eight different dysfunctional proteins were used in functional assays against the substrates activated Cls, plasma kallikrein, activated forms of factor XII and plasmin, each Cl-inhibitor mutant showed a
unique spectrum of activity (169,176). Each protein blocked the activities of the enzymes tested to a different degree, most showing lesser degrees of inhibition compared with normal Cl-inhibitor. Limited structural analysis which has been carried out on a number of mutant Cl-inhibitor proteins also suggests a degree of structural heterogeneity (177,178,179). This degree of heterogeneity at a structural and functional level has been interpreted by Donaldson as indicating likely heterogeneity of Cl-inhibitor gene mutations causing Type II HAE. This has not been confirmed by molecular genetic studies.

1.4.8 Molecular genetics of HAE

The logical investigative progression in HAE based on the foregoing data was to identify and define the Cl-inhibitor gene mutations responsible for the two hereditary forms of the disease. This was not possible until the relatively recent isolation of a number of complementary DNA (cDNA) clones for human Cl-inhibitor including an exon 2-8 Cl-inhibitor cDNA which represents the full protein coding sequence (67,180,181,182,183). In addition, the normal Cl-inhibitor gene was cloned and was shown to comprise eight exons and seven introns (Figure 2) covering a total of approximately 17 kilobases of DNA on chromosome 11 (67,180,183). As described in the previous section, evidence existed to suggest that there may be more than one structural gene mutation responsible for Type II HAE. In Type I HAE, although a similar state of affairs was suspected, there was less certainty about the outcome of structural gene analysis. Currently the identification and characterisation of the
FIGURE 2
Structure of the normal Cl-inhibitor gene. There are eight exons shown as rectangles and seven introns shown as a line connecting the exons and marked IVS (for intervening sequence). The gene is approximately 17kb in length although the 5' boundary of the gene and of the first exon has not yet been fully characterised. The position of the sequence coding for the reactive site is marked in exon 8.
molecular genetic lesions responsible for both Type I and Type II HAE are being undertaken in a number of laboratories worldwide. Already certain facts have been established.

In Type II HAE Cl-inhibitor gene mutations affecting the reactive site of the molecule have been characterised. Most of these mutations change the Pl residue of the reactive site, commonly producing an arginine (CGC) to cysteine (TGC) or arginine to histidine (CAC) substitution (184,185). From a total of 14 Type II patients examined, nine (approximately 70%) have been shown to have one of these Pl mutations (185). Curiously the four other amino-acids which could be expected from point mutations in this area have not been seen. The hypermutability of this area is most probably explained by the presence of a CpG dinucleotide in the normal codon for the Pl arginine residue (186). Confirmation of these amino-acid changes derived from nucleotide sequence data was obtained by limited proteolysis of dysfunctional Cl-inhibitor proteins and amino-acid sequencing. Aulak et al (185) noted that these Pl mutations would render the peptide bond between the Pl residue and the Pl' threonine residue insensitive to trypsin cleavage. However, the peptide bond sensitive to an elastase derived from Pseudomonas aeruginosa lying three residues upstream from the Pl residue would be unaffected by the mutation. If a Pl mutated protein was digested with this elastase and the released C-terminal peptide subjected to N-terminal amino-acid sequencing, the nature of the mutated Pl residue could be determined.

Mutation of the Pl reactive site residue is not a surprising finding in Type II HAE. It was already known that some dysfunctional
Cl-inhibitor proteins had either negligible anti-protease activity or else had activity that was significantly different from normal Cl-inhibitor. A mutated reactive site in these proteins helps to explain these observations. However, an as yet unresolved problem exists. This is exemplified by two of the mutant dysfunctional proteins which had been reported as having markedly differing enzyme inhibitory profiles but have been shown recently to have identical PI mutations. Either this mutation is not the sole cause of the enzyme inhibitory profile and a second gene mutation exists or, alternatively, the inhibitory profile differences are purely in vitro phenomena. This latter possibility could have been produced by contaminating proteases or by contaminating normal Cl-inhibitor protein in the dysfunctional protein preparations (169). Based on current evidence this latter possibility seems more likely.

In six of the 14 dysfunctional Cl-inhibitor genes and proteins examined no PI residue mutation was identified. It therefore seems that mutations elsewhere in the gene can be responsible for Type II HAE. To date one other mutated site has been identified in Type II HAE. This is a G to A substitution within the eighth exon that alters amino-acid 436 from alanine to threonine (187). This altered amino-acid lies eight amino-acids N-terminal to the PI residue in the P9 position. It is thought that this position contributes to the maintenance of the stressed conformation of the active centre which is required for normal functioning of the Cl-inhibitor protein. Overall it is likely that in Type II HAE a limited number of critically placed mutations will be found to be responsible for the disease.
The molecular genetic basis of Type I HAE, as it is currently understood, comprises two main categories of Cl-inhibitor gene mutation. Firstly, Stoppa-Lyonnet et al demonstrated deletion/insertion events in the 5' half of the gene in approximately 16% of their Type I HAE patients (188,189). The mutations described were mainly exon 4 deletions but deletion of the third exon and exon 4 duplication have also been observed. The second type of mutation reported by Ariga et al affected the 3' end of the Cl-inhibitor gene and took the form of an exon 7 deletion (190). The observation of a shortened Cl-inhibitor mRNA species was the initial clue which led to characterisation of this gene mutation. Both forms of mutation were shown to co-segregate absolutely with Type I HAE in affected families. It is now thought that these two forms of gene abnormality are generated by a very similar molecular mechanism. Sequence analysis of the normal Cl-inhibitor gene has revealed the presence of tandemly arranged clusters of ALU repeat sequences in the third and fourth introns which flank the fourth exon and in the sixth and seventh introns which flank the seventh exon (191). It is proposed that these ALU sequences allow non-homologous crossing over at meiosis and so lead to deletion/insertion events centred round the fourth and seventh exons. Precedents for such an event are recognised in familial hypercholesterolaemia where duplication of the 5' end of the low density lipoprotein receptor gene occurred following unequal crossing over between intronic ALU repeat sequences (192). In Tay-Sachs disease a partial deletion of the β-hexosaminidase gene due to ALU repeat sequences has also been described (193). Interestingly
Stoppa-Lyonnet has not observed an exon 7 deletion and Ariga has never detected a 5' deletion/insertion event in their patient populations. The explanation for this is unclear but ethnic differences in the two populations studied have been considered significant in this regard. No methodological differences could account for it. Characterisation of the gene lesions responsible for HAE heralds the possibility of prenatal diagnosis for the disease.

In summary, the molecular genetic abnormalities responsible for Type I and Type II HAE are gradually being characterised. Further work is needed in order to establish the full spectrum of Cl-inhibitor gene mutations that cause HAE.
1.5 Aims of the study

1) Identify Cl-inhibitor gene restriction fragment length polymorphisms (RFLPs) in the normal population.
2) Identify Cl-inhibitor gene RFLPs in families with Type I HAE and Type II HAE.
3) Localise and characterise the genomic mutations responsible for the RFLPs.
4) Establish whether these mutations co-segregate with HAE and therefore whether they could be used as disease markers.
5) Establish the technique of genomic library construction using a bacteriophage vector in preparation for isolating and nucleotide sequencing mutated Cl-inhibitor genes.
6) Carry out a clinical survey of HAE in Scotland in order to establish disease incidence, symptom profiles, treatment regimes, disease-related morbidity and mortality and finally to establish the level of interest in prenatal diagnosis within HAE families.

At the outset of this investigation there were no published data on the nucleotide sequence of the normal Cl-inhibitor gene nor was there any information on the nature of Cl-inhibitor gene mutations in HAE. The availability of a number of Cl-inhibitor cDNAs, including the exon 2-8 cDNA, made RFLP analysis a logical initial investigative step for the following reasons - 1) It was relatively simple to carry out. 2) It would allow rapid analysis of the normal gene and its flanking sequences. 3) It had the potential
to identify and permit localisation of disease-specific gene mutations in a number of HAE families.
### MATERIALS AND METHODS

#### MATERIALS AND METHODS INDEX

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2.1 Materials

2.1.1 Chemicals

Agarose (ultra pure)  
Alpha-32P-dCTP (PB10205)  
Ammonium acetate  
Ammonium chloride  
Ampicillin  
Adenosine 5’-triphosphate (ATP)  
Bacteriophage DNA (lambda wild type)  
Bactoagar  
Bactotryptone  
Bactoyeast extract  
Biogel A-1.5 (100-200 mesh)  
Boric acid  
Bowtowel 40 (white)  
Bovine serum albumin  
Bromophenol blue  
Butan-2-ol  
Caesium chloride (ultra pure)  
Calcium chloride  
Chloroform  
Collodion bags  
Dextran blue  
Dextran sulphate  
Diethyl ether  
Disodium hydrogen orthophosphate  

GIBCO-BRL Ltd.  
Amersham International PLC.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
Sigma Chemical Company  
Sigma Chemical Company  
Northumbria Biologicals Ltd.  
Difco Laboratories  
Difco Laboratories  
Difco Laboratories  
Bio-rad Laboratories  
BDH Chemicals Ltd.  
Bowater Scott Ltd.  
Sigma Chemical Company  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
GIBCO-BRL Ltd.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
Sartorius Ltd.  
Pharmacia Chemicals  
Sigma Chemical Company  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.
Dithiothreitol
Ecosint A
EMBL 3 cloning kit
Ethanol (95%)
Ethidium bromide
Ethylene diamine
tetra-acetic acid (EDTA)
Ficoll 400
Filter paper (3MM)
Formamide
Gelatin powder
Gigapack Gold Bacteriophage
Packaging Kit
Glucose
Glycerol
Heparin sodium
Hybond-N hybridisation
membranes
Hydrochloric acid
(concentrated)
Hydroxyquinoline
Kodak X-Omat AR X-ray film
Magnesium chloride
Magnesium sulphate
Maltose
Millex-GS filter units
Phenol crystals
Phenol red
Phosphate buffered saline
tablets

Sigma Chemical Company
National Diagnostics
Stratagene
BDH Chemicals Ltd.
Sigma Chemical Company
BDH Chemicals Ltd.
Sigma Chemical Company
Whatman
Fluka Garantie Ltd.
BDH Chemicals Ltd.
Stratagene
BDH Chemicals Ltd.
BDH Chemicals Ltd.
Leo Laboratories Ltd.
Amersham International PLC.
BDH Chemicals Ltd.
Sigma Chemical Company
Kodak (U.K.) Ltd.
BDH Chemicals Ltd.
BDH Chemicals Ltd.
Sigma Chemical Company
Millipore
Koch Light Ltd.
Sigma Chemical Company
Oxoid Ltd.
Pipette tips (yellow and blue)  
Polaroid type 57 high speed film  
Polyadenylic acid  
Polycytidylic acid  
Polyvinylpyrrolidone 360  
Potassium acetate  
Potassium chloride  
Potassium hydrogen carbonate  
Random Priming Kit  
Salmon sperm DNA  
Screw cap polypropylene microtubes (1.5 ml)  
Seaplaque agarose  
Sodium acetate  
Sodium chloride  
Sodium citrate  
Sodium dihydrogen orthophosphate  
Sodium dodecyl sulphate (SDS)  
Sodium hydroxide pellets  
Sodium lauroyl sarcosine (sarcosine)  
Spermidine  
Sucrose (ultra pure)  
TRIS-base  
TRIS-HCL  

Costar  
Polaroid Ltd.  
Sigma Chemical Company  
Sigma Chemical Company  
Sigma Chemical Company  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
Boehringer Mannheim Corp.  
Sigma Chemical Company  
Sarstedt Ltd.  
FMC Bioproducts  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
BDH Chemicals Ltd.  
Sigma Chemical Company  
Sigma Chemical Company  
Schwartz/Mann Biotech.  
Boehringer Mannheim Corp.  
Sigma Chemical Company
2.1.2 Enzymes

Calf intestinal alkaline phosphatase
Klenow fragment of *E. coli* DNA polymerase 1
Lysozyme
Proteinase K
Restriction endonucleases
RNase A
RNase T1
T4 DNA ligase

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<th>Enzyme</th>
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<tr>
<td>phosphatase</td>
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<tr>
<td>Klenow fragment of <em>E. coli</em></td>
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<td>DNA polymerase 1</td>
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<tr>
<td>RNase T1</td>
<td>Sigma Chemical Company</td>
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<tr>
<td>T4 DNA ligase</td>
<td>Boehringer Mannheim Corp.</td>
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2.1.3 Recombinant

Exon 2-8 Cl-inhibitor cDNA (pCl1)

This cDNA cloned into the Bam HI site of pUC 13 was a generous gift from Dr. S.C. Bock, Rockefeller University, New York. The cDNA is 1.8 kilobases (kb) in length and represents the full protein coding sequence of the Cl-inhibitor gene (67). Characterisation of this recombinant molecule is described in Section 3.1.

2.1.4 Control and patient blood samples

Blood samples from 25 unrelated control individuals were obtained during the course of blood donation to the Scottish Blood Transfusion Service. During the normal processing of a donated unit
of whole blood a leukocyte rich "buffy coat" was prepared and was usually discarded. This "buffy coat" was used as a source of high molecular weight DNA from these individuals. All the controls were healthy Caucasians between the ages of 18 and 60 years. The DNA from each of these 25 samples was used to establish whether Cl-inhibitor gene RFLPs were present in the normal population for the restriction endonucleases employed.

Blood samples from 12 families with Type I HAE and from two families with Type II HAE were collected. The purpose of the study was explained to each individual and consent obtained before venesection was carried out. A total of 45 individuals from the 12 Type I HAE families were venesected. Serum Cl-inhibitor and C4 levels were measured for each individual by Mrs. J. Veitch, Department of Pathology, Western Infirmary, Glasgow. This showed that of the 45 individuals venesected 26 were Type I HAE patients. A more detailed description of each RFLP positive Type I HAE family will be given in Section 3.5. A total of four blood samples were obtained from two Type II HAE families. Measurement by Mrs. J. Veitch of serum Cl-inhibitor and C4 levels in addition to Cl-inhibitor functional activity in these samples showed that in one family the samples were from two affected and one normal family member and that the single sample from the second family was from a Type II HAE patient, no sample being available from a normal family member. Initially DNA samples from one affected family member from each Type I and Type II HAE kindred were screened for Cl-inhibitor gene RFLPs. When an abnormality was found DNA samples from all available normal and affected family members were then examined in
order to establish whether the RFLP co-segregated with the disease in that family.

2.2 Methods

2.2.1 Collection of blood samples and preparation of peripheral blood leukocytes

Those blood samples collected from HAE family members were placed in sterile tubes and anticoagulated with sodium heparin at a concentration of approximately 50 international units of heparin per millilitre of blood. The "buffy coats" were received in a sterile bag from the Blood Transfusion Service having been anticoagulated with sodium citrate.

Harvesting the peripheral blood leukocytes from each sample involved lysing the erythrocytes in red cell lysis buffer (0.83% (w/v) ammonium chloride, 0.1% (w/v) potassium hydrogen carbonate and 0.1mM EDTA). Lysis was achieved by adding three volumes of sterile lysis buffer to one volume of blood or buffy coat, mixing, then incubating for 20 minutes at room temperature. Centrifugation of the mixture at 1000 x gmax for 10 minutes in an MSE 4L centrifuge at room temperature pelleted the leukocytes and any unlysed erythrocytes. The supernatant was removed and the cell pellet resuspended in 20 mls of fresh lysis buffer. Repetition of the whole process twice gave a virtually pure white cell pellet. Following the third centrifugation step the leukocytes were resuspended in 20 mls of sterile phosphate buffered saline (one
phosphate buffered saline tablet dissolved in deionised water to a final volume of 100 mls), pelleted by centrifugation and washed once more to remove the last traces of the lysis buffer. The leukocytes could then be used directly for genomic DNA preparation or frozen as a pellet in liquid nitrogen for use at a later date.

2.2.2 Preparation of high molecular weight genomic DNA from peripheral blood leukocytes

The method employed was an adaptation of the one described by Gross-Bellard et al (194). Peripheral blood leukocytes derived from 20 mls of whole blood were resuspended in 10 mls of sterile 10mM TRIS-HCl (pH 8.0), 10mM NaCl, 10mM EDTA in a sterile polypropylene tube. An even cell suspension with no clumps was best. Sodium lauroyl sarcosine was then added to 4% (w/v) and proteinase K to 100 µg/ml. Following gentle tumbling of this mixture on a rotating mixer for 16 hours at 37°C, an equal volume of phenol/chloroform (1/1 v/v) saturated with TE buffer (10mM TRIS-HCl (pH 7.6), 1mM EDTA) was added. Gentle tumbling on a rotating mixer at room temperature followed until the aqueous and phenol phases were completely mixed (20–30 minutes). Centrifugation at 250 x g max for 10 minutes in an MSE 4L centrifuge at room temperature separated the two phases allowing the upper, viscous, aqueous phase to be removed with a wide bore pipette and transferred to a sterile glass flask. Care was taken to ensure that the creamy looking aqueous/phenol interface was not transferred. Sterile 3M sodium acetate (pH 5.2) was added to the aqueous fraction to a concentration of 0.3M followed by 2.5 volumes of ice-cold 95% ethanol. The ethanol was
stirred slowly into the aqueous phase using a sterile glass rod and the strands of DNA precipitating at the ethanol/aqueous interface were wound onto the glass rod. Fluid was expelled from the DNA by pressing it against the side of the flask. Further dehydration of the DNA was achieved by washing it in 70% (v/v) ethanol followed by 95% ethanol and finally a wash each in chloroform then diethyl ether. Each wash lasted 2 minutes. Air drying of the DNA for 10 minutes followed and once dry it was resuspended overnight in sterile 0.1 x SSC (15mM NaCl, 1.5mM sodium citrate). Treatment with boiled RNase A added to a final concentration of 50 µg/ml and with RNase T1 added to 2 µg/ml was then carried out for 3 hours at 37°C. The reaction was stopped by the addition of 0.5M EDTA pH 7.0 to a concentration of 10mM, sarcosine to 4% (w/v) and proteinase K to 50 µg/ml. Gentle tumbling for a further 3 hours at 37°C was carried out followed by a repetition of the phenol/chloroform extraction step twice and extraction with chloroform alone twice. DNA precipitation, dehydration and drying were carried out as described before. Finally, the DNA was resuspended in sterile TE buffer (10mM TRIS-HCL (pH 7.6), 1mM EDTA) the volume of which was adjusted to give a concentration of DNA of 200-400 µg/ml. The DNA was stored at 4°C until needed.

2.2.3 Restriction endonuclease digestion of genomic DNA

Usually 6 µg of leukocyte genomic DNA was digested to completion with each of the 38 restriction endonucleases at a concentration of 10 units of enzyme/µg DNA in a total digest volume of 160 µl under the conditions recommended by the manufacturer for
each enzyme. A total digest time of 6 hours was employed with half of the total amount of endonuclease being added for the first 3 hours and an equal amount of enzyme added for the second 3 hour period. Following digestion, each DNA sample was precipitated by adding 16 μl of sterile 7.5M ammonium acetate followed by 440 μl of 95% ethanol. After mixing, each sample was stored overnight at -20°C and the DNA then pelleted by centrifugation at 6000 x gmax for 20 minutes at 4°C in an Eppendorf 5412 microfuge. The supernatant was discarded, the DNA pellet washed in 1 ml of ice-cold 70% ethanol and repelleted by a further 5 minutes of centrifugation. Again the supernatant was discarded and the DNA pellet vacuum dried for 30 seconds before being resuspended in 13 μl of sterile TE buffer (10mM TRIS-HCL (pH 7.6), 1mM EDTA) ready for gel electrophoresis.

When a DNA sample required to be digested by two endonucleases each digest was carried out separately, the DNA being ethanol precipitated and resuspended in sterile TE buffer between digests as well as after the second digest. This protocol for double digests prevented the occurrence of partial digests by the second endonuclease used. The addition of sterile spermidine to a concentration of 10mM in the second digest reaction also helped ensure complete digestion of the DNA.

2.2.4 Gel electrophoresis

Agarose gel electrophoresis was the standard technique employed. Routinely a 200 mm x 135 mm x 9 mm gel containing 150 mls of 0.8% (w/v) ultra pure agarose in TBE buffer (89mM TRIS-base (pH 8.2), 90mM boric acid, 2.5mM EDTA) was used. To prepare an
agarose solution the agarose was added to the TBE buffer and the mixture autoclaved in a domestic style pressure cooker at full pressure for 10 minutes. The mixture was then allowed to cool to approximately 40°C before pouring into a perspex mould sealed by biohazard tape at either end. A 20 tooth comb was positioned such that it sat 3mm clear of the base of the mould and was 20mm from the top edge of the gel. After the gel had set, usually around 30 minutes, the comb was removed and the gel placed in the electrophoresis tank along with 1.6 litres of TBE buffer, the buffer covering the surface of the gel to a depth of 10mm. Electrophoresis of genomic DNA samples was carried out using a Kingshill stabilised power supply (model 15A01C) for a period of 16 hours at 40 volts with the current control at its maximum setting giving a current of 2 milliamps. If separation of DNA fragments of similar molecular size was required the electrophoresis run times were lengthened up to a maximum of 64 hours at 25 volts.

2.2.5 Southern blotting of genomic DNA

Gel electrophoresis was carried out as detailed above with 6 μg of DNA per lane. Before loading each DNA sample into the gel 0.1 volume of sterile dye mix containing 10mM TRIS–HCl (pH 8.0), 10mM EDTA, 0.1% (w/v) bromophenol blue and 30% (w/v) sucrose was added to each sample. Standard molecular weight markers were routinely run in the two outside lanes of each gel. These markers were lambda bacteriophage DNA restricted with Hind III or a combination of Eco RI and Hind III giving sets of molecular weight marker bands as follows - lambda Hind III 23.1kb, 9.4kb, 6.4kb,
4.4kb, 2.3kb, 2.0kb and 0.5kb; lambda Eco RI/Hind III 21.2kb, 5.1kb, 4.2kb, 3.5kb, 2.0kb, 1.9kb, 1.5kb, 1.3kb, 0.9kb, 0.8kb, 0.5kb and 0.1kb. At the end of the electrophoresis run the gels were photographed and the DNA transferred to a hybridisation membrane according to the method of Southern (195).

Before photography the DNA in the gel was stained by immersing the gel in 500 mls of TBE running buffer containing ethidium bromide at a concentration of 0.5 \( \mu \text{g/ml} \) for 15 minutes. Excess ethidium bromide was removed by soaking the gel in 500 mls of TBE for 10 minutes and the gel was then photographed lying alongside a 300mm ruler while being illuminated by a 320nm UV light box. Polaroid type 57 high speed film was used for photography. Prior to DNA transfer the gel containing the electrophoresed DNA was treated as follows -

1) The gel was immersed in 500 mls of 0.25M HCl for 20 minutes to depurinate the DNA.

2) The DNA was then denatured by immersing the gel in 500 mls of 1.5M NaCl, 0.5N NaOH for two periods of 20 minutes each.

3) The gel was neutralised by immersion in 500 mls of 0.5M TRIS–HCl (pH 7.5), 3M NaCl for three periods of 30 minutes each.

Following this series of treatments capillary transfer of the DNA was undertaken by placing the gel on a glass plate which was raised on rubber bungs over the centre of a pyrex pie dish containing approximately one litre of 20 x SSC (3M NaCl, 0.3M sodium citrate). Three wicks of Whatman 3MM filter paper ran under the gel and dipped into the 20 x SSC. Care was taken to ensure no air bubbles were trapped between the filter paper wicks and the gel. Four pieces of old X-ray film were placed along the edges of the gel to fill and seal the gap between the gel edges and the edges of the dish. Their
presence ensured that no 20 x SSC could pass round the edges of the gel. A sheet of precut and presoaked (20 x SSC for 5 minutes) Hybond-N hybridisation membrane was now laid over the surface of the gel and was overlapped onto the X-ray film round its edges. Again it was essential to ensure no air bubbles became trapped between the membrane and the gel. Two layers of thin Whatman filter paper soaked in 20 x SSC were then placed on top of the hybridisation membrane and a 6 inch high stack of white Bowtowels placed on top ensuring the whole surface of the filter paper was covered. Finally, a glass plate was placed on top of the Bowtowels and a full 500 ml bottle placed on the plate. This construction was left for at least 16 hours to allow DNA transfer, soaked Bowtowels being replaced when necessary. Following DNA transfer the hybridisation membrane was removed after marking on it the position of the wells of the underlying gel and after removing the top left hand corner of the membrane which corresponded to the top left corner of the gel. After air drying, the membrane was exposed to a weak 320nm UV light source in order to cross-link the DNA to the membrane. The membrane was now ready for prehybridisation and hybridisation.

2.2.6 Prehybridisation and hybridisation

Central to the detection of a DNA or RNA sequence of interest is the process of hybridising a suitably labelled specific DNA or RNA probe to the test nucleic acid sample. In this study the genomic DNA under investigation was immobilised on Hybond-N membranes as described in Section 2.2.5.

Initially all the membranes were prehybridised in order to
block any non-specific nucleic acid binding sites. This process involved incubating the membranes for 16 hours at 42°C in a sealed polythene bag with a suitable volume of prehybridisation fluid containing 50% (v/v) formamide, 0.75M NaCl, 75mM sodium citrate, 0.1% (w/v) ficoll, 0.1% (w/v) polyvinylpyrolidone, 0.02% (w/v) bovine serum albumin, 50mM sodium dihydrogen phosphate (pH 6.8), 0.1% (w/v) SDS, 100 µg/ml sheared salmon sperm DNA, 10 µg/ml polyadenylic acid and 10 µg/ml polycytidylic acid. The volume of prehybridisation fluid used was tailored to the size of membrane being prehybridised. Thereafter hybridisation could be carried out under the same incubation conditions and in the same volume of hybridisation fluid. This fluid comprised 50% (v/v) formamide, 0.75M NaCl, 75mM sodium citrate, 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrolidone, 0.004% (w/v) bovine serum albumin, 20mM sodium dihydrogen phosphate (pH 6.8), 0.1% (w/v) SDS, 10% (w/v) dextran sulphate, 100 µg/ml sheared salmon sperm DNA, 10 µg/ml polyadenylic acid and 10 µg/ml polycytidylic acid. Radiolabelled probe was added to the hybridisation fluid such that 1 million cpm/ml was attained. Before addition to the hybridisation fluid the probe was boiled for 10 minutes then rapidly cooled on ice to ensure denaturation of the probe nucleic acid secondary structure.

On completion of the hybridisation period the Hybond-N membranes were placed in 200 mls of 0.3M NaCl, 30mM sodium citrate, 0.1% (w/v) SDS for 1 hour at 65°C in a gently shaking water bath. Repetition of this process under progressively more stringent conditions, namely 75mM NaCl, 7.5mM sodium citrate, 0.1% (w/v) SDS then 15mM NaCl, 1.5mM sodium citrate, 0.1% (w/v) SDS followed.
Washing to this stringency was possible because of the absolute homology of the human Cl-inhibitor cDNA probe with the Cl-inhibitor gene sequences of interest in the human DNA samples being studied.

2.2.7 Autoradiography

The washed Hybond-N membranes were sealed in a thin polythene bag while still damp and placed along with a sheet of Kodak X-Omat AR X-ray film in an X-ray cassette containing two Dupont Lightening Plus intensifying screens. After storage at -70°C for up to seven days the X-ray film was developed in a Kodak 90 second X-ray processor.

2.2.8 Radiolabelling of DNA fragments by random priming

In this study random priming was the sole method used to produce radiolabelled probes. The process was routinely carried out using the Boehringer Mannheim Random Priming Kit which supplied 10x concentrated reaction buffer containing the hexanucleotide primers, the deoxynucleotides and the Klenow fragment of DNA polymerase 1. A standard reaction mixture comprised 2.5 µl (25 ng) of DNA to be labelled, 2 µl of the 10x concentrated reaction buffer, 1 µl of 2'-deoxyadenosine 5'-triphosphate (0.5mM), 1 µl of 2'-deoxyguanosine 5'-triphosphate (0.5mM), 1 µl of 2'-deoxythymidine 5'-triphosphate (0.5mM), 1 µl of Klenow, 5 µl (50μCi) of alpha-32P-2'-deoxycytidine 5'-triphosphate and 6.5 µl of sterile distilled water. Before addition to the reaction mixture the DNA was boiled for 10 minutes then cooled rapidly an ice to ensure denaturation of secondary structure. The whole reaction was incubated at 37°C for 30 minutes.
then stopped by placing on ice. Radiolabelled DNA could now be separated from unincorporated isotope by passing the reaction mixture over a small column containing Biogel A-1.5 (100-200 mesh). This was achieved by first adding 0.1 volume of sterile dye mix containing 50mM TRIS-HCl (pH 7.6), 50mM EDTA, 0.02% (w/v) dextran blue, 0.02% (w/v) phenol red, 30% (w/v) sucrose and 250 μg/ml sheared salmon sperm DNA to the random primed reaction mixture. The whole mixture was then added to the Biogel column which was held in a Pasteur pipette and washed through with 15mM NaCl, 1.5mM sodium citrate, 0.1% (w/v) SDS. Fractions of 0.5 ml were collected and 5 μl aliquots of each fraction were added to 5 mls of Ecosint A ready for counting on an LKB 1217 liquid scintillation counter. The first eluted peak of radioactivity represented the radiolabelled DNA, the second peak being unincorporated counts. Routinely the isotope incorporation was sufficient to achieve 100-1000 million cpm/μg of input DNA. This radiolabelled DNA could then be used for hybridisation studies as described in Section 2.2.6.

2.2.9 Preparation of agar plates

Sterile agar plates were required for preparing single colonies of host bacterial strains to be used for plasmid transformation and bacteriophage growth. The agar requirements for each of these situations differed. For simple bacterial streaking of host strains before and after plasmid transformation a 1.5% (w/v) agar plate was used whereas for bacteriophage growth a 1.2% (w/v) agar base overlaid by a 0.6% (w/v) agar top layer was employed.

Agar plates were prepared by dissolving the appropriate amount
of agar in L-broth (pH 7.5) which contained 1% (w/v) bactotryptone, 0.5% (w/v) bactoyeast extract and 1% (w/v) NaCl. Dissolution and sterilisation of the agar/L-broth mix necessitated autoclaving it at a pressure of 15 pounds per square inch for 15 minutes. The autoclaved solution was allowed to cool to approximately 40-50°C before adding sterile 1M magnesium sulphate to a concentration of 10mM and sterile 20% (w/v) maltose to a concentration of 0.2% (w/v). This mixture was then poured into 90mm diameter Sterilin plastic petri dishes to a thickness of 5mm. When necessary ampicillin was added to the autoclaved, 40-50°C agar solution to a concentration of 100 \mu g/ml. The plates were left to cool sitting on a perfectly flat surface to ensure an even 5 mm thickness of agar. Before use, particularly for bacterial streaking to obtain single bacterial colonies, it was important to ensure that any condensation that had formed inside the lid of the newly poured plate was allowed to evaporate off to give "dry" plates. Prepared agar plates were stored inverted at 4°C until use or for four weeks whichever was the shorter period.

2.2.10 Preparation of competent cells

The host strain of Escherichia coli (E. coli) chosen for transformation with the recombinant plasmid pClI described earlier was JM83. A single bacterial colony which had been prepared by streaking out on a 1.5% (w/v) agar plate without ampicillin was inoculated into a 10 ml volume of L-broth and grown overnight in an orbital 37°C incubator (New Brunswick Scientific Orbital Incubator). Thereafter 1 ml of this culture was added to 100 mls of L-broth.
which had been preheated to 37°C. This mixture was grown at 37°C until the OD600nm reading of the bacterial suspension was 0.4 - 0.6, this usually taking approximately 2 hours. Following transfer to sterile 50 ml centrifuge tubes the bacterial suspension was cooled on ice and the bacteria pelleted by centrifugation at 1000 x gmax for 5 minutes at 4°C in an MSE 4L centrifuge. The supernatant was then decanted and the bacteria gently resuspended in 25 mls of sterile, ice-cold 10mM magnesium sulphate and left on ice for 30 minutes. The bacteria were repelleted by centrifugation and gently resuspended in 12.5 mls of sterile, ice-cold 50mM calcium chloride. After storage on ice for a further 15 minutes the bacteria were pelleted again by centrifugation before gentle resuspension in 2 mls of sterile, ice-cold 50mM calcium chloride. These bacteria could be used immediately for transformation giving an efficiency of approximately 500,000 colony forming units/µg of recombinant plasmid DNA. However, if stored at 4°C overnight a 5-fold increase in transformation efficiency was seen.

2.2.11 Transformation of competent JM83 with pClI

Using an overnight sample of competent JM83, 50 ng of pClI in 10 µl of TE buffer (10 mM TRIS-HCl (pH 7.6), 1 mM EDTA) were added to 90 µl of the JM83 in 50 mM calcium chloride. Controls which included TE buffer alone added to JM83, non-recombinant pUC13 DNA added to JM83 and JM83 alone were also prepared. Each mixture was kept on ice for 25 minutes, heat shocked at 42°C for 2 minutes then kept at room temperature for 10 minutes. Thereafter 1 ml of L-broth at 37°C was added to each transformation reaction and the mixture incubated with
shaking for 90 minutes at 37°C. A 100 μl aliquot of each reaction was now spread thinly over the surface of 1.5% (w/v) agar plates containing 100 μg/ml ampicillin before being inverted and incubated overnight at 37°C. Only the ampicillin-resistant plasmid carrying bacteria grew as colonies on the plates and were selected for plasmid DNA isolation and characterisation.

2.2.12 Small scale isolation of plasmid DNA

Plasmid DNA can be isolated from a transformed bacterial culture by the method of Birnboim and Doly (196). The protocol described below allowed the purification of 0.5-1 μg of plasmid DNA which could then be characterised to ensure its identity before bulk preparation. The method is dependent upon the fact that alkali treatment of a mixture of E. Coli DNA and plasmid DNA produces irreversible denaturation of most of the bacterial DNA, however, the plasmid DNA can be renatured by neutralisation following the alkali treatment.

A single transformed bacterial colony containing the presumed plasmid DNA of interest was inoculated into 2 mls of L-broth containing the appropriate antibiotic, in this case ampicillin at a concentration of 100 μg/ml. After an overnight incubation in a shaking incubator at 37°C, 1.5 mls of the bacterial suspension was placed in a sterile microfuge tube and centrifuged for 15 seconds at 6000 x g max in an Eppendorf 5412 microfuge to pellet the bacteria. All the supernatant was carefully removed and the bacteria resuspended in 100 μl of sterile lysis solution (25mM TRIS-HCl (pH8.0), 50mM glucose, 10mM EDTA) before the addition of lysozyme as
a powder to a concentration of 2 mg/ml. Once the lysozyme was dissolved the samples were left at room temperature for 5 minutes then 200 µl of freshly prepared, sterile alkaline/SDS (0.2N NaOH, 1% (w/v) SDS) added to each tube. The tubes were inverted sharply several times and incubated on ice for 5 minutes before the addition of 150 µl of neutralising, ice-cold, sterile 5M potassium acetate (pH 4.8). Mixing by tube inversion was carried out before the samples were incubated on ice for 10 minutes. The precipitated E. coli DNA and protein could now be pelleted by centrifugation at 6000 x gmax for 5 minutes. The supernatant was transferred to a fresh, sterile microfuge tube and an equal volume of phenol/chloroform (1/1 v/v) saturated with TE buffer (10mM TRIS-HCl (pH 7.6), 1mM EDTA) added. The tube contents were mixed by tube inversion for 1 minute after which the tube was centrifuged at 6000 x gmax for 1 minute. The upper aqueous phase was removed and transferred to a fresh, sterile microfuge tube and boiled RNase A was added to a concentration of 100 µg/ml. The mixture was incubated at 37°C for 30 minutes to ensure that any RNA present was degraded. Addition of 0.1 volume of sterile 7.5M ammonium acetate followed by the addition of two volumes of ice-cold 95% ethanol precipitated the plasmid DNA. After 15 minutes at room temperature the plasmid DNA was pelleted by centrifugation at 6000 x gmax for 10 minutes. The pellet was washed once in 70% (v/v) ethanol, recentrifuged for 5 minutes, the supernatant removed and the DNA pellet vacuum dried for 30 seconds before resuspension in 20 µl of sterile TE buffer (pH 7.6). The plasmid DNA was characterised by restriction endonuclease digestion.
2.2.13 Large scale isolation of plasmid DNA

The initial steps were almost identical to those used above for small scale preparations, however, to ensure greater purity of plasmid DNA product equilibrium centrifugation on a caesium chloride cushion containing ethidium bromide was carried out following the alkali denaturation protocol. The efficacy of the equilibrium centrifugation is based on the fact that supercoiled plasmid DNA binds less of the ethidium bromide compared with open circular DNA or linear DNA. The supercoiled plasmid DNA therefore forms a discrete band further down the caesium chloride gradient (197). Using this protocol 500-1000 µg of plasmid DNA could be isolated from 1 litre of bacterial culture assuming the plasmid was present in reasonably high copy number within the bacterial host strain.

A single bacterial colony containing the required plasmid was inoculated into 10 mls of L-broth containing the appropriate antibiotic which for pC11 was ampicillin at a concentration of 100 µg/ml. The culture was incubated overnight at 37°C in an orbital incubator after which the whole bacterial suspension was inoculated into 500 mls of L-broth containing ampicillin (100 µg/ml) and the incubation continued for 20 hours. The 500 ml culture was placed in a sterile 500 ml centrifuge bottle and the bacteria pelleted by centrifugation at 4°C for 10 minutes in a Sorval RC 5B centrifuge at 4000 x gmax. The supernatant was discarded in its entirety and the bacterial pellet fully resuspended in 20 mls of sterile lysis solution (25mM TRIS-HCl (pH8.0), 50mM glucose, 10mM EDTA) before the addition of lysozyme as a powder to a concentration of 5 mg/ml. Incubation at room temperature for 5 minutes ensured extensive
bacterial lysis and was followed by the addition of 40 mls of freshly prepared, sterile alkaline/SDS (0.2N NaOH, 1% (w/v) SDS) to irreversibly denature the bacterial DNA. This mixture was incubated for 10 minutes on ice before 30 mls of ice-cold, sterile 5M potassium acetate (pH 4.8) were added. After mixing, the sample was left on ice for a further 10 minutes. Precipitated bacterial DNA and protein were pelleted by centrifugation at 16000 x gmax for 10 minutes at 4°C in a Sorval RC 5B centrifuge. The supernatant was carefully removed to a fresh tube so ensuring that it was not contaminated by the precipitate. Isopropanol (0.6 volumes) was added to the supernatant and after mixing an incubation at room temperature for 15 minutes allowed the plasmid DNA to precipitate. Centrifugation at 16000 x gmax for 10 minutes at room temperature in a Sorval RC 5B centrifuge pelleted the DNA which was then washed in 5 mls of 70% (v/v) ethanol before vacuum drying for approximately 2 minutes. The DNA was now resuspended in 5 mls of sterile TE buffer (10mM TRIS–HCl (pH 8.0), 1mM EDTA) and for every 1 ml of suspension, 1 gram of solid caesium chloride added. Dissolution of the caesium chloride was followed by the addition of ethidium bromide to a concentration of 600 μg/ml and the refractive index of the whole solution was adjusted to 1.393. This mixture was then centrifuged at 45000 revolutions per minute for 36 hours at 20°C in a 10 x 10 fixed angle rotor of a Centricon ultracentrifuge. The lower band of plasmid DNA was harvested and the ethidium bromide removed from the DNA solution by repeated extraction (4-6 times) with an equal volume of isopropanol saturated with caesium chloride. Removal of the caesium chloride could then be carried out by dialysis of the
plasmid DNA solution in a collodion bag against several changes of 500 ml of TE buffer (pH 7.6) over 24 hours. The DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 95% ethanol. Mixing was followed by incubation at -20°C for at least 1 hour and centrifugation at 16000 x g max for 15 minutes at 4°C in a Sorval RC 5B centrifuge. The pelleted DNA was washed twice in 5 ml of ice-cold 70% (v/v) ethanol, vacuum dried for approximately 2 minutes and resuspended in 1 ml of sterile TE buffer (pH 7.6). Care was taken to optimise DNA recovery by washing the sides of the centrifuge tube with the TE buffer. The plasmid DNA was divided into aliquots and stored at -20°C until needed.

2.2.14 Purification of DNA from low melting point agarose gel

This method was employed to isolate specific fragments of the recombinant plasmid pCli (Section 3.2) for use as probes when localising Cl-inhibitor gene mutations already detected by RFLP analysis using the complete exon 2-8 pCli as probe.

Restriction enzyme digestion of pCli was followed by electrophoresis through a 0.8% (w/v) Seaplaque agarose gel as previously described. The gel was examined following ethidium bromide staining by illumination under 320nm UV light. The appropriate DNA band was excised and the gel slice placed in a centrifuge tube containing 2 ml of sterile distilled water. The tube was heated to 70°C until the gel slice had melted and dispersed through the water. An equal volume of phenol saturated with TE buffer (10mM TRIS-HCl (pH 7.6), 1mM EDTA) was added and vigorously
mixed with the aqueous phase until an emulsion formed. Thereafter the aqueous and phenol phases were separated by centrifugation at 4000 x g_{max} for 5 minutes at room temperature in an MSE 4L centrifuge. The clear aqueous phase was collected and placed in a fresh, sterile centrifuge tube. To minimise DNA loss in the thick creamy interface that had formed between the aqueous and phenol phases after the first phenol extraction, a "back extraction" procedure was performed. Sterile distilled water (0.5 ml) was added and after mixing and recentrifugation the aqueous phase was removed and added to the first aqueous phase. This pooled aqueous DNA solution was re-extracted with phenol a further 4-6 times until the interface between the aqueous and phenol phases after centrifugation was clear. At this stage two chloroform extractions were performed. An equal volume of chloroform was used each time. Before DNA precipitation the volume of the DNA solution was reduced to 200 \mu l by repeated butan-2-ol extractions at room temperature as follows. The DNA solution was inverted several times with an equal volume of butan-2-ol, the phases allowed to separate and the butan-2-ol removed. A fresh equal volume of butan-2-ol was added and the whole process repeated until the aqueous phase was reduced to the required 200 \mu l. The DNA was then precipitated by adding 0.1 volume of sterile 7.5M ammonium acetate, 2.5 volumes of 95% ethanol, the solution mixed and stored overnight at -20°C. Pelleting the DNA could now be carried out by centrifugation at 6000 x g_{max} for 15 minutes at 4°C in an Eppendorf 5412 microfuge. The pellet was washed twice in ice-cold 70% (v/v) ethanol, vacuum dried for 30 seconds and resuspended in sterile TE buffer (pH 7.6).
Reprecipitation and resuspension of the DNA in sterile TE buffer (pH 7.6) was then carried out before use in a random priming reaction. Long term storage of the DNA fragments in TE buffer (pH 7.6) purified in this way was frozen at -20°C in small aliquots.

2.2.15 Construction of human genomic libraries in bacteriophage vector EMBL 3

(a) The critical starting point for human genomic library construction is the isolation of very high molecular weight human genomic DNA (198). The method used for this differed from that described in Section 2.2.2. After isolation of the peripheral blood leukocytes, as described in Section 2.2.1, the leukocytes were lysed in 5 mlS of sterile lysis buffer containing 100mM TRIS-HCl (pH 7.5), 100mM NaCl, 10mM EDTA and 1% (w/v) sarcosine. Lysis was achieved by very careful mixing of the cells with the lysis buffer for 2-3 minutes before the addition of proteinase K to a concentration of 100 μg/ml. This mixture was then incubated for 2 hours at 55°C with periodic gentle inversion. Thereafter an equal volume of phenol saturated with TE buffer (10mM TRIS-HCl (pH 7.6), 1mM EDTA) was added and gently mixed with the DNA solution for 15 minutes to ensure formation of an even emulsion. Centrifugation at 250 x g max for 10 minutes at room temperature in an MSE 4L centrifuge separated the aqueous and phenol phases. The aqueous phase was now removed into a fresh, sterile centrifuge tube using a wide bore pipette ensuring minimal contamination by the creamy looking aqueous/phenol interface. Extraction with an equal volume of phenol/chloroform (1/1 v/v) saturated with TE buffer (pH 7.6) and then with an equal
volume of chloroform followed. The final aqueous DNA containing phase was dialysed against four changes of 2 litres of sterile TE buffer (pH 7.6) containing 100mM NaCl over a period of 24 hours followed by an identical period of dialysis against four changes of 2 litres of sterile TE buffer (pH 7.6). All dialysis steps were carried out at 4°C. Estimation of DNA concentration was achieved by running a small aliquot of the DNA against known concentrations of lambda bacteriophage DNA in an ethidium bromide stained 0.5% (w/v) agarose gel. This was necessary because reading the UV adsorption of the DNA solution at 260 nm would have given a markedly inaccurate measurement of the DNA concentration due to the content of RNA within the DNA preparation.

(b) Partial endonuclease digestion of the high molecular weight genomic DNA was performed using the restriction enzyme Sau 3AI in order to generate 15-20kb fragments with cohesive ends suitable for ligation into the EMBL 3 bacteriophage vector. Sau 3AI cuts widely throughout the human genome to generate a set of restriction fragments which are representative of the vast majority of the sequences present in the human genome. Test digests of the genomic DNA were set up initially in which the DNA concentration, digest time, digest volume and temperature were kept constant and the amount of Sau 3AI varied. Electrophoresis of the resulting mixtures of DNA restriction fragments through a 0.5% (w/v) agarose gel allowed estimation of the concentration of enzyme which generated the maximum number of 15-20kb fragments. This concentration +/- 50% was then used to digest larger amounts of genomic DNA ready for bulk isolation of the 15-20kb fragments by
sucrose gradient ultracentrifugation.

(c) Before ultracentrifugation the Sau 3AI digested genomic DNA was extracted once with an equal volume of phenol saturated with TE buffer (pH 7.6), followed by extraction with an equal volume of chloroform. The DNA was then ethanol precipitated by the addition of 0.1 volume of sterile 7.5M ammonium acetate and 2.5 volumes of 95% ethanol. Storage of this mixture for at least one hour at -20°C was followed by centrifugation at 6000 x g_{max} for 10 minutes in an Eppendorf 5412 microfuge at 4°C in order to pellet the DNA. The DNA pellet was washed in 1 ml of ice-cold 70% (v/v) ethanol, vacuum dried for 30 seconds and resuspended in sterile TE buffer (pH 7.6) at a concentration of 300 μg/ml. The DNA was now ready for loading onto a sucrose gradient.

Sucrose gradients, 10% to 40% sucrose (w/v) in 20mM TRIS-HCl (pH 7.1), 1M NaCl, 20mM EDTA were used. Each gradient was made by successively underlayering equal volumes of 10%, 15%, 20%, 25%, 30%, 35% and 40% sterile sucrose solutions into a 14 ml ultracentrifuge tube and leaving this to stand for 16 hours at room temperature to allow diffusion. A maximum of 100 μg of the digested DNA was layered on top of each gradient and the gradients centrifuged for 24 hours at 26000 revolutions per minute at 10°C in a Centricon ultracentrifuge using a 6x14 swing out rotor. Each gradient was harvested by piercing the bottom of the centrifuge tube with a sterile 21 gauge needle and collecting six drop fractions. A 10 μl aliquot of each of these fractions was electrophoresed through a 0.5% (w/v) agarose gel along with standard molecular weight size markers in order to identify which fractions contained the 15-20kb DNA
restriction fragments. It was important to adjust the sucrose and salt concentration of the molecular weight size markers to that of the gradient samples by the addition of a suitable volume of the sucrose gradient buffer to the marker samples since the high salt concentration markedly affected DNA fragment mobility through the gel. The gel was photographed and the fractions containing the 15-20kb DNA fragments were identified. The DNA was precipitated separately from each of these fractions by the addition of two volumes of 95% ethanol. Mixing with the ethanol was followed by storage of the samples at \(-20^\circ\)C for 48 hours before pelleting the DNA by centrifugation at 6000 x g max for 30 minutes in an Eppendorf 5412 microfuge at \(4^\circ\)C. The DNA pellet was then carefully washed twice with 1 ml of ice-cold 70% (v/v) ethanol, vacuum dried for 30 seconds and resuspended in sterile TE buffer (pH 7.6) to give an approximate concentration of 0.5 \(\mu\)g/\(\mu\)l. A small aliquot (0.5-1.0 \(\mu\)l) of each DNA sample was electrophoresed through a 0.5% (w/v) agarose gel along with suitable molecular weight size markers and the gel photographed. Only those fractions which contained DNA fragments within the 15-20kb size range were reselected and pooled for further use in library construction.

(d) In order to prevent the insert DNA ligating to itself before being ligated into the EMBL 3 vector arms the 15-20kb insert fragments were phosphatased in order to remove their 5' phosphate groups. The phosphatasing protocol was as follows. The DNA fragments were ethanol precipitated by the addition of 0.1 volume of sterile 7.5M ammonium acetate and 2.5 volumes of ice-cold 95% ethanol. Incubation at \(-20^\circ\)C for 1 hour was followed by centrifugation at
6000 x gmax for 10 minutes at 4°C in an Eppendorf 5412 microfuge to pellet the DNA which was now washed once in 1 ml of ice-cold 70% (v/v) ethanol. The DNA was repelleted by centrifugation at 6000 x gmax for 5 minutes, vacuum dried and resuspended in sterile 10mM TRIS-HCl (pH 7.5), 10mM EDTA at a concentration of approximately 200 μg/ml. Calf intestinal alkaline phosphatase was added to a concentration of 0.5 unit/μg of DNA and the mixture incubated at 37°C for 20 minutes. Thereafter sterile 1M NaCl was added to a final concentration of 200mM and the mixture extracted twice with an equal volume of phenol saturated with TE buffer (pH 7.6) and twice with an equal volume of chloroform. The DNA was ethanol precipitated, as just described, washed once in 1 ml of ice-cold 70% (v/v) ethanol, vacuum dried for 30 seconds and finally resuspended in sterile TE buffer (pH 7.6) at a concentration of 0.5 μg/μl.

(e) The next stage in library construction was the ligation of the purified, phosphatased 15-20kb insert fragments into the EMBL 3 bacteriophage vector arms. For this stage commercially available EMBL 3 arms were used. An initial check was made to ensure firstly that the T4 DNA ligase to be used was active and secondly to ensure that the insert DNA had been properly phosphatased. A 1 μl aliquot containing 0.5 μg of either the unphosphatased or the phosphatased insert DNA was added to a 9 μl ligation reaction containing 1 unit of T4 DNA ligase, 1mM ATP (pH 7.5), 50mM TRIS-HCl (pH 8.0), 7mM magnesium chloride and 1mM dithiothreitol. This mixture was incubated for 16 hours at 22°C then electrophoresed through a 0.5% (w/v) agarose gel and visualised by ethidium bromide staining of the gel. Generation of a high molecular weight band in the
unphosphatased lane and maintenance of a 15-20kb smear in the phosphatased sample proved the activity of the ligase and the effectiveness of the phosphatase respectively. The same batch of ligase was used for construction of the library as was used for these control ligations.

Library ligations were set up as follows. A ligation reaction volume of 5 μl was standardly used for all reactions and contained the same relative concentrations of T4 DNA ligase, ATP, TRIS-HCl, magnesium chloride and dithiothreitol as just described. The optimal ratio for predigested EMBL 3 vector arms to insert DNA in this system was found to be 1 μg of EMBL 3 arms to 0.4 μg of insert DNA in each ligation reaction. Ligations were incubated at 22°C for 1 hour then at 4°C for 16 hours.

(f) The number of packageable and therefore viable recombinant EMBL 3 bacteriophage DNA molecules generated by the vector/insert ligation reaction now had to be assessed. Packaging of the recombinant bacteriophage DNA molecules was carried out by using the commercially available high efficiency bacteriophage packaging kit, Gigapack Gold. Each packaging kit contained "freeze-thaw" and "sonic" bacterial extracts which provided all the bacteriophage structural proteins necessary for packaging "naked" bacteriophage DNA. Following ligation, 4 μl of the ligation reaction were added to 10 μl of the "freeze-thaw" extract which had just been thawed and this mixture was placed immediately on ice. The "sonic" extract which had been kept frozen at -70°C until this stage was now quickly thawed and 15 μl of it added to the "freeze-thaw"/DNA mixture on ice. Stir mixing was immediately carried out and the whole mixture
then incubated at 22°C for 2 hours. At the end of this period 0.5 ml of sterile bacteriophage dilution buffer (50mM TRIS-HCl (pH 7.5), 100mM NaCl, 10mM magnesium sulphate and 0.01% (w/v) gelatin) was added to the reaction followed by 20 μl of chloroform. The chloroform was mixed gently through the solution by repeated inversion and the precipitate which formed during this process pelleted by a 10 second centrifugation step in an Eppendorf 5412 microfuge. The content of viable, packaged bacteriophage particles in the supernatant now had to be calculated by titrating the bacteriophage on a suitable bacterial host strain. A number of essential controls were included in the ligation and packaging steps in order to be able to accurately assess the number of viable recombinant bacteriophage particles created. These controls included - 1) a ligation reaction containing predigested EMBL 3 bacteriophage arms alone without insert DNA, 2) insert DNA alone without arms and 3) arms plus a test insert. These reactions were subsequently packaged in order to assess both the background of the system and also to test the efficiency of the ligation and packaging reactions. The number of packageable, viable, non-recombinant bacteriophage that were generated by ligation of the EMBL 3 arms alone was particularly important when calculating the efficiency of generation of genuine recombinant bacteriophage. In addition, it was essential to know how many viable bacteriophage the packaging reagents themselves created without any DNA input. For the commercial kits used in this study this background was uniformly zero.

(g) Titrating the viable bacteriophage generated by the
ligation/packaging reactions was now undertaken using the restrictive bacterial host *E. coli* NM539 which allowed the growth of only recombinant bacteriophage. A single colony of NM539 was picked from a 1.5% (w/v) agar plate on which the bacterial species had been streaked out. This colony was inoculated into 20 mls of L-Broth supplemented with magnesium sulphate to 10mM and maltose to 0.2% (w/v) and incubated with shaking at 37°C until the O.D600 reading of the bacterial suspension reached 0.5. The bacteria were then pelleted by centrifugation at 1000 x gmax for 5 minutes at 4°C in an MSE 4L centrifuge, the supernatant poured off and the bacteria resuspended in the same volume of ice-cold, sterile 10mM magnesium sulphate. Aliquots (100 µl) of this bacterial suspension were added to 100 µl of the bacteriophage suspension to be titrated. This latter suspension comprised 1 µl of the packaging reaction supernatant diluted to 100 µl in sterile bacteriophage dilution buffer (50mM TRIS-HCl (pH 7.5), 100mM NaCl, 10mM magnesium sulphate, 0.01% (w/v) gelatin). Gentle mixing of the bacterial suspension with the bacteriophage dilution was followed by a 15 minute incubation at 37°C to allow the bacteriophage to adsorb onto the surface of the bacteria. Thereafter 2.5 mls of sterile 0.6% LAM agar (0.6% w/v bactoagar in L-Broth (pH 7.5) supplemented with 10mM magnesium sulphate and 0.2% (w/v) maltose) at 48°C was added to each adsorption reaction, mixed by inversion then poured onto the surface of a 1.2% (w/v) agar plate prewarmed to 37°C. This top agar layer was spread evenly over the surface of the 1.2% bottom agar and allowed to set on a perfectly flat surface for 15 minutes at room temperature. The plates were now inverted and incubated for 16 hours.
at 37°C. Counting the number of lytic bacteriophage plaques on the lawn of bacteria allowed calculation of the number of bacteriophage present in the packaging reaction supernatants and so the efficiency of recombinant bacteriophage production per µg of input EMBL 3 arms. It was then possible to work out the number of subsequent ligation and packaging reactions that would have to be carried out to complete the library based on the following guidelines. For a human genomic library using a 15-20kb insert, approximately 1 million recombinants were deemed sufficient to give a 99% chance of the library containing the required sequence (199). Once created the recombinant bacteriophage were stored at 4°C until needed.

2.2.16 Clinical survey of HAE in Scotland

All Scottish hospital consultants in medicine and its major subspecialities, dermatology, paediatrics, haematology, clinical immunology, biochemistry and blood transfusion were circulated with a letter enquiring whether they had patients with Type I or Type II HAE under their care along with an explanation of the reasons for the survey. If they were currently managing HAE patients each consultant was contacted again and requested to provide the following details - number of patients, names, sex, age and clinical history. Included in the clinical history was the following information - age at diagnosis; symptoms at diagnosis; duration of symptoms before diagnosis; treatment; effect of treatment on symptoms; factors which precipitate acute disease attacks; remission in pregnancy if appropriate; family history of the disease; disease related deaths; interest in prenatal diagnosis. This allowed an
estimation of overall disease incidence and allowed the construction of a disease profile for each affected patient and family. It was then possible to examine the main problems facing each kindred. When no reply was received from a consultant regarding the presence or absence of HAE patients under their care, the consultant was contacted personally by telephone to obtain the necessary information.
RESULTS

RESULTS INDEX

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3.1 Characterisation of the Cl-inhibitor cDNA (pCli)

An essential prerequisite for using a recombinant DNA molecule as a probe is to confirm its identity. One of the most convenient ways of doing this is to carry out a number of restriction enzyme digests on the intact molecule in order to demonstrate generation of a fragment pattern that can be predicted from its known restriction map. For the exon 2-8 Cl-inhibitor cDNA cloned into the Bam HI site of pUC13 (pCli), as described in Section 2.1.3, three sets of digests were chosen as follows - (a) pCli digested with Hind III, (b) pCli digested with Eco RI and (c) pCli digested with Hind III/Eco RI. The restriction maps for the exon 2-8 Cl-inhibitor cDNA (67) and for pUC13 (200) predict approximate fragment sizes of 4.4kb for digest (a), 3.9kb and 0.5kb for digest (b) and 2.6kb, 1.3kb and 0.5kb for double digest (c). Confirmation of these fragment patterns and sizes is shown in Figure 3. Digest (c) excises the entire Cl-inhibitor cDNA from pUC13 in the form of the 1.3kb and 0.5kb fragments as described by Bock et al (67).
FIGURE 3
Characterisation of pCli. Lanes 1 and 5 represent the molecular weight markers generated when lambda bacteriophage DNA is restricted with Eco RI/Hind III. Lane 2 represents pCli restricted with Hind III which linearises the recombinant and generates a 4.4kb fragment. Lane 3 represents pCli restricted with Eco RI which generates two fragments of 3.9kb and 0.5kb. Lane 4 represents pCli restricted with Hind III/Eco RI which generates three fragments of 2.6kb, 1.3kb and 0.5kb. The 1.3kb and 0.5kb fragments in lane 4 comprise the whole of the exon 2-8 Cl-inhibitor cDNA insert. These restriction fragment patterns and sizes are those predicted from the published restriction map of pCli (67) and pUCl3 (200).
3.2 Isolation of specific pCli restriction fragments

It was necessary to isolate certain pCli restriction fragments which could then be used as probes specific for the 5', middle and 3' ends of the Cl-inhibitor gene. As shown in Figure 4, the 5' fragment corresponds to the 5' end of the Cl-inhibitor cDNA up to the Bgl II recognition site in exon 4. The middle fragment extends from this Bgl II site to the Eco RI site at the very beginning of exon 8 and the 3' fragment is the whole of exon 8. For ease of purification the 3' fragment was isolated first following Eco RI digestion of pCli and electrophoresis of the whole digest reaction through a low melting point Seaplaque agarose gel (Figure 5). The two DNA fragments generated by Eco RI were then isolated from the gel as described in Section 2.2.14. The 527 base pair (bp) fragment was retained for use as the 3' exon 8 specific probe while the larger 3.9kb fragment was digested using the endonuclease Bgl II. This generated a 584bp fragment representing the middle (exon 4-7) probe and a 3.3kb fragment representing the 5' exon 2-4 probe with attached pUC13 DNA (Figure 4). The entire 3.3kb fragment was used as the 5' probe without removal of the pUC13 DNA.
FIGURE 4

Restriction map of pCli. The Bgl II and Eco RI restriction sites show where the cDNA was cleaved to generate the 5' (exon 2-4), middle (exon 4-7) and 3' (exon 8) probes that were used to localise the Cl-inhibitor gene mutations.
Isolation of specific pCli restriction fragments. Lanes 1 and 9 represent the molecular weight size markers generated when lambda bacteriophage DNA is restricted with Eco RI/Hind III. Lane 2 represents pCli restricted with Hind III generating the 4.4kb linearised form. Lane 3 represents pCli restricted with Eco RI generating 3.9kb and 0.5kb (527bp) fragments. Lanes 4 and 5 show the 3.9kb fragment and 527bp fragment respectively after purification of each separately from a low melting point agarose gel. The 527bp fragment represents exon 8 of pCli as shown in Figure 4. Lane 6 represents the purified 3.9kb fragment shown in lane 4 restricted with Bgl II to generate the 3.3kb and 0.5kb (584bp) fragments. Each of these two fragments was purified separately from a low melting point agarose gel as shown in lanes 7 and 8. The 584bp fragment represents the middle (exon 4-7) fragment of pCli and the 3.3kb fragment is the 5' (exon 2-4) fragment of pCli with attached pUC13 DNA. Each of the fragments shown in Lanes 5, 7 and 8 were used as the 3', 5' and middle probes respectively.
3.3 Restriction fragment analysis of the normal Cl-inhibitor gene

A total of 38 separate restriction endonucleases were used to study the Cl-inhibitor gene in this investigation. These enzymes are listed in Table 4. The restriction fragments generated by each of these enzymes as detected by using the exon 2-8 Cl-inhibitor cDNA as probe are shown in Figures 6 to 18. The restriction fragment sizes generated by each enzyme are marked beside the autoradiographs. Five of these enzymes generated multiple small fragments due to the high frequency of their recognition sites within the Cl-inhibitor gene (Table 5). In addition, some of the bands consisted of multiple similarly sized restriction fragments from a number of areas of the gene. Identification of a mutation affecting just one of these constituent fragments could therefore be difficult with the electrophoretic conditions routinely used. In contrast, the enzymes listed in Table 6 had few recognition sequences within the Cl-inhibitor gene and so many generated a single high molecular weight fragment which spanned much of the gene. For these large fragments it would require a major gene deletion or insertion to produce a detectable alteration in fragment size using 0.8% agarose gel electrophoresis. Alternatively, it would need a critically placed mutation destroying or creating a recognition site for that enzyme. Overall, therefore, the restriction enzymes with either very frequent recognition sites within the Cl-inhibitor gene or with very infrequent recognition sites were less likely to be recognisably informative in RFLP analysis of the Cl-inhibitor gene using these electrophoretic conditions. A substantial proportion of the enzymes (Table 7) generated multiple well-spaced, easily resolved fragments
which were more likely to generate identifiable RFLPs when used for analysis of the Cl-inhibitor gene. Indeed, as will be described in the following sections, a significant number of these enzymes did generate identifiable RFLPs. By comparison only a small number of the enzymes listed in Tables 5 and 6 contributed useful data.
<table>
<thead>
<tr>
<th>RESTRICTION ENDONUCLEASE</th>
<th>RECOGNITION SEQUENCE</th>
<th>RESTRICTION ENDONUCLEASE</th>
<th>RECOGNITION SEQUENCE</th>
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<tr>
<td>Alu I</td>
<td>AG'CT</td>
<td>Hpa I</td>
<td>GIT'AAC</td>
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<td>Msp I</td>
<td>C'CGG</td>
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<td>CGTCA'G</td>
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<td>Bcl I</td>
<td>T'GATCA</td>
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<td>CGAT'C'G</td>
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<td>Bst EII</td>
<td>G'GTNACC</td>
<td>Sal I</td>
<td>G'TCGAC</td>
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<tr>
<td>Cla I</td>
<td>AT'CGAT</td>
<td>Sau 3AI</td>
<td>'GATC</td>
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<tr>
<td>Dde I</td>
<td>C'TNAG</td>
<td>Sca I</td>
<td>AGT'ACT</td>
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<td>Dra I</td>
<td>TT'TAAA</td>
<td>Sma I</td>
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<td>GG'CC</td>
<td>Stu I</td>
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<td>Sty I</td>
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<td>Hinc II</td>
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<td>Hinf I</td>
<td>G'ANTC</td>
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</table>

Recognition sequences are written 5' to 3' and the point of cleavage is indicated by '. N represents any nucleotide. Py and Pu indicates pyrimidine and purine nucleotides respectively. (A/T) is A or T at that position.
FIGURE 6
Restriction fragment patterns and sizes generated by the restriction endonucleases Alu I, Apa I and Ava I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 7
Restriction fragment patterns and sizes generated by the restriction endonucleases Bam HI, Bcl I and Bgl I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 8
Restriction fragment patterns and sizes generated by the restriction endonucleases Bgl II, Bst EII and Cla I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 9
 Restriction fragment patterns and sizes generated by the restriction endonucleases Dde I, Dra I and Eco RI for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 10
Restriction fragment patterns and sizes generated by the restriction endonucleases Hae II, Hae III and Hha I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 11

Restriction fragment patterns and sizes generated by the restriction endonucleases Hgi AI, Hinc II and Hind III for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 12
Restriction fragment patterns and sizes generated by the restriction endonucleases Hinf I, Hpa I and Kpn I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 13
Restriction fragment patterns and sizes generated by the restriction endonucleases Msp I, Pst I and Pvu I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 14
Restriction fragment patterns and sizes generated by the restriction endonucleases Pvu II, Rsa I and Sal I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 15

Restriction fragment patterns and sizes generated by the restriction endonucleases Sau 3AI, Sca I and Sma I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 16
Restriction fragment patterns and sizes generated by the restriction endonucleases Sst I, Sst II and Stu I for the Cl-inhibitor gene and detected by using the exon 2–8 Cl-inhibitor cDNA as probe.
FIGURE 17
Restriction fragment patterns and sizes generated by the restriction endonucleases Sty I, Taq I and Xba I for the Cl-inhibitor gene and detected by using the exon 2-8 Cl-inhibitor cDNA as probe.
FIGURE 18
Restriction fragment patterns and sizes generated by the restriction endonucleases Xho I and Xmn I for the Cl-inhibitor gene and detected by using the exon 2–8 Cl-inhibitor cDNA as probe.
### Table 5

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<tr>
<th>Restriction Endonuclease</th>
<th>Fragment Sizes (kb)</th>
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<tr>
<td>Hae III</td>
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<td>Hinf I</td>
<td>0.9, 0.8, 0.6, 0.2</td>
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<tr>
<td>Sau 3AI</td>
<td>0.9, 0.8, 0.5, 0.3</td>
</tr>
<tr>
<td>RESTRICTION ENDONUCLEASE</td>
<td>FRAGMENT SIZES (kb)</td>
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<td>Bcl I</td>
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<tr>
<td>Hgi AI</td>
<td>2.4, 2.2, 1.7, 1.2, 0.85, 0.4, 0.3</td>
</tr>
<tr>
<td>Hinc II</td>
<td>7.6, 4.8, 2.8, 2.6, 1.8</td>
</tr>
<tr>
<td>Hind III</td>
<td>14.1, 4.2, 0.6</td>
</tr>
<tr>
<td>Kpn I</td>
<td>24.0, 13.0, 4.9</td>
</tr>
<tr>
<td>Msp I</td>
<td>3.5, 2.3, 1.9, 1.7, 1.3</td>
</tr>
<tr>
<td>Pst I</td>
<td>4.1, 3.1, 2.6, 2.4, 0.4</td>
</tr>
<tr>
<td>Pvu II</td>
<td>5.0, 3.2, 2.5, 2.3, 0.9</td>
</tr>
<tr>
<td>Rsa I</td>
<td>2.0, 1.9, 1.5, 1.2, 0.7, 0.5</td>
</tr>
<tr>
<td>Sst I</td>
<td>12.0, 6.0, 3.0, 1.6</td>
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<tr>
<td>Stu I</td>
<td>11.1, 5.5, 5.2</td>
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<tr>
<td>Sty I</td>
<td>4.8, 3.7, 0.9, 0.7, 0.2</td>
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<tr>
<td>Taq I</td>
<td>4.3, 3.7, 2.6, 2.4, 1.4</td>
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<tr>
<td>Xba I</td>
<td>21.0, 10.5, 1.3</td>
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<tr>
<td>Xmn I</td>
<td>16.0, 8.2, 2.3, 1.8</td>
</tr>
</tbody>
</table>
3.4 Cl-inhibitor gene RFLPs in the normal population

The restriction fragment patterns for all 38 restriction enzymes were established for genomic DNA samples from 25 normal individuals. Two of the restriction enzymes generated identifiable Cl-inhibitor gene RFLPs in these normal individuals. The enzymes involved were Kpn I and Hgi AI.

The enzyme Kpn I generated an RFLP which had not been reported previously. The polymorphism has two alleles which are represented by 30kb and 13kb hybridising fragments (Figure 19). The allele frequency for each was 0.2 and 0.8 respectively for a total of 50 chromosomes examined (Table 8). Studies to localise the genomic mutation responsible for this polymorphism showed that both the middle (exon 4-7) and the 3' (exon 8) probes detected the polymorphic fragments as shown in Figure 20 for the 3' probe. The 5' probe detected only the 24kb restriction fragment appropriate for the 5' end of the gene (Figure 21). These data in conjunction with known Cl-inhibitor gene sequence (183) indicated that the RFLP arose due to a mutation involving the first Kpn I recognition site that lies approximately 10kb downstream of the 3' end of the Cl-inhibitor gene as shown in Figure 22. No further investigations were carried out to try and characterise the nature of the mutation. Serum Cl-inhibitor levels were measured for all 25 normal individuals whose DNA samples had been used. No relationship could be demonstrated between possession of a particular Kpn I allelic combination and the serum Cl-inhibitor concentration. Family studies were consistent with co-dominant Mendelian inheritance for this polymorphism.
Detection of the Hgi AI RFLP in the normal population served to confirm the observations of Bock et al in the North American population (67). The polymorphism has two alleles represented by 0.7kb and 0.4kb hybridising fragments as shown in Figure 23. The allele frequency was 0.38 and 0.62 respectively for the 50 chromosomes studied (Table 9). This frequency is very similar to the 0.32 and 0.68 reported by Bock et al (67). Bock and her colleagues established that this polymorphism was due to a G to A mutation in the triplet codon for amino-acid 458 in the Cl-inhibitor protein. This mutation destroyed the Hgi AI recognition sequence and caused a valine (GTG) to methionine (ATG) amino-acid substitution in the protein. In this study a relationship could not be demonstrated between possession of a particular Hgi AI allelic combination and serum Cl-inhibitor concentration or Cl-inhibitor functional activity. A co-dominant mode of inheritance was observed in family studies.

None of the remaining 36 restriction enzymes showed identifiable RFLPs with the electrophoretic conditions employed.
FIGURE 19
Cl-inhibitor gene RFLP generated by Kpn I and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lanes 1, 2 and 7 represent a homozygote for the 13kb allele, a heterozygote and a homozygote for the 30kb allele respectively. All the remaining lanes represent homozygotes for the 13kb allele except lane 5 which is a heterozygote. The faint 4.9kb fragment which is normally present in a genomic Kpn I digest (Figure 12) had migrated off the bottom of this gel due to the prolonged electrophoresis time employed to ensure good separation of the 30kb and 24kb fragments. There is a 50% reduction in the intensity of the 13kb fragment in the heterozygote lanes 2 and 5 with complete absence of this fragment in lane 7 which represents a homozygote for the 30kb allele.
**TABLE 8  POPULATION FREQUENCIES OF Kpn I ALLELES**

<table>
<thead>
<tr>
<th>Kpn I ALLELIC FRAGMENTS</th>
<th>NUMBER OBSERVED</th>
<th>PHENOTYPE FREQUENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>30kb + 30kb</td>
<td>2</td>
<td>0.08</td>
</tr>
<tr>
<td>30kb + 13kb</td>
<td>6</td>
<td>0.24</td>
</tr>
<tr>
<td>13kb + 13kb</td>
<td>17</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**ALLELE FREQUENCY**

<table>
<thead>
<tr>
<th>ALLELE</th>
<th>FREQUENCY</th>
</tr>
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<tr>
<td>30kb</td>
<td>0.2</td>
</tr>
<tr>
<td>13kb</td>
<td>0.8</td>
</tr>
</tbody>
</table>
FIGURE 20
Cl-inhibitor gene RFLP generated by Kpn I and detected by the 3' (exon 8) probe. Lanes 1, 2 and 6 represent a homozygote for the 13kb allele, a heterozygote and a homozygote for the 30kb allele respectively. Lanes 3, 4 and 5 represent genomic DNA samples from each of these individuals digested with Kpn I/Bgl II generating 6.5kb and 1.9kb fragments. These are the fragment sizes expected from Cl-inhibitor gene sequence data for the 3' end of the gene as shown in Figure 22 (183). Lane 7 represents Bgl II digested DNA generating the 6.5kb and 1.9kb fragments as expected for the 3' end of the gene.
FIGURE 21

Cl-inhibitor gene restriction fragment generated by Kpn I and detected by the 5' (exon 2-4) probe. Lane 1 represents the invariant 24.0kb Kpn I fragment which is appropriate for the 5' end of the gene (183). Lane 2 represents Bgl II digested DNA from the same individual. There is a 10.8kb fragment detected in this lane which is appropriate for the 5' end of the gene and which confirms the 5' origin of the 24kb Kpn I fragment.
FIGURE 22

Restriction map of the 3' end of the Cl-inhibitor gene. The fragment sizes generated by Kpn I and Bgl II digests as detected by the 3' (exon 8) probe are shown. Exons are represented by open rectangles containing the exon number and introns by the line connecting the exons. The 30kb fragment is derived from the 13kb fragment by loss of the first Kpn I recognition site downstream (3') of the Cl-inhibitor gene. The positions of the 6.5kb and 1.9kb fragments generated by the Bgl II and Kpn I/Bgl II digests are shown.
FIGURE 23

Cl-inhibitor gene RFLP generated by Hgi AI and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lane 1 represents a heterozygote for the 0.7kb and 0.4kb alleles and lane 2 represents a homozygote for the 0.4kb allele. A 50% reduction in intensity of the 0.4kb fragment is seen in lane 1 compared with lane 2 as expected for this two allele system.
<table>
<thead>
<tr>
<th>Allelic Fragments</th>
<th>Number Observed</th>
<th>Phenotype Frequency</th>
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<tbody>
<tr>
<td>0.7kb + 0.7kb</td>
<td>1</td>
<td>0.04</td>
</tr>
<tr>
<td>0.7kb + 0.4kb</td>
<td>17</td>
<td>0.68</td>
</tr>
<tr>
<td>0.4kb + 0.4kb</td>
<td>7</td>
<td>0.28</td>
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</table>

**Allele Frequency**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7kb</td>
<td>0.38</td>
</tr>
<tr>
<td>0.4kb</td>
<td>0.62</td>
</tr>
</tbody>
</table>
3.5 Cl-inhibitor gene RFLPs in hereditary angio-oedema

A total of 12 kindred with Type I HAE and two kindred with Type II HAE were screened for Cl-inhibitor gene RFLPs using the exon 2-8 Cl-inhibitor cDNA as probe. Four of the Type I HAE kindred were found to have Cl-inhibitor gene RFLPs which co-segregated with the disease. Neither of the Type II HAE kindred showed an identifiable abnormality. Once identified, the location of the Cl-inhibitor gene mutation responsible for each RFLP was established by using the 5', middle and 3' fragments of the exon 2-8 Cl-inhibitor cDNA as probes. Each of these Cl-inhibitor gene RFLPs will be described in turn.

Family A - The Cl-inhibitor gene RFLP in this family was generated by the restriction enzyme Sty I and was represented by an additional 1.1kb fragment accompanied by a 50% reduction in the intensity of the 0.9kb fragment as shown in Figure 24. A total of 23 family members from three generations of the family were tested with only the nine DNA samples from Type I HAE patients showing the polymorphism (Figure 25). Localisation studies showed that the RFLP was detected by the middle (exon 4-7) probe (Figure 26). When the 5' (exon 2-4) and the 3' (exon 8) fragments were used as probes only the normal Sty I restriction fragments appropriate for the 5' and 3' ends of the gene were detected in both normal and patient samples as shown in Figure 27 and Figure 28 respectively. This information in conjunction with known Cl-inhibitor gene sequence (183) indicated that the polymorphism was due to a mutation within one Cl-inhibitor allele that produced loss of the Sty I recognition site that lies very close to the 3' end of the sixth exon (Figure 29). A major deletion or insertion event did not appear to be responsible for the
mutation. This conclusion was based on two sets of observations. Firstly, no changes in the sizes of restriction fragments spanning the mutated area of the gene were apparent at the resolution of Southern analysis. Furthermore, the size of the 1.1kb fragment was derived by simply adding the sizes of its constituent fragments together (0.9kb + 0.2kb). Secondly, the positions of the restriction enzyme recognition sites for Rsa I and Hinf I (Figure 29), both of which lie within 40 base pairs of the mutated Sty I site, were unaffected. The likely significance of the position of this mutation at an exon/intron boundary will be discussed.
FIGURE 24
Cl-inhibitor gene RFLP generated by Sty I in Family A and detected by using the exon 2–8 Cl-inhibitor cDNA as probe. Lane 1 represents the restriction fragment pattern for a family member with Type I HAE. Lane 2 represents the pattern for a normal family member. There is an additional 1.1kb fragment in lane 1 with a 50% reduction in the intensity of the 0.9kb fragment.
FIGURE 25
Detailed family tree for Family A showing the six main subdivisions with males indicated by a square and females by a circle. Individuals with Type I HAE are indicated by a star underlying their symbol. A dot within the symbol indicates those family members with Type I HAE who underwent RFLP analysis.
FIGURE 26
Cl-inhibitor gene RFLP generated by Sty I in Family A and detected by the middle (exon 4-7) probe. Lane 1 represents the restriction fragment pattern for a normal family member and lane 2 represents the pattern for a family member with Type I HAE. There is an additional 1.1kb fragment in lane 2 with a 50% reduction in the intensity of the 0.9kb fragment. Lane 3 represents Sty I/Bgl II digested DNA from the same HAE patient as in lane 2. The polymorphic fragments are unaffected however the 3.7kb fragment is reduced in size to 2.0kb as predicted from sequence data for this region of the Cl-inhibitor gene (183).
FIGURE 27
Cl-inhibitor gene restriction fragments in Family A as detected by the 5' (exon 2-4) probe. Lanes 1 and 2 represent Sty I digested DNA from a normal family member and a Type I HAE family member respectively. A 4.8kb fragment is generated in both lanes as expected for the 5' end of the gene (183). Lanes 3 and 4 represent Sty I/Bgl II digested DNA from the same Type I HAE patient and normal family member respectively. A 4.8kb fragment is generated in both lanes as expected for the 5' end of the gene. Lane 5 represents DNA from a Type I HAE family member digested with Bgl II. This generates a 10.8kb fragment which is appropriate for the 5' end of the gene.
FIGURE 28
Cl-inhibitor gene restriction fragments in Family A as detected by the 3' (exon 8) probe. Lanes 1 and 2 represent Sty I digested DNA from a normal family member and a Type I HAE family member respectively. A 0.7kb fragment is generated in both lanes as expected for the 3' end of the gene (183). Lanes 3 and 4 represent Sty I/Bgl II digested DNA from the same Type I HAE patient and normal family member respectively. A 0.2kb fragment is generated in both lanes as expected for the 3' end of the gene. The 0.2kb fragment is very faint in lane 4 as a result of a smaller amount of DNA being present in this lane compared with lane 3. Lane 5 represents DNA from a Type I HAE family member digested with Bgl II. This generates 6.5kb and 1.9kb fragments which are appropriate for the 3' end of the gene.
Restriction map of the 3' half of the Cl-inhibitor gene as detected by the middle (exon 4-7) probe for Family A. Loss of the Sty I site at the 3' end of exon 6 generates the 1.1kb fragment (0.9kb + 0.2kb) present in family members with Type I HAE. The positions of the recognition sites for the restriction enzymes Rsa I and Hinf I which flank the mutated Sty I site are shown. These sites are unaffected by the mutation in the Cl-inhibitor gene in Family A. The 0.2kb fragment was not detected by the exon 2-8 or middle (exon 4-7) probe since the nucleotide composition of this fragment includes only four nucleotides derived from the sixth exon. The faint signal present in
FIGURE 29 (continued)

Figure 26 of approximately 0.2kb in size represents more than one similarly sized fragment (0.1-0.2kb) derived from the 5' half of the gene (183). It does not represent the 0.2kb fragment from the 3' end of the sixth exon and 5' end of the sixth intron. The 5' (exon 2-4) probe does not apparently detect the 0.1-0.2kb fragments derived from the 5' end of the gene in Figure 27 due to the fact that the exposure time for the autoradiograph was too short for these low intensity fragments to be recorded.
Family B - Analysis of Bgl II digested DNA from the one affected and two available normal family members showed a Cl-inhibitor gene RFLP in the Type I HAE patient. The RFLP comprised an additional 8.4kb fragment with a 50% reduction in the intensity of the 6.5kb and 1.9kb fragments as shown in Figure 30. Localisation studies for this gene mutation showed that the RFLP was detected by the 3' (exon 8) probe (Figure 31). When the middle and 5' cDNA fragments were used as probes only the normal restriction fragments appropriate for those areas of the gene were detected as shown in Figure 32 for the middle probe. This information in conjunction with known Cl-inhibitor gene nucleotide sequence (183) indicated that the polymorphism was due to a mutation within one Cl-inhibitor allele that produced loss of the Bgl II recognition site present in exon 8 towards its 5' end (Figure 33). A major deletion or insertion event did not appear to have given rise to the mutation since no alteration in the sizes of restriction fragments spanning the mutated area was seen and the size of the 8.4kb fragment was derived by simply adding the sizes of its constituent fragments together (6.5kb + 1.9kb). In addition, the positions of the adjacent restriction sites for Taq I and Pst I (Figure 33) were preserved.
Cl-inhibitor gene RFLP generated by Bgl II in Family B and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lane 1 represents the restriction fragment pattern for a normal family member. Lane 2 represents the pattern for the Type I HAE family member. There is an additional 8.4kb fragment in lane 2 with a 50% reduction in the intensity of the 6.5kb and 1.9kb fragments. The 1.9kb fragment is so faint at this autoradiograph exposure that the reduction in its intensity cannot be appreciated.
FIGURE 31

Cl-inhibitor gene RFLP generated by Bgl II in Family B and detected by the 3' (exon 8) probe. Lanes 1 and 3 represent the restriction fragment pattern for two normal family members. Lane 2 represents the pattern for the family member with Type I HAE. There is an additional 8.4kb fragment in lane 2 with a 50% reduction in the intensity of the 6.5kb and 1.9kb fragments. Lane 4 represents the fragment pattern for Bgl II digested DNA from an unrelated control. Lanes 5, 6 and 7 represent the restriction fragment patterns for Bgl II/Hind III digested DNA from the same two normal family members and from the Type I HAE patient (lane 6). An additional 4.2kb fragment is present in lane 6 with a 50% reduction in the intensity of the 3.7kb and 0.5kb fragments. These are the fragment sizes expected for a Bgl II/Hind III digest of the 3' end of the mutated and normal gene (183).
FIGURE 32
Cl-inhibitor gene restriction fragments in Family B as detected by the middle (exon 4-7) probe. Lanes 1 and 3 represent DNA from two normal family members digested with Bgl II/Hind III. Lane 2 represents DNA from the Type I HAE patient digested with Bgl II/Hind III. The 9.6kb and 0.6kb fragments generated in all three lanes are as expected for the middle region of the gene (183).
FIGURE 33
Restriction map of the 3' end of the Cl-inhibitor gene for Family B. The 3' (exon 8) probe detects the 6.5kb and 1.9kb Bgl II fragments in normal family members. Loss of the Bgl II site in exon 8 generates the 8.4kb fragment (6.5kb + 1.9kb) present in the Type I HAE family member. The positions of the recognition sites for the restriction enzymes Taq I and Pst I which flank the mutated Bgl II site in exon 8 are shown. These sites are unaffected by the mutation in the Cl-inhibitor gene from Family B.
Family C - A total of eight family members, including six Type I HAE patients, were examined for Cl-inhibitor gene RFLPs. RFLPs were identified in the six Type I HAE patients with nine separate restriction enzymes. There was loss of a Bgl II recognition site with generation of an additional 20kb fragment and a 50% reduction in the intensity of the 10.8kb and 10.2kb fragments as shown in Figure 34. The enzymes Bam HI, Bgl I, Bst EII, Dra I, Hinc II, Sst I and Stu I all generated an additional fragment which was consistently about 1kb smaller than the corresponding normal fragment whose intensity was reduced (Figure 34). Finally, the restriction fragment pattern for the enzyme Taq I showed a 50% reduction in the intensity of the 1.4kb fragment (Figure 35). There was no apparent generation of an additional fragment by Taq I. Localisation studies for this gene mutation showed that the RFLPs were detected by the 5' and middle probes as shown in Figure 36 for the 5' probe. The 3' probe detected identical patient and control restriction fragments appropriate in size for the 3' end of the Cl-inhibitor gene (Figure 37). These findings interpreted along with known Cl-inhibitor gene structure (183) indicated that the mutated Bgl II site lay in the fourth exon of one Cl-inhibitor allele (Figure 38). Furthermore, when combined with the fact that there was a 1kb reduction in the size of restriction fragments spanning the fourth exon and its flanking introns, this indicated that the gene lesion most probably represented an example of the exon 4 deletion described recently by Stoppa-Lyonnet et al in Type I HAE (188). This was confirmed by the 50% reduction in intensity of the 1.4kb Taq I fragment since this fragment spans only the fourth exon and portions
of its adjacent introns as shown in Figure 38. The apparent lack of generation of an additional fragment by Taq I is due to the fact that although a complete exon 4 deletion does produce an abnormal sized Taq I fragment, this fragment is purely intronic in origin and so does not hybridise to the cDNA probe.
FIGURE 34

Cl-inhibitor gene RFLPs in Family C detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lanes 1 and 2 represent Bgl II digested DNA from a normal family member and from a Type I HAE family member respectively. There is an additional 20kb fragment in lane 2 with a 50% reduction in the intensity of the 10.8kb and 10.2kb fragments. Each of the lane pairs 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16 represent normal and Type I HAE patient DNAs digested with the following enzymes - Bam HI, Sst I, Hinc II, Bgl I, Bst EII, Stu I and Dra I respectively. There is an additional fragment in lanes 4, 6, 8, 10, 12, 14 and 16 which correspond to each of the patient lanes for each enzyme. Each additional fragment
FIGURE 34 (continued)
is approximately 1kb smaller than the corresponding normal fragment
which is reduced in intensity by 50% except for the enzymes Bam HI
and Sst I where the reduction in intensity is less than this due to
the fact that the normal "fragment" for each enzyme is a closely
spaced doublet whose constituent fragments have not been resolved.
In lanes 1 and 2 the normal 1.9kb fragment generated by Bgl II is
not visible due to the relatively short exposure time for this
autoradiograph.
FIGURE 35

Cl-inhibitor gene RFLP generated by Taq I in Family C and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lane 1 represents the restriction fragment pattern for a normal family member and lane 2 is the pattern for a family member with Type I HAE. There is a 50% reduction in the intensity of the 1.4kb fragment in lane 2. No additional fragment is detected.
Cl-inhibitor gene RFLPs generated by Bam HI and Bgl II in Family C and detected by the 5' (exon 2-4) probe. Lane 1 represents the Bam HI restriction fragment pattern for a normal family member. Lane 2 represents the Bam HI fragment pattern for a Type I HAE family member. An additional 6.0kb fragment is present in lane 2 with a 50% reduction in the intensity of the normal singlet 7.0kb fragment. Lanes 3 and 4 represent the restriction fragment patterns for Bam HI/Bgl II digested DNA from the same Type I HAE patient and the same normal family member respectively. The normal, expected fragment pattern for the 5' end of the gene is generated in lane 4 (183) and the 6.0kb polymorphic fragment is maintained in lane 3 as expected. Lane 5 represents the restriction fragment pattern for Bgl II digested DNA from the same Type I HAE patient DNA as used in lanes 2 and 3. There is a 20kb fragment in addition to the 10.8kb fragment appropriate for the 5' end of the gene.
Cl-inhibitor gene restriction fragment patterns generated by Bam HI and Bgl II in Family C and detected by the 3' (exon 8) probe. Lanes 1 and 2 represent Bam HI digested DNA from a normal family member and a Type I HAE patient. The 7.0kb fragment present in both lanes is the fragment size expected for the 3' end of the gene (183). Lanes 3 and 4 represent Bam HI/Bgl II digested DNA from the same Type I HAE patient and normal family member respectively. The 6.7kb and 0.3kb fragments generated in both lanes are as expected for the 3' end of the gene.
Restriction map of the 5' end of the Cl-inhibitor gene for Family C. The 5' (exon 2-4) probe detects the 10.8kb Bgl II fragment in normal family members. Loss of the Bgl II recognition site in exon 4 due to a 1kb deletion involving exon 4 generates the additional 20kb fragment (10.8kb + 10.2kb - 1.0kb) as shown in lane 5 of Figure 36. The size of this deletion is indicated by the reduction in size of the normal 7.0kb Bam HI fragment to 6.0kb as shown in lane 2 of Figure 36. The position of the 1.4kb Taq I fragment which spans the fourth exon is shown.
Family D - The single Type I HAE patient in this family of three showed Cl-inhibitor gene RFLPs with the following restriction enzymes - Bam HI, Bgl II, Eco RI, Hind III, Kpn I, Pst I, Pvu II, Sst I, Stu I and Xmn I. The most informative enzymes were Hind III, Kpn I, Pst I, Sst I, Stu I and Xmn I. All of this group, except Hind III, generated an obvious additional fragment as shown in Figures 39, 40, 41 and 42. For Hind III the only apparent abnormality was a 50% reduction in the intensity of the 0.6kb fragment (Figure 39). Depending on which enzyme was being used this complex pattern of altered restriction fragments was detectable by both the middle probe and the 3' probe but not by the 5' probe as shown in Figures 43, 44 and 45 for the enzymes Sst I and Stu I. These RFLP data combined with the known restriction map of the Cl-inhibitor gene (183) were consistent with the following Cl-inhibitor gene abnormalities as summarised in Figure 46. 1) Loss of the Sst I site which is normally present at the 5' end of the seventh intron. 2) Loss of the two Pst I sites at the 3' end of the sixth intron and 5' end of the seventh exon. 3) Loss of the Kpn I recognition site which lies within the seventh exon towards its 5' end. 4) Deletion of approximately 1.7kb of DNA affecting the same area of the gene as shown by the reduction in size of the restriction fragments spanning the seventh exon for each of the enzymes Stu I and Xmn I. The 50% reduction in intensity of the 0.6kb Hind III fragment was very useful in exactly localising the deletion to the seventh exon since the affected 0.6kb fragment comprises only the seventh exon and 400bp of flanking sixth and seventh introns. These changes are all entirely consistent with the Cl-inhibitor gene
mutation in this Type I HAE family being a complete deletion of the seventh exon and portions of its flanking introns as described by Ariga et al (190). The subtle restriction fragment abnormalities generated by the enzymes Bam HI, Eco RI and Pvu II also helped to confirm the exon 7 deletion. There was a 50% reduction in the intensity of one of the normal fragments for each enzyme but without the generation of an additional fragment (data not shown). In each case this normal fragment with a reduced intensity comprised only the seventh exon plus variable lengths of the adjacent sixth and seventh introns. Deletion of the seventh exon from 50% of the fragments prevented these fragments from hybridising to the cDNA probe with a resultant 50% reduction in fragment intensity. The absence of an additional fragment was due to the fact that all these enzymes cleaved again within both the sixth and seventh introns outwith the limits of the deletion so the abnormal sized fragments generated were purely intronic in origin. These intronic fragments would not hybridise to the cDNA probe. Interestingly this was not the explanation for the apparent lack of an additional fragment generated by Hind III. Gene sequence data for the normal Cl-inhibitor gene (183) indicated that an additional fragment should have been generated by Hind III, the size of this fragment being 14.3kb. A 50% reduction in the intensity of the normal 14.1kb fragment should also be seen. With the electrophoretic conditions employed, however, this 14.3kb fragment would not be resolved from the normal 14.1kb fragment (Figure 39).
FIGURE 39

Cl-inhibitor gene RFLPs in Family D generated by Pst I and Hind III and detected by using the exon 2–8 Cl-inhibitor cDNA as probe. Lanes 1 and 2 for the Pst I digest represent DNA from a normal family member and from the Type I HAE patient respectively. There is an additional 1.7kb fragment in lane 2 with a 50% reduction in the intensity of the 2.6kb fragment. Lanes 1 and 2 for the Hind III digest represent DNA from a normal family member and from the Type I HAE patient. There is a 50% reduction in the intensity of the 0.6kb fragment in lane 2. No additional fragment is present.
FIGURE 40
Cl-inhibitor gene RFLP in Family D generated by Xmn I and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lane 1 represents DNA from a normal family member and lane 2 represents DNA from the Type I HAE patient. There is an additional 14.3kb fragment in lane 2 with a 50% reduction in the intensity of the 16.0kb fragment.
FIGURE 41

Cl-inhibitor gene RFLP generated by Kpn I in Family D and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lane 1 represents DNA from a normal family member. Lane 2 represents DNA from the Type I HAE patient. There is an additional 16.3kb fragment with a 50% reduction in the intensity of the 13kb fragment in lane 2.
FIGURE 42
Cl-inhibitor gene RFLPs generated by Sst I and Stu I in Family D and detected by using the exon 2-8 Cl-inhibitor cDNA as probe. Lanes 1 and 2 represent Sst I digested DNA from a normal family member and from the Type I HAE family member respectively. There is an additional 7.0kb fragment in lane 2 with a 50% reduction in the intensity of the 3.0kb fragment and approximately a 25% reduction in intensity of the 6kb doublet "fragment". Lanes 3 and 4 represent Sst I/Bgl II digested DNA from the same patient and normal family member respectively. There is an additional 6.4kb fragment in lane 3 with a 50% reduction in the intensity of the 5.7kb and faint 1.9kb fragments. Lanes 5 and 10 represent Bgl II digested DNA from a
normal family member and from the Type I HAE patient respectively. In lane 5 a faint 1.9kb fragment is present, however, in lane 10 this fragment is not visible. This represents a 50% reduction in the intensity of the 1.9kb fragment in lane 10 but at this autoradiograph exposure time no signal is apparent. In lane 10, although an additional 10.4kb fragment is created, this cannot be resolved from the normal 10.2kb fragment under these electrophoretic conditions. Lanes 6 and 7 represent Stu I digested DNA from a normal family member and the Type I HAE patient. An additional 9.4kb fragment is present with a 50% reduction in the intensity of the 11.1kb fragment. Lanes 8 and 9 represent Stu I/Bgl II digested DNA from the patient and a normal family member respectively. No difference in fragment pattern is present. Lanes 11 and 12 represent Sst I/Stu I digested DNA from a normal family member and the HAE patient. An additional 6.3kb fragment is present in lane 12 with a 50% reduction in the intensity of the 5.9kb fragment.
FIGURE 43

Cl-inhibitor gene RFLPs in Family D as detected by the middle (exon 4-7) probe. Lanes 1 and 2 represent Sst I digested DNA from a normal family member and the Type I HAE patient respectively. An additional 7.0kb fragment is present in lane 2 with a 50% reduction in the intensity of the singlet 5.7kb fragment. Lanes 3 and 4 represent Sst I/Bgl II digested DNA from the Type I HAE patient and a normal family member respectively. There is an additional 6.4kb fragment in lane 3 with a 50% reduction in the intensity 5.7kb singlet fragment. Lane 5 represents Bgl II digested DNA from a normal family member. There is a 10.2kb fragment which is appropriate for this region of the gene. Lanes 6 and 7 represent Stu I digested DNA from a normal family member and from the HAE patient respectively. An additional 9.4kb fragment is present in lane 7 with a 50% reduction in the intensity of the 11.1kb fragment.
FIGURE 44

Cl-inhibitor gene RFLP generated by Sst I in Family D and detected by the 3' (exon 8) probe. Lane 1 represents Sst I digested DNA from a normal family member. Lane 2 represents Sst I digested DNA from the Type I HAE patient. There is an additional 7.0kb fragment in lane 2 with a 50% reduction in the intensity of the 3.0kb fragment.
FIGURE 45

Cl-inhibitor gene restriction fragment patterns in Family D as detected by the 5' (exon 2-4) probe. The order of DNA samples and the restriction enzymes used to digest each sample are identical to those described for Figure 42. No polymorphic fragments are detected, the fragment patterns being identical for the normal and patient DNA samples. In addition, the fragment sizes are appropriate for the 5' end of the gene (183).
FIGURE 46

Restriction map of the 3' half of the Cl-inhibitor gene for Family D. The middle (exon 4-7) probe detects the 5.7kb Sst I restriction fragment in normal family members. However, loss of the Sst I site just downstream of the 3' end of the seventh exon due to a 1.7kb deletion generates the additional 7.0kb fragment (5.7kb + 3.0kb - 1.7kb) as shown in lane 2 of Figure 43. Using the 3' (exon 8) probe the 3.0kb Sst I fragment is detected in normal family members whereas an additional 7.0kb fragment is seen in the Type I HAE patient (Figure 44, lane 2). The size of the gene deletion is indicated by the reduction in size of the normal 11.1kb Stu I fragment to 9.4kb as shown in lane 7 of Figure 43. In addition, the deletion produces loss of the two Pst I sites and loss of the Bgl II site which are marked at opposite ends of the seventh exon.
3.6 Kpn I and Hgi AI RFLPs in Type I and Type II HAE families

In each Type I and Type II HAE family the pattern of Kpn I and Hgi AI alleles present in both patients and normal family members was studied in order to establish whether it was possible to associate the mutated Cl-inhibitor gene with a particular Kpn I and Hgi AI allele. The results are shown in Table 10. It can be seen that for all the families an allele association with the mutant gene could be made with at least one of the polymorphic endonucleases. Furthermore, for nine of the Type I HAE families the abnormal Cl-inhibitor gene had an identifiable allele association with one allele generated by both polymorphic endonucleases. The commonest associations were with the 13kb Kpn I and 0.4kb Hgi AI alleles which reflects the overall commoner population frequency of each of these alleles as described in Section 3.4. In five of the families a definite allele association could not be established for one of the polymorphic endonucleases. The potential usefulness of these observations will be discussed.
<table>
<thead>
<tr>
<th>HAE FAMILY</th>
<th>Kpn I</th>
<th>Hgi AI</th>
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<tbody>
<tr>
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<td></td>
<td></td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Family I</td>
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<tr>
<td>Family B</td>
<td>13kb</td>
<td>not established</td>
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3.7 Clinical survey of HAE in Scotland

(a) Disease incidence

This survey identified a total of 46 HAE patients derived from 22 kindred. Type I HAE was present in 21 of these kindred whereas Type II disease affected in only one kindred. The disease incidence in Scotland is therefore approximately 1 in 110,000 since the current population of Scotland is 5,090,700. Furthermore, a male:female patient ratio of 0.58:0.42 and a 43% incidence of HAE patients in the affected kindred was entirely compatible with an autosomal dominant mode of inheritance for the disease. A patient age range of one year to 65 years of age existed.

(b) Diagnosis and symptoms

Detailed clinical histories have so far been obtained for 21 HAE patients from 17 kindred. The following analysis is based on the findings in these patients. The diagnosis of HAE was made in 17 out of 21 patients before the age of 30 years with virtually equal numbers being diagnosed in each of the three decades. Only four patients had the diagnosis made later than this, three being in their thirties and one patient being 50 years of age. This last individual had no symptoms prior to the episodes of angio-oedema which led to diagnosis. It was commonplace for a number of years to elapse between the onset of symptoms and diagnosis. Indeed, for one individual the delay was in excess of 20 years. This length of delay was exceptional in this study, however, for approximately 20% of patients the diagnostic delay exceeded four years. The delay in the remaining 80% of patients was often not significantly less than four
years. Mild and infrequent initial symptoms appeared to have a significant role to play in causing this delay.

In the majority of patients, 16 out of 21, the symptoms experienced were a combination of angio-oedema of the skin and gastro-intestinal upset, although not necessarily simultaneously. For the remaining five patients gastro-intestinal symptoms were not a feature of their disease. The commonest cutaneous sites involved by angio-oedema were the hands, face and feet whereas gastro-intestinal oedema usually manifested itself as colicky abdominal pain, nausea, vomiting and abdominal distension. In five individuals gastro-intestinal involvement was prominent but was never the only manifestation of the disease. A total of six patients experienced oedema of the upper respiratory tract with resultant respiratory difficulty of variable degree. This represented the most serious effect of HAE since laryngeal oedema with resultant asphyxia is the only recorded way in which the disease can be fatal.

Considerable variation between patients was seen in the length of acute disease attacks with the range being 36 hours up to four days. Likewise the frequency of attacks varied markedly between individual patients and in some cases varied at different times for the same individual. In those patients with frequent attacks, sometimes two or three attacks every month with each attack lasting approximately three days, the result could be up to nine days illness each month for that patient. When regarded in these terms it is easy to appreciate how disruptive HAE can be for the patients and for their families. Precipitating factors for acute disease attacks were identified for ten of the 21 patients. Trauma was the commonest
trigger, being reported by eight of the ten patients, with dental procedures representing the single most potent event. Premenstrual accentuation of the disease and acute attacks following emotional stress were evident in the remaining two patients.

Disease remission during pregnancy was experienced by one patient in this study. Remission was complete in that no symptoms or signs of the disease were apparent during the second and third trimesters. This quiescent disease state also persisted for a number of months after delivery. The same degree of remission occurred in each of the patient's three pregnancies with each "disease-free" period representing a very welcome relief from her usual frequent and severe symptoms.

(c) Treatment

Drug therapy was being prescribed for 19 of the 21 patients. The reasons for two patients not receiving treatment were that one patient was an asymptomatic six year old diagnosed biochemically during a family study and the second patient refused therapy. Treatment consisted of an attenuated androgen (danazol or in a single case stanazolol) for 13 patients, the anti-fibrinolytic agent tranexamic acid for three patients and a combination of androgen and tranexamic acid for the remaining three patients. Combination therapy for these three patients was an attempt to control their severe symptoms, however, in each case disease control remained incomplete, one patient in particular still experiencing frequent attacks of cutaneous angio-oedema and abdominal pain. The effective dose of danazol varied for each patient with therapy being regularly
adjusted to achieve the minimum effective dosage for each individual. This helped to minimise the side-effects of these potent drugs. Despite this, drug-induced weight gain and, for women, a variable degree of menstrual irregularity were very commonly seen. Other more severe side-effects which included hepatic dysfunction and depression were confined to individual cases. When interpreting the apparent effectiveness of attenuated androgens in HAE it should be remembered that of the 13 individuals treated with them three had mild symptoms before treatment and five patients had periods of breakthrough symptomatic disease during treatment. For these five individuals drug compliance was not thought to be a problem. The use of tranexamic acid alone as a form of therapy in three patients was for a specific reason in each case. The prepubertal status of one patient precluded the use of androgens, one patient was unwilling to take androgens because of their potential side-effects and finally one patient developed hepatic side-effects due to danazol therapy thereby necessitating its withdrawal. All three of the patients receiving tranexamic acid alone had their symptoms only partly controlled by the drug, although in one patient drug compliance was poor.

The extent to which Cl-inhibitor concentrate had been used was ascertained in this study. It was found that concentrate had been administered to six patients from six kindred in two main situations. In five cases it was used to prevent an acute disease attack following elective dental surgery. In a sixth patient its use successfully interrupted a spell of severe disease activity that was not being controlled by increasing doses of danazol and tranexamic
acid in combination.

Disease-related deaths do still occur in HAE despite modern drug therapy. Two kindred were identified in whom a definite asphyxial disease-related death had occurred in recent years and a further two kindred had each recorded a possible disease-related death.

(d) Clinical genetic considerations

A definite parental history of HAE was documented for eight patients and for a further two patients the disease affected at least one brother or sister so indicating parental origin. For the remaining 11 patients there was a complete lack of family history of the disease. This absence of a family history raises the possibility that the genetic abnormality in each of these patients represents a spontaneous gene mutation. Before this possibility can be accepted, however, the presence of asymptomatic parental disease must be excluded. This can be achieved by testing parental blood samples for Cl-inhibitor and C4 levels. Both parents of two patients were shown to be biochemically normal in this way so indicating the spontaneous nature of the Cl-inhibitor gene mutation in these two patients. In the remaining nine patients with no parental clinical history of HAE there was no documented evidence of parental complement system status. It was therefore possible that a spontaneous mutation accounted for the disease in these nine patients, however, the likelihood is that for a significant number of them the parent carrying the mutant Cl-inhibitor gene was asymptomatic or else the parental disease was mild and had never been diagnosed.
Excluding the four children, 16 of the remaining 17 patients had married with 14 of these married patients subsequently having children. One married patient decided against having a family because of the risk of the children inheriting the disease and one married patient had not yet started a family but was prepared to do so despite the risk. Potential interest was shown in the concept of prenatal diagnosis by nine patients with a definite decision to use it, should it become available, being made by two of these individuals. The four children were too young to be questioned about prenatal diagnosis.
3.8 Genomic library construction

At the outset generating sufficient numbers of recombinant bacteriophage proved difficult. These problems resolved when the protocol for high molecular weight DNA preparation described in Section 2.2.15 was adopted. Prior to this, prolonged proteinase K digestion and phenol extraction steps had been employed in addition to multiple ethanol precipitations and RNase A treatment of the DNA preparation. Cumulatively all of these manoeuvres appeared to produce an unacceptable degree of damage to the DNA in the form of random breaks. This damage was not apparent when an aliquot of the final DNA preparation was visualised after agarose gel electrophoresis. However, following endonuclease digestion by Sau 3AI a significant proportion of the 15-20kb fragments purified by sucrose gradient ultracentrifugation would have an end which had been created by this random cleavage and not by the action of the endonuclease. Such fragments would be unable to ligate completely into the vector and so their presence would markedly reduce cloning efficiency (201). Once this problem had been overcome two genomic libraries were constructed, the first from a normal individual and the second from Type II HAE patient DNA. An input of approximately 1.2 μg of size-fractionated insert DNA and 3 μg of EMBL3 DNA was sufficient to generate 47,000,000 genuine recombinant bacteriophage in the normal library and 27,000,000 genuine recombinants in the HAE library. Both libraries are to be used to isolate Cl-inhibitor genomic clones.
### DISCUSSION INDEX

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4.1 Restriction fragment analysis of the normal Cl-inhibitor gene

The initial selection of the restriction endonucleases to be used in this RFLP study of the Cl-inhibitor gene was determined by a number of factors, the most important of which were as follows - published statistical likelihood of each endonuclease detecting an RFLP (202); enzyme availability; cost. The enzymes selected were the ones which combined a high likelihood of detecting an RFLP based on recognition sequence characteristics with a previous record of success, ready availability and reasonable cost since relatively large amounts of enzyme would be necessary. Since no Cl-inhibitor gene sequence data were available at the outset of the investigation the use of a wide range of restriction enzymes helped to ensure the generation of sets of DNA fragments which were both representative of the whole gene and its flanking sequences and which were of a size that could be accurately examined by standard 0.8% agarose gel electrophoresis and Southern blotting. A total of 38 restriction endonucleases were used with 20 of these enzymes generating multiple well spaced, easily resolved fragments as shown in Table 7, Section 3.3. Subsequently it was members of this group that were informative in RFLP analysis of the Cl-inhibitor gene. The other 18 enzymes listed in Tables 5 and 6 of Section 3.3 were largely uninformative due to the nature of their fragment patterns. Analysis of the restriction map of the normal Cl-inhibitor gene indicated that many of these latter enzymes did generate polymorphic fragments in two of the RFLP positive Type I HAE families. However, due to the fact that these enzymes generated either very large fragments or else multiple small fragments, the polymorphic fragments generated

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could not be resolved from the normal fragments with the electrophoretic conditions employed. Major alterations in electrophoretic methodology would have been necessary to produce identifiable abnormalities in the fragment patterns generated by these enzymes.

4.2 Cl-inhibitor gene RFLPs in the normal population

The restriction enzymes Kpn I and Hgi AI both generated RFLPs for the Cl-inhibitor gene in the normal population as described in Section 3.4. The Kpn I polymorphism (203) was previously unreported since no other investigator had used this restriction enzyme. To obtain good resolution of the 30kb polymorphic fragment from the invariant 24kb fragment it was necessary to use a prolonged period of electrophoresis through 0.8% agarose gels. Routinely 64 hours at 25 volts was employed to achieve good separation. Standard electrophoretic conditions of 16 hours at 40 volts produced only a single, wide 24kb fragment which meant that identification of a heterozygote was dependent upon recognition of a 50% reduction in the intensity of the 13kb fragment (Figure 19, Section 3.4). Only the homozygote for the 30kb allele would be immediately apparent due to the complete absence of the 13kb fragment. Recognition of a 50% reduction in the intensity of a fragment can be difficult particularly when an autoradiograph is overexposed or an adjacent control sample does not match exactly the test sample with regard to the quantity of DNA loaded into the gel. Detection of the Kpn I polymorphism emphasised the importance of adequate fragment resolution in RFLP analysis. In this study the use of 200mm long
agarose gels and 16 hour electrophoresis runs helped to ensure reasonable fragment resolution.

Localisation of the mutation responsible for the Kpn I RFLP showed that it lay approximately 10kb downstream of the Cl-inhibitor gene. No attempt was made to further characterise the mutation responsible for the RFLP, however, since no alterations were seen in the sizes of restriction fragments which extended this distance downstream of the Cl-inhibitor gene, it could be inferred that the mutation was not a major deletion. Lying at this distance from the Cl-inhibitor gene the mutation was unlikely to be able to quantitatively affect the gene product. This was borne out by the fact that no relationship could be demonstrated between possession of a particular allelic combination and serum Cl-inhibitor protein level.

Demonstration of the Hgi AI RFLP in the normal population confirmed the observations of Bock et al (67) in the North American population. No relationship could be demonstrated between possession of a particular Hgi AI allelic combination and serum Cl-inhibitor protein level or Cl-inhibitor functional activity despite the fact that the gene mutation responsible for the RFLP produced an amino-acid substitution in the Cl-inhibitor polypeptide. This substitution was very close to the carboxy-terminus of the protein, distal to its reactive site in an area which has no known role to play in maintaining the stressed loop of the reactive site or in forming part of the "bait sequences" of the molecule. Thus a lack of biological effect for such a mutation was not entirely surprising.

In a study of HAE the relevance of Cl-inhibitor gene RFLPs in
the normal population is their potential application as indirect genetic markers of the mutated Cl-inhibitor allele responsible for the disease (204). Such an application is dependent upon a low level of recombination between the disease locus and the RFLP locus and upon the presence of a reasonable degree of polymorphism. For both the Hgi AI and Kpn I polymorphisms the chances of recombination between the disease locus and the RFLP locus will be very low. This is particularly true for the Hgi AI RFLP since the mutation responsible for it resides within the Cl-inhibitor gene itself. For the Kpn I RFLP the risk of recombination is also very low since 10kb is a relatively short distance in terms of recombination when it is considered that there is only a 1% chance of recombination between two loci separated by 1,000kb (204). Furthermore, since the distance between the two loci responsible for the RFLPs is only marginally greater than 10kb, the chance of recombination occurring between these two loci is also very small. Detection of more than one RFLP in the normal population makes it possible to combine the information from each independent RFLP to construct a simple haplotype for the Cl-inhibitor gene locus in each individual. The whole haplotype can then be used as an "allele" for that locus. The Kpn I and Hgi AI RFLP system as detected by the exon 2-8 cDNA probe allows the generation of four different haplotype "alleles" each of which comprises a different arrangement of cutting or non-cutting at each of the two restriction sites. Since the individual alleles for the Kpn I and Hgi AI RFLPs are not excessively rare it is likely that each of the four haplotype "alleles" will be present in the general population and so will be potentially useful as indirect
genetic markers for the disease. Identification of additional Cl-inhibitor gene RFLPs in the normal population would be worthwhile since it would markedly increase the number of potentially informative haplotypes. One additional RFLP would increase the number of haplotypes from four to eight and if two additional RFLPs were identified 16 different haplotype "alleles" could theoretically be generated. In summary, it is apparent that the potential usefulness of multiple RFLPs is more than the simple addition of the usefulness of each individual RFLP.

The use of both the Kpn I and Hgi AI RFLPs as indirect genetic disease markers will be of particular importance to those Type I and Type II HAE families for whom no disease-specific RFLP can be identified. Indeed, in this and in other studies at least 70% of the Type I HAE families fall into this category. In Type II HAE current evidence suggests that the gene mutations responsible for the disease remain to be identified in only 30% of families (185). As shown in Table 10 of Section 3.6, allele allocation of the disease locus for at least one of the polymorphic endonucleases has been made in all the Type I and Type II HAE families. In addition, for five of the Type I families without a disease-specific RFLP, allele allocation for both endonucleases was possible. More detailed and extensive studies would need to be undertaken in each family, however, before the Kpn I and Hgi AI RFLPs could be used in a predictive capacity for disease presence in a particular member of these families.
4.3 Disease-specific Cl-inhibitor gene RFLPs in HAE

In this study a total of four out of 12 Type I HAE kindred showed identifiable, unique Cl-inhibitor gene RFLPs which co-segregated with the disease. In each case the RFLP affected one Cl-inhibitor allele as expected for an autosomal dominant, heterozygous disease. Neither of the Type II HAE kindred showed an abnormality with the endonucleases employed. This detection rate of 33% in Type I HAE is higher than that reported in other published studies where approximately 16% was the previous maximum (188,189). The large number of restriction endonucleases employed in this study appeared to be the most important reason for this higher detection rate. For example, the enzyme Sty I was used by just one other investigator to examine DNA from a single Type I HAE family (67). A second potential source of variation in the RFLP detection rate between studies was the size of the cDNA probe employed to screen for abnormalities. Since some investigators used shorter cDNA probes which lacked 5' exonic sequence compared with the exon 2-8 cDNA, it was possible that these shorter probes may not have been able to detect all the gene mutations. This was not the case. For the two previously published disease-specific gene mutations (188,190) and the two new mutations reported herein the shorter probes would have detected them all had the appropriate restriction enzymes been employed. Finally, it should be remembered that in this study 12 Type I HAE kindred were analysed. In percentage terms a single extra kindred with an identifiable RFLP produces a relatively large increase in the overall detection rate. With the analysis of greater numbers of HAE families the detection rate may fall.
The diversity of disease-specific gene mutations identified by RFLP analysis in this study was greater than that described by other authors. Four distinct gene mutations were characterised, one lying in the 5' half of the gene and three mutations located in the 3' half of the gene. Two of these mutations were small, possibly point mutations and neither had been reported previously in HAE. The other two mutations, which were the same as those characterised recently by Stoppa-Lyonnet (188) and Ariga (190), comprised an exon 4 deletion and an exon 7 deletion respectively. In both cases sizeable lengths of DNA had been deleted from the Cl-inhibitor gene. Stoppa-Lyonnet observed only 5' deletion/insertion events and Ariga detected only the exon 7 deletion. This contrasts with the findings in this study in which four widely separated gene abnormalities were identified within the one patient population. As described in Section 1.4.8 the reasons for this lack of overlap between the findings of Stoppa-Lyonnet and Ariga were thought to be the different ethnic origins of the two populations they studied rather than methodological differences since both investigators studied reasonable numbers of kindred, used a very similar range of restriction enzymes and probed with Cl-inhibitor cDNAs capable of detecting both forms of mutation. The diversity of genetic origins present in our patient population may have had a role to play in producing the heterogeneity of Cl-inhibitor gene mutations observed in this study particularly when combined with the large number of restriction enzymes used.

In Families A and B small mutations involving the 3' end of the sixth exon and the eighth exon respectively were present. This
represents the first report of abnormalities affecting these areas of the Cl-inhibitor gene in Type I HAE. The gene mutation in Family A lay at the boundary between the sixth exon and the sixth intron. Sty I, the restriction enzyme which generated the polymorphism, has the recognition sequence 5' CCAAGG 3'. The first five nucleotides of this recognition sequence represent the last five nucleotides of the sixth exon and the sixth nucleotide is the first nucleotide in the sequence GT which is the donor splice site for excision of the sixth intron during RNA processing (205). It is therefore possible that a mutation in this sequence could interfere with normal RNA splicing and gene expression from this allele so giving rise to Type I HAE. A similar mechanism is well recognised in other genetic diseases including ß-thalassaemia (206), haemophilia A (207), haemophilia B (208) and Tay-Sachs disease (209). Nucleotide sequence analysis of the mutated area should confirm the likely mechanism and will define the extent of the mutation. In this kindred it may also be informative to examine patient RNA. Theoretically, unless the mutant RNA species is unstable and rapidly degraded intracellularly, a Cl-inhibitor RNA species of abnormal size should be present. In Family A it is highly likely that the Cl-inhibitor gene mutation detected by the Sty I RFLP is the cause of the disease since the RFLP co-segregated absolutely with the disease in the six main branches of the family shown in Figure 25. No recombination events were identified.

In Family B the Cl-inhibitor gene mutation pinpointed by the Bgl II RFLP was in the eighth exon approximately 50 base pairs from its 5' end. The effect of this mutation on the gene product remains
uncertain, however, if this mutation is responsible for the disease the most likely effects would be 1) to alter the reading frame of the eighth exon; 2) to create a premature termination codon; 3) to adversely affect the stability of the Cl-inhibitor mRNA as has been reported for the oncogene c-fos (210). Nucleotide sequence analysis of the abnormal area should resolve this uncertainty. It should be remembered, however, that since only three family members were available for genetic linkage analysis the possibility exists that sequencing will indicate that the RFLP is detecting a mutation in the eighth exon which is not responsible for the disease. Should this be the case the RFLP could still be used in this family as an indirect disease marker along with the Kpn I and Hgi AI RFLPs. Furthermore, if the RFLP is not disease-specific it must represent a very infrequent Cl-inhibitor gene RFLP since it was not observed in any other individual in this study nor has it been seen by other investigators within their test populations.

In Families C and D the gene mutations identified had been characterised by other investigators (188,190). Each mutation involved the deletion of a sizeable length of DNA from the Cl-inhibitor gene, approximately 1kb in Family C and 1.7kb in Family D. Contained within the deleted segment was the entire fourth exon for Family C and the entire seventh exon for Family D. Based on detailed normal Cl-inhibitor gene structure and the RFLP data, it appears that both deletions extend into the sets of ALU repeat sequences that are present in the introns which flank the fourth and seventh exons (191). This observation is entirely compatible with the pathogenesis of these deletions being a process of non-
homologous crossing over at meiosis between the ALU repeat sequences. Recently Tosi (191) reported the genomic cloning and sequencing of the abnormal area of the Cl-inhibitor gene in two families with a complete exon 4 deletion. This paper convincingly demonstrated that the limits of each deletion lay within intronic ALU sequences and that in each case the 5' and 3' deletion boundaries, although different in each family, both lay within short runs of identical sequence in the 5' and 3' ALU elements. For both families the 5' deletion boundary was within the same ALU element however different 3' ALU elements contained the 3' boundary for each family. Recombination between ALU elements is a recognised cause of gene mutation in other genetic diseases such as familial hypercholesterolaemia in which there is duplication of the whole 5' end of the gene as a result of unequal crossing over between intronic ALU sequences (192). In some cases of Tay-Sachs disease there is partial deletion of the β-hexosaminidase gene as a result of the same pathogenetic mechanism (193). Demonstration of the extent and so likely mechanism of the exon 4 and exon 7 deletions in Family C and D respectively will require sequence analysis of the abnormal alleles. In Family C the Taq I RFLP data suggest that the 5' deletion boundary does not lie within the same ALU element as that reported by Tosi (191). The reasons for this are as follows. The Taq I recognition site at the 5' end of the 1.4kb polymorphic Taq I fragment shown in Figure 38 must be preserved or else an additional 4.9kb Taq I fragment would be seen in Figure 35. Since this 5' Taq I site lies in the third intron within the ALU element that is immediately 3' to the ALU element which forms the 5' boundary of the
deletion described by Tosi (191), this Taq I site would be deleted if the 5' deletion boundary for Family C was within the same ALU element as that of Tosi (Figure 47). The inference is that since this 5' Taq I site is preserved, the 5' deletion boundary for Family C lies within either the 3' half of the ALU element containing the Taq I site or else it lies within the ALU element 3' to this. Confirmation of this will have to await sequence analysis of the abnormal Cl-inhibitor allele.

Messenger RNA transcripts derived from exon 4 and exon 7 deleted alleles have been reported by Ariga (190) and by Tosi (191). In each case the mRNA species was shorter than the normal Cl-inhibitor mRNA by the length of the deleted exon. The failure to detect mutant Cl-inhibitor protein in the sera of their patients indicated that either the shortened mRNAs were not translated or that if a protein was produced, it was unstable and was degraded intracellularly or could not be secreted from the cell of origin. Stability of the protein derived from a Cl-inhibitor allele with a deleted fourth exon would certainly be suspect since this exon codes for a cysteine residue that participates in one of the intramolecular disulphide bonds (67). Further work is required to establish the exact mechanism through which exon 4 or exon 7 deletions cause Type I HAE. Recently an mRNA species shortened by approximately 200 base pairs has been identified in Family D (K. Whaley, personal communication).

In this study the gene mutations in Family C and Family D co-segregated with the disease although in Family D there was only one HAE patient. Stoppa-Lyonnet and Ariga analysed extended families
FIGURE 47

Limited restriction map of the Cl-inhibitor gene in the area of the third and fourth exons. The three ALU elements in the third intron are marked "a", "b" and "c". The position of the 1.4kb Taq I fragment is indicated. It can be seen that the Taq I site at the 5' end of this fragment lies within ALU element "b". If the 5' boundary of the exon 4 gene deletion for Family C lay within ALU element "a" as described by Tosi (191) the Taq I site in ALU element "b" would be lost. This would create an additional 4.9kb Taq I fragment which would extend in the 5' direction beyond exon 3 so allowing it to hybridise to the cDNA probe. Since no such fragment is seen (Figure 35), the 5' exon 4 deletion boundary must be 3' to the ALU element "b" Taq I site. Since there are a total of three Taq I sites distributed along the 4kb length of the fourth intron the abnormal Taq I fragment created by the exon 4 deletion would now be purely intronic in origin and so would not hybridise to the cDNA probe. This is in keeping with Figure 35.
with exon 4 and exon 7 deletions respectively and showed absolute co-segregation with the disease (188,190). No genetic recombination events were observed and lod score analysis carried out by Stoppa-Lyonnet for the exon 4 deletion was entirely compatible with a causative relationship between the deletion and Type I HAE.

RFLP analysis of the Cl-inhibitor gene in eight out of 12 Type I HAE kindred and in both Type II HAE kindred failed to show an RFLP which co-segregated with the disease. In Type II HAE this was not a surprising result since all the reactive site Pi point mutations and the single P9 point mutation characterised at the nucleotide sequence level and which account for 70% of all Type II HAE cases produced no alteration in a restriction enzyme recognition site. Although the nature of the mutations in the remaining 30% of cases are currently unknown, the likelihood is that they will be small since no disease-specific RFLP was identified. It is probable that further RFLP analysis will be unhelpful in localising the remaining Type II HAE gene defects. For the eight Type I HAE kindred with no identifiable disease-specific RFLPs it is also highly likely that no major deletion or insertion event has occurred within the Cl-inhibitor gene. As in Type II HAE, the remaining gene mutations will most probably be small but critically placed. A good example of this type of abnormality is seen in Family A where a small mutation is centred around an exon/intron boundary. It was simply fortuitous that the mutation altered a restriction enzyme recognition site. In summary, it is felt that RFLP analysis is unlikely to make a major contribution to defining the remaining Cl-inhibitor gene mutations.
responsible for HAE. The investigative approaches necessary to achieve this will be discussed in Section 4.6.

4.4 Clinical survey of HAE in Scotland

A reported disease incidence for HAE of approximately one patient for every 110,000 of the population (Section 3.8a) represents the first documented attempt to calculate this figure for Scotland. It should be remembered, however, that this figure will almost certainly be an underestimate of the actual disease incidence for the following reasons -

1) A proportion of individuals with the disease will remain undiagnosed as a result of being asymptomatic or as a result of having mild symptoms for which they never seek medical advice. The discovery during family studies of completely asymptomatic individuals who have all the characteristic biochemical features of HAE is strongly supportive of this possibility.

2) Mild, infrequent symptoms will predispose to either no diagnosis or to an incorrect diagnosis being made despite medical advice being sought.

3) After diagnosis patients may be treated in a primary care setting without involving the hospital based specialists contacted in this survey. The number of HAE patients not detected for this reason is likely to be small.

4) Failure to disclose HAE patients to the survey. This was known to have occurred with two kindred whose existence only became apparent through their involvement with a second medical practitioner
contacted in the survey. It is possible that further instances of this could exist.

The clinical data regarding age at onset, precipitating factors, symptoms and treatment, as described in Sections 3.8b and 3.8c, were in general agreement with other published clinical surveys of HAE (126,128,130,168,211,212). Despite this a few of the observations deserve more detailed discussion. It was apparent that the 29% incidence of symptomatic respiratory tract oedema in this survey was markedly lower than the 70% incidence reported by Frank et al (126) and Cicardi et al (128). By comparison the 21% incidence reported in Finland more closely resembled this survey (211). Although the reasons for this variation are not entirely clear, the clinical threshold for diagnosing significant respiratory tract oedema and improvements in treatment appear to have an important role to play in this regard.

A definite precipitating factor for acute attacks of HAE could be identified in 48% of the patients in this survey, a figure which is very similar to other studies (126,128). Trauma represented the single commonest trigger with minor trauma not infrequently being capable of inducing an attack. Typing or standing upright for a significant period of time are examples of this minor trauma. It is therefore likely that a 48% incidence is an underestimate since a number of these precipitating events of a minor traumatic nature will remain unrecognised by the patient.

The clinical surveys of HAE which studied patients treated with androgens or Cl-inhibitor concentrate (128,130,168,211), including this survey, were in agreement with the following
statements regarding treatment.

1) The most effective long term therapy is attenuated androgens, danazol being the most widely employed. Approximately one third of patients will continue to experience significant symptoms despite taking androgens. Their potentially severe side-effects make it important to establish the minimum effective dose of androgen for each patient.

2) The anti-fibrinolytic agent tranexamic acid is less effective in the treatment of HAE compared with attenuated androgens. The majority of patients treated with this drug show definite symptomatic improvement, however, only the minority of patients become symptom free. Side-effects due to tranexamic acid are uncommon. It is most commonly used when a definite contraindication or objection to the use of attenuated androgens exists or else as an addition to androgen therapy in patients experiencing severe disease activity that is only partly responsive to androgens.

3) Cl-inhibitor concentrate is a very valuable form of therapy because of its specificity and rapidity of action. Its main uses include the prevention of acute disease attacks when a patient has to undergo surgery, particularly dental surgery. Furthermore, it is very effective at relieving life threatening disease attacks or can be used to interrupt a spell of debilitating disease activity. Limited availability, cost and the potential risk of transmitting viral infections such as hepatitis and the human immunodeficiency virus, especially in non-heat-treated concentrate, are the main problems associated with its use. To date, no such side-effects have been reported following its administration.
One of the most significant effects of improved treatment regimes has been the reduction in mortality statistics for the disease. Mortality rates of 56% and 33% were reported by Frank et al (126) and Landerman et al (212) respectively based largely on observations made before the advent of modern drug therapy. By comparison, this survey demonstrated that approximately 10% of deaths were directly attributable to the disease. Although this is a very significant reduction, 10% still represents a high mortality rate for the young adults most commonly affected.

In summary, the medical management of HAE has greatly improved in recent years with the use of attenuated androgens and Cl-inhibitor concentrate. Despite this there is still an unacceptable level of patient morbidity and mortality. The recent advances in the molecular genetics of the disease create a further potential therapeutic avenue for patients and their families to consider, namely prenatal diagnosis.

4.5 Prenatal diagnosis and HAE

The prenatal diagnosis of HAE represents a potential application for the RFLPs which co-segregate with the disease in Families A, B, C and D and for the Kpn I and Hgi AI RFLPs in the remaining families. It has been argued that the application of prenatal diagnostic techniques to HAE is inappropriate and unnecessary because of the much improved prognosis that exists for patients as a result of modern therapeutic regimes (213). This is
true to an extent, however, as discussed in Section 4.4 a number of clinical problems do still exist for HAE patients - 1) The death of HAE patients in their early adult years due to the disease still occurs at an unacceptable rate. 2) Patients are faced with the prospect of long term androgenic steroid therapy which in approximately one third of cases will produce incomplete disease control. 3) Compliance with drug therapy can be variable. 4) For women, who constitute 42% of the patients in Scotland, androgens can have particularly unpleasant masculinising side-effects. 5) If a woman wishes to become pregnant therapy has to be interrupted to facilitate pregnancy as well as to obviate any teratogenic effects of the drugs on the embryo. Early pregnancy also represents a period of increased frequency of acute disease attacks independent of drug therapy. 6) There are the potentially distressing and psychologically damaging effects on patients of the knowledge that there is a 50% risk of a child inheriting the disease. For these reasons hereditary angio-oedema is not fully under medical control and the contribution that prenatal diagnosis can make to patients and their families is potentially very substantial. Since it is a disease which affects relatively few kindred, a high acceptance rate for prenatal diagnosis could reduce the disease incidence to very low levels with resultant greatly diminished disease associated morbidity and mortality in future generations. Such a marked impact is probably rather optimistic and currently with the molecular genetics of the disease still in its infancy, such an impact is unrealistic. A reasonable certainty, however, is that in the future with careful family studies and fuller elucidation of the gene
mutations responsible for HAE, there will be no need for non-curative, potentially harmful medical treatment in those families who wish to avail themselves of prenatal diagnosis.
4.6 Suggestions for future study

The first priority will be to establish the nature and extent of the Cl-inhibitor gene mutations in the four RFLP positive Families A, B, C and D. For Families A and B where the mutation is small, the most effective way to do this will be by using the polymerase chain reaction (PCR) followed by nucleotide sequencing of the amplified product either directly or after cloning it into bacteriophage M13 (214). The entire normal Cl-inhibitor gene sequence is now known so selection of suitable PCR oligonucleotide primers which flank the mutated areas is straightforward. Following amplification, restriction enzyme digestion of the PCR product using Sty I or Bgl II for Family A and B respectively will establish whether the appropriate area of the gene has been amplified. Since patients are heterozygous, Sty I or Bgl II digestion will cleave the PCR product from the normal allele into two fragments whereas the PCR product from the mutated allele will not be cut. The mutated fragment can then be purified for cloning or direct sequencing as required. For both Family A and B nucleotide sequencing will establish the likely effect of the gene mutation on allele expression. PCR results have already confirmed the sites of the Cl-inhibitor gene mutations in Families A and B and preliminary nucleotide sequencing of the mutated area in Family A indicates a G to T point mutation affecting the first nucleotide of the GT donor splice site at the beginning of the sixth intron (K. Whaley, personal communication).

Defining the limits of the exon 4 and exon 7 deletions in Families C and D respectively will require genomic library
construction and isolation of clones containing the mutated area of the gene. The technique of genomic library construction has already been established as described in Section 2.2.15 and Section 3.8. Clones containing Cl-inhibitor gene sequence will be identified by probing the library with the exon 2-8 Cl-inhibitor cDNA followed by restriction analysis of the positive clones to identify their site of origin within the gene and to identify which of the clones is carrying gene sequence from the mutated allele. Subsequent subcloning and nucleotide sequence analysis will be carried out. Use of the polymerase chain reaction to define exon 4 and exon 7 deletions from the Cl-inhibitor gene poses problems because choosing primers is difficult when the exact limits of the deletion are not defined. Furthermore, the primers chosen would almost certainly lie within intronic ALU repeat elements which could markedly interfere with amplification specificity.

The main challenge in HAE is the identification and characterisation of the Cl-inhibitor gene mutations which lie outwith the categories described in the preceding pages but which are responsible for the majority of Type I disease and approximately 30% of Type II disease. As stated earlier the remaining mutations are likely to be small, critically placed abnormalities which RFLP analysis is unlikely to detect. Other techniques will have to be employed. Sequencing the entire Cl-inhibitor gene to detect a small abnormality would be tedious, time-consuming and expensive. What is required is a selection procedure which can be carried out initially to detect and localise a mutation. To date a number of methods have been developed to pinpoint base changes in genes. These include the
analysis of RNase A cleavage products of RNA:DNA heteroduplexes or RNA:RNA heteroduplexes between wild type and mutant molecules (215,216) or else the detection of base pair mismatches between DNA:DNA heteroduplexes detected by their reaction with carbodiimide (217). These methods have limitations in that they do not detect all mutations. Recently a relatively straightforward chemical cleavage reaction has been described using osmium tetroxide and hydroxylamine to chemically modify the mismatched bases between the strands of a defined DNA:DNA heteroduplex followed by piperidine cleavage at the modified bases (218). This method potentially detects all possible base pair mismatches depending on the conditions used and allows the position of the mismatch and so mutation to be identified following polyacrylamide gel electrophoresis and autoradiography of the radiolabelled cleavage products. Application of this detection system to HAE in order to identify Cl-inhibitor gene mutations seems a potentially very informative investigative step to take. Exonic and exon/intron boundary mutations will be sought at the outset. The source of exonic material to form the defined heteroduplexes will be the PCR product of the appropriate exon being examined. Since HAE is a heterozygous disease the PCR product from patient genomic DNA will contain an equal parts mixture of amplified normal exon and mutant exon which is ideal for the formation of normal:mutant heteroduplexes during the initial stages of the chemical cleavage reaction. Having identified a potential mutation, nucleotide sequence analysis of the appropriate PCR product can be carried out.

In one of the Type I HAE families for whom no disease-specific RFLP could be demonstrated, a shortened Cl-inhibitor mRNA species
has recently been identified (K. Whaley, personal communication). An exon deletion from the Cl-inhibitor gene cannot be responsible for this shortening which is approximately 200-300 bases since an RFLP would have been identified. The most likely alternative explanations are either a small mutation causing premature termination of transcription or else a mutation creating an alternative splice site. Confirmation of the first possibility can be achieved by sequence analysis of the seventh and eighth exons following amplification of each exon by PCR. Establishing the existence of an alternative splice site would be more difficult but PCR amplification of each exon using patient Cl-inhibitor mRNA as the PCR template may identify the missing exonic material and so direct further investigation to the appropriate area of the gene where the mutation could be exonic or intronic.

It can be argued that a justifiable alternative approach for identifying some of the remaining Cl-inhibitor gene mutations responsible for HAE would be to "blindly" sequence only the exonic component of the Cl-inhibitor gene from a patient in each HAE kindred, each exon having been prepared by PCR. In this way a number of abnormalities would be found which would represent a proportion of the mutations responsible for Type I HAE and all of the mutations that cause Type II HAE. Since the exonic component of the gene is only 12% of its total length the sequencing task would not be too daunting. Unfortunately with this investigative protocol it is likely that a number of the Cl-inhibitor gene mutations that cause Type I HAE would remain undetected. In these cases the chemical cleavage reaction would have to be applied to patient DNA.
In conclusion, it is probable that a multiplicity of Cl-inhibitor gene mutations responsible for Type I and Type II HAE will be characterised in the near future. From a molecular genetic standpoint HAE will then more closely resemble other better characterised genetic diseases such as haemophilia A (207), haemophilia B (208) and β-thalassaemia (219), all of which are caused by a wide range of gene mutations.
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APPENDIX

Publications arising from the work described in this thesis.

PAPERS


ABSTRACTS