Clinical monitoring of 'autoimmune' chronic active hepatitis

Hoek, Bart van

CLINICAL MONITORING OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

A HISTOLOGICAL, BIOCHEMICAL AND IMMUNOLOGICAL STUDY

BART VAN HOEK

CLINICAL MONITORING OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

A histological, biochemical and immunological study

STELLINGEN

1. Although physicians are intuitively aware of the impact of different chronic conditions on functioning and well-being, they need precise measures of these outcomes that are also practical for use in the office setting... We still know very little about the relative impact of different chronic conditions on patients' functioning and well-being.
   (Stewart AL, Greenfield S, Hays R, et al. JAMA 1989; 262: 907-913).

2. Het is zeer wel mogelijk dat idiopathische 'autoimmune' CAH een 'final common pathway' is met een uiteenlopende etiologie, zoals wellicht: hepatitis C, Epstein Barr virus, mazelenvirus en 'inborn errors of metabolism'.

3. Een klassiek geval van 'piecemeal necrose' en regeneratie van de lever vinden we in de Griekse mythologie bij Prometheus.

4. De bepaling van de meeste niet orgaan-specifieke antistoffen heeft bij CAH slechts een beperkte waarde.
   (dit proefschrift)

5. Bepaling van N-terminale procollageen-III-peptide in serum maakt het, in tegenstelling tot vele gangbare methoden, vaak mogelijk de diagnose remissie of actieve CAH te stellen zonder leverbiopsie.
   (dit proefschrift)

6. Het is gewenst om bij het verstrekken van immunosuppressieve therapie aan een patiënt met 'autoimmune' chronisch actieve hepatitis te streven naar: een histologische activiteitsscore (0-18) gelijk aan of lager dan 1, normalisering van N-terminale procollageen-III-peptide in serum, afwezigheid van detecteerbare anti-LSP antistoffen, en normale eiwitsynthese door de lever.
   (dit proefschrift)

7. Indien gepoogd wordt immunosuppressie geleidelijk te stoppen of op een lager niveau te brengen bij een patiënt met 'autoimmune' chronisch actieve hepatitis is het de beste methode dit in korte tijd te doen (bijv. 6-12 weken) opdat frequente (bijv. 2-wekelijkse) controle met meting van anti-LSP, PIIINP en ALT (met een individuele referentie limiet) mogelijk is.
   (dit proefschrift)

8. Na het bereiken van histologische remissie van idiopathische 'autoimmune' CAH dient deze remissie veelal twee jaar gehandhaafd te blijven met behulp van immunosuppressieve medicatie, teneinde volledig herstel van de synthesefunctie van de lever te bewerkstelligen.
(dit proefschrift)

9. Bij 'autoimmuun' chronisch actieve hepatitis lijkt een vroeg stadium van hepatorenaal falen, ook wel functionele nierinsufficientie genoemd, reversibel te ziin, niet alleen door middel van levertransplantatie, maar ook met behulp van immunosuppressieve therapie indien de hepatitis daarop in remissie komt.
(dit proefschrift)

10. Antistoffen tegen cardiolipine komen vaak voor bij chronisch actieve hepatitis, de kruisreactie met anti-DNA antistoffen is beperkt, en er is bij deze ziekte geen correlatie met thrombose of recidiverende spontane abortus aangetoond.
(dit proefschrift)

11. Er zijn geen aanwijzingen dat het CMV een etiologische rol speelt via 'molecular mimicry' tussen door CMV op de celmembraan geïnduceerde antigenen en antigenen in LSP.
(dit proefschrift)

12. De bepaling van anti-DNA antistoffen met Crithidia luciliae als substraat kan behulpzaam zijn in de differentiaaldiagnose van SLE met leverproefstoornissen en CAH met extrahepatische manifestaties.
(dit proefschrift)

13. Bij controle van patiënten met leverziekten wordt vaak te weinig aandacht geschonken aan de gestoorde functies van dit orgaan.

14. Het vragen naar nachtblindheid hoort bij de anamnese van een patiënt met leverziekte.

15. Het is nog zeer de vraag of de 74 kD antimitochondriale antistoffen van het type M2, gericht tegen pyruvaat-dehydrogenase E2, een (additionele) rol spelen in de pathogenese van primaire biliaire cirrose.

16. Daar waar de verstrekking van delen van de gezondheidszorg het monopolie wordt van enkelen, dreigt het belang voor de gemeenschap verloren te gaan.

17. Een 'muzikaal gehoor' is de stemvork voor slechts één persoon.

18. Het kind van 'tweeverdieners' krijgt vaak niet wat het verdient.

19. Aanwezigheid van het 'human immune deficiency virus' (HIV) bij de recipient is geen absolute contra-indicatie tegen orgaantransplantatie. Het kan wel een relatieve contra-indicatie hiertegen zijn in een situatie van beperkte beschikbaarheid van donororganen.

20. In de jaren 1965 tot 1980 was er in de Duitse Bondsrepubliek een evidente correlatie tussen de daling van het aantal menselijke geboorten per jaar en van het aantal broedende paren ooievaars.
(Nature, 7 april 1988)

21. Het toenemend aantal giframpen werkt 'riooljournalistiek' in de hand.

22. Bij het voornemen tot opheffing van de grenzen tussen de EEG-lidstaten per 1992 lijkt het gewenst om te anticiperen op mogelijke protectionistische neigingen bij EEG-handelspartners.

23. Vele clinici zijn slechts in letterlijke zin 'voordeurdelers'.

24. Kunst wordt gemaakt door 'kenners'.

25. Nederlanders die in het buitenland verblijven tellen voor verkiezingen niet mee.

26. De functie van paranimfen bij een promotie komt pas ten volle tot zijn recht als de promovendus zich in het buitenland bevindt.

Stellingen behorende bij proefschrift

CLINICAL MONITORING OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

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BART VAN HOEK

22 november 1989

RIJKSUNIVERSITEIT TE GRONINGEN

CLINICAL MONITORING OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

A histological, biochemical and immunological study

PROEFSCHRIFT

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit Groningen
op gezag van Rector Magnificus Dr. L.J. Engels,
in het openbaar te verdedigen op woensdag 22 november 1989
des namiddags te 14.45 uur precies.

door

BART VAN HOEK
geboren te Voorburg

PROMOTORES:	prof. dr. C.H. Gips prof. dr. T.H. The
REFERENTEN:	dr. A.J.K. Grond dr. C.G.M. Kallenberg

PROMOTIECOMMISSIE:	prof. dr. J.D. Elema prof. dr. H.S.A. Heymans prof. J.H.P. Wilson

aan mijn ouders

Cataloging-in-Publication DATA
ROYAL LIBRARY, THE HAGUE, THE NETHERLANDS.

Hoek, Bart van

Clinical monitoring of 'autoimmune' chronic active hepatitis : a histological, biochemical and immunological study / Bart van Hoek. - [S.l. : s.n.] (Groningen : Computekst). - Ill.
Thesis Groningen. - With ref.
ISBN 90-9003047-6
SISO 605.13 UDC 616.36-002(043.3)
Subject heading: hepatitis.

Cover drawing: Huib Waterbolk. (after Arkesilas-painter, *Prometheus and Atlas*, interior of black-figure Laconian kylix from Cerveteri, 565-555 B.C., Vatican Museums, Rome)

Computer Typeset by COMPUTEKST tekstverwerking, Groningen, The Netherlands.
Printed and bound in The Netherlands by Dijkhuizen van Zanten, Groningen.

The printing costs were supported by kind contributions of Merck, Sharp & Dohme and Glaxo.

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'Autoimmune' chronic active hepatitis

Chronic active hepatitis of various aetiologies:

VOORWOORD/ACKNOWLEDGEMENTS

Het onderzoek, beschreven in dit proefschrift, werd verricht binnen de afdeling Hepatologie (hoofd: Prof. Dr. C.H. Gips) en de afdeling Klinische Immunologie (hoofd: Prof. Dr. T.H. The) van de Kliniek voor Inwendige Geneeskunde (hoofd: Prof. Dr. G.K. van der Hem) van het Academisch Ziekenhuis te Groningen.

Bij het tot stand komen van dit proefschrift waren de hulp en de bijdragen van velen onontbeerlijk. Een aantal van hen ben ik bijzondere dank verschuldigd.

Mijn ouders dank ik voor hun warme belangstelling, steun en aanmoediging die zij altijd gegeven hebben, waardoor ik het gestelde doel kon bereiken.

De fundamenten voor dit proefschrift zijn reeds vele jaren geleden, ruim voor de periode waarin dit onderzoek werd verricht, gelegd door Prof. Dr. C.H. Gips. Beste Chris, je aanstekelijk enthousiasme in combinatie met je diepgaande kennis van de pathofysiologie van de lever hebben mij getroffen en steeds aangespoord mij verder te verdiepen in dit interessante gebied van de interne geneeskunde. Je vriendschap waardeer ik zeer.

Prof. Dr. T.H. The ben ik zeer erkentelijk voor de vele stimulerende gesprekken en het scheppen van ruime mogelijkheden voor het doen van onderzoek. Hij introduceerde mij in het fascinerende gebied van de Klinische Immunologie. De plezierige en persoonlijke wijze waarop hij mij steeds begeleidde was een belangrijke stimulans op vele momenten.

Dr. A.J.K. Grond (afdeling Pathologische Anatomie) was een voortdurende bron van tomeloze energie. Beste Joris, voor de wijze waarop jij mede de aanzet en later de uitwerking van dit proefschrift mogelijk maakte wil ik je hartelijk danken. Je kennis van de leverpathologie bewonder ik zeer. Ik ben je erkentelijk voor de wijze waarop je mijn huidige verblijf in de Mayo Clinic gestimuleerd hebt.

Dr. C.G.M. Kallenberg (afdeling Klinische Immunologie) opende wegen voor nieuw onderzoek, en was verantwoordelijk voor vele suggesties en verbeteringen. Beste Cees, van je kennis van autoimmuunziekten heb ik veel geleerd.

Ik ben Prof. Dr. E. Mandema, Prof. J.H. Scholten en Prof. Dr. W.D. Reitsma bijzonder erkentelijk voor mijn opleiding tot internist, en ik dank Prof. Dr. G.K. van der Hem voor de genoten gastvrijheid binnen de Kliniek voor Inwendige Geneeskunde van het Academisch Ziekenhuis te Groningen.

De leden van de promotie-commissie, Prof. Dr. J.H.P. Wilson (Rotterdam), Prof. Dr. J.D. Elema (Groningen) en Prof. Dr. H.S.A. Heymans (Groningen) dank ik voor het kritisch doornemen van het manuscript.

En speciaal woord van dank betreft de heer J.R. (Reint) Huizenga. Reint, de plezierige sfeer die jij wist te creëren in het door jou geleide laboratorium hepatologie was voor mij en anderen steeds een stimulans om door te gaan. De nauwgezette wijze waarop je sinds 1969 een uitgebreid serumarchief hebt opgezet is uniek en was een belangrijke basis voor dit onderzoek. Jij zorgde ervoor dat vanuit de U.S.A. toch steeds alles geregeld kon worden. In de loop der jaren heb je een uitgebreide kennis van de hepatologie verworven. Aan de vele discussies die wij voerden zowel in werksfeer als daarbuiten denk ik met plezier terug. Dit alles heeft geresulteerd in een mij dierbare vriendschap.

Aan mevrouw T.H. (Tineke) Lijnema wil ik mijn bewondering uitspreken voor de snelle en intelligente wijze waarop zij zich nieuwe onderwerpen en technieken eigen wist te maken. Tineke, je was een onmisbare steun, en ik hoop dat je huidige aanstelling in het kader van het project "chronisch actieve hepatitis" een vastere basis krijgt.

Dat het Academisch Ziekenhuis de heer drs. A.J.(Arja) Mackor ook in de gelegenheid stelt om binnen de afdeling hepatologie onderzoek naar dit ziektebeeld te verrichten verheugt mij zeer.

Mevr. Mr. A.K. (Annie) van Zanten wil ik bijzonder hartelijk danken voor de gastvrijheid en plezierige samenwerking bij het opzetten van de anti-LSP bepaling en het verrichten van vele PIIINP bepalingen.

De heer Dr. V. Fidler verzorgde op deskundige wijze de statische analyse van gegevens betreffende overleving en prognostische parameters. Zeer hartelijk dank.

Mevrouw E. (Els) Hummel-Tappel, mevrouw C. (Carla) Terpstra en de heer C. (Chairul) Effendi wil ik hartelijk danken voor het verrichten van een groot deel van de anticardiolipine, ANA, a-ENA en anti-DNA-bepalingen, alsmede de plezierige sfeer waarin dit verliep.

Van de heer Dr. P.C. (Piet) Limburg en collega Drs. B.P.C. (Bauke) Hazenberg kreeg ik steeds op plezierige wijze een acute fase respons op nieuwe versies van het artikel betreffende CRP en SAA. Prof. Dr. M.H. (Martin) van Rijswijk dank ik voor verhelderende discussies over dit onderwerp, alsmede voor het scheppen van de mogelijkheden voor dit gezamenlijk onderzoek. De heer J. (Johan) Bijzet dank ik voor de vele bepalingen die hij hiervoor verrichtte.

De heer S. (Siep) Postma en mevrouw J. (Coba) van Zanten dank ik voor het binnen zeer korte tijd verrichten van vele ELISA's voor het onderzoek betreffende CMV. Mevrouw Dr. M. (Marijke) van der Giessen speelde een belangrijke rol in het interpreteren van deze uitslagen. Dr. L.F.M.H. (Lou) de Leij, mevrouw J.G. (Anita) ter Haar, en mevrouw H.A. (Harmke) de Vries-Huiges dank ik voor mijn introductie in het laboratorium van de afdeling Klinische Immunologie.

I would like to thank Dr. R. (Roger) Williams, FRCP, for the opportunity he gave me to work in his excellent laboratory in King's College Hospital, London, Great-Brittain. Dr. I.G. (Ian) McFarlane and Dr. B.M. (Barbara) McFarlane-Wojcicka showed me how to perform the radioimmunoassay for anti-LSP and anti-ASGR. I very much appreciate their help and friendliness during and after my stay in London.

De Nederlandse Lever Darm Stichting ondersteunde dit verblijf in London financieel.

I wish to thank Prof. Dr. Dr. K.-H. Meyer zum Büschenfelde and Prof. Dr. M. (Michael) Manns for their hospitality and help during the weeks we spent in their well-known laboratory in the Clinic of the Johannes Gutenberg University in Mainz, West-Germany. I also thank mrs. S. Bratfish and Mr. Hodawand for technical assistance.

I thank Dr. J. (Juha) and Dr. L. (Leila) Risteli, Collagen Research Unit, Department of Medical Biochemistry, University of Oulu, Oulu, Finland, who did part of the determinations with the new human PIIINP assay they developed.

De dames K. (Karin) Cazemier en A.M. (Anne Marie) Zoelman en de heren H. (Henk) Oost en K.H.P. (Peter) Geres verrichtten als stagiaires vele cholinesterase bepalingen en hielpen met het serum-archief. Dank!

Mevrouw H.J. (Rina) de Jong (laboratorium immunochemie, hoofd Dr. J. Marrink) assisteerde op deskundige wijze bij het isoleren van LSP.

De heer J.G.J. (Joan) Vos dank ik voor het vliegensvlug verrichten van, onder andere, hepatitis B serologie.

De heer F. (Frans) Broekhuizen (polikliniek hepatologie) en de registratie-assitentes van afdeling A2 waren essentieel voor het continueren van dit archief.

Dr. J. Trommel, voormalig geneesheer-direkteur van het Talmahuis te Veenwouden, verstrekte gegevens betreffende een relatief groot aantal met oxyphenisatine behandelde patiënten, waarvan degenen bij wie een in het AZG verrichte leverbiopsie CAH liet zien in dit onderzoek zijn opgenomen.

De heer Dr. J.A. Kramps, Academisch Ziekenhuis te Leiden, (lab. longziekten) dank ik voor het verrichten van α_1-antitrypsine fenotyperingen bij alle patiënten.

Op mevrouw M.H. (Kiki) Bugter, mevrouw J.A. (Jetty) Renkema, mevrouw M.G.H. (Riet) van den Broek en mevrouw M. (Martha) Messchendorp deed ik nooit een vergeefs beroep. Mevrouw R. (Renske) Wekema was een belangrijke schakel in de overzeese communicatie.

De dames en heren van het statusarchief zorgden ervoor dat gegevens snel beschikbaar waren.

De heren K. (Klaas) van der Linde en G. (Gerard) Glade verrichtten, naast hun studie in de geneeskunde, belangrijk werk bij het verzamelen van gegevens. Dr. H. (Herman) Kreeftenberg en Drs. J. (Jan) Jager dank ik voor de opbouwende discussies tijdens diverse besprekingen. Dit gold ook voor mevrouw P. (Pauline) Schuur en de heren N. (Niele) Bhairo, G.M. (Go) van Dam, R. (Rik) Reekers, en drs G.J. (Gerrit Jan) Westerveld, die hun studie goed wisten te combineren met het verrichten van onderzoek in de afdeling hepatologie.

De patiënten waarop dit onderzoek gebaseerd is wil ik hartelijk danken voor hun medewerking en de toestemming een serumarchief aan te leggen voor later onderzoek. Ik hoop van ganser harte dat de resultaten van dit onderzoek hun behandeling ten goede zal komen.

Vele internisten, huisartsen en patholoog-anatomen uit Nederland en daarbuiten stelden gegevens en leverbiopsieën te beschikking van patiënten die zowel afwisselend elders als in het Academisch Ziekenhuis te Groningen behandeld werden. Ik dank hen zeer hartelijk voor deze medewerking. Ook de medici die in het Academisch Ziekenhuis Groningen in de loop der jaren de zorg voor de beschreven patiënten hadden dank ik voor de verstrekte gegevens. Diverse patiënten verstrekten gegevens. Gemeentehuizen gaven waardevolle informatie. Dank daarvoor.

Alle medewerkers van het CKCL (in het bijzonder G. (Greetje) Sienot en de heer E.E. (Eddie) Ligon), het haematologie laboratorium, en het stollings-laboratorium dank ik voor het verrichten van de routine bepalingen die opgenomen zijn in dit proefschrift.

De heer A. Huizer van de afdeling Medische Fotodienst van de RUG dank ik voor de snelle en correcte wijze waarop hij grafieken "drukklaar" maakte.

De heer A.J. (Bert) Schaalma assisteerde waar mogelijk bij het verzamelen van literatuur.

Dr. K. H. Brandt, internist, wijlen Dr. F. Bronkhorst, patholoog-anatoom (Ziekenhuis Rijnstate, lokatie Gemeenteziekenhuis te Arnhem), Prof. Dr. S.W. Schalm (Academisch Ziekenhuis Dijkzigt, Rotterdam), Prof. Dr. G.P. van Berge Henegouwen (Academisch Ziekenhuis Utrecht), Dr. R.A. Janssens (voorheen Academisch Ziekenhuis Leiden) en Dr. P. Niermeyer (Majella Ziekenhuis, Bussum) dank ik voor de vele stimulerende discussies omtrent chronisch actieve hepatitis, onder andere tijdens de voorbereiding van een cursus en een symposium.

De firma's Behring en Imphos dank ik voor het gratis ter beschikking stellen voor radioimmunoassay-kits voor de bepaling van PIIINP. De uitkomst dat resultaten van beider assays goed correleren lijkt mij voor beide firma's een verheugend gegeven.

De Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) dank ik voor het ter beschikking stellen van een beurs, die mij momenteel in de gelegenheid stelt onderzoek naar, onder andere, chronisch leverziekten en rejectie na levertransplantatie te verrichten in de Mayo Clinic te Rochester, Minnesota, U.S.A.

I wish to thank Dr. A.J. Czaja, M.D. and Dr. R.H. Wiesner, M.D., Dept. of Gastroenterology and Internal Medicine, Mayo Clinic, Rochester MN, U.S.A., for valuable comments after critically reviewing the manuscript of this book.

I thank Suzan and Anthony Hayes for editing the English language in this thesis. You did a marvelous job, and I hope our friendship will last forever.

De vriendschap van mevrouw Mr. J. (Jeanne) Wesseling-Lubberink en van de heer J.R. (Reint) Huizenga is mij zeer waardevol, en hun vele inspanningen als paranimfen waardeer ik zeer.

"Last but not least" wil ik de gecompliceerde interactie noemen die Beryl, dit proefschrift en ik met elkaar hadden tijdens de wording van dit boek. Steeds wist je mij op originele wijze ervan te overtuigen dat er nog vele andere belangrijke zaken in het leven bestaan. Dit heeft op paradoxale wijze bijgedragen aan de voltooiïng van dit werk.

De vriendschap en hulp van velen worden in dankbaarheid gememoreerd.

COLLABORATION

Chapter 4	J. Risteli, L. Risteli, J.R. Huizenga
Chapter 5	T.H. Lijnema, A.K. van Zanten, J.R. Huizenga, I.G. McFarlane, B.M. McFarlane
Chapter 6	G. Glade, V. Fidler
Chapter 9	J.R. Huizenga, T.H. Lijnema
Chapter 10	B.P.C. Hazenberg, P.C. Limburg, J. Bijzet, J.R. Huizenga, M.H. van Rijswijk
Chapter 11	J. Risteli, J.R. Huizenga, L. Risteli
Chapter 12	J.R. Huizenga, T.H. Lijnema
Chapter 13	T.H. Lijnema, A.K. van Zanten, J.R. Huizenga
Chapter 14	T.H. Lijnema, A.K. van Zanten, J.R. Huizenga
Chapter 15	M. Manns, J.R. Huizenga, K.-H. Meyer zum Büschenfelde
Chapter 16	C.G.M. Kallenberg, C. Effendi, P.C. Limburg, E. Hummel-Tappel
Chapter 17	T.H. Lijnema, C.G.M. Kallenberg, M. van der Giessen, A.K. van Zanten, J.R. Huizenga

LIST OF ABBREVIATIONS

AAA	=	aromatic amino acids
ABT	=	aminopyrine breath test
AI-CAH	=	'autoimmune' CAH
ALB	=	albumin
A1AT	=	$alpha_1$ antitrypsin
ALT	=	alanine aminotransferase
AMA	=	antimitochondrial antibodies
ANA	=	antinuclear antibodies
anti-DNA	=	antibodies against dsDNA
anti-HBs	=	antibodies against HBsAg
anti-HBs	=	antibodies against HBcAg
anti-M2	=	AMA against M2 antigen
anti-Mx	=	AMA against Mx antigen
APTT	=	activated partial prothrombin time
ASGR	=	asialoglycoprotein receptor
AST	=	aspartate aminotransferase
ATIII	=	antithrombin III
AZA	=	azathioprine
BCAA	=	branched chain amino acids
BUN	=	blood urea nitrogen
CAH	=	chronic active hepatitis
CB	=	conjugated bilirubin
CHE	=	(pseudo-)cholinesterase
CLH	=	chronic lobular hepatitis.
CMV	=	cytomegalovirus
CPH	=	chronic persistent hepatitis
CREAT	=	creatinin
CRP	=	C-reactive protein
CTL	=	cytotoxic T-lymphocyte
CyA	=	cyclosporin A
DI	=	drug-induced
dsDNA	=	double-stranded desoxyribonucleic acid
EA	=	early antigen
ELISA	=	enzyme-linked immunosorbent assay
ENA	=	extractable nuclear antigens
EO	=	eosinophil granulocytes
GG	=	gamma globulin
GEC	=	galaktose elimination clearance
HAS	=	Histological Activity Score (= P+PP+L)
Hb	=	hemoglobin
HBcAg	=	hepatitis B virus core antigen
HBD	=	healthy blood donors
HBeAg	=	hepatitis B virus e-antigen
HBsAg	=	hepatitis B virus surface antigen
HDL_3	=	high density lipoprotein type 3
HFS	=	Histological Fibrosis Score
Ht	=	hematocrit

IAI-CAH	=	idiopathic 'autoimmune' chronic active hepatitis
ICH	=	immunecompromised host
IEA	=	immediate early antigen
IFN	=	interferon
IL-1ß	=	interleukin 1ß
IL-2	=	interleukin 2
IL-2R	=	interleukin 2 receptors
IL-6	=	interleukin 6
K cells	=	killer cells
kD	=	kiloDalton
K-M	=	Kaplan-Meier
L	=	lobular inflammation score
LA	=	late antigen
LAK cells	=	lymphokine-activated killer cells
LEU	=	leukocytes
LC-1	=	liver cytosol antigen(s) type 1
LKM-1	=	liver kidney microsomal antigen type 1
LMA	=	liver membrane antigen
LSP	=	'liver-specific membrane lipoprotein'
LT	=	liver transplantation
LYMPHO	=	lymphocytes
MA	=	membrane antigen
Na	=	sodium
NK cells	=	natural killer cells
nRNP	=	an extractable nuclear antigen
OLT	=	orthotopic liver transplantation
P	=	portal inflammation score
PP	=	periportal inflammation score
PLASMA	=	plasma cells
PMN	=	polymorphonuclear leucocytes
PRED	=	prednisolone
PT	=	prothrombin time
PIIINP	=	N-terminal propeptide of collagen type III
RIA	=	radioimmunoassay
SAA	=	serum amyloid precursor A
SAP	=	serum amyloid P-component
SBA	=	serum bile acids
SLA	=	soluble liver antigen
Sm	=	an extractable nuclear antigen
SS-A	=	an extractable nuclear antigen
SS-B	=	an extractable nuclear antigen
UB	=	unconjugated bilirubin
TB	=	total bilirubin (=UB+CB)
THR	=	thrombocytes
TNF-α	=	tumor necrosis factor α

SCOPE OF THIS STUDY

The scope of this work was to gain insight into the effect of an accepted standardized therapeutic regimen for 'autoimmune' chronic active hepatitis, consisting of prednisolone and azathioprine in a small group of patients treated according to the same standardized protocol in a specialized hepatological unit of a university hospital. In some parts of the study these patients were analyzed against the background of a larger group of patients with CAH referred within the same period of time. This represented a limited number of measurements as survival, antibodies to cytomegalovirus, anticardiolipin, anti-DNA and antinuclear antibodies. The items we studied can be divided into three groups:

1) parameters of inflammation and derived parameters such as fibrogenesis,
2) parameters of repair and restoration of liver function, and
3) immunological parameters.

With regard to immunological parameters we studied pathogenetic factors like anti-LSP autoantibodies, as well as other (epiphenomenal?) autoantibodies. Antiviral antibodies were determined in a search into etiology of 'autoimmune' chronic active hepatitis. The value of these parameters for diagnosis, therapy and follow-up of 'autoimmune' chronic active hepatitis is discussed. The work is introduced by a review of the literature and is concluded with an analysis of our observations and suggestions for future lines of research.

INTRODUCTION

§1 CHRONIC ACTIVE HEPATITIS IN A HISTORICAL PERSPECTIVE

In 1907 Noël Fissinger described a disease in which the immune system does no longer defend but attacks the liver ("le sujet ne défend plus son foie, il se défend contre son foie"), raising the concept of autoimmune liver disease.[1] The concept of idiopathic, often recurrent, jaundice in association with severe chronic necrosis and inflammation of the liver emerged in the late 1930's and early 1940's.[2,3] The components of a syndrome, however, were not identified until the 1950's, when cirrhosis with plasma cell infiltration of the liver was recognised in young women who had hypergammaglobulinaemia, fever, arthralgias, acne, and delayed menstruation and amenorrhoea, splenomegaly, obscure febrile reactions, and Cushingoid facies.[4-9] The term *active chronic hepatitis* was first applied in 1953[10] and was later modified to *lupoid hepatitis* in 1956[11] when the frequency of association with the lupus erythematosis cell phenomenon was appreciated.[12] Subsequently, immunoserologic findings were regarded as having etiologic importance and an 'autoimmune' concept of the disease developed.[13-19] The emergence of additional immunoserologic tests, including assays for smooth muscle antibodies (SMA), antimitochondrial antibodies (AMA), and hepatitis B surface antigen (HBsAg), allowed subclassification of patients with the same generic disease but different clinical and laboratory manifestations. Subgroups were distinguished by, for instance, specific immunoserologic findings,[18-21] presence of HBsAg,[22-24] associated autoimmune diseases[25-26] and sex.[27] Prognosis and multiple morphologic patterns were also described.[28] Eventually a single classification was proposed which comprised all disorders of similar clinical, biochemical, and histological features of periportal hepatitis,[29,30,31] and the term *chronic active hepatitis* (CAH) became current. Although incorrectly regarded as specific for CAH, the histologic pattern of *piecemeal necrosis* is regarded as the sine qua non of all forms of CAH.

§2 TERMINOLOGY

Although chronic viral hepatitis was described in 1947,[32] the first histopathological description of CAH dates from 1966.[33] De Groote *et al.* defined and classified chronic hepatitis based on histopathological findings into chronic aggressive hepatitis (CAH), chronic persistent hepatitis (CPH), and chronic lobular hepatitis (CLH).[29,31] With the gaining of knowledge the classification of chronic liver diseases underwent some modifications, the most important being the recognition of primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) as distinct entities.[34] This implies that in the earlier descriptions of chronic hepatitis, cases were included that we would now classify as PBC or PSC. The term chronic hepatitis incorporates CAH, CPH and CLH but not PBC or PSC. We will use this terminology, although CAH, CPH and CLH probably belong to the spectrum of one disease. Moreover, the distinction between CAH

and PBC or PSC can be difficult or even impossible, suggesting some overlap as well. Another problem in interpreting the early studies on CAH is that in these trials patients with chronic hepatitis B and other causes of CAH or 'CAH-related liver disease' (table 1) were included. Terminology like "chronic active liver disease", "chronic active cirrhosis", "chronic aggressive hepatitis", and "subacute hepatitis" has been abandoned now, because it does not serve a special purpose and produces confusion.[35,36]

Table 1 Common causes of chronic active hepatitis (CAH) and 'CAH-related disorders'.

	Abbreviation used:
CAH	
'Autoimmune', aetiology unknown	Idiopathic 'autoimmune' CAH
'Autoimmune', after hepatitis B (anti-HBs positive)	Anti-HBs positive 'autoimmune' CAH
Hepatitis B virus (HBsAg positive)	HBsAg-positive CAH
Hepatitis C virus (and other parenterally transmitted, but unknown non-A,non-B viruses)	HCV-CAH or NANB-CAH
Hepatitis delta	HDV-CAH
CAH-related disorders	
drug-induced	DI-CAH
Wilson's disease	Wilson-CAH
α_1-antitrypsin deficiency	A1AT-CAH
'small-duct primary sclerosing cholangitis'/ CAH/PSC 'overlap'	CAH/PSC
cholestatic CAH ('overlap' of CAH with primary biliary cirrhosis)	CAH/PBC
alcohol related	
haemochromatosis	
anti-chymotrypsin deficiency	

§3 HISTOLOGY

The traditional description of the histology of CAH has its roots in the autoimmune form.[37] The hallmark of CAH is portal and periportal inflammation with erosion of the limiting plate, piecemeal necrosis, and fibrosis. The classic or lymphocytic form of piecemeal necrosis was originally described by Popper *et al.*[38] It may be defined as inflammatory destruction of single or small groups of liver cells at a mesenchymal/

parenchymal interface (periportal, periseptal or at the margin of confluent necrosis) associated with lymphohistiocytic inflammatory cells.[31] Bianchi states that a close contact between the membranes of clustered lymphocytes/macrophages and hepatocytes (*peripolesis*[39]) is a prerequisite for the diagnosis of piecemeal necrosis and hence of chronic active hepatitis, while periportal hepatitis without peripolesis is referred to as *'CAH-related liver disease'*.[40] Others do not include peripolesis in the definition of CAH. One of the reasons is that recognizing the presence or absence of peripolesis can be difficult, even for experienced pathologists.[35,36,41] In *chronic persistent hepatitis (CPH)*, the predominantly lymphocytic infiltrate is restricted to widened portal tracts, leaving the parenchymal limiting plate preserved.[42] A special form of CPH, *chronic septal hepatitis*[43] is characterized by the formation of septa with conspicuous inflammatory activity but without piecemeal necrosis. This may represent a regressive stage of CAH, as seen spontaneously or after immunosuppressive therapy[44] and will be referred to as CPH or *CAH in remission*. When acute lobular features of the degree seen in acute hepatitis are present over a period of more than 6 months, this is termed *chronic lobular hepatitis (CLH)*.[45,46] For more extensive descriptions of histology the reader is referred to Ludwig, Bianchi and Scheuer.[35,40,41]

§4 DEFINITION

In general, the definition of CAH includes only CAH with parenteral hepatitis viruses as cause, or with unknown cause, with or without 'autoimmune' phenomena.

Conditions such as α_1-antitrypsin deficiency or Wilson's disease are excluded, and are labelled *CAH-related disorders*.[35,36,40]. Other authors however, state that these diseases are other causes of the same pattern of periportal hepatitis.[47]

We will adhere to the first definition of CAH, being:

- a primarily hepatocytic liver disease
- characterized by a common pattern of progression, that is: (episodes of) periportal and portal inflammation and piecemeal necrosis, with the potential to progress to cirrhosis or being associated with cirrhosis
- a condition which will last 6 months or longer,
- caused by a presumed autoimmune disorder,
  idiopathic or cryptogenic CAH caused by unknown aetiology, or
  by hepatitis virus type B (HBV) (with or without type D), or
  a parenteral hepatitis virus type non-A,non-B (e.g. hepatitis C).

This definition of CAH includes 1) morphology, 2) various aetiologies as mentioned above and 3) a clinical observation time.

Certain liver diseases, listed in table 1, can have histopathological features resembling CAH with periportal hepatitis and piecemeal necrosis. Such cases are termed *chronic hepatopathy with features of CAH, CAH-related disorder,* or *CAH-related liver disease.*[35,36,40]

We will define 'autoimmune' CAH as

- idiopathic CAH, in the absence of known causes of CAH
- in the presence of at least one immunologic disorder.

This excludes patients with e.g. HB_sA_g, but includes patients with only anti-HB_s that fulfill these criteria. Patients can have AMA, but not of subtype M_2. The immunologic disorder can include presence of other autoantibodies, like ANA or SMA or elevated gamma globulin, and can also be associated extrahepatic autoimmune disease.

§5 CLINICAL SYMPTOMS AND PHYSICAL FINDINGS

Initial reports by Waldenström and Kunkel emphasized the occurrence of CAH in young women with amenorrhoea, hepatosplenomegaly, hyperglobulinaemia, cushingoid facies, pigmented abdominal striae and arthritis.[4,5] 70-80% of the patients with idiopathic 'autoimmune' CAH (IAI-CAH) are women.[48-50] The disease is usually detected in the 3th to 5th decade of life, but young children and adults in their 60s and 70s may also be affected.[48-50] In approximately one third of the patients the disease begins abruptly and mimics acute viral hepatitis. The majority of the patients described in the literature had a more insidious onset of disease , however, with progressive jaundice (61%), abdominal pain (35%), epistaxis (21%), acne (19%), persistent fever (11%) and arthralgia (11%).[48] Fatigue, jaundice, arthralgias, and abdominal pain are the most frequently occurring symtoms. Between first symptom and diagnosis a delay of several months frequently occurs.[48-50] Sometimes patients present with extrahepatic manifestations of the disease, or develop such features later in the disease.[25,48-51] Up to 20% of the CAH-patients fulfill the revised ARA-criteria for SLE.[52]

§6 PROGNOSIS OF UNTREATED SEVERE CAH

Early assessments of natural history and prognosis of severe CAH were recently summarized.[53] Mortality ranged from 20% in 3 years,[54] 40% in 4 years,[55] 80% within 5 years[56] or 70% in 6 years,[57] to 50% within 10 years.[8] Prospective studies have indicated that mortality approximates 40 percent within 6 months of diagnosis if corticosteroids are not administered in severe CAH.[50]

Prognostic factors

- Histology and presence/absence of cirrhosis:

It has been demonstrated that cirrhosis occurred in 90% of untreated patients within 4 years.[48,58,59] Cirrhosis at presentation was associated with the highest mortality.[60] Untreated patients with morphologic evidence of bridging or multilobular necrosis had an 82% incidence of cirrhosis within 5 years and a mortality of 45%. In contrast, patients with only periportal necrosis had less than a 17 % incidence of cirrhosis and a normal life expectancy.[61] Cirrhosis frequently develops in early survivors,[57] and late mortality usually is related to the consequences of portal hypertension rather than liver failure, the main cause of early death in CAH.[48] Survival beyond 3 years in untreated CAH was frequently associated with a spontaneous reduction in inflammatory activity, and with transition to an inactive macronodular cirrhosis in most survivors.[48]

The pattern of fluctuating inflammatory activity was later recognized as a behavioral characteristic of CAH. Spontaneous clinical improvement occurs in 40% of untreated patients within 6 months of diagnosis, and histologic resolution has been described in 20% of patients surviving from 1 to 3 years after diagnosis.[50] Of the patients entering remission, 67% sustained the improvement. Death from liver failure eventuated in 41% and cirrhosis developed in 59%.[61] Patients who improved without corticosteroids could not be distinguished from others on accession by disease duration, age, sex, initial histologic findings, associated autoimmune disease (with exception of colitis ulcerosa, which probably indicates that PSC and not CAH is present), immunoserologic studies (presence of LE-cell phenomenon, antinuclear antibody, smooth muscle antibody) , or biochemical abnormalities (AST, bilirubin, gamma globulin, albumin).[53,62] Patients with mild disease improve without therapy as frequently as those with severe disease (13 percent versus 20 percent).[63] Although less than severe CAH has a better prognosis than the more severe forms of CAH, its course is not always a benign one. In a study of 80 asymptomatic patients , including 34 patients with HBsAg, 20% developed cirrhosis and 9% died of liver failure, while the 5- and 10-year survival probabilities were 91 and 81 percent respectively.[64] The presence of nonsuppurative cholangitis seems to have prognostic significance by increasing the likelihood of cirrhotic transformation.[65]

- Aetiology:

Aetiology is an important prognostic factor in CAH: The prognosis of untreated IAI-CAH has been mentioned above. Although it has been described,[66,67] HCC in patients with IAI-CAH is rare. 70% of HBsAg-positive patients developed cirrhosis and 30 percent died within 10 years.[68,69] These HBsAg-positive patients have an increased risk for the development of hepatocellular carcinoma (HCC),[70] even if a patient has become an asymptomatic carrier.[71] In HCV-hepatitis most patients are anicteric and

35% asymptomatic during the initial episode. Nevertheless, the majority (55-65%) develop progressive liver disease with CAH, with or without cirrhosis[72,73] In transfusion-related HCV-CAH however, spontaneous improvement is usual, a complete resolution having been described in half of the patients within 3 years after diagnosis.[74,75] Cirrhosis developed in 10-60 percent, immediate survival was excellent and HCC was absent.[73,75,76] HCC after HCV-hepatitis has been reported, however.[77,78]

- Other determinants of prognosis

The abrupt onset of symptoms, persistent jaundice, hepatic encephalopathy, ascites, and ulcerative colitis were associated with poor prognosis.[58] Serum aminotransferase elevation of greater than tenfold normal, or fivefold normal in conjunction with twofold elevation of gamma globulin level, was associated with mortality of greater than 50% within 3 years of diagnosis.[30] The presence of extensive hepatocellular necrosis with or without cirrhosis presaged a fatal outcome within 5 years in untreated patients.[58,59] Patients presenting on first admission with bleeding from varices, hepatic encephalopathy, ascites, greater prolongation of prothrombin time, cirrhosis or confluent necrosis in the liver biopsy, or HBsAg-positivity, have a worse prognosis than patients without these features.[79]

§7 TREATMENT REGIMENS IN CAH

In 1964, Page et al demonstrated that therapy with *6-mercaptopurine (6-MP)* could improve the clinical, biochemical and histologic manifestations of CAH, without reducing the level of hypergammaglobulinaemia.[60] Cook *et al.*,[80] Kirk *et al.*,[81] and Vogten[82] showed that *variable doses of prednis(ol)one* improved certain liver tests and survival.[80,81] Appropriate downward *titration* of corticosteroids was used to reduce drug-toxicity. The Copenhagen Study Group for Liver Diseases comparing 10 mg prednisone daily and placebo in histologically confirmed cirrhosis showed that survival was not enhanced by prednisone (45% after 4 years and 20% after 8 years), although extrapolation from these results suggested that a subgroup of patients with clinical characteristics commonly associated with IAI-CAH could be improved by corticosteroid therapy.[83,84] Therapy with a *fixed dose of prednisone* (15 mg daily) appeared to be more effective than *azathioprine* (75 mg daily) for increasing the life expectancy of patients with CAH with or without cirrhosis.[85] Soloway *et al.* proved that therapy with a fixed dose of prednisone 20 mg, or a *combination of prednisone 10 mg with azathioprine 50 mg* improved survival and secured clinical, biochemical and histological remission of CAH significantly better than azathioprine (100 mg) or placebo.[50] Subsequently Summerskill *et al.* showed that this combination therapy with prednisone 10 mg and azathioprine 50 mg is the initial treatment of choice for CAH (fulfilling criteria for treatment. The resolution of disease activity occurred as often as

with prednisone 20 mg, whereas side-effects were significantly less frequent, and because with both treatment regimens histological remission occurred more often than in the group receiving 'dose-titration' of prednisone.[86] Re-analysis of this study, however, showed no difference between the remission rates in fixed dose prednisone and the titration regimen. Remission was more frequent in patients receiving fixed dose prednisone and azathioprine, however.[87] Although this retrospective analysis also showed that in the untreated control group cirrhosis was significantly more common in patients with treatment failure (65%) than in those who achieved remission (37.5%). This might have made therapy appear more effective than it would have been with an equal distribution of cirrhosis between the control and treatment groups. The main conclusions of the Mayo study remain unaffected,[87] and this study is the basis of most current immunosuppressive treatment regimens in CAH.

Current absolute criteria for treatment of CAH (in the absence of contra indications) are AST>10N or AST>5N with GG>2N (N = upper reference limit). Arthritis, fatigue, itching or other severe subjective complaints are all relative indication for treatment. At present it is uncertain whether less severe CAH should be treated or not. It is also unknown whether compromised liver synthetic function or the presence of functional heparorenal failure should be included in the criteria for treatment. Further problems will be mentioned in § 10 of this introduction.

Two treatment options have been recommended in the management of idiopathic 'autoimmune' CAH: Prednisone in a high initial dose (60 mg daily) tapered over a 4-week period to a fixed maintenance dose (20 mg daily) is as effective as prednisone (30 mg daily) tapered to a smaller dose (10 mg daily) in conjuction with a fixed maintenance dose of azathioprine (50 mg daily).[88,89] Hepatic conversion of prednisone to prednisolone, the metabolically active compound, is not greatly impaired in patients with and without cirrhosis and there is no reason to prefer either one.[90,91] Withdrawal of azathioprine in patients maintained on a combination of azathioprine and prednisolone, leaving the dose of prednisolone unchanged, increases the probability of relapse.[92,93] After induction of remission some patients can be maintained on azathioprine alone to prevent relapse.[94] Relapse occurs in up to 80% of patients when an attempt to withdraw corticosteroid therapy is made, while a minority enters sustained remission.[95-97]

Immunosuppression for viral CAH ?

The clinical data regarding steroid treatment of HBsAg-positive CAH are contradictory. Controlled studies have suggested that steroids are ineffective and even deleterious in HBsAg-positive hepatitis.[98,99] Prednisone delays loss of HBeAg and seroconversion to anti-HBe[98] and does not affect[98] (and possibly increases[99,100]) mortality in HBsAg-positive patients, especially in those with cirrhosis. Corticosteroid treatment in HBsAg-positive patients may even delay biochemical remission and hastened biochemical relapse.[100] Some reports indicate that short courses of corticosteroid treatment may be beneficial in some patients with acute clinical and biochemical exacerbations of chronic

HBsAg-positive hepatitis.[101-104] These reports should be interpreted with caution, however: Immunosuppression may lead to an increase in amount of hepatitis B virus in the hepatocyte and/or expression of virally coded antigens on the hepatocyte,[103,105] frequently resulting in a hyperimmune rebound state after withdrawal of immunosuppression.[103,106] This presumed effect is used in current evaluation of treatment of hepatitis B, in combination with subsequent immunostimulatory therapy. A recent controlled trial of a 4-week course of prednisolone in chronic hepatitis-B demonstrated that even short courses of corticosteroid treatment can be potentially detrimental in hepatitis B.[107] While it is uncertain whether prednisone increases the level of viral replication, it has been demonstrated that corticosteroid therapy increases the average duration of active HBV replication,[108,109] which might result in progression of the liver disease and might increase the likelihood of integration of viral DNA sequences into the host genome.[110] These data combined with the fact that steroid therapy can give rise to serious side effects, lead to the conclusion that steroids should be only considered in patients with severe and potentially life-threatening hepatitis B, and even then may be contra-indicated.
In anti-HBe-positive patients with liver disease, however, steroids plus azathioprine may be beneficial. This may also apply in less active disease,[98] especially if associated immunoserologic markers are present.[110,111]

An uncontrolled trial of prednisolone and azathioprine in patients with HCV-hepatitis, and of cyclophosphamide in chimpanzees suggested some beneficial effect in HCV-CAH.[112-114]
Preliminary data indicate that treatment of HCV-hepatitis by immunostimulation with alpha interferon may be beneficial.[115,116] It is possible that a small subgroup of patients with severe HCV-hepatitis, but without active viral replication, benefit from corticosteroids, as do some anti-HBe positive patients in HBV-hepatitis. Recently the genome of a specific HCV-antigen has been cloned[117] and an assay for circulating antibodies to the antigen of this toga- or flavivirus will become available.[118] Thus future trials will probably indicate the best strategy in HCV-CAH.
Treatment options for viral CAH have been summarized recently.[119,120]

Other treatment options for non-viral CAH

Alternate-day steroids were originally tested by suddenly lowering the dose of steroids to 5 mg every other day after 3 months of treatment, and treating biochemical relapses by increasing therapy only on alternate days.[86] The present method for treatment with alternate-day steroids in other diseases is to double the dose on one day and then gradually reduce the other day's dose to zero. This approach has not been fully tested in CAH and may need examination, for example in patients with osteoporosis.[121]

Pulse prednisone therapy with high-dose prednisone (90 mg daily) given in 3- to 5-day courses repeated at 4-week intervals can inhibit B-cell synthesis of immunoglobulin G

and reduce inflammatory activity. Experience with this regimen is limited, however,[122,123] and it was recently reported that such pulse therapy failed to control the symptoms and biochemical findings of relapse in severe CAH, and is less effective than combination therapy with 10 mg prednisone and 50 mg azathioprine daily.[123]

Cyclosporine (CsA) was effective in some young patients with 'autoimmune' CAH,[124,125] and may be tried in a clinical setting if high-dose combination-therapy with prednisolone and azathioprine fails, or if contraindications to the use of corticosteroids exist (e.g. severe growth failure in children). Close monitoring, especially of renal function and CsA blood levels will be mandatory, and will probably preclude its routine use in the long term, although further evaluation of CsA therapy in corticosteroid-unresponsive CAH may be justified. A trial with cyclophosphamide yielded disappointing results.[126]

Cytoprotective therapies like polyunsaturated phosphatidyl choline or arginine thiazolidinecarboxylate suggested some effectiveness in limited trials, but the risks and advantages of such treatment are uncertain.[127,128]

Experience with *antifibrotic therapy* (other than corticosteroids and penicillamine) in liver disease is limited. Almost every report on D-penicillàmine in CAH has demonstrated some efficacy, and it was used for several years. Its high toxicity and lack of superiority over prednisolone, however, did not allow this treatment to be an option.[129] Colchicine appeared effective in a heterogeneous group of patients with liver disease,[130] also in primary biliary cirrhosis,[131] although it probably does not have a direct inhibitory effect on collagen synthesis. Other promising drugs for the treatment of hepatic fibrosis, like 2-oxoglutarate analogs and prostaglandins are currently tested *in vitro* and in animal models.[132] The value of antifibrotic drugs in CAH remains to be established. Since ursodeoxycholic acid appears an effective therapy for PBC,[133] the rare patients with cholestatic CAH ('CAH/PBC overlap') might benefit from such therapy if a therapy trial with corticosteroids[134] is not, or only partially, effective. However, these treatment options require further investigation.

The value of bed rest in CAH

This item remains controversial. Some studies report that exercise does not have any harmful effect on the course of hepatitis, even in the acute phase,[135] or in patients with CAH in remission while receiving immunosuppressive therapy.[136] This confirms earlier observations.[137,138] Others, however, report deterioration of patients with CAH after exercise.[139-141] Currently bedrest is advised only during active disease.

Liver transplantation in CAH

This item will be discussed in the section on therapy failure.

§8 PROGNOSIS OF TREATED CAH

Corticosteroid therapy induces remission in 60 percent of patients with severe autoimmune CAH within 3 years and 5-year survival is approximately 90 percent.[51] The cumulative remission rate for therapy of IAI-CAH with a combination of fixed dose prednisolone and azathioprine is 90%.[87] From 151 treated HBsAg-negative patients, 92% survived 5 years and 86% survived 10 years, while in 22 HBsAg-positive (65% also HBeAg-positive) patients, survival was lower: 73% and 65% at the same intervals ($p<0.01$). While the survival curve in treated HBsAg-negative CAH declines gradually, highest mortality in HBsAg-positive CAH occurred within the first 2 to 3 years and was quite low thereafter.[110] In retrospect, several patients with severe HBsAg-positive CAH who entered the Mayo study had in all likelihood an exacerbation of CAH in relation to 'immune clearance' of virally infected hepatocytes.[110] These results underline what has already been mentioned, namely that the indication for immunosuppressive therapy in viral CAH is very limited, while this therapy leads to a marked enhancement of survival in non-viral (idiopathic 'autoimmune') CAH.

Determinants of prognosis in treated CAH

- Prognostic value of histology:

In 1964 Page *et al.* demonstrated that patients without cirrhosis had the greatest chance of improvement during immunosuppressive therapy.[60] The presence of bridging necrosis (moderate CAH) or multilobular necrosis (severe CAH) in the initial biopsy was associated with a worse prognosis than periportal hepatitis in the absence of these features (minimal CAH).[61] In fact, some authors even state that the sole presence of periportal hepatitis is a benign condition. In the Mayo experience, the 10-year probability of survival without hepatic failure is 98 percent for patients without cirrhosis at presentation. For patients with cirrhosis prior to treatment, the likelihood of survival without hepatic failure is 80 percent after 5 years and 65 percent after 10 years.[142,143] Interestingly, patients who develop cirrhosis during therapy have a 5-year survival after the development of cirrhosis that is similar to that of patients who do not develop cirrhosis,[144] indicating that the propensity to develop cirrhosis in IAI-CAH depends more on the rapidity and completeness of the response to treatment than on any histologic feature at presentation.[144] This observation suggests that the ability to suppress inflammatory activity may be the most important prognostic factor.

- Prognostic value of markers for 'autoimmunity':

Page *et al.* also observed that patients with an acute onset of disease, evidence of multisystemic involvement, the LE cell phenomenon, and extreme hypergammaglobulinaemia were more likely to respond to corticosteroids than others.[60] Later on it was

demonstrated, however, that the presence or absence of autoimmune markers does not influence the response to corticosteroids in patients with severe CAH, screened for drug-induced or virus-related disease. The frequency of remission, treatment failure, hepatic death, and progression to cirrhosis were similar in patients with and without the LE cell phenomenon or antinuclear antibody (ANA). Therefore the same program of management for all patients with idiopathic, presumably autoimmune disease is recommended, regardless of immunologic findings, emphasizing that treatment decisions be based on clinical, biochemical and histologic assessments of disease severity.[145] Patients with LE cell or ANA who entered remission, however, were less likely to relapse after medication was discontinued, suggesting that corticosteroids had a greater ability to disrupt the underlying pathogenetic mechanisms in these patients. However, death from hepatic failure (4 versus 12 percent) and 5-year survival (92 versus 87 percent) were not statistically different between the two groups with and without LE cell or ANA.[145]

- To conclude, the degree of inflammation will determine the extent of liver failure and is connected with the induction of cirrhosis and its complications. The inflammation influences hepatocyte-function via cytokines and causes cell-necrosis and hence reduction of the number of functional hepatocytes. The duration of the hepatitis will especially influence prognosis via these mechanisms. This means that in idiopathic 'autoimmune' CAH, the initial reaction to immunosuppressive therapy must be the most important prognostic factor. Indeed, a recent study confirmed that patients who resolved at least one pretreatment laboratory abnormality, improved a pretreatment hyperbilirubinaemia, or did not experience biochemical deterioration after two weeks of corticosteroid therapy (with or without azathioprine) survived for at least 6 months in 98 percent of instances.[146]

§9 IMMUNOLOGY OF IAI-CAH

Pathogenetic mechanisms leading to CAH and 'CAH-related liver disease' can be diverse,[147] as has been pointed out earlier. In IAI-CAH, there is considerable evidence that cytotoxic reactions of the antibody dependant (ADCC-)type are directed at hepatocyte plasma membrane-derived epitopes in the macromolecular, lipid-associated complex called 'liver specific membrane lipoprotein' (LSP).[148,149] In 1972, Meyer zum Büschenfelde *et al.* reported the finding that rabbits repeatedly immunized with a high molecular weight fraction (LP1) of normal human liver developed liver lesions remarkably similar to those seen in CAH in man.[150] LP1 appeared to be a lipoprotein containing a liver-specific antigen derived from the hepatocellular plasma membrane, and consequently this fraction became known as liver-specific membrane lipoprotein (LSP).[151] Lymphocytes from patients with a variety of acute and chronic liver diseases are able to 'kill' heterologous[152-156] or autologous[157-160] hepatocytes, or LSP-coated

avian erythrocytes[161] *in vitro*. These cytotoxic reactions are apparently directed at antigens in LSP, while cytotoxicity is blocked by addition of LSP.[160] The hepatic asialoglycoprotein receptor (ASGR) is an important constituent of LSP.[162] This is a liver-specific, species cross-reactive hepatic receptor, also known as hepatic lectin. Anti-LSP antibodies appear to be predominantly directed at the ASGR, although a role for anti-LSP directed at other antigenic specificities has not yet been excluded. Anti-LSP is usually present in IAI-CAH patients requiring immunosuppressive therapy according to accepted criteria, and anti-ASGR is found in many of these anti-LSP positive CAH-patients.[163] It has also been shown that anti-ASGR antibodies preferentially coat periportal hepatocytes in the ante- and retrogradely perfused rat liver,[164] which is consistent with the concept of ADCC.

Composition of LSP

At present LSP is perhaps most accurately described as a macromolecular, lipid-associated complex containing a number of hepatocyte plasma membrane-derived antigens. Some of these antigens are non-organ-specific and others are liver-specific (including at least one that is species-specific and at least one other that is species cross-reactive).[165] There is now convincing evidence[166,167] indicating that LSP is composed to a large extent of vesicles (40-1600 nm diameter) and other fragments of the hepatocellular plasma membrane. It also contains some cytoplasmic fragments.[168] Furthermore, it has been demonstrated that LSP contains liver-specific antigens and non-specific determinants. Both of these are localized in the low-density subfraction after CsCl density gradient centrifugation, using both heterologous and autologous sera.[169] Attempts have been made to employ hybridoma techniques to identify antigens in LSP[170-174] and one monoclonal antibody has been produced[171] that reacts with a liver-specific, species-specific, plasma membrane antigen in rabbit LSP. However, it might eventually be proved that liver-specific but species cross-reactive determinants are the most important target antigens in CAH. The molecular weights of four LSP-determinants, recognized by monoclonal antibodies, in the study of Wiedmann *et al.*[172] ranged from 22 kiloDalton (kD) to 164 kD. LSP contains lecithin, lysolecithin, sphingomyelin, phosphatidyl ethanolamine, fatty acids, and cholesterol. SDS-PAGE of LSP from man, cattle, and rabbits shows essentially identical patterns in each, with four main bands corresponding to molecular weights of 50, 55, 60 and 66 kD, and a number of minor bands of 29 to 350 kD.[175] In addition it is now known that LSP contains the ASGR, as has been mentioned above.[162]. This lectin was found to comprise only 0.25% of the total protein in LSP, which may explain why it is difficult to demonstrate liver-specific LSP components by such techniques as sodium dodecyl sulphate/ polyacrylamide (SDS-PAGE)-gel electrophoresis.[176]

Regulation of the immune response in IAI-CAH

Con A induced non-specific T-suppressor cell activity in monocytes from blood of patients with IAI-CAH or HBV-CAH is impaired.[177-180] If IAI-CAH is treated with corticosteroids, this T-suppressor activity normalizes[181]. Some authors doubt whether this T-suppressor defect is antigen-specific or not. If the monocytes isolated from IAI-CAH patients are preincubated with prednisolone, T-suppressor function improves, while the function of T-suppressor cells from HBV-CAH patients does not improve on addition of corticosteroids.[182] This may indicate that the T-suppressor defect in IAI-CAH and HBV-CAH may be of a different nature, and it reflects the difference in response to corticosteroids between these diseases. In the indirect migration inhibition test, monocytes of an IAI-CAH patient produce T-lymphocyte migration inhibition factors in the presence of LSP. These factors were absent if these cells were cultured in the presence of T-cells of healthy donors or HBV-CAH patients. These inhibitory factors were also absent in the same assay with monocytes derived from HBV-CAH patients. This implies a defect in the LSP-specific suppressor T-cell function in IAI-CAH,[183] and recent studies indicate that the basal defect probably lies in the inducers of these suppressor cells.[184] These observations were supported by findings in thymectomized mice with experimental IAI-CAH.[185]
In addition to triggering factors, a genetic predisposition appears a prerequisite for the development of IAI-CAH. The histocompatibility antigens A1, B8 and D3 and the GM-ax allotype marker are all more frequent in IAI-CAH.[186-190] Relatives of patients with IAI-CAH show an increased incidence of serological abnormalities, various auto-immune diseases, and suppressor T-cell defects.[184,191] Whether these suppressor T-cell defects in relatives, linked with HLA-type, are only non-antigen specific or also antigen specific is still uncertain.

To summarize[192,193] in IAI-CAH a genetically determined defect exists in T-cell inducers of antigen-specific T-suppressor lymphocytes. It is possible that these patients are provoked into ADCC by LSP, or more specifically, the ASGR. Corticosteroids can improve the non-antigen specific T-suppressor cell functions in IAI-CAH, resulting in reversal of histological and biochemical manifestations of disease. This is sufficient to induce 'remission' according to currently accepted criteria, but from the King's College's experience it appears that corticosteroids have no effect on the genetically determined LSP-specific T-suppressor cell defect. This latter defect could allow for continued stimulation of anti-LSP producing B lymphocytes.

No defects in the secretion of interleukins and interpherons have been reported to play a role in IAI-CAH. This is unlike chronic HBV-infection.[184-186] A number of immuno-regulatory factors have been identified in human liver and sera, including liver-derived inhibitory protein (LIP),[197-199] rosette inhibitory factor (RIF),[200-203] serum inhibition factors (SIF),[204-206] and an immunoregulatory subset of low-density lipoprotein (LDL)

which has been designated as LDL-In.[207-209] The functions of these factors have been reviewed recently.[210] It is not currently known whether altered secretion of these factors might be pathogenetically involved in CAH.

Effector mechanisms of the immune response in CAH

Evidence for cell-mediated immunity to LSP has been found in 26 out of 29 patients with IAI-CAH, but in only one out of 21 patients with HBV-CAH.[183] The presence of cytotoxic T-cells and natural killer cells (Pit cells) in contact with hepatocytes in the periportal inflammatory infiltrate of CAH has been demonstrated,[211-214] suggesting a pathogenetic role for these cells in both IAI-CAH, HBV-CAH and HCV-CAH. Cytotoxicity in CAH shows antigen-[152] and target cell[215] specificity. Although direct evidence for the role of these effector cells *in vivo* is lacking at present, indirect evidence for their role is abundant in *in vitro* experiments.[153,216-225] Unfortunately many of the *in vitro* studies have been plagued by methodological difficulties, such as the small numbers of autologous cells available for testing, the problems related to different methods for evaluating target cell death, and the failure in some studies to allow for the constraints of HLA restriction when testing for T-cell cytotoxicity.[226] The properties of these mononuclear cells *in vitro* do not necessarily reflect those of the cells in the inflammatory infiltrate of the liver, of course.

To summarize, CAH can be the final common pathway of several aetiologies, and partly shared pathogenetic mechanisms. Cytotoxicity for autologous hepatocytes by mononuclear cells from the peripheral blood has been found in both viral-associated and idiopathic autoimmune CAH. Cytotoxicity mediated by lymphokine-activated killer (LAK)-cells directed to LSP (probably of the ADCC type) has been shown in most patients with IAI-CAH and HBV-CAH. The main type of cytotoxicity in HBV-CAH is an attack by cytotoxic T-cells (CTL), possibly directed to HBcAg and HBeAg on the membrane of hepatocytes.

Putative role for exogenic factors in triggering IAI-CAH

Besides a genetically determined immunological abnormality, an exogenous factor may be necessary to induce 'autoimmune' CAH. Increased antibody titres to exogenous antigens, notably bacterial and viral, were reported.[227,228] High antibody titres against gut-derived bacteria such as E. Coli, Bacterioides, and Salmonella can occur.[227,229] Elevated antibody titres were described for measles, rubella[230-233] and cytomegalovirus (CMV).[233] Elevated antibody titres to CMV early antigen[234] were also described in oxyphenisatin-induced CAH.[235] Anti-measles antibodies in CAH can belong to the IgM-class of antibodies,[236] suggesting persistent production of measles virus antigens.

Recently, persistent measles virus genome was identified in the lymphocytes from 12/18 patients with IAI-CAH, in contrast to 1/45 controls.[237] This finding correlated strongly with the presence of high antibody titres to measles.
These findings suggest the possibility that viruses and bacteria may be involved in the aetiology and pathogenesis of IAI-CAH.
Other authors postulated as intrinsic defect caused by spontaneous mutation of immunocytes. A third theory involves as environmental toxin. This theory was - like the infections theory - supported by the presence of autoantibodies in the sera of spouses of CAH-patients.

§10 PROBLEMS IN THE DIAGNOSIS AND MANAGEMENT OF CHRONIC ACTIVE HEPATITIS

These have been extensively dealt with by Czaja.[238] The most important problems he mentions are:

1. Determining chronicity

No reliable methods exist to determine whether hepatitis will follow a chronic course or not , even when a histology of CAH is present.[238] Spontaneous improvement of CAH can occur, as already mentioned. Determination of chronicity is necessary to prevent administration of potentially toxic or unnecessary medication. Only a subgroup of CAH-patients requires immunosuppressive therapy, while institution of such therapy in other CAH-subgroups can be contra-indicated, as discussed. Therefore, duration of illness was relied on as a criterion of chronicity.

Continuation of hepatitis for at least 10 weeks without clinical or biochemical improvement excludes self-limited acute hepatitis in most instances and suggests chronicity.[239] Demonstration of ongoing activity for at least 6 months stresses further the unresolving nature of the disease and satisfies international criteria for chronic disease.[34,36,240] However, morphologic features of acute viral infection in a patient with disease of more than 6 months duration can challenge classification. The presence of hypoalbuminaemia, hypergammaglobulinaemia, and ascites in a patient with disease of acute onset and of less than 6 months' duration may justify corticosteroid treatment, even though the disease is not fully chronic by international criteria. Initially, exclusion of a viral aetiology of hepatitis was difficult, because sensitive and specific markers were lacking. Now that sensitive and specific methods for detection of hepatitis B and - recently- hepatitis C have been developed, known causes of CAH (including viral) can be excluded earlier in the disease, and with greater reliability. Immunosuppressive therapy might therefore, when indicated, be instituted earlier in a patient fulfilling criteria for the diagnosis of idiopathic 'autoimmune' CAH (duration excluded).

2. Establishing the diagnosis

Known causes of CAH and CAH-related liver disorders, as mentioned in table 1, must be excluded to allow diagnosis of idiopathic 'autoimmune' CAH.

3. Deciding to treat

Patients who most clearly deserve immunosuppressive therapy are those with IAI-CAH and sustained elevation of AST at least 10-fold normal or 5-fold increases in conjunction with at least twice-normal serum gamma-globulin concentrations.[50] There is a high mortality among these patients if untreated.[53] Presence of bridging or multilobular necrosis on liver biopsy also connotes severity of necrotic inflammation, and therefore motivates initiation of treatment.[28,61] Serious clinical deterioration with development of ascites, endogenous encephalopathy, or other features of liver failure warrant therapeutic intervention.[89] The decision to initiate treatment in patients with mild to moderately active hepatitis (CAHmin and CAHa) is difficult. That disease of mild to moderate severity can progress to cirrhosis and liver failure is unquestioned: 33% of HBsAg-negative patients with CAHa progress to cirrhosis, and the 7-year mortality of these patients is 14%.[68,69] In patients not fulfilling biochemical, histological or clinical criteria for severe IAI-CAH the potential benefits of treatment have to balanced against the risks of therapy and the likelihood of spontaneous resolving of the disease in the individual patient. Controlled clinical trials have not yet been conducted in this area. Immunosuppressive treatment of CAH of subgroups other than IAI-CAH, like anti-HBe positive patients, sometimes can be justified as has been discussed already.

4. Drug toxicity

Development of major steroid-induced side-effects is dose-related: Only 11% of patients receiving 10 mg/day of prednisolone developed major side effects after 24 months of therapy, while this percentage approximated 75% when the dose was higher than 20 mg/day.[240] Azathioprine enables dose-reduction of corticosteroids.[50,86] Corticosteroid-related cosmetic changes (truncal obesity, facial rounding, dorsal hump formation, acne or hirsutism) are frequent, weight gain in excess of 20 pounds, hypertension, diabetes mellitus, cataracts, osteoporosis[242] and severe skeletal complications (vertebral compression fractures and aseptic necrosis of the hip) can occur, while psychosis or serious infections are rare.[87] Peptic ulcer disease is no more frequent in patients with CAH treated with prednisone than in those receiving placebo,[243] although peptic ulcer and gastritis are more frequent in CAH than in the general population, especially in those with portal hypertension.[244] Diabetes mellitus is not only a complication of treatment, but is also more frequent in patients with untreated CAH than in the general population.[245,246,247] Of all the complications, about 70% developed during the first

12 months.[87] Antacids significantly reduce prednisone bioavailability, and therefore should not be ingested simultaneously.[248] Oral antidiabetic therapy should be used with caution in CAH, because these drugs can induce chronic active hepatitis.[249] Calcium supplementation of 1 gram/day can reduce steroid-induced bone resorption without detectable suppression of bone formation.[250] 25-hydroxy(OH)vitamin D administration can improve parameters of mineral and bone metabolism in steroid-induced osteoporosis,[251] but 1,25 $(OH)_2$ vitamin D appears ineffective.[252] Another treatment option for corticoid-induced osteoporosis may be the use of salmon calcitonin,[253] while other investigators recently reported that administration of 150 mg 3-amino-1-hydroxypropylidene-1,1-biphosphonate (APD) daily in 'enteric coated' form, in combination with calcium supplementation 1 gram/day effectively prevented development of steroid-induced osteoporosis, and even reversed it after development.[254] Further studies would establish whether this therapy is effective in the long term prevention and therapy of steroid-induced osteoporosis, and whether it can be used safely in CAH.

Azathioprine toxicity occurs in fewer than 10% of patients receiving low-dose therapy (50 mg daily). It usually manifests early with hepatotoxicity, gastrointestinal upset, skin rashes, leukopaenia, and thrombocytopaenia and is reversed by reduction in dosage or discontinuation of medication.[89,50] The risks of oncogeneity after low-dose azathioprine therapy are low but probably increased. What has been reported underlines the efficacy and safety of low-dose azathioprine, the benefits outweighing the long-term risks of extrahepatic neoplasm.[87,255-257]
In patients for whom azathioprine has increased potential toxicity (children, patients with severe leukopaenia or severe thrombocytopaenia (e.g. $<50x10^9/l$), during pregnancy) an initially high dose of steroids with a rapid taper to 20 mg daily has been recommended,[258] although during pregnancy in CAH-patients no important side-effects could be attributed to azathioprine, as will be discussed.

5. Complications of CAH

The frequent development of cirrhosis has already been mentioned. Following institution of treatment development of ascites or encepahalopathy rarely occurs, and if it does this is an ominous prognostic sign.[259] This probably also applies to progression of liver insufficiency despite therapy. The presence or development of oesofageal varices is less threatening in treated patients, however: Oesofageal varices bleed infrequently in treated CAH, and their presence appeared to be not associated with mortality. Bleeding from gastritis or gastric ulcer, however, is more likely in patients with varices than in others.[260]

If a sudden decompensation occurs despite compliance with the treatment regimen after long-standing stable disease, without recent changes in other medication, the possibility of presence of primary hepatocellular carcinoma should be considered.[66,67]

Serious infection is an uncommon occurrence during standard maintenance therapy.[87] However, systemic mycosis can complicate high dose corticosteroid therapy given for treatment-failure,[261] and therefore this possibility should be considered if a patient does not improve despite such therapy.

6. Pregnancy in CAH

Many patients with IAI-CAH are in the child-bearing age. One retrospective analysis of pregnancy in CAH compared to pregnancy in normal (Australian) controls has been reported.[262] Pregnancy did not adversely influence the behaviour of maternal liver disease. A higher than expected rate of fetal loss and obstetric complications occurred, but the majority of deliveries were successful, and the babies normal. These findings suggest that conception is not absolutely contraindicated by the presence of CAH, but that close monitoring (especially obstetric) is necessary during pregnancy. Therapy during pregnancy has been discussed above.

7. When and how to stop therapy?

Criteria for discontinuing therapy in patients after induction of remission are arbitrarily defined. The current practice is to confirm histological remission by liver biopsy after a fixed period of sustained remission during therapy, e.g. two years, and then try to withdraw therapy in a period of variable length. Remission is defined arbitrarily as absence of clinical symptoms, biochemical resolution of AST to less than twofold normal, with normal bilirubin and gamma globulin, and histologic remission to a histology of chronic persistent hepatitis or complete resolution of the inflammatory changes, with or without cirrhosis. In some studies even minimal parenchymal inflammatory activity (grade 1 piecemeal necrosis) did not preclude classification as remission.[50,86,263] Relapse occurs in 49-87 percent of patients within 6 months after remission and withdrawal or discontinuation of treatment, and the majority of patients who experience relapse do so within the first 3 months after termination of treatment.[263,264,265] As will be discussed, it therefore will be necessary to find better endpoints of therapy and early indicators of relapse.

Intolerable cosmetic changes, weight gain of at least 40 pounds, inability to satisfactorily control diabetes mellitus or hypertension, osteoporosis with vertebral collapse, persistent severe cytopaenia, psychosis, or hypersensitivity skin eruption are current indications for dose modification or drug withdrawal. Some consider pregnancy, upper gastrointestinal bleeding of unknown origin or severe gastrointestinal upset also as indications for modification of therapy.[143]

8. Managing the sub-optimal response of CAH to therapy.

Sub-optimal responses of CAH to therapy can be divided into:

- treatment failure
- slow response
- multiple relapses

Treatment failure

Treatment failure was defined as death or deterioration characterized by the development of hepatic encephalopathy or increasing ascites with a twofold increase in serum bilirubin to greater than 12 mg/dl.[50,86] After 1971, the criteria were revised to include a 67% increase in bilirubin (if earlier >4mg%) or of SGOT (if earlier >59 IU/l).[259] At present, patients requiring reassignment of therapy because of complications, or with a fatal outcome due to complications from therapy, are also classified as treatment failures.[87]

Patients who fail standard treatment usually do so within 3 months after institution of therapy. They are more likely to have evidence of hepatic insufficiency (ascites or hypoprothrombinaemia)[259] and histologic evidence of confluent necrosis and cirrhosis at presentation.[61] Although the mean serum AST level is higher in patients who fail treatment than in those who do not,[259] treatment failure is not more common in individual patients with marked serum aminotransferase elevations (>1000 IU/l).[266] For patients meeting criteria for treatment failure, initial therapy with high-dose prednisolone (60 mg per day) or a combination of prednisolone 30 mg daily in conjunction with azathioprine 150 mg daily has been recommended, and the results after this high-dose treatment (81 percent survival) were better than previously observed.[79] The likelihood of subsequent sustained remission in these patients is low (25 percent) however,[88] and prolonged administration of high-dose corticosteroid therapy may carry an increased risk of side-effects. Liver transplantation may be the ultimate option for some of these patients.[143]

Slow or incomplete response

Within 3 years of therapy 65% of patients enter remission, and 20% fail treatment . The other 15% of patients improve during therapy, but fail to satisfy remission criteria after this period of treatment.[267] Continuation of treatment beyond 3 years in these patients is associated with a per annum probability of remission of only 7 percent, while the risk of side effects accrues at a similar rate.[267] The current policy in these patients is to administer conventional maintenance doses of medication until early evidence of drug intolerance.

Relapse

Relapse is defined as return of symptoms, a threefold or greater elevation of serum AST level or increase in serum gamma globulin concentration to at least 2 gram/100 ml, and histologic evidence of moderate to severe CAH (CAHa or CAHb).[44,50,263] Relapse occurs in 49-87 percent of patients within 6 months after remission and during or after discontinuation of treatment. The majority of patients who experience relapse do so within the first 3 months after termination of treatment.[263-265] Although relapse is usually accompanied by symptoms like fatigue, arthralgias, pruritus, anorexia, and abdominal pain, one-quarter of patients will have no clinical symptoms to signal recurrence of inflammatory activity.[263] It has been found that elevation of the AST level to at least threefold the upper reference limit during or after drug-withdrawal is invariably associated with histologic findings of CAH and indicates relapse.[268] The histologic pattern and degree of inflammation are related to the length of period between the onset of relapse and diagnosis, and may differ from the initial findings before therapy.[44] Patients who develop cirrhosis during therapy regularly relapse (85 to 90 percent), but patients with cirrhosis prior to treatment are as eligible for sustained remission after treatment as patients without cirrhosis before institution of therapy.[143,144,263] At present no other features predict the likelihood of relapse after treatment. Recently in a trial of treatment withdrawal the appearance of antibodies to liver-specific membrane lipoprotein (LSP) (with the asialoglycoprotein-receptor as a major antigenic determinant) preceded (in 15 out of 22 patients) or occurred concurrent with (in one patient) biochemical and histologic relapse. All 6 patients who sustained their remission remained anti-LSP negative.[269] If these results can be confirmed, testing for the presence of anti-LSP antibodies might (partially) obviate the need for sequential liver biopsies, and provide a more appropriate endpoint of therapy. In patients experiencing relapse, the pathogenetic mechanisms of the disease are probably not, or not permanently, disrupted and/or the inflammatory activity inadequately suppressed. Indeed, relapse occurs in only 28 percent of patients who revert to normal liver tissue as compared to a relapse frequency of 76 percent in patients with residual portal hepatitis at the time of drug-withdrawal.[270] In patients with cirrhosis whose inflammatory activity ceased, the frequency of relapse is still 79 percent, consistent with our knowledge that many patients develop cirrhosis after diagnosis of CAH, especially during the first two years of treatment when hepatic inflammation is most severe.[143,144] Once relapse has occurred, the probability of re-entering remission with successive courses of corticosteroid treatment remains similar to that experienced during initial therapy.[263] The likelihood of relapse within 6 months after a second course of treatment, however, increases from 49 percent to 60 percent, and after a third course becomes 86 percent.[143,263] 27 percent of patients followed longitudinally after remission will experience multiple (three or more) relapses.[263] Currently, therapy is reinstituted after each relapse until remission, treatment failure, or drug-intolerance necessitates treatment adjustments and improvisations.[143]

Liver transplantation in CAH

Orthotopic liver transplantation (OLT) may be the ultimate option for patients failing to respond to treatment of IAI-CAH.[143] Current criteria for determination of prognosis may not be entirely adequate, however.[271-274] Development of new survival models for decision making in these patients may be required (similar to PBC).[275,275a] It is felt that transplantation should be offered before the patient develops preterminal liver insufficiency, severe coagulopathy, variceal haemorrhage or hepatorenal syndrome and before the patient becomes severely catabolic. The odds of surviving transplantation have to be weighed against the probability of surviving the liver disease in every individual patient.[276] As has been mentioned already, most patients with IAI-CAH respond to immunosuppressive therapy, and do not need liver transplantation. The patient who, despite optimal immunosuppressive therapy, develops decompensated liver disease with spontaneous encephalopathy, progressive jaundice, diuretic-resistant ascites, uncorrectable and worsening coagulopathy, repeated uncontrollable variceal haemorrhage, or recurrent septicaemia ought to be considered as a transplantation candidate.[121] Recurrence of IAI-CAH after OLT has been reported once.[277] Partial heterotopic 'auxiliary' liver transplantation has been reported to be an alternative option in high-risk patients with end-stage chronic liver disease.[278]

§11 MONITORING ACTIVITY OF DISEASE AND LIVER FUNCTION IN CAH

Liver biopsy

In a liver biopsy of sufficient size[279,280] the observer variability in scoring the degree of hepatitis in CAH is small with a 94% consistency rate for overall morphologic interpretation. Inter- and intraobserver variability in grading the degree of piecemeal necrosis in other forms of liver disease is greater than 50 and 40%, respectively[281].

The diagnosis of cirrhosis by needle biopsy is dependant on the different criteria for diagnosing cirrhosis histologically. When complete regeneration nodules are required for diagnosis, false negative results are common. In many instances only a tentative diagnosis of cirrhosis can be made on the base of fragmentation, liver cell appearance, parenchymal regeneration, and fibrosis.[40,41] The combination of liver biopsy with laparoscopy increases the accuracy of diagnosing cirrhosis.[282]

However, CAH is defined principally by its activity rather than by structural changes. The classifications of the degree of severity of CAH are variable. The recommendations by an international group are still widely used.[31,40,42] Activity may be variable from location to location in one and the same biopsy, and the most severely affected area will

determine the degree of activity reported. Sample error in CAH can be less than 10 percent when scoring the severity of inflammation.[283] Recently, Knodell *et al.* defined a numerical scoring system to assess histological activity in asymptomatic chronic active hepatitis.[284]

Laboratory testing

Liver morphology in CAH has to be considered in combination with clinical and biochemical findings in order to enable therapeutic decision-making.[285] Although it is a minor procedure, a liver biopsy is still invasive and has its own -low- morbidity and mortality.[286] This precludes its use for frequent routine monitoring of activity of disease. Furthermore, morphology does not reflect hepatic functional capacity.

Standard tests

Aspartate aminotransferase (AST) and gamma globulin (GG) to some extent reflect hepatocellular necro-inflammation.[287-293] Currently, these two routine tests are used to determine sufficient severity of disease to require treatment,[89] help to assess response to treatment,[89,268] define treatment failure,[259,266] resolution of disease,[44,263,270] and disease relapse.[44,89,95,96,263] However, the accuracy in predicting morphologic activity of CAH is limited, especially for normal values of AST and/or GG when using standard reference ranges.[268] In addition, these tests do not reflect liver function, and they are of no great help in differential diagnosis of CAH.

Data is limited on changes in functional routine laboratory parameters like albumin, coagulation parameters, and water- and salt homeostasis, in relation to activity of disease and hepatic function.

No single clinical or standard laboratory parameter such as serum albumin, prothrombin time or platelet count, was able to predict the presence of cirrhosis. The combination of such parameters, however, increased sensitivity and specificity.[294]

Specialized tests

Serum amino acids

Specific plasma acid patterns have been described in patients with chronic liver disease,[295-302] including CAH.[299,303,304] Elevation of the aromatic amino acids (AAA) phenylalanine, tyrosine, and methionine, with concomitant reduction of the branched-chain amino acids (BCAA) valine, leucine, and isoleucine appear in cirrhosis. These

abnormalities have been explained by imbalances between hepatic clearance and peripheral utilization of the amino acids.[305,306] The BCAA are decreased in chronic liver disease due to increased peripheral oxidation,[305] increased ketogenesis,[307] increased renal gluconeogenesis,[308] increased distribution volume,[309] and/or increased intracellular distribution, [310,311] while AAA are increased due to enhanced proteolysis [312,313] and altered hepatic metabolism.[314-316] In several studies the molar ratio of BCAA to AAA levels has been shown to correlate with severity of inflammation in hepatic histology,[298,300,318,319] liver function,[297,302,320,321] and prognosis.[298,301,319] However, prospective studies did not confirm the high predictive value of the BCAA/AAA ratio for these parameters.[297] These differences in results may in part be explained by the many other conditions that influence the BCAA/AAA ratio, like portal systemic shunting,[322] nutritional status,[323-325] alcohol use,[325] and gender.[326] A normal plasma molar ratio, however, is of value in excluding the presence of cirrhosis.[295,298,300,317]

Serum bile acids

The same problems in interpretation are encountered when assessing the usefulness of serum bile acids (SBA) in the evaluation of liver disease. Results are influenced by the analytical method used, gallbladder contraction, digestive and interdigestive gastrointestinal motility, enterocyte absorption, hepatocyte clearance, and the function of the sphincter of Oddi and iliocecal valve. Only competitive binding radioimmunoassays[327,328] and heterogeneous[329] and homogeneous[330] enzyme assays have been shown to be sufficiently sensitive to accurately measure SBA concentrations.

Some studies suggest that SBA determinations correlate well with severity of histologically active liver disease in CAH-patients.[331,332] Fasting SBA levels have been reported to discriminate active from inactive CAH equally or even better than did postprandial SBA-levels[333-335] and were shown to be superior to conventional liver tests in this respect.[334-339] Hepatic clearance of SBA in liver disease is dependant upon portosystemic shunting as well as hepatic function.[340] SBA levels appear to correlate better with quantitative liver function tests such as bromosulfophtalein and indocyanine green clearance tests[341] and the aminopyrine breath tests,[342] than with standard liver tests. These correlations explain the described ability of SBA levels to predict mortality risks in cirrhosis.[343] SBA levels might thus prove useful as one of the parameters in the selection of patients for liver transplantation,[344] despite the many factors that influence SBA levels. However, these studies need further confirmation.

Quantitative measurements of hepatic function

Aminopyrine breath test

Like SBA, the aminopyrine breath test (ABT)[345] is an infrequently used quantitative measure of hepatic function.[346-348] It measures the combination of elimination and clearance rates,[349] and as such is influenced by the functional microsomal mass, and

complex biochemical reactions that affect CO_2 formation and elimination. These are influenced by alterations in levels of folic acid, vitamin B_{12}, reduced glutathione, and NAD/NADH redox status. The ABT is difficult to standardize, because each of these factors may or may not be independantly impaired during liver disease, and the ABT is also influenced by such factors as gastric emptying[350] and patient age.[351] It is a relatively easy and non-invasive test, however.[350,352] The ABT has been shown to be abnormal in non-cholestatic liver diseases, particularly cirrhosis.[346,350-356] The ABT reflects the severity of inflammation in chronic hepatitis, but a significant overlap between mild and severe CAH and between controls and patients with mild liver disease limits its use in this respect.[342,356,357] In addition some investigators reported that ABT was no better than[352,356] or worse than[358] standard liver tests in predicting histologic severity. ABT was reported to discriminate between CAH and PBC,[359,360] which might be of use in CAH with prominent cholestatic features. There is some evidence that the ABT may have prognostic value in predicting mortality[361] prior to surgery and it may aid in the selection of patients for liver transplantation.

Other tests of function
The hepatic extraction fraction of many substances can be used as a measure of liver function. Galactose elimination clearance (GEC) has the advantage that it is less influenced by disturbing variables than some of the other tests. However, especially the second phase of galactose elimination varies with variations in the hepatic flow rate. The place of GEC in diagnosis and follow-up of liver diseases like CAH remains to be established.[361a]

Portal pressure measurements

Portal pressure measurements can probably only give insight into the degree of portal hypertension, but there is no reason to assume that portal pressure reflects histologic activity.[285]

Procollagen peptides

Fibrosis and formation of cirrhosis are sequelae of untreated CAH,[362,363] and can develop during therapy.[364,365] About 70% of liver collagens consist of collagen types I and III.[366] Collagen type III is predominant in early fibrosis; in late cirrhosis, type I, hard collagen" prevails.[366] Type III collagen has a more rapid turnover than type I collagen.[367] The C- and the N-terminal procollagen-III-peptide (PIIINP) are split from the procollagen-III molecules before extracellular assemblage into fibers in the extracellular matrix. The amount of free PIIINP that circulates predominantly stochiometrically reflects deposition of collagen-III.[368-370] Some of the serum PIIINP is derived from PIIINP which was retained in the deposited fibers.[371,372] Decreased degradation of PIIINP due to impaired liver function can account for a rise in serum

PIIINP in the anhepatic situation during liver transplantation.[373] However, these two factors probably do not materially affect the relationship of serum PIIINP with deposition of collagen type III in the liver during CAH. Some studies have indicated that serum PIIINP parallels the activity of inflammation in acute hepatitis, and returns to normal with transaminases and bilirubin[374] and in alcoholic hepatitis PIIINP correlated well with periportal and intralobular inflammation.[375-379] Several studies suggested a relation between activity of CAH and PIIINP,[380-383] although this could be masked in children with CAH by elevated PIIINP due to growth.[384] Persistent elevation of PIIINP in serum of patients with viral hepatitis for more than 6.5 months correlated with chronicity.[374,385] Therapy with corticosteroids can lower an elevated PIIINP in CAH,[386-390] and recently it was shown that PIIINP changed in accordance with other liver tests and normalized when remission of CAH had been achieved.[391,392]

Autoimmune phenomena

Anti-LSP antibodies

It was reported that patients with IAI-CAH requiring immunosuppressive therapy invariably have anti-LSP antibodies,[393,394] as already discussed above. In several studies, titres of anti-LSP antibodies were shown to correlate with presence and severity of periportal inflammation in CAH.[393,395,396] Moreover, in a trial of treatment withdrawal, patients without anti-LSP who remained negative did not relapse, while patients with these antibodies or in whom anti-LSP reappeared, relapsed.[269] Therefore it appears that testing for anti-LSP could be important in monitoring of CAH.

Other immunological phenomena

Several autoantibodies, like antinuclear antibodies (ANA), antibodies to liver membrane antigens (LMA), anti smooth muscle antibodies (SMA), anti soluble liver antigen (SLA), anti liver-kidney microsomal antibodies (LKM1), and other antibodies, including those against exogeneous agents like measles and cytomegalovirus, have been identified in IAI-CAH and CAH of other aetiologies. The value of many other tests like the concentration of circulating interleukin-2 (IL-2) receptors,[397] and tests for measuring cytotoxicity and T-suppressor function for routine monitoring of IAI-CAH remains to be established.

To conclude, there is great need for non-invasive parameters for following the response to therapy, determining the optimum duration of treatment, for monitoring and predicting disease activity after cessation of therapy, and for help in differential diagnosis of CAH.

More information on long-term prognosis in CAH is needed, and data on accurate assessment of early response to treatment are scarce.[146] Elucidation of the aetiologies of idiopathic 'autoimmune' CAH may lead to more specific therapies.

Section 1

Patients and Methods

CHAPTER 1

PATIENTS

Between January 1969 and January 1988, a histological diagnosis of chronic active hepatitis (CAH)[398] was made in 186 patients referred to the division of Hepatology at Groningen University Hospital, a tertiary care center. These are the patients studied in this thesis. During this period sera were frozen and stored at -20°C on all examinations and admissions, and the records of all liver biopsies and revisions of liver biopsies were kept systematically. The 186 CAH-patients were found by reviewing all pathological descriptions of liver biopsies from this period, with continued follow-up, and by reviewing our own patient database and the computerarchives of the hospital. Patient records were reviewed and, where necessary, liver biopsies were reviewed. Patients with chronic persistent hepatitis (CPH), but no biopsy with CAH, were excluded, as were patients where a probable diagnosis of CAH was made without taking a liver biopsy. All patients with cryptogenic cirrhosis and no biopsy with CAH were excluded,

Table 1.1 CAH-patients grouped according to aetiology.

Aetiology*	Number of patients	Sex M:F
IAI-CAH	69	12: 57
anti-HBsCAH	13	3: 10
HBsAgCAH	51	37: 14
HCV-CAH	10	4: 6
DI-CAH	20	3: 17
Other CAH	23	6: 17
Total	186	65: 121

ad *)

IAI-CAH	=	idiopathic 'autoimmune' CAH
HBsAg CAH	=	CAH with circulating HBsAg
Anti-HBs CAH	=	CAH with circulating anti-HBs
HCV-CAH	=	CAH due to parenteral non-A,non-B hepatitis (probably due to hepatitis C virus)
DI-CAH	=	drug-induced 'CAH'

Other CAH:

- 9x CAH/PBC= 'cholestatic CAH' or 'mixed form'CAH/PBC
- 5x CAH/PSC= 'small-duct' PSC and/or CAH
- 3x Wilson-CAH= 'CAH' associated with Wilson's disease
- 5x A1AT-CAH= 'CAH' related to α_1-antitrypsin deficiency
- 1x 'CAH' in multicystic liver

even if serology, clinical findings or reaction to corticosteroids suggested CAH. Furthermore, patients with acute hepatitis B and a histology of CAH were only included if hepatitis perpetuated for more than six months after the onset of disease. Patients with "CAH-related disorders" and a histology indistinguishable from CAH, e.g. α_1-antitrypsin deficiency + CAH (see introduction), were included. Classification as to aetiology and age, sex and therapy of the patients is shown in tables 1.1, 1.2 and 1.3, and age is also shown in figure 1.1.

Table 1.2 Classification of CAH patients into etiological and therapeutic categories.

Therapy	IAI	HBsAg	anti-HBs	HCV	CAH/PBC	CAH/PSC	A1AT	Wilson	cysts	drugs	TOTALS
P+A $_1$	25(21)	0	5(3)	1(1)	0	1(0)	0	0	0	1(0)	33(25)
P+A $_2$	7(6)	0	0	0	0	0	2(1)	0	0	0	9(7)
P+A $_3$	4(4)	0	0	0	1(1)	2(1)	2(1)	0	1(1)	0	10(8)
P $_4$	1(1)	0	0	0	0	0	0	0	0	0	1(1)
N $_5$	32(25)	51(17)	8(7)	9(5)	8(7)	2(0)	1(1)	3(3)	0	19(17)	133(82)
TOTALS	69(57)	51(17)	13(10)	10(6)	9(8)	5(1)	5(3)	3(3)	1(1)	20(17)	186(123)

Numbers denote therapy groups and number of patients, numbers between brackets denote number of women, P=prednisolone, A=azathioprine, N=(group 5)no immunosuppressive therapy Therapy-groups 1 and 2: standardized immunosuppression, with resp. without prior low-dose immunosuppression, therapy-groups 3 and 4: not standardized immunosuppression. For further explanation :see text.

Table 1.3 Age at onset of CAH.

Age(yrs)	Idiop	HBsAg	antiHBs	HCV	CAH/PBC	CAH/PSC	A1AT	Wilson	cysts	drugs	TOTALS
0- 9	0	0	0	0	0	0	0	0	0	0	0
10-19	9	2	1	1	0	1	1	1	0	0	16
20-29	5	15	3	3	0	1	2	2	0	3	34
30-39	11	8	1	1	1	2	1	0	1	1	27
40-49	8	12	2	1	1	0	1	0	0	1	26
50-59	19	9	3	3	5	0	0	0	0	3	42
60-69	13	4	2	0	1	1	0	0	0	7	28
70-79	4	2	0	1	1	0	0	0	0	5	13
80-89	0	0	1	0	0	0	0	0	0	0	1
TOTALS	69	51	13	10	9	5	5	3	1	20	187

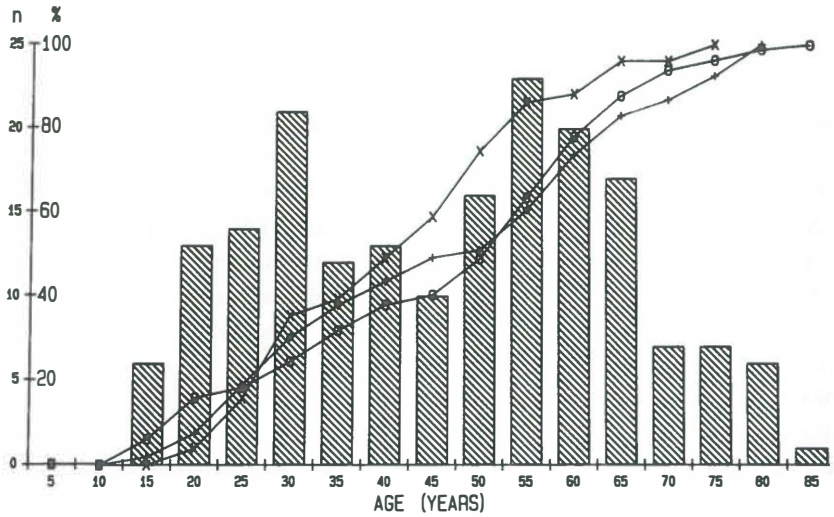


Figure 1.1. Age of 186 patients with CAH. Bars show age at 5-year intervals. Lines give cumulated percentages of categories of these patients: o = IAI-CAH without anti-HBs, x = HBsAg-positive CAH, + = other types of CAH.

Some patients (shown in table 1.4) took drugs during the course of CAH that could themselves induce CAH, but the drug was assumed to be the cause of CAH only if a rechallenge proved an aetiological role, or if relation in time to the appearance and disappearance of CAH were very suspect in this regard.

A diagnosis of non-A,non-B CAH, probably due to the hepatitis C virus, (HCV-CAH) was only made if the onset of disease was within half a year of documented blood-transfusion (9 patients) or intravenous drug-abuse (1) without evidence of hepatitis B infection; otherwise the CAH was classified as idiopathic.

Documented risk factors for transmission of hepatitis B and non-A,non-B virus(es) in several patients are shown in table 1.5, which also shows that 2 patients abused alcohol in addition to the other aetiological factors.

All patients were tested for HBsAg, anti-HBs and anti-HBc-IgG. Where tested, HBsAg and HBcAg in liver biopsies were only positive in the group with circulating HBsAg. 42 out of 51 HBsAg-positive patients were positive for serum-HBeAg, 9 had anti-HBe antibodies.

In 9 patients (table 1.2), histological distinction between CAH and primary biliary cirrhosis (PBC) was impossible; 6 of these patients had high titres of antimitochondrial antibodies (AMA), but in 3 no AMA was detected. A diagnosis of PBC was later made in one of these AMA-positive patients when the liver was examined after orthotopic liver transplantation (OLT). One other patient showed a favorable reaction to therapy with prednisolone and azathioprine.

Table 1.4 **Factors that might have played an additional etiological role in 13 patients.**

CAH-classification*	Therapy*	Additional factors
IAI-CAH	1	sulphonamides
HBsAg CAH	5	MZ-A1AT deficiency
HBsAg CAH	5	oxyfenisatin
HBsAg CAH	5	sulphonamides
Anti-HBs CAH	5	nitrofurantoin
HCV-CAH	5	salicylates
HCV-CAH	5	nitrofurantoin
CAH/PBC	5	sulfonamides
CAH/PSC	2	MZ-A1AT deficiency
CAH/PSC	2	oxyfenisatin+sulfonamides
CAH/PSC	2	methyldopa
A1AT-CAH	5	nitrofurantoin
DI-CAH (oxyphenisatin)	5	sulfonamides

Table 1.5 **Documented risk factors in patients with 'viral' CAH.**

CAH-classification*	Risk factor(s)	Number of patients
HBsAg CAH	iv drug abuse	6
(20 patients)	iv drug abuse, alcohol abuse	2
	transfusion	3
	renal Tx, transfusion	3#
	haemodialysis, transfusion	1
	haemodialysis, transfusion, renal TX	3
	homosexual+changing contacts	1
	homosexual+changing contacts and alcohol abuse	1
Anti-HBs CAH	iv drug abuse	2
(4 patients)	iv drug abuse and homosexual+changing contacts	1
	haemodialysis, transfusion	1
HCV-CAH	transfusion	9$,&
(10 patients)	iv drug abuse	1

ad *)see table 1.2 Tx=transplantation iv=intravenous
#=one of these patients also used sulphonamides (see table 1.4).
$=one of these patients also used salicylates (see table 1.4).
&=one of these patients also used nitrofurantoin (see table 1.4).

In 3 patients (table 1.2) distinction between CAH and small- duct primary sclerosing cholangitis (PSC) could not be made with a combination of liver biopsy and endoscopic retrograde cholangiopancreaticography (ERCP). One reacted favorably to standardized immunosuppression, one remained stable and well while untreated, and in one patient histology and serum parameters did not improve despite all kinds of immunosuppression (including cyclosporin) and finally at liver transplantation a diagnosis of PSC was made. Two other patients were classified as CAH/PSC because of the presence of colitis ulcerosa and the impossibility to exclude PSC in the era before endoscopie retrograde cholangiopancreaticography (ERCP).
A diagnosis of Wilson's disease was established in 3 patients with CAH. One of these patients is alive and well with tri-ethylene-tetra-amine therapy (TETA), which was given because she was allergic to d-penicillamine. The other 2 patients died: one patient with disturbed swallowing (related to Wilson's disease) choked on food and died, despite previous improvement of the disease with TETA. The other patient had very severe therapy-resistant neurological disturbances and - in the pre-OLT-era - died of aspiration pneumonia.
Seven patients with α_1-antitrypsin (A1AT) deficiency were detected by phenotyping using immunoelectrophoresis. In two of these patients with MZ-phenotype, the A1AT deficiency was probably not the main cause of CAH; one HBsAg-positive patient had an acute hepatitis B, followed by CAH; the other had high AMA-titres and CAH/PBC histology. These two patients were classified accordingly. In the other 5 patients the A1AT deficiency was the only abnormality that could be aetiologically associated with the CAH in these patients (table 1.1). The phenotypes were ZZ in one, MZ in three, and MS in one patient. One patient with phenotype MS and one with MZ were only detected after A1AT phenotyping of the whole group of idiopatic CAH patients for reasons of this study, while the other patients were detected before then by phenotyping. The patient with ZZ phenotype received an orthotopic liver graft and is doing well.
One patient had a multicystic liver and histology of CAH, reacting favorably to short-term prednisolone and she is in remission without therapy for years now.
Drug-induced CAH was due to oxyphenisatin in 14, nitrofurantoin in 1, methyldopa in 3, sulphonamides in 1, and diclophenac in 1 patient(s).

From 176 patients stored sera were available. These are the patients investigated in the chapters on anti-cardiolipin and on anti-cytomegaloviral antibodies.

From the patients with IAI-CAH (with or without anti-HBs) CAH, 21 patients receiving standardized immunosuppressive therapy fulfilled inclusion criteria for the studies on effects of therapy.

CHAPTER 2

METHODS

All methods will be described or referred to in the chapter where they are used.

Some methods were investigated in detail to select or establish the best method for the purpose under investigation. These are described in chapters 3, 4 and 5.

CHAPTER 3

NUMERICAL VERSUS CONVENTIONAL HISTOLOGICAL SCORING OF ACTIVITY IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

SUMMARY

A histological activity index (HAI) generating a numerical score facilitates analysis of serial changes in liver histology of patients with chronic active hepatitis (CAH). In the present study, we compare the HAI as designed by Knodell *et al.* for asymptomatic CAH, with conventional pathological descriptions in the study of 164 liver biopsies from 38 patients with 'autoimmune' CAH requiring immunosuppressive therapy. In numerical scoring, periportal(PP), lobular(L), and portal(P) inflammation and fibrosis/cirrhosis (HFS=histological fibrosis score) were scored both separately and in combination (HAS=histological activity score=PP+L+P) (Knodell's HAI=histological activity index=HAS+HFS). HAS,HAI and PP correlated best with conventional scores (Spearman R .88,.86, and .92 respectively). Between conventional activity categories there is more overlap in HAI than in HAS, due to relative staticity of HFS. 10% of biopsies with chronic persistent hepatitis (CPH) when scored conventionally and 64% of biopsies with minimal CAH (CAHmin.) had a HAS>3. We conclude that HAS most accurately reflected activity of CAH in this patient group, and appeared to be a better parameter for monitoring activity during immunosuppression than conventional description. On the basis of the current experience we consider CAH histologically to be in 'partial remission' with a HAS ≤ 3 , and propose to reserve the term 'complete histological remission' for HAS ≤ 1.

INTRODUCTION

Histological scoring of inflammation in CAH

Chronic active hepatitis (CAH) is defined principally by its activity rather than by structural changes. In diagnosis and follow-up of CAH, an accurate and reproducible scoring system would be of great help. The original definition of CAH by de Groote *e t al.*, revised [399] and extended by Bianchi *et al.*,[400] is widely used. In this classic conventional pathological description, three degrees of activity of CAH are discerned: *(1) Minimal activity (CAH*-min): In these borderline cases periportal piecemeal necrosis may not be present *in all* portal tracts, or in a given portal tract may be seen only in a segment of the perimeter of the portal tract. *(2) Moderate activity (CAHa)* is characterized by dense portal infiltration with extensive piecemeal necrosis involving

substantial parts of the perimeter of almost every portal tract, but still restricted to periportal areas. *(3) Severe activity (CAHb)* includes cases with piecemeal necrosis in the periportal region and along fibrous septa (periseptal piecemeal necrosis), or cases with confluent bridging hepatic necrosis in addition to features of periportal piecemeal necrosis. To establish a more objective assessment of histological activity in asymptomatic CAH, Knodell *et al.* recently proposed a numerical scoring system, the Histological Activity Index (HAI).[401] In the current study, we applied this Histological Activity Index in symptomatic 'autoimmune' CAH and compared the data with conventional scoring of activity.

MATERIALS AND METHODS

Patients and liver biopsies

164 liver biopsies from 38 patients with CAH requiring immunosuppressive therapy according to accepted criteria[402-404] were reviewed. 28 patients were classified as primary 'autoimmune' CAH, 10 patients belonged to other aetiological CAH-categories with predominant secondary autoimmune features (table 3.1). All patients in groups A and B (table 3.1) had been maintained on standardized immunosuppression: 15 mg. prednisolone and 75 mg. azathioprine for two months, followed by 10 mg. prednisolone and 50 mg. of azathioprine for two years.[403] Low-dose immunosuppression preceded standardized therapy in therapy-group B, while patients in group A did not receive immunosuppressive therapy on referral. Patients in group C (table 3.1) had received non-standardized therapy with prednisolone alone (1 patient) or combined with azathioprine (4 patients). Needle biopsies obtained before and during immunosuppressive therapy with the Menghini technique were fixed in formalin and embedded in paraffin. Four micron sections were routinely stained with hematoxylin-eosin, periodic acid-Schiff with previous diastase digestion, Perl's iron stain, Gomori's reticulin stain, and Azan collagen stain.

Scoring activity

For the purpose of this study, all biopsies were re-examined under code by two of us (J.G. and B.v.H.), without knowledge of the patient's identity or previous histological diagnosis. In case of persisting disagreement the scores of the liver pathologist were used.

It is well-known that in a biopsy of sufficient size,[404a,404b] the observer variability in scoring the degree of hepatitis in CAH is small with a 94% consistency rate for overall morphologic interpretation.[404c]
In order to further minimalize intra-observer error we scored the biopsies in a short period of time using standardized forms.

Table 3.1 **Patients (37) and biopsies (164)**

	AETIOLOGY:	IAI	OTHER	TOTAL
THERAPY:				
A	patients	21	6	27
	biopsies	92	24	116
B	patients	5	1	6
	biopsies	24	4	28
C	patients	2 *	3	5
	biopsies	8	12	20
TOTAL	patients	28	10	38
	biopsies	124	40	164

ad Therapy:
A= standardized (see text) immunosuppression without prior therapy,
B= standardized immunosuppression with prior low-dose corticosteroid therapy,
C= non-standardized therapy with prednisolone (2.5-30 mg) and azathioprine (25-100 mg).
ad*) 1 patient (2 biopsies) with IAI-CAH in group C received prednisolone without azathioprine

ad Aetiology: IAI: idiopathic 'autoimmune' CAH

Other:
- A:
  - (a) 3 patients (16 biopsies): CAH with anti-HBs or/and anti-HBc in serum without signs of viral replication.
  - (b) 1 patient (5 biopsies): like (a), combined with use of oxyphenisatin.
  - (c) 1 patient (1 biopsy): HCV-CAH.
  - (d) 1 patient (2 biopsies): probably IAI-CAH, but 'small-duct' primary sclerosing cholangitis cannot be excluded (CAH/PSC).
- B: 1 patient (4 biopsies) MZ-α_1-antitrypsin deficiency.
- C:
  - (a) 1 patient (7 biopsies) MS-α_1-antitrypsin deficiency.
  - (b) 1 patient (3 biopsies) 'cholestatic CAH' (CAH/PBC).
  - (c) 1 patient (2 biopsies) CAH/PSC.

The conventional description of histology according to Bianchi [400] was applied on all biopsies. To enable Spearman rank correlation between classical and numerical scoring, the conventional score of severity of CAH was assigned a number as follows: 0=(almost) no inflammation, scored normal=N, 1=chronic persistent hepatitis (CPH), 2=CAHmin., 3=CAHa, 4=CAHb. Conventional descriptions of the presence of fibrosis/ cirrhosis were scored as follows : 0=no fibrosis/cirrhosis detected, 1=fibrosis/cirrhosis probable, 2=fibrosis /cirrhosis present with certainty.

Table 3.2 HAS, HFS and HAI for numerical scoring of liver biopsy specimens.

HAI[a]

HAS						HFS	
I. Periportal +/− bridging necrosis	Score	II. Intralobular degeneration and focal necrosis[b]	Score	III. Portal inflammation	Score	IV. Fibrosis	Score
A. None	0	A. None	0	A. No portal inflammation	0	A. No fibrosis	0
B. Mild piecemeal necrosis	1	B. Mild (acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis in <½ of lobules or nodules)	1	B. Mild (sprinkling of inflammatory cells in <½ of portal tracts)	1	B. Fibrous portal expansion	1
C. Moderate piecemeal necrosis (involves *less* than 50% of the circumference of most portal tracts)	3	C. Moderate (involvement of ½–⅔ of lobules or nodules)	3	C. Moderate (increased inflammatory cells in ½–⅔ of portal tracts)	3	C. Bridging fibrosis (portal-portal or portal-central linkage)	3
D. Marked piecemeal necrosis (involves *more* than 50% of the circumference of most portal tracts)	4	D. Marked (involvement of >⅔ of lobules or nodules)	4	D. Marked (dense packing of inflammatory cells in >⅔ of portal tracts)	4	D. Cirrhosis[c]	4
E. Moderate piecemeal necrosis *plus* bridging necrosis[d]	5						
F. Marked piecemeal necrosis *plus* bridging necrosis[d]	6						
G. Multilobular necrosis[e]	10						

[a] HAI score is the combined scores for necrosis, inflammation, and fibrosis.
[b] Degeneration—acidophilic bodies, ballooning; focal necrosis—scattered foci of hepatocellular necrosis.
[c] Loss of normal hepatic lobular architecture with fibrous septae separating and surrounding nodules.
[d] Bridging is defined as ≥2 bridges in the liver biopsy specimen; no distinction is made between portal-portal and portal-central links
[e] Two or more contiguous lobules with panlobular necrosis.

Numerical scoring was performed exactly as described by Knodell *et al.* (table 3.2).[401] This system uses four histological categories: (I)periportal inflammation (piecemeal necrosis-PMN-) with or without bridging necrosis; (II) intralobular degeneration and focal necrosis; (III) portal inflammation; and (IV) fibrosis/cirrhosis. A numerical score was assigned ranging from 0 (no alteration) to 10 for caregory I, and from 0 to 4 for categories II to IV. As the score for inflammation (Categories I to III) and the score for fibrosis/cirrhosis (=HFS) (Category IV) might vary in the opposite direction during follow-up, the four categories were monitored separately in the individual patient, and the addition of categories I, II, and III was termed "Histological Activity Score" (HAS). In doing so the combined score for piecemeal necrosis and bridging necrosis weighed more heavily than the separate scores for the other three categories.

Statistical analysis

Statistical analysis was performed with Spearman's rank correlation, Mann Whitney U test and analysis of variance where appropriate, with $p<0.05$ as the level of significance. If a feature could not be scored it was assigned a missing value code in the statistical analysis.

RESULTS

Results of comparison of conventional and numerical scoring of activity of CAH and relation with fibrosis/cirrhosis are shown in figures 3.1 and 3.2 and in tables 3.3 and 3.5. Analysis of variance revealed no differences in the measured parameters between primary 'autoimmune' CAH and the other forms of CAH with autoimmune features meeting requirements for immunosuppressive therapy. The combined data are presented. In 6 biopsies, fibrosis/cirrhosis could not be scored and here fibrosis/cirrhosis was assigned a missing value code. Scoring of inflammation was possible in all biopsies.

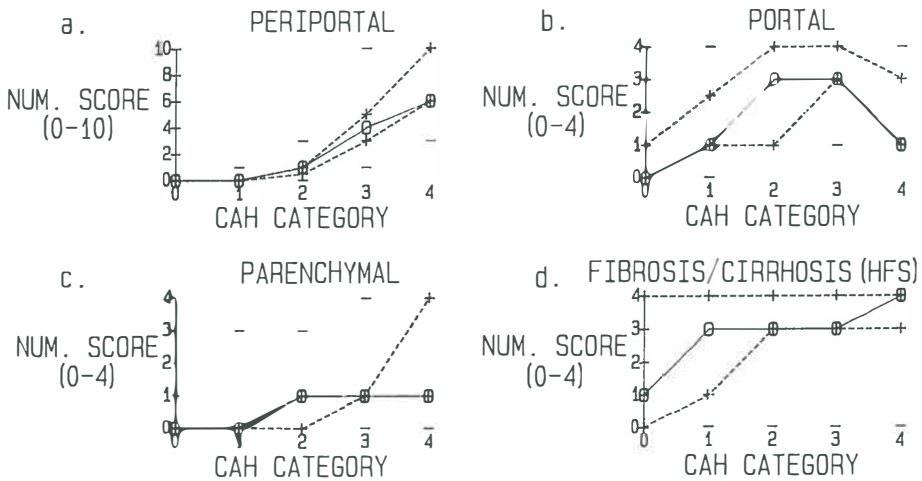


Figure 3.1 Comparison of conventional with numerical scoring of activity of CAH and its relation with fibrosis/cirrhosis. Conventional CAH-categories: 1 = normal histology, 2 = CPH, 3 = CAHmin, 4 = CAHa, 5 = CAHb.

Within each conventional category, there is a wide range of numerical scores of periportal,portal and lobular inflammation (figures 3.1 a,b,c) and fibrosis/cirrhosis (figure 3.1d), resulting in wide ranges for HAI and HAS in each of the conventional categories (figures 3.2a and b). In general, HAS is different between all conventional categories of severity while HAI does not differ significantly between CAHa and CAHb and between N and CPH. This is due to lack of significant difference in HFS (fibrosis/cirrhosis), a parameter included in the HAI, between subsequent conventional categories of CAH (fig. 3.2d). A higher degree of inflammation generally coincides with a higher HFS, indicating more fibrosis/cirrhosis when more inflammation was present. 78% of the biopsies contained some degree of fibrosis/cirrhosis (HFS≥1).
Significant differences in lobular inflammation could only be detected between CAHa and CAHb and not between less severe categories or between less severe categories and CAHa. Portal inflammation shows an increase between N and CPH, and between CPH

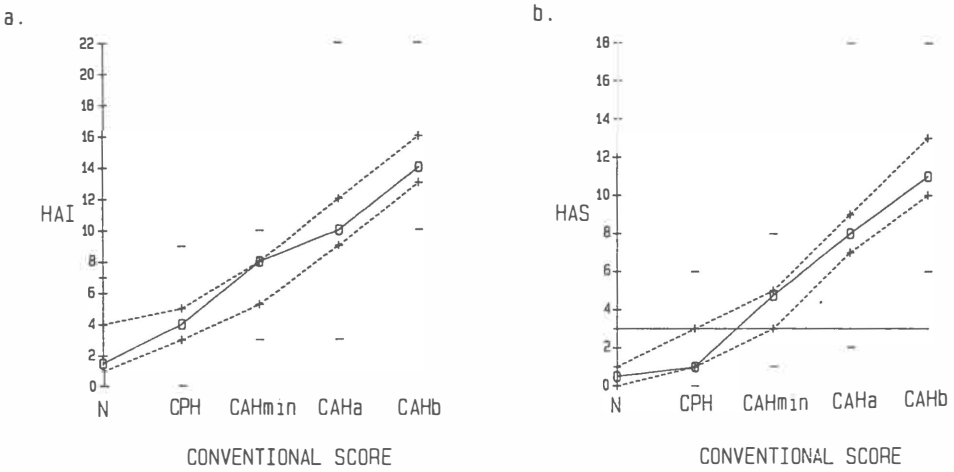


Figure 3.2 Comparison of conventional with numerical scoring of activity of CAH: a) histological activity index, b) histological activity score.

Table 3.3 Correlation (R) between conventional and numerical scoring of histological activity of CAH (Spearman rank) with p-values; 1=p<0.05, 2=p<0.001, 3=p<0.0005, 4=p<0.0001.

	CONVENTIONAL			
	ACTIVITY		FIBROSIS/CIRRHOSIS	
	R	p	R	p
NUMERICAL				
periportal (PP)	.92	3	.28	2
lobular (L)	.69	4	.20	1
portal (P)	.55	4	--	NS
HAS (=PP+L+P)	.88	4	--	NS
HFS	.32	3	.78	4
HAI (=HAS+HFS)	.86	4	.37	4
CONVENTIONAL				
cirrhosis	.30	2		

n=164 (for HFS and HAI and fibrosis n=158)

and CAHmin., but not between more severe conventional categories. Scores of periportal inflammation obtained for each conventional category of severity were different (except between N and CPH, as could be expected), although scores covered a wide range of values within each of these categories (figure 3.1a).
Spearman rank correlation coefficients between HAI and HAS and its components on one side, and conventional scoring of severity of inflammation and cirrhosis on the other side are depicted in table 3.3. Spearman rank correlation coefficients between the components of the numerical scoring system versus each other and versus the resulting HAS and HAI are shown in table 3.4.

Table 3.4. Correlation (R) between the components of numerical scores of histology in CAH (Spearman rank).

	PERIPORTAL (PP)		LOBULAR		PORTAL		HAS		HFS	
	R	p	R	p	R	p	R	p	R	p
LOBULAR (L)	.75	2								
PORTAL (P)	.51	2	.44	2						
HAS (=PP+L+P)	.91	2	.79	2	.72	2				
HFS	.34	2	.27	1	.07	ns	.22	1		
HAI (=HAS+HFS)	.90	2	.74	2	.59	2	.91	2	.54	2

Significance level: 1=p<0.01, 2=p<0.0001. n=164 (for HFS and HAI n=158)

Table 3.5 shows that 10% of biopsies with chronic persistent hepatitis (CPH) and 64% of biopsies with minimal CAH (CAHmin.) had a HAS >3 when scored conventionally.

Table 3.5. Conventional versus numerical (HAS) score of activity (n=164).

conventional	numerical	
	HAS≤3	HAS>3
N	14 (100%)	0 (0%)
CPH	57 (90%)	**6 (10%)**
CAHmin.	10 (36%)	**18 (64%)**
CAHa	2 (5%)	40 (95%)
CAHb	0 (0%)	17 (100%)

DISCUSSION

A histological activity index generating a numerical score was proposed by Knodell and coworkers in 1981 to facilitate the analysis of serial changes in liver histology of patients with asymptomatic CAH of various aetiologies.[401] Very recently, Lindh *et al.* demonstrated that this new way of histological activity scoring was also a valuable tool in monitoring activity of chronic hepatitis B.[405] Our current data in liver biopsies of patients with symptomatic 'autoimmune' CAH and CAH of other aetiologies with predominant autoimmune features and requiring immunosuppression, demonstrates the value of such a numerical score in this category of chronic liver disease. The histological activity score (HAS), which is an addition of periportal, portal and parenchymal (lobular) inflammation, and excludes the more static parameter of fibrosis (which is included in the HAI), correlated best with conventional scoring, and reflects most exactly the degree of inflammation in a liver biopsy in these patients.

Periportal inflammation may not be the only component expressing the severity of CAH. Patients with dense portal infiltrates, but only slight or no piecemeal necrosis are considered in remission in classical scoring systems. One could question whether all conventionally scored 'CAH in remission' really indicates remission. Investigators from the Mayo Clinic observed relapse rates of 28% in patients who reverted to normal liver biopsies, but 76% if residual portal hepatitis was present at the time of drug-withdrawal.[406,407] In our patients with CAH there was a significant overlap in HAS between conventional histologic categories of severity of CAH (figure 3.2b). A number of 6/63 (10%) and 18/28 (64%) out of biopsies scored as CPH and CAHmin conventionally had a HAS >3, indicating significant inflammation in portal - and sometimes lobular- areas. As will be discussed in chapter 11, almost all of the patients with HAS >3 display elevated serum collagen-split products predominantly indicating ongoing increased deposition of collagen type III in the liver. Therefore, we propose to term HAS ≤3 'partial remission', and HAS ≤1 'complete remission'.
Although CAHb tended to have a lower score of portal inflammation than CAHa, this was not a significant difference. This observation may indicate a tendency to underscore portal inflammation in the presence of severe periportal inflammation.

In conclusion, the advantages of the numerical scoring system are that the HAS more accurately reflects activity of CAH when compared to conventional scoring of activity in following the natural history and treatment responses of CAH. It facilitates the comparison of histological and serological assessments of severity of disease. On the basis of the current experience we consider CAH histologically to be in 'partial remission' with a HAS ≤3, and propose to reserve the term 'complete histological remission' for HAS ≤1. In future studies these new criteria for remission must be validated by testing them against outcome.

CHAPTER 4

SERUM N-TERMINAL PROPEPTIDE OF COLLAGEN TYPE III IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

Comparison of three radioimmunoassays and relation with histology.

SUMMARY

We compared three radioimmunoassays (RIAs) for determination of PIIINP in autoimmune chronic active hepatitis (AI-CAH), and the relation between serum PIIINP values and numerical scores of histological activity of CAH.
90 random sera with synchronous coded biopsies in 31 AI-CAH patients were investigated. Histological activity was numerically scored (0-18). Sera, obtained during all stages of activity of disease at the time of these liver biopsies, were tested in 1) the 'PIIIP RIA-gnost®' and 2) 'PIIIP Fab assay®'(Behring) which are based on a reference bovine PIIINP antigen (col 1-3) and antibodies raised in rabbits, and 3) a recently described rapid equilibrium type RIA which is based on the human propeptide ('human PIIINP RIA'). This rapid equilibrium type of assay is not sensitive to smaller degradation products. Therefore, unlike the other two assays, no serial dilutions of the sera and no 50% intercept method for calculating the results were necessary, since the standards and the serum samples give parallel inhibition curves.
Correlation between the 'human PIIINP RIA' and the 'PIIIP RIA-gnost®' was excellent: r=.96. Between the 'PIIIP RIA-gnost®' and the 'PIIIP Fab assay' this was r=.78, between the 'human PIIINP RIA' and the 'PIIIP Fab assay' r=.76. The correlation between PIIINP in the 'human PIIINP RIA' and histological activity score (HAS) was r=.67. Correlation of all three tests with fibrosis was only weak (.27<r<.33).
With the human PIIINP RIA, a rapid non-invasive way for measuring deposition of collagen III has become available, which circumvents the problems of the assays based on the bovine PIIINP. The role for determination of PIIINP in CAH requires further study.

INTRODUCTION

Although its exact place in monitoring diseases has not yet been established, measuring the serum N-terminal propeptide of collagen type III (PIIINP) as a parameter of deposition of collagen type III appeared to be useful in several diseases,[408] including chronic active hepatitis. Now that new therapeutic strategies for e.g. hepatic fibrosis

may become available in the near future, the need for a rapid and accurate PIIINP determination increases.

Type III collagen is a major constituent of most dense and loose connective tissues in the body, although bone, tendon and cartilage are devoid of it. Type III collagen is synthesized as a procollagen which contains propeptide extensions at both ends of the molecule.[409] Most of the aminoterminal propeptides are set free during the synthesis and deposition of type III collagen, while some are retained in the molecules which remain on the surface of the collagen fibrils.[410] This antigen, when found in serum, can thus be derived from the posttranslational modification after synthesis of new type III collagen or from the degradation of PIIINP retained in the collagen fibrils.[411,412] Smaller degradation products related to PIIINP are found in serum and excreted in urine.[413] The accurate analysis of serum antigens related to PIIINP has been difficult because of non-parallelism between the inhibition curves obtained with standard and serum samples, which is mainly due to the fact that the 'PIIIP RIA-Gnost' is sensitive to smaller degradation products.[411,412]

The 'PIIIP Fab assay' (Behring) is also based on a reference bovine PIIINP antigen (col 1-3) and antibodies raised in rabbits. This assay uses Fab fragments with identical affinity for col 1-3 and col 1,[413,414] 'PIIIP Fab assay®'(Behring). Therefore there is the need for serial dilutions in this assay. However, since the 'PIIIP Fab assay®' measures both antigen and degradation products, results do not necessarily parallel those obtained with the 'PIIIP RIA-Gnost'.

Recently a new radioimmunoassay (RIA) for the determination of PIIINP was developed by Risteli and Risteli.[415] This rapid equilibrium type of assay is based on the human propeptide and is not sensitive to smaller degradation products.[416] Thus, unlike the 'PIIIP RIA-gnost®',[417] in this new PIIINP-assay no serial dilutions of the sera and no 50% intercept method[418] for calculating the results are necessary, since the standards and the serum samples give parallel inhibition curves.

MATERIALS AND METHODS

Patient sera

90 sera from 31 patients with autoimmune CAH with and without immunosuppressive therapy had been frozen and stored (-20°C) until use. The only criterium for selection of these sera for this chapter was the availability of a liver biopsy performed at the same time.

Liver biopsies

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[419] In contrast to the original system,[420] presence or absence of fibrosis/cirrhosis is not included in the HAS, but expreved as a different score (0-4), the histological fibrosis score (HFS) - which was also measured -.

The HAS is expressed as a numerical score (0-18), and is the summation of portal (score 0-4), periportal (score 0-10) and lobular (score 0-4) inflammation and necrosis.

Serum PIIINP

The 'PIIIP RIA-gnost®'[417], the 'PIIIP Fab assay' (Behring),[413,414] and the new radioimmunoassay based on the human propeptide, developed by Risteli and Risteli,[415] were performed as described. Storage of sera at -20°C,[421] and bilirubin- and prednisolone-levels [422,423] in serum do not affect results of PIIINP-determination. PIIINP can be elevated in renal insufficiency.[423] One patient had a 40% lowered glomerular filtration rate due to membrano-proliferative glomerulonephritis, but PIIINP values in this patient paralleled those found in the other CAH-patients. The age of all the patients was above 16 years, so that the normal reference values for adults (upper limit 4.2 µg/l)[415] could be applied. The intra-assay variation of the new equilibrium-type RIA, which is based on the human propeptide, was 4.1%, and PIIINP values were determined in one assay.

Statistical analysis

Since the populations showed similar (near-)normal distributions, statistical analysis was performed with Pearson's correlation with $p<0.001$ as the level of significance.

RESULTS

Correlations are shown in table 4.1. Between the 'human PIIINP RIA' and the 'PIIIP RIA-Gnost®' there was an excellent correlation of r=.96, as shown in figure 4.1A. Correlation between the 'human PIIINP RIA' and the 'PIIIP Fab RIA' was r=.78 (figure 4.1B). Between the PIIIP RIA-Gnost® and the 'PIIIP Fab RIA' this was r=.76 (figure 4.1C). The correlation between the new 'human PIIINP RIA' and histological activity (HAS) was r=.67 (figure 4.2). In all but 3 cases a PIIINP within the reference range was related to HAS≤3. All three tests had only a week correlation with HFS.

Table 4.1 Correlation between serum PIIINP-values obtained with three different assays, and between these values, histological activity scores (HAS) and histological fibrosis scores (HFS) in 'autoimmune' CAH (n=90) (Pearson's correlation, p<.001) (p<.05 for HFS).

	Human RIA	RIA-Gnost	Fab-assay
RIA-Gnost	.96		
Fab-assay	.78	.76	
HAS	.67	.64	.55
HFS	.31	.33	.27

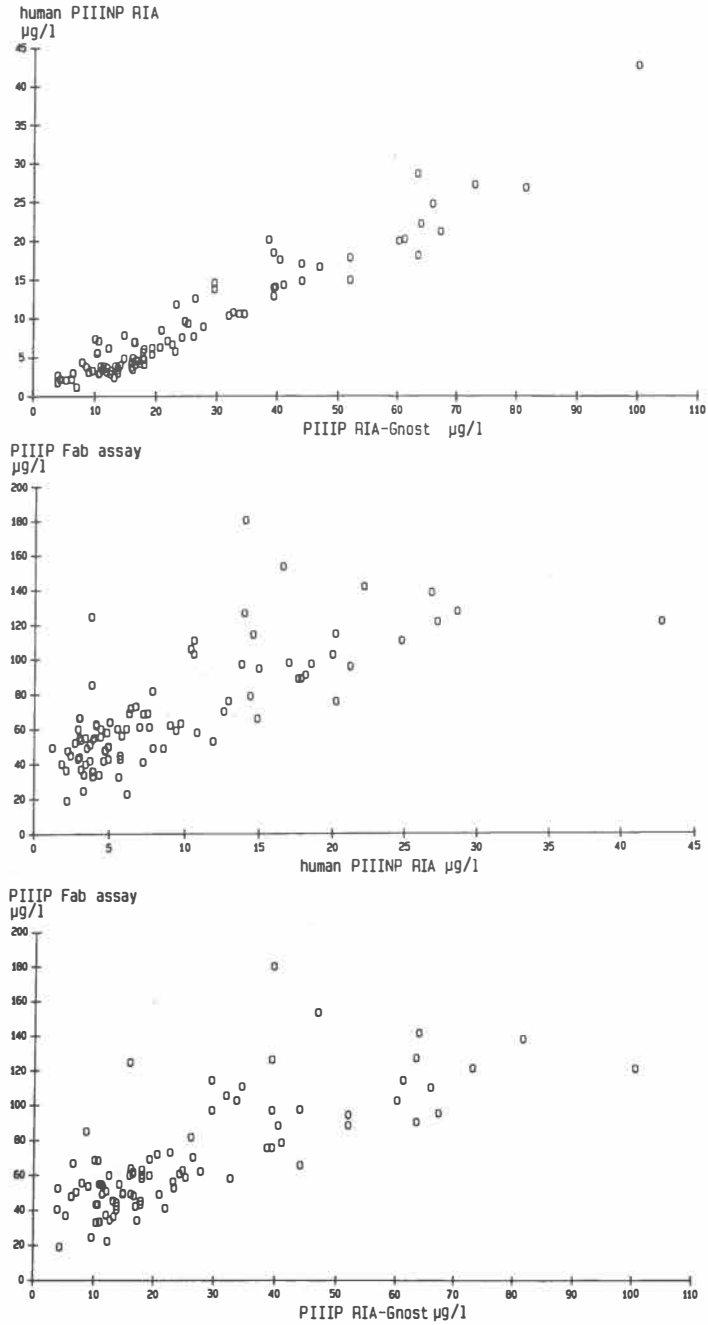


Figure 4.1 Correlation (Pearson) between serum PIIINP-values (µg/l) obtained with three different assays in 'autoimmune' CAH (n=90) (p<.001). A)Human PIIINP RIA vs. PIIIP RIA-Gnost. R=0.96, y=-0.376 + 0.370x, B)Human PIIINP RIA vs. PIIIP Fab-assay. R=0.78, y=-37.17 + 1.24x, C)PIIIP RIA-Gnost vs. PIIIP Fab-assay. R=0.76, y=39.73 + 3.21x.

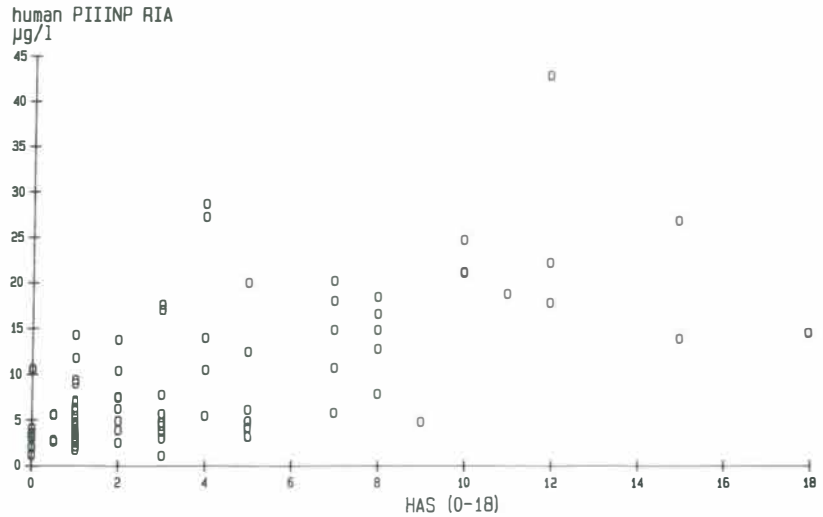


Figure 4.2 Correlation between PIIINP (human PIIINP RIA) and histological activity (HAS).(Pearson's correlation R=0.67, p<.001, n=90).

DISCUSSION

The current study shows that results obtained with the new PIIINP-assay, which is based on the human propeptide, correlate very well with the standard 'PIIIP RIA-gnost®'. The 'PIIIP Fab assay' measures both antigen and degradation products, which probably explains why results do not parallel those obtained with the two other assays. The fact that PIIINP is only weakly correlated to HFS probably reflects the fact that serum PIIINP reflects (dynamic) fibrogenesis rather than (static) fibrosis.

Serum PIIINP, predominantly reflecting deposition of collagen type III in the liver in CAH, correlated to some extent with activity of hepatic inflammation. Some studies indicated that serum PIIINP values parallel the activity of inflammation in acute hepatitis, and return to normal with transaminases and bilirubin.[424] In alcoholic hepatitis, serum PIIINP correlated well with periportal and intralobular inflammation.[425-429] Several studies suggested a relation between activity of CAH and PIIINP,[430-433] although this could be masked in children with CAH by elevated PIIINP due to growth.[434] Persistent elevation of PIIINP in serum of patients with viral hepatitis for more than 6.5 months correlated with chronicity.[424,435] Therapy with corticosteroids can lower an elevated PIIINP in CAH,[422,436-439] and in a recent study PIIINP changed in accordance with other liver tests and normalized when remission of CAH had been

achieved.[421,440] In the current transversal study, the correlation between serum PIIINP and HAS was only r=.67. The correlations between PIIINP and seperate scores of periportal inflammation were similar (data not shown). Many factors influence fibrogenesis, which may explain why this correlation is not better than 0.67. Another explanation may be a lay-time in the decline, of the inflammatry infiltrate. This will be discussed in chapter 11 as well. Some of the data represent several tests done in the same patients, so that we might have missed a correlation in the region with higher HAS and PIIINP.
Human PIIINP values above 20 µg/l were related to HAS>3 (active disease), while PIIINP values below 2 µg/l were related to HAS≤3 (partial histological remission). Serum PIIINP might be helpful in monitoring activity of CAH. Between 2 and 20 µg/l, however, there was a 'gray zone' where the serum PIIINP was less predictive for the HAS. Apart from a few exceptions, however, it can be stated that a PIIINP within the reference range is related to histological remission (HAS≤3).

With the 'human PIIINP RIA', a rapid non-invasive way of measuring deposition of collagen III has become available, which circumvents the problems[441] of the assays based on the bovine PIIINP. The role for determination of PIIINP in monitoring CAH and possible future therapies for increased hepatic fibrogenesis and collagen-deposition[442] requires further study. Further studies aimed at delining the value of testing for PIIINP will follow in chapters 11 and 12.

CHAPTER 5

ESTABLISHMENT OF A MODIFIED QUANTITATIVE RADIOIMMUNOASSAY FOR THE DETERMINATION OF ANTIBODIES AGAINST 'LIVER-SPECIFIC PROTEIN' (LSP)

SUMMARY

Antibodies against 'liver specific membrane lipoprotein' (anti-LSP) play an important role in pathogenesis of idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH), and may be useful for immune monitoring this disease. Quantitative determination of anti-LSP, however, required dilution in several steps, which limited routine use in IAI-CAH. We here describe a modification of the original anti-LSP assay. Binding percentages as determined in the previously described assay and in our modified assay did not differ. The inter-assay variation was intra-labelling <10%, inter-labelling 15%, and the intra assay variation <10%. Using 3 dilutions calculated anti-LSP titres did not differ from measured titres. The normal binding range was 5-24%. We developed a modified quantitative, reproducible radioimmunoassay for determination of anti-LSP, facilitating studies on monitoring IAI-CAH.

INTRODUCTION

'Liver-specific protein' (LSP) is a macromolecular, lipid-associated complex containing a number (as yet unidentified) of hepatocyte plasma membrane-derived antigens, some of which are non-organ-specific and others are liver-specific (including at least one that is species-specific and at least one other that is species cross-reactive).[443] The high incidence of autoantibodies against LSP in chronic active hepatitis (CAH), especially in idiopathic 'autoimmune' CAH (IAI-CAH) and its role in antibody-dependant cytotoxicity have been discussed in the introduction. In order to assess the value of anti-LSP in our population of standardized treated IAI-CAH patients, we established a modified radioimmunoassay for the determination of anti-LSP.

METHODS

Preparation of LSP has been described.[444] To establish a radioimmunoassay for determination of anti-LSP antibodies, rabbit-LSP prepared as described (with slight modifications) was used. A modification in preparation had been the dialysis of LSP

against sucrose for rabbit-LSP, and acetazolamide for human LSP, for concentration of the material. This was an alternative to ultrafiltration through an Amicon PM 30 membrane. The rabbit-LSP (1 mg/ml) used for this study was a gift from B.M. McFarlane-Wojcicka and I.G. MacFarlane (King's College Hospital -KCH-, London). This facilitated comparison with the original assay. Recently human LSP was prepared in the department of hepatology, University Hospital Groningen, and the following also applies to the assay using human LSP as an antigen.

The essentials of the radioimmunoassay (RIA) have been described.[445] Our modifications concern both the labelling and the assay itself:

Labelling

Instead of older labelling techniques[446] or labelling with ^{125}I coupled to Sephadex,[447] we labelled the LSP with Iodobeads® (Pierce Chemical Company, Rockford Ill, U.S.A.). These are N-chloro-benzenesulfonamide (sodium salt) derivatized uniform 2.8 mm diameter nonporous polystyrene spheres, with the structure as shown in figure 5.1. These beads facilitate smooth and reproducible iodination without the preparation of

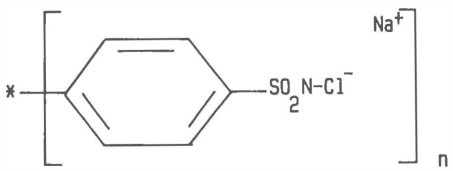


Figure 5.1 Structure of 'Iodobeads'.

additional solid phase chemical agents, and without the harsh effects of chloramine T. They also enable labelling over a broad pH and temperature range.[448] After washing the beads twice with borate-buffer 0.1 M, with 1mM EDTA with or without azide (no difference in results), 200 µl BSA-borate buffer was added to the beads. Then 5 µl Na-^{125}I solution (Radiochemical Centre, Amersham), was added (0.5-1 mCi), and after 5 minutes at room temperature, 20 µl of LSP was added. After 10 minutes, the reaction volume was separated from the Iodobeads to terminate iodination. Then, to remove free iodide, the reaction contents were directly transferred to an equilibrated Sephadex G-25 column, and the radiolabelled LSP was recovered in the usual way in the void volume with the help of a fraction collector. Every fraction was counted in the gamma counter as depicted in figure 5.2. The first (LP1-) peak contains the LSP as has been previously described. In contrast to the original assay, where labelled LSP was diluted 1:1 with unlabelled LSP, we pooled the fractions that contained LSP and added buffer with BSA 1% and azide up to 5 ml. In the 10 µl fractions with LSP, the percentage LSP-bound ^{125}I (*LSP) was determined by the trichloric acid (TCA) precipitation technique as

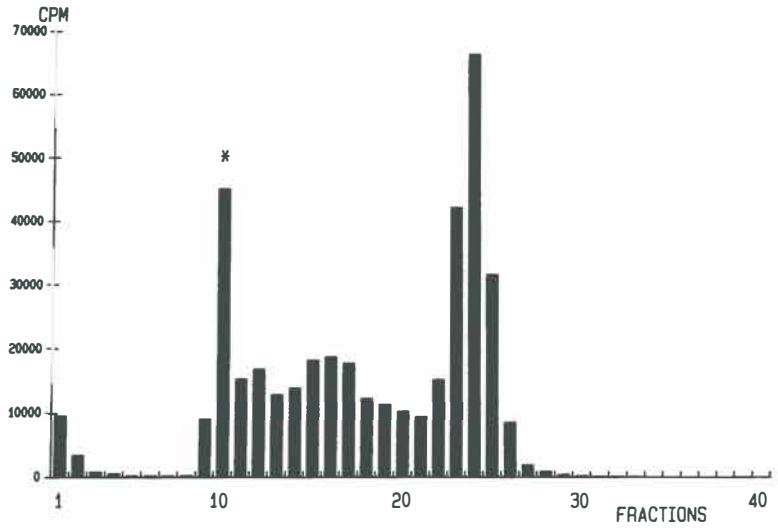


Figure 5.2 Recovery of labelled LSP from a Sephadex G-25 column after iodination. Counts per minute minus background (CPM) as determined by a gammacounter in the fractions collected are shown.
ad *) Fractions 9,10,11 and 12 form the LPI-peak that contain LSP.

described,[449] and the usual pattern obtained is shown in fig 5.2. TCA-precipitation was done each time before an assay was performed, and the *LSP only used if the precipitatable *LSP was >75%. Before use, 1 ml of *LSP was purified on a Sepharose 6B column, and the LSP-peak determined as described above. Again, the first peak was collected and 0.1% buffer with BSA and buffer with azide added up to 5 ml. In the calculations after performance of an assay, the activity of this *LSP was set at 100%.

Assay

Essentially, the assay was performed as described earlier,[445] but we made some modifications: Patient sera were thawed at 4-7°C, and 50-100 µl aliquots were inactivated for 30 minutes at 56°C using glass tubes. Sera were diluted 1:50 with 1% BSA-borate (pH 8.5) in duplicate. If quantitation was required, additional doubling dilutions 1:100 and 1:200 were made. Pooled normal serum was used as a negative control, blanks were added, and a titrated serum containing high anti-LSP titres (binding percentages also determined in KCH, London) was used as a positive control. 25 µl of diluted sera and 25 µl of *LSP (without further dilution with unlabelled LSP) were put into plastic tubes. Two tubes for the precipitation test contained only 25 µl *LSP. After mixing and incubating overnight at 4-7°C 100 µl of dried staphylococcal cells suspended in 1% BSA-borate pH 8.5 (concentration 10 mg/ml) was added to each tube. To the tubes containing only *LSP and no serum, 475 µl 1% BSA-borate and 500 µl 40% TCA was added. The contents were mixed and incubated for 1 hour at 4-7°C with

additional mixing at the half hour. 850 µl 0.25 M sucrose (pH 8.5) was added to the tubes with sera, and all tubes centrifuged for 10 minutes at 3500 rpm. Instead of counting one tube with the infranatant and half of the supernatant, and one tube with the other half of the supranatant, we removed all supranatant and counted only the infranatant. We counted 4 minutes/tube in an LKB gammacounter linked to a computer to calculate titres (according to the formulas given in the results), and binding-percentages (%B) as follows:

$$\%B = \frac{[\text{cpm(patient serum)} - \text{cpm(blank)}]}{[\text{cpm(*LSP)} - \text{cpm(background activity)}]} * 100\%$$

(cpm=counts per minute)

The effect of reducing the counting time to 2 minutes per tube was assessed. Calculated titres were compared with measured titres. For titration of the positive control, we determined dilutions from 1:50, 150, 200, 250, 300, 350, 400, 450, 500 and 1:1000. The consequences of our modifications for the results were assessed, by simultaneous comparison with the results obtained in the original method as described.[445] Statistical analysis was performed with the Wilcoxon two-sample test and Spearman's rank correlation where appropriate, and p-values <0.05 were considered significant.

RESULTS

There was no significant difference betweem binding percentages (%B) as determined in London (KCH) with the original assay, and those determined in our assay.
The correlation between %B in these two assays was r=.80. The inter-assay coefficient of variation in our assay was <10%, inter-labelling this was 15%, and the intra-assay variation was <10%. Our modifications in labelling and in the assay (dilution with buffer instead of unlabelled LSP, and counting only the infranatant) did not affect the results. Binding percentages obtained after 2 minutes of counting did not differ from those obtained after 4 minutes counting per tube (although we usually count 4 minutes). Curves indicating %B obtained in increasing dilutions of sera could be linearized by $\log_{10}$-transformation of %B, as shown in figure 5.3. For dilutions above 1:50 the correlation between dilution and $\log_{10}$(%B) was median r=0.98 (range 0.95-0.99).

This enabled us to calculate titres with only three doubling dilutions of a serum (1:50, 1:100, 1:200). The upper limit of normal was set at $\log_{10}(\%B_0 + 2SD)$ (SD=standard deviation, $\%B_0$=mean %B of 32 negative controls). The best fitting straight line through the %B obtained in 1:50, 1:100 and 1:200 dilutions was determined, and the abscissa at

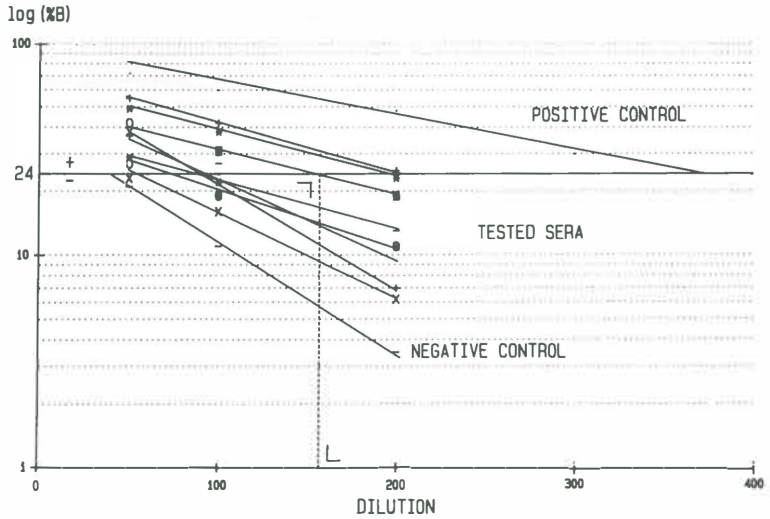


Figure 5.3 Calculation of anti-LSP titres. (N+2SD = median + 2 times standard deviation of reference values). See text for further explanation.

its intercept with the upper reference limit yielded the anti-LSP titre. Only on the rare occasion that the correlation between dilution and %B was lower than r=0.95 was the measurement repeated. In formulae:

$x_1 = 50x \quad y_1 = \log_{10}(\%B_1) \quad x_2 = 100x \quad y_2 = \log_{10}(\%B_2) \quad x_3 = 200x \quad y_3 = \log_{10}(\%B_3)$

$D_1 = (y_1 - y_2)/(x_1 - x_2)$
$D_2 = (y_2 - y_3)/(x_2 - x_3)$
$D = (D_1 + D_2)/2$

$x = (x_1 + x_2 + x_3)/3 \quad y = Dx + A \quad A = y - Dx$
$y = (y_1 + y_2 + y_3)/3 \quad T = (C - A)/D$

where D= the gradient of the standard line.
A= constant
C= $\log_{10}$(%B + 2SD) of negative control
T= titre of anti-LSP

These formulae were entered into a computer linked to the gamma-counter, so that results were obtained immediately in %B and titres. Anti-LSP titres calculated in this way did not differ significantly from measured titres: the correlation between measured titres and calculated titres was r=.98. The reference range of normal binding was 5-24%, and titres >1:50 were considered positive.

DISCUSSION

The importance of a reproducible anti-LSP assay lies in the predictive value of anti-LSP antibodies for the outcome of therapy withdrawal in IAI-CAH,[450] and in its help in distinguishing AI-CAH from non-A,non-B CAH (HCV-CAH).[451] Determination by means of a radioimmunoassay appeared to be superior to measurement with a micro-ELISA.[452]

We established a modified, reproducible radioimmunoassay for the determination of anti-LSP that yields quantitative results with 3 dilutions. Modifications did not affect the results. The method used labelling, and the dilution of *LSP with buffer instead of unlabelled LSP gave higher cpm, enabling labelling with less radioactivity and/or counting for a shorter time. Having to count only the infranatant, the linkage of a computer to the gamma-counter to calculate %B and titres, and the use of a pipetting-automate were further time-saving steps.

The difference in gradients of the regression line connecting binding percentages of the dilutions 1:50, 1:100 and 1:200 between patients, indicates that among patients different antibodies directed at more than one antigenic site within the LSP-complex may be present. Although it appears that the asialoglycoprotein receptor (hepatic lectin) is one of the important antigenic determinants within LSP,[453-455] other antigenic sites may play a role. For immune monitoring of IAI-CAH this is not a problem, and it may even be preferable to measure all anti-LSP antibodies involved, to assess 'immunological activity' of IAI-CAH.

The present assay enables rapid, reproducible and quantitative determination of anti-LSP antibodies, facilitating studies on monitoring IAI-CAH. This assay was used for the studies described in chapters 13 and 14. For further details on immunology in CAH the reader is referred to the introduction of this thesis.

SECTION 2

Analysis of survival in chronic active hepatitis of various aetiologies

CHAPTER 6

LONG-TERM PROGNOSIS IN CHRONIC ACTIVE HEPATITIS

SUMMARY

We assessed long-term survival and prognostic factors in chronic active hepatitis (CAH). Methods: Survival (Kaplan-Meier) and prognostic factors, using Cox's proportional hazards estimation were evaluated, in 186 patients with CAH, consecutively referred from 1969 through 1988. End of follow-up was November 1988. Information was retrieved from charts, other physicians and public registry offices. All HBsAg-positive patients and 3/4 of anti-HBs-positive patients received no therapy. Results: Ages among therapy- and aetiological groups did not differ. Kaplan-Meier probability of survival was:

SURVIVAL(%)	n	Years: 5	10	15	20
All patients	186	85(±3)	70(±4)	55(±5)	40(±10)
AETIOLOGY:					
HCV	10	100	100	100	100
IAI (no anti-HBs)	69	90(±4)	76(±7)	60(±11)	-
Anti-HBs-positive IAI	13	50(±17)	15(±13)	15(±13)	15(±13)
HBsAg-positive	51	80(±6)	72(±7)	65(±9)	-
other aetiology	43	85(±6)	67(±8)	45(±10)	22(±12)
THERAPY:					
standardized therapy	42		85(±8)		85(±8)
none/non-standardized	144		67(±5)		35(±9)
BOTH:					
no/n-s therapy + anti-HBs	8	17(±15)	0		
standard + anti-HBs	5		50(±35)	50(±35)	
standard, no anti-HBs	39	97(±3)	90(±7)		90(±7)
none/non-standard + no anti-HBs	136	84(±3)	70(±5)	55(±6)	

Most causes of deaths registrated in patients with CAH were not related to the liver disease. Remarkably, there was a high incidence of plasmocytoma. In Cox's regression analysis independant riskfactors for mortality were: age (increase in risk-IR- x1.3/10 years), presence of anti-HBs (IR x6.7), inability to institute standard immunosuppressive therapy (IR x3.6), and abnormal values of serum alanineaminotransferase (ALT) (IR x3.6). Sex, year of diagnosis, duration of disease until the end of follow-up, serum values of gamma globulin, cholinesterase, albumin, and presence or absence of ANA and SMA at the time of diagnosis were not risk factors.

INTRODUCTION

Early assessments of natural history and prognosis in CAH have been summarized recently.[456] Mortality ranged from 20% in 3 years,[457] 40% in 4 years,[458] 80% within 5 years[459] or 70% in 6 years,[460] to 50% within 10 years.[461] Prospective studies have indicated that mortality approximates 40 percent within 6 months from diagnosis if corticosteroids are not administered.[462] In the era before the introduction of immunosuppressive therapy, cirrhosis, bridging or multilobular necrosis at presentation was associated with the highest mortality.[463-466] Late mortality was related to the consequences of portal hypertension rather than liver failure, which is the main cause of early death in CAH.[467] Survival beyond 3 years in untreated CAH was associated with a spontaneous reduction in inflammatory activity, and with transition to an inactive macronodular cirrhosis.[467] The abrupt onset of symptoms, persistent jaundice, hepatic encephalopathy, ascites, and ulcerative colitis were associated with poor prognosis.[463] Serum aminotransferase elevation of greater than tenfold normal, or fivefold normal in conjunction with twofold elevation of gamma globulin level, was associated with mortality of greater than 50% within 3 years of diagnosis.[468] Corticosteroid therapy with or without azathioprine has been shown to induce remission in 60 percent of patients with severe autoimmune CAH within 3 years, with an increase of 5-year survival to approximately 90 percent.[469,470] Treated IAI-CAH patients without cirrhosis on admission had the best prognosis, while development of cirrhosis during therapy -which occurred in 40% of patients without cirrhosis on entry- did not adversely affect the probability of survival. Other results indicate that prognosis in transfusion-related non-A,non-B CAH -probably due to hepatitis C virus (HCV-CAH)- appeared best, followed by that of treated IAI-CAH, with the worst prognosis in HBsAg-positive CAH.[456]

In the present study, we analyzed survival in 186 consecutive patients with liver biopsy proven chronic active hepatitis. The analysis was in relation to aetiology of CAH, instituted therapy, age, sex, year of diagnosis, duration of disease until the end of follow-up, cause of death, serum values of alanineaminotransferase, gamma globulin, cholinesterase, albumin, and presence or absence of ANA and SMA at the time of diagnosis.

MATERIALS AND METHODS

Patients

From November 1969 to November 1988, a total of 186 consecutive patients referred to the division of hepatology, State University Hospital Groningen, were diagnosed as having liver biopsy proven chronic active hepatitis. During this period liver biopsy records were kept systematically, and from 175 patients sera were frozen (-20°C). Patient-characteristics were described in chapter 1. Where necessary, biopsies (sometimes from other hospitals) and diagnoses were reviewed, hepatitis B serology redone, and in all patients alpha-1-antitrypsin phenotyping was performed. For the purpose of this study, we discern five aetiological groups: 1) idiopathic 'autoimmune' CAH without anti-HBs (IAI-CAH), 2) hepatitis B surface antigen-positive CAH (HBsAg-CAH), 3) CAH with circulating antibodies to HbsAg (anti-HBs CAH), 4) transfusion- or needlestick- and iv drug-abuse related non-A,non-B CAH (HCV-CAH), 5) other aetiologies (table 6.1).

Table 6.1 CAH-patients grouped according to aetiology.

Aetiology*	Number of patients	Sex M:F	Age (years) median	range
1)IAI-CAH	69	12: 57	51	11-78
2)HBsAg-CAH	51	37: 14	42	18-74
3)anti-HBs-CAH	13	3: 10	46	17-81
4)HCV-CAH	10	4: 6	37	18-77
5)DI-CAH	20	3: 17	61	24-78
other CAH	23	6: 17	34	15-72
Total	186	65: 121	46	11-81

ad *)

IAI-CAH = idiopathic 'autoimmune' CAH without anti-HBs
HBsAg CAH = CAH with circulating HBsAg
Anti-HBs CAH= CAH with circulating anti-HBs
HCV-CAH = CAH due to parenteral non-A,non-B hepatitis (probably due to hepatitis C virus)
DI-CAH = drug-induced 'CAH' due to: oxyphenisatin(12), α-methyl-dopa(3), nitrofurantoin(1), diclofenac(1).

Other CAH:
9x CAH/PBC = 'cholestatic CAH' or 'mixed form'CAH/PBC
5x CAH/PSC = 'small-duct' PSC and/or CAH 3x Wilson-CAH= CAH associated with Wilson's disease
5x A1AT-CAH = CAH related to α_1-antitrypsin deficiency 1X CAH in multicystic liver

Five therapeutic groups were present: standardized immunosuppression with prednisolone (P) and azathioprine (A) as described in chapter 1, a) without and b) with preceding low-dose (2.5-10 mg prednisolone) immunosuppression, c) P in conjunction with A, but not according to a standardized regimen, d) no immunosuppressive therapy, e) P only incidentally during exacerbations.

Methods

In this analysis of survival we recorded: age (in years) at the time of the diagnostic liver biopsy , sex of the patient, aetiology of CAH, the kind of therapy for CAH which was instituted, the serum values of alanine aminotransferase (ALT), gamma globulin (GG), cholinesterase (CHE), albumin (ALB), and presence or absence of antinuclear antibodies (ANA) and anti-smooth muscle antibodies (SMA) at the time of diagnosis, the year of diagnosis, duration (in days) between diagnosis and the end of follow-up (November 1988, or time of death or liver transplantation), and cause of death. Duration of survival was related to these characteristics. In our study we assumed an equal distribution of cirrhosis over the several groups, since sufficient data to exclude the presence of cirrhosis were lacking. If serum ALT or total bilirubin were continuously elevated more than twofold for more than a month preceding the diagnostic liver biopsy, the onset of these abnormalities in liver tests were regarded as the moment of diagnosis. The family practitioners and specialists were asked whether a patient was still alive, and, if not, what the cause of death had been. Where necessary, data were derived from public registry offices.

Statistical analysis

Survival curves were plotted by the Kaplan-Meier productlimit method.[471] Results are expressed as probabilities ± standard error. Differences between subsets of patients were analysed by means of the log rank test. For identification of individual prognostic factors we used Cox's proportional hazards estimation.[472]

RESULTS

We assessed survival probability in 186 consecutive patients with chronic active hepatitis. This assessment was in relation to aetiology of CAH, therapy undertaken, age, sex, year of diagnosis, duration of disease until the end of follow-up, cause of death, serum values of alanineaminotransferase, gamma globulin, cholinesterase, albumin, and presence or absence of ANA and SMA at the time of diagnosis. 85(±3) percent of all 186 patients with CAH survived 5 years, 70(±4) percent survived 10 years, 55(±5) survived 15 years, and 40(±10) survived 20 years after diagnosis (figure 6.1). Ages between therapygroups and aetiological groups did not differ significantly.

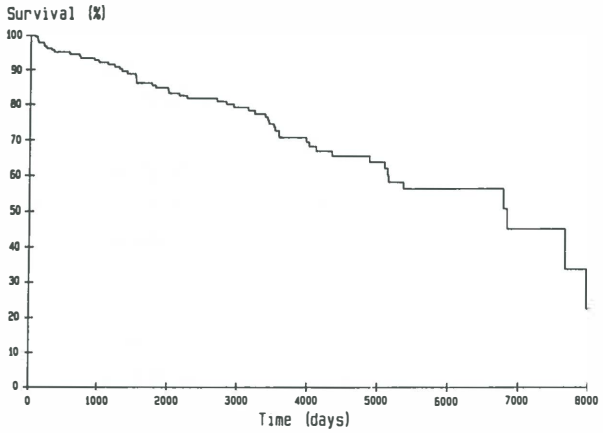
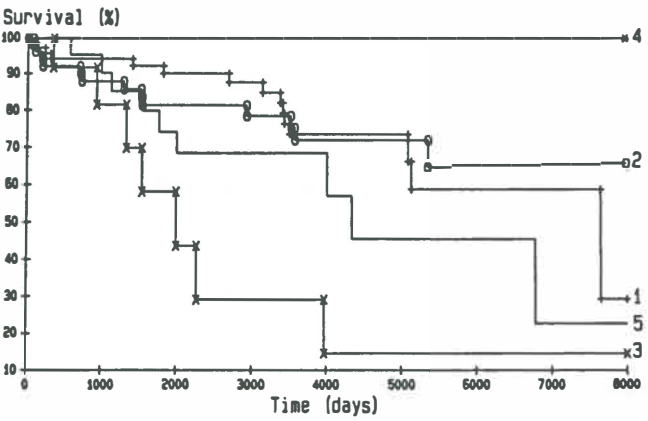


Figure 6.1 Probability of survival in all 186 patients with CAH.

On stratification of the patients according to aetiologies (figure 6.2), survival markedly differed between groups (p=.001): None of the ten HCV-CAH patients in group 4) died,

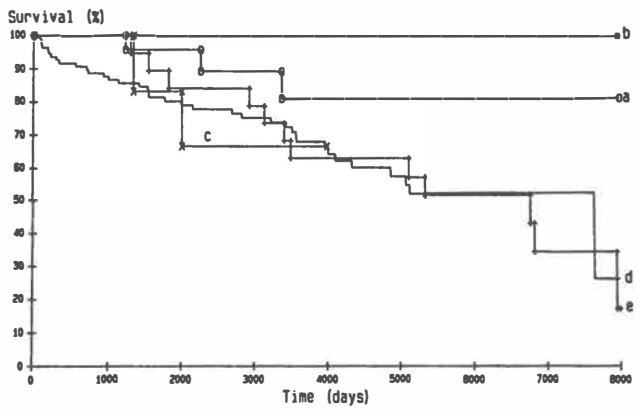


Numbers at risk	Aetiology				
1	69	44	27	9	1
2	51	38	23	11	11
3	13	4	2	2	2
4	10	10	10	10	10
5	23	13	6	5	1

Figure 6.2 Probability of survival stratified on aetiology of CAH.
1=idiopathic 'autoimmune' CAH
2=HBsAg-positive CAH
3=autoimmune CAH with anti-HBs
4=non-A,non-B (or HCV)-CAH
5=CAH of other aetiology (not drug-induced)

leading to a probability of survival of 100% after 20 years. The probability of survival in groups 1) and 2) after 5 years in IAI-CAH (treated and untreated) was 90(±4) percent and in HbsAg-CAH (always untreated) 80(±6) percent. After 10 years this was 76(±7) percent and in HbsAg-CAH 72(±7) percent, and after 15 years 60(±11) and 65(±9) percent. In the anti-Hbs positive patients (75 percent of them untreated) of group 3), the Kaplan-Meier (K-M) probability of survival was worst of all patients: 50(±17) percent after 5 years, 15(±13) percent after 10 and 20 years. The group 5) with CAH of other aetiology had a probability of survival of 85(±6) percent after 5 years, 67(±8) percent after 10 years, 45(±10) percent after 15 years, and 22(±12) percent after 20 years.

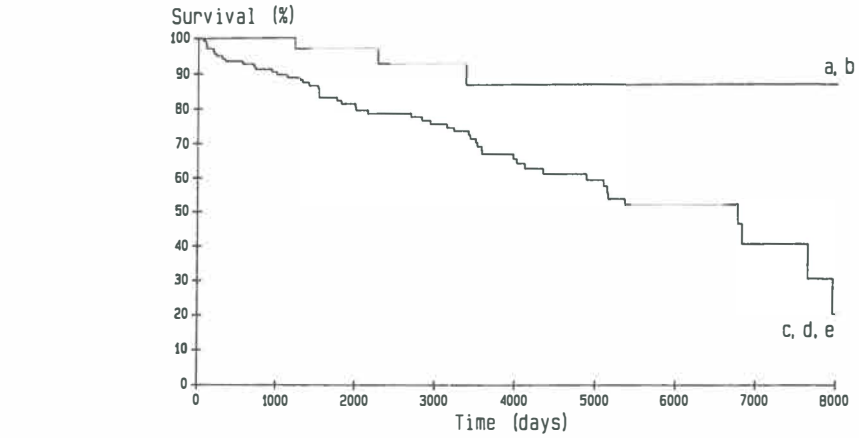
When stratifying the patients according to therapies (figures 6.3 and 6.4), survival between therapy groups a and b versus therapy groups c,d and e differed significantly. There was a 85(±8) percent 10 and 20-year survival in the first group versus 67(±5) percent 10-year survival and 35(±9) percent 20-year survival in the latter group (p=.01). No differences were detected between groups a and b, nor between groups c,d, and e.



Number of patients at risk	Aetiology					
	a	33	15	11	11	11
	b	9	9	9	9	9
	c	11	6	5	5	5
	d	112	66	37	20	2
	e	17	17	17	10	2

Figure 6.3 Probability of survival stratified on therapy.
a=standard therapy prednisolone+azathioprine without pretreatment
b=standard therapy prednisolone+azathioprine with pretreatment
c=non-standard therapy prednisolone+azathioprine
d=untreated
e=prednisolone only during exacerbations of CAH

Stratification of the patients according to both aetiological groups and therapy groups also yielded significant differences (figure 6.5): Anti-HBs positive patients in therapy groups c,d, or e had a survival probability of 17(±15) percent after 5 years and zero after 10 years.



Number of patients at risk	Aetiology					
	a,b	42	24	20	20	20
	c,d,e,	144	89	59	35	9

Figure 6.4 Probability of survival stratified on therapy. (a and b combined, c + d + e combined).
See also legend of figure 3.

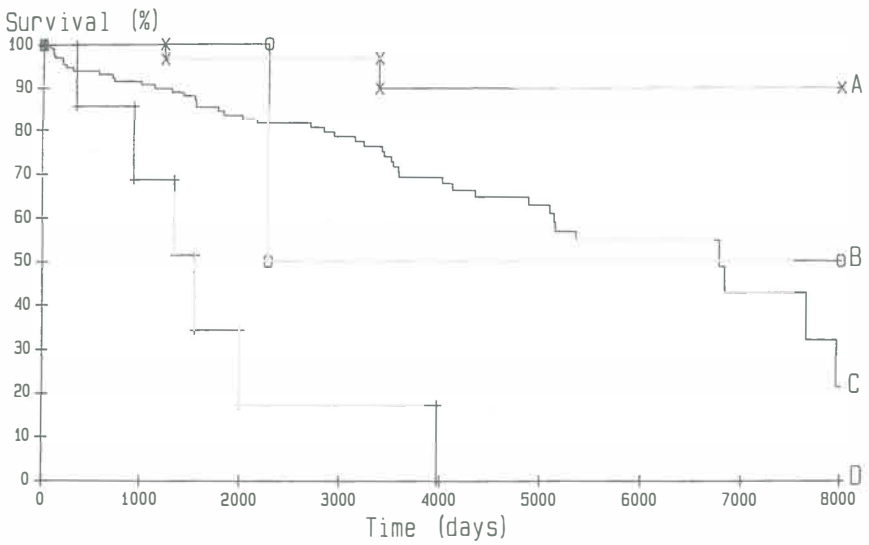


Figure 6.5 Probability of survival stratified on both aetiology and therapy.
A= no anti-HBs, standardized therapy
B= anti-HBs, standardized therapy
C= no anti-HBs, no standardized therapy
D= anti-HBs, no standardized therapy

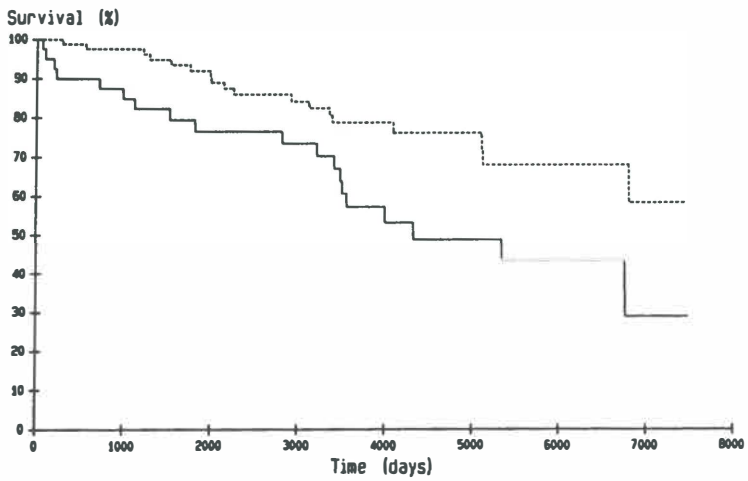
Table 6.2 Causes of death in CAH and CAH-related disorders (n=186).

aetiology	therapy	cause of death	n=
1	a	lung metastases of adenocarcinoma	1
1	d	pulmonary embolism + liver metastases	1
1	d	myocardial infarction	2
1	d	hepatic coma	1
1	e	3' years after OLT, cause unknown	1
1	d	digestive tract bleeding 1 1 4 cause unknown	4
1	e	cause unknown	5
1			total 16
2	d	bleeding from oesophageal varices	2
2	d	cardiac death	1
2	d	plasmocytoma (Kahler's disease)	1
2	d	HCC + plasmocytoma (Kahler's disease)	1
2	d	cause unknown	6
2	e	cause unknown	3
2			total 14
3	a	myocardial infarction + tamponade	1
3	d	traumatic subdural haematoma	1
3	d	during OLT (coagulation disorders)	1
3	d	during OLT (intraoperative problems)	1
3	d	6 days after OLT:bleeding from ulcus ventriculi	1
3	c	cause unknown	1
3	d	cause unknown	1
3			total 7
4			total 0
5A	e	plasmocytoma (Kahler's disease)	1
5A	d	cause unknown	3
5A	e	cause unknown	1
5B	c	acute liver failure	1
5B	e	bleeding from oesophageal varices, HCC with portal thrombosis	1
5B	d	cause unknown 1	
5C	d	choking on food caused by abnormal swallowing in CAH' related to Wilson's disease	1
5C	d	pneumonitis in 'CAH' related to Wilson's disease with severe neurological abnormalities	1
5D	a	perforated sigmoiditis	1
5D	d	acute liver failure and pulmonary carcinoma	1
5D	d	urosepsis	2
5D	d	myocardial infarction + rhythm disorder	1
5D	d	cause unknown	4
5			total 19
			Total 56

Aetiology:
1) IAI-CAH, 2) HBsAg-positive CAH, 3) anti-HBs positive CAH, 4) non-A,non-B (HCV) CAH 5) other CAH: 5A) CAH/PBC, 5B) CAH/PSC, 5C) 'CAH' related to Wilson's disease, 5D) oxyphenisatin-induced CAH.
Therapy: a) standardized prednisolone (P) and azathioprine (A), no pretreatment, b) idem with pretreatment (2.5-10 mg P, one with 25 mg A), c) non-standardized P+A, d) untreated, e) P only during exacerbations.

Five anti-HBs positive patients in therapy-groups a and b, however, had a probability of survival of 50(±35) percent after 10 and 15 years. Patients with CAH, but without anti-HBs, had the best survival probabilities: in therapy-groups a and b this was 97(±3) percent after 5 years and 90(±7) percent after 10 and 20 years. In therapy-groups c, d, and e this was 84(±3) percent after 5 years, 70(±5) percent after 10 years, and 55(±6) percent after 15 years. Most causes of deaths registered in patients with CAH were not related to the liver disease. Remarkably, there was a high incidence of plasmocytoma (table 6.2).

Patients with ALT above the upper reference limit had a worse long-term and short-time prognosis than patients with an ALT within the reference range at the time of diagnosis ($p=<0.05$).



Number of patients at risk	ALT normal	86	61	30	18	7
	ALT elevated	41	27	14	9	3

Figure 6.6 Probability of survival in patients with ALT above the upper reference limit (solid line) and patients with ALT within the reference range (dashed line) at the time of diagnosis ($p<0.05$) ($n=127$)

Cox's proportional hazards estimation for identification of individual prognostic factors yielded a model with age, presence/absence of anti-HBs, possibility to institute standardized immunosuppressive therapy and elevated ALT as independant risk factors for mortality (table 6.2). No other prognostic factors were identified among the other parameters mentioned above. Using these data individual prognosis in CAH can be determined.

12 CAH patients underwent a liver transplantation (LT), as shown in table 6.3. The results in anti-HBs positive patients contrasted with survival probability in patients from the same aetiological group receiving immunosuppression, as stated above. One patient

Table 6.3 CAH-patients undergoing liver transplantation (LT).

aetiology of CAH	therapy before LT	LT performed x days after diagnosis	from LT until end of follow-up	status at end of follow-up (days)
1	d	778	1105	alive and well
1	d	259	3163	alive and well
1	d	152	1326	alive and well
1	e	3504	1356	died after OLT
2	d	46	2388	alive and well
3	d	341	0	died during OLT
3	d	1547	0	died during OLT
3	d	934	6	died after OLT
4	d	1241	6	alive and well
5A	c	3597	3487	alive and well
5B	c	30 *	537	alive and well
5E	d	176	1281	alive and well

ad *) This patient underwent an auxilary heterotopic transplantation in Rotterdam, and histology from the acceptor-liver changed the diagnosis from CAH/PSC to PSC lateron. The other patients underwent orthotopic liver transplantation (OLT) in Groningen. Causes of death: see table 2. For aetiology- and therapy-numbers: see table 2.

with 'CAH' in relation to the homozygous ZZ-phenotype α_1-antitrypsin deficiency needed a liver graft soon after diagnosis, despite a short course of non-standardized immunosuppression. This contrasts with three patients with partial α_1-antitrypsin deficiency who all responded to immunosuppressive therapy with prednisolone and azathioprine. LT in patients without anti-HBs was successful. The incidence of LT in anti-

Table 6.4 Final model of long-term prognostic factors for chronic active hepatitis of various aetiology.

Higher age, presence of anti-HBs in serum without HBsAg, no institution of standardized immunosuppressive therapy and elevated ALT increased the risk of mortality.

Co-variate	Relative risk of mortality	95% confidence interval lower	upper	T-stat	p-value
Age	x 1.3/10 years	1.04	1.5	2.420	<0.001
anti-HBs	x 6.7	1.3	34.2	2.277	<0.001
No St-therapy	x 3.6	1.03	13.6	1.910	<0.015
ALT elevated	x 2.0	1.04	3.9	-2.083	<0.010

HBs-negative IAI-CAH was low (only 4 patients, of whom 3 without therapy and 1 on non-standardized immunosuppression). This probably reflects the excellent survival probability in those patients selected for immunosuppressive therapy.

DISCUSSION

From the present study it follows that in chronic active hepatitis, long-term prognosis is determined by aetiology of CAH -especially the presence of anti-HBs-, the decision whether to administer (fixed-dose) immunosuppressive therapy or not, as well as the age of the patient and the ALT at diagnosis. The difference in survival between patients with high and low ALT became clear early, because of liver failure in some of the patients with higher ALT. Remarkably, CHE at diagnosis, and presence/absence of ANA and SMA were found not to be risk-factors. Patients with putative non-A,non-B CAH -probably due to HCV- had the best prognosis. One of these patients had fulfilled requirements for standardized immunosuppression and had been treated accordingly. Untreated anti-HBs positive patients with CAH had the worst prognosis. Besides causes of death unrelated to liver disease, this was due to a high incidence of OLT with high per- and postoperative mortality in the subgroup of untreated anti-HBs positive patients. These patients had more advanced disease. CAH in these patients may also have been caused by HCV.
Intermediate between the likelihood of survival of these 'aetiological groups' lies the survival of (untreated) HBsAg-positive CAH and (both treated and untreated) IAI-CAH. Patients with IAI-CAH without anti-HBs and anti-HBs-positive CAH maintained on standardized immunosuppressive therapy with fixed doses of prednisolone (P) and azathioprine (A) had a much better survival probability than untreated patients from the same 'aetiological groups'. Their outcome was also better than that of patients receiving non-standardized immunosuppressive therapy, e.g. only given during exacerbations of the disease. Survival was as good as in treated IAI-CAH in two 'CAH'-patients with partial α_1-antitrypsin deficiency of the MZ-phenotype on standardized therapy with P and A, and in one patient with partial α_1-antitrypsin deficiency of the MS-phenotype on non-standardized therapy with P and A.

In more than half of the deceased patients with known cause of death, fatality was not related to the liver disease, and death from gastrointestinal bleeding was uncommon. The incidence of plasmocytoma was quite high, while HCC occurred infrequently.

In the era before the introduction of immunosuppressive therapy, cirrhosis,bridging or multilobular necrosis at presentation was associated with the highest mortality.[463-466] Late mortality was related to the consequences of portal hypertension rather than liver failure, which is the main cause of early death in CAH.[467] Survival beyond 3 years in untreated CAH was associated with a spontaneous reduction in inflammatory activity, and with transition to an inactive macronodular cirrhosis.[467] Other studies indicated that

patients that improved without corticosteroids could not be distinguished from others on accession by disease duration, age, sex, initial histologic findings, associated autoimmune disease (with exception of colitis ulcerosa), immunoserologic studies (presence of LE-cell phenomenon, antinuclear antibody, smooth muscle antibody) , or biochemical abnormalities (AST, bilirubin, gamma globulin, albumin).[456,474] It was shown that patients with mild disease improve without therapy as frequently as those with severe disease (13 percent versus 20 percent).[474] In a study of 80 asymptomatic patients , including 34 with HBsAg, 20% developed cirrhosis and 9% died of liver failure, while the 5- and 10-year survival probabilities were 91 and 81 percent.[475] The abrupt onset of symptoms, persistent jaundice, hepatic encephalopathy, ascites, and ulcerative colitis were associated with poor prognosis.[465] Serum aminotransferase elevation of greater than tenfold normal, or fivefold normal in conjunction with twofold elevation of gamma globulin level, was associated with mortality of greater than 50% within 3 years of diagnosis.[468]

Some other studies indicate that aetiology is an important prognostic factor in CAH,[456] and that non-A,non-B (or HCV)-CAH usually is a self-limiting process. During the initial episode of HCV-hepatitis most patients are anicteric and 35% asymptomatic. Nevertheless, the majority (55-65%) of these patients develop progressive liver disease with CAH, with or without cirrhosis.[476,477] In transfusion-related HCV-CAH however, spontaneous improvement is usual, a complete resolution having been described in half of the patients within 3 years after diagnosis.[478,479] Cirrhosis developed in 10-60 percent, immediate survival was excellent and HCC was absent.[477,479,480]
Our finding that aetiology is an important determinant influencing survival may be explained by several factors. A different propensity to develop hepatocellular carcinoma (HCC) among several aetiologies is one of these. Although it has been described, HCC in patients with IAI-CAH[481,482] and HCV-CAH[483,484] is rare. However, 70% of HBsAg-positive patients develop cirrhosis and 30 percent died within 10 years.[485,486] These HBsAg-positive patients have an increased risk of the development of hepatocellular carcinoma (HCC),[487] even if a patient has become an asymptomatic carrier.[488] During the current study HCC was detected in two of the 186 CAH-patients: one with HBsAg-positive CAH, the other with CAH/PSC.

Other studies showed that corticosteroids in severe autoimmune CAH induce remission in 60 percent of the patients within 3 years, while 5-year survival is approximately 90 percent.[471] The cumulative remission rate for therapy of IAI-CAH with a combination of fixed dose prednisolone and azathioprine is 90%.[470] From 151 treated HBsAg-negative patients, 92% survived 5 years and 86% survived 10 years, while in 22 HBsAg-positive (65% also HBeAg-positive) patients, survival was lower: 73% and 65% at the same intervals ($p<0.01$). While the survival curve in treated HBsAg-negative CAH declines gradually, highest mortality in HBsAg-positive CAH occurred within the first 2 to 3 years and was quite low thereafter.[489] Our findings in untreated HBsAg-

positive CAH and treated IAI-CAH were similar. Untreated IAI-CAH patients however, followed a course similar to HBsAg-positive CAH. Immunosuppressive therapy in IAI-CAH leads to a marked enhancement of survival in non-viral (idiopathic 'autoimmune') CAH: In the Mayo experience, the 10-year probability of survival without hepatic failure is 98 percent for patients without cirrhosis at presentation. For patients with cirrhosis prior to treatment, the likelihood of survival without hepatic failure is lower: 80 percent after 5 years and 65 percent after 10 years.[490,491] Interestingly, patients who develop cirrhosis during therapy have a 5-year survival after the development of cirrhosis that is similar to that of patients who do not develop cirrhosis,[492] indicating that the propensity to develop cirrhosis in IAI-CAH depends more on the rapidity and completeness of the response to treatment than on any histologic feature at presentation.[492] However, the presence of cirrhosis can not be excluded by a liver biopsy,[493,494] so that prospective studies using other methods, are required to assess the prognostic value of the presence of cirrhosis. The ability to suppress inflammatory activity with subsequent repair and restoration of liver function may be a more important prognostic factor, since in the current study the decision to institute immunosuppressive therapy is among the most important prognostic factors.

The fact that autoimmune features had no prognostic significance, neither in our findings nor in a previous report[495], may be due to selection of patients with the greatest abnormality of these factors for therapy. The same applies to CHE levels in the current study. Indeed, Page *et al.* observed that patients with an acute onset of disease, evidence of multisystemic involvement, the LE cell phenomenon, and extreme hypergammaglobulinaemia were more likely to respond to corticosteroids than others.[465] Later on it was demonstrated, however, that the presence or absence of autoimmune markers does not influence the response to corticosteroids in patients with severe CAH, screened for drug-induced or virus-related disease. The frequency of remission, treatment failure, hepatic death, and progression to cirrhosis were similar in patients with and without the LE cell phenomenon or antinuclear antibody (ANA). Our study supports the policy of applying the same program of management to all patients with idiopathic, presumably autoimmune, CAH of sufficient severity, regardless of immunologic findings.[495] It also favors immunosuppressive treatment of patients with other aetiology, as long as no replicating pathogenetically involved virus like HBV or HCV is present. The current study also suggests that anti-HBs positive patients should be treated like IAI-CAH patients.

To conclude, the degree of inflammation will determine the extent of liver failure by influencing hepatocyte function via cytokines, and by cell necrosis and hence reduction of the number of functional hepatocytes, and is connected with the induction of cirrhosis and its complications. Hepatitis will especially influence prognosis via these mechanisms. The ability to institute immunosuppressive therapy is the most important prognostic factor. This means that in idiopathic 'autoimmune' CAH, the initial reaction

to immunosuppressive therapy must be the most important prognostic factor. The same applies to anti-HBs positive patients, and probably also to other CAH-patients without replicating HBV or HCV, who fulfill requirements for treatment. An accurate analysis of the early effects of immunosuppressive therapy -e.g. on liver function- has been published recently, confirming the importance of initial reaction to immunosuppressive treatment for prognosis.[496] The combination of that study and the current data on long-term survival can hopefully lead to improved decision making in CAH. The long-term prognostic model we derived from the current study has the advantage over a different recently published model[497] in that it is confined to CAH and does not need liver biopsy data. Our survival model has to be cross-validated on an independant data set from other patients, however. Therefore, further studies in this field are warranted.

Section 3

'Autoimmune' chronic active hepatitis: Activity of disease and repair during standarized treatment

CHAPTER 7

ROUTINE BLOOD TESTS IN STANDARDIZED IMMUNOSUPPRESSIVE THERAPY OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

§7.1

ROUTINE 'LIVER BIOCHEMISTRY' IN STANDARDIZED IMMUNOSUPPRESSIVE THERAPY OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

SUMMARY

We analyzed routine liver biochemistry from 21 patients with idiopathic auto-immunechronic active hepatitis (IAICAH), before and during standardized immuno-suppression. ALT , AST and gammaglobulin (GG) (medians 337 U/l, 359 U/l, and 28 g/l at start respectively) improved considerably (median 20 U/l, 20 U/l and 12 g/l after two years), while conjugated bilirubin (median 22.5 µmol/l) normalized within two months to median 2.5 µmol/l. Median unconjugated bilirubin was normal (14 µmol/l), but declined to 9 µmol/l in the first two months of therapy. Results from the current study indicate that standardized immunosuppression with a fixed dose schedule was able to improve biochemistry within 2 months, with further improvement thereafter.

INTRODUCTION

Idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) usually reacts favorably to immunosuppressive therapy,[498] and immunosuppression has been shown to enhance survival in this type of CAH.[499-503] Remission has been induced in 60% of patients within 2.5 years of treatment in the Mayo Clinic studies.[501,504] A combination of fixed dose prednisone and azathioprine daily proved superior to fixed dose, titrad or alternate day therapy with prednisone alone, when regarding efficacy and adverse effects of therapy.[499] Azathioprine alone cannot induce remission in IAI-CAH,[499] but can maintain remission without prednisone in a selected group of patients.[505] In order to assess the effects of a combination of fixed dose prednisolone and azathioprine on histology and routine liver tests we analysed protocol liver biopsies and biochemistry at 0,2,14 and 26 months after commencement of accordingly standardized immunosuppression in 21 patients with symptomatic 'autoimmune' CAH.

MATERIALS AND METHODS

Patients

We studied 21 untreated patients with idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH)- 3 of them with antibodies to hepatitis B surface antigen: 4 men and 17 women. These patients required immunosuppressive therapy according to accepted criteria.[499] Their median age was 52 years (range 16-71), and median duration of disease 75 days (range 0 months to 3 years). All were subsequently treated with oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (2 patients) or two years (19 patients) of therapy with 10 mg prednisolone and 50 mg azathioprine daily. Serial routine liver biopsies and sera were studied at commencement and at two months, one and two years after the beginning of therapy. One biopsy at 2 months, one at 14 months and 6 at 26 months were missing, although biochemistry was available.

Serum biochemistry

Sera were obtained on the same day on which the liver biopsies were performed. Alanine aminotransferase (ALT) and aspartate aminotransferase (upper reference limit 30 U/l), total and conjugated bilirubin (UB and CB, with upper reference limits of 26 and 5 µmol/l respectively), and total protein (TP)(g/l) were determined automatically (Technicon SMA-C). Unconjugated bilirubin (UB) values were calculated by subtracting CB from TB (upper reference value 21 µg/l). The summation of gammaglobulin (GG) was determined in percentage of TP by electrophoresis, and values were calculated by multiplication with TP (upper reference limit 20 g/l).

Statistical analysis

Non-parametric statistical analysis was performed with Friedman's analysis of variance (ANOVA), Wilcoxon's matched pairs test (p-values shown in figures), and Mann-Whitney U test where appropriate, with $p<0.05$ as the level of significance.

RESULTS

Before therapy, ALT levels were markedly elevated in all patients (median 337 U/l). These declined during the first two months of therapy to median 32 U/l ($p<.001$), with stabilization thereafter at median 20 U/l, both at 14 and 26 months (figure 7.1.1A).
AST values at commencement were median 359 U/l, with reduction to 32 U/l after two months ($p<.001$), and a trend to further improvement over the next two years ($p=.06$), with median values of 24 and 20 at 14 and 26 months respectively (figure 7.1.1B).

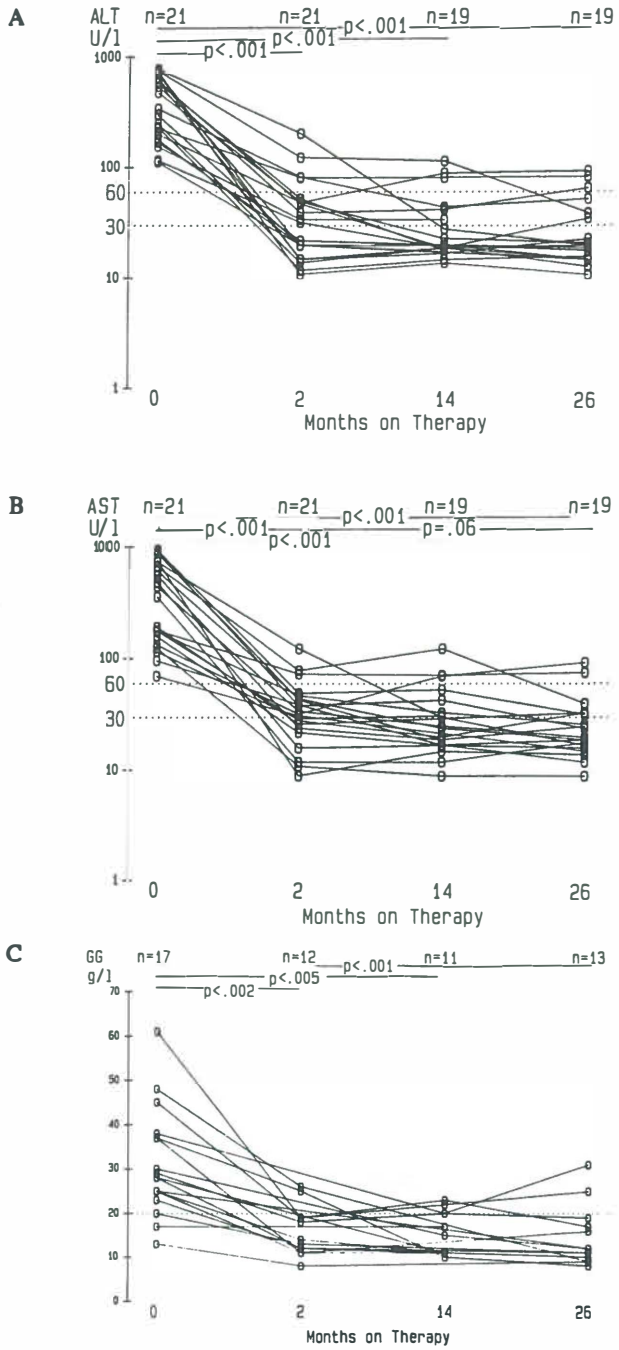


Figure 7.1.1. A) ALT, B) AST and C) gammaglobulin (GG) in 'autoimmune' CAH and changes during immunosuppression. Dotted lines indicates upper reference limits (N), upper dotted lines in ALT and AST indicate 2xN.

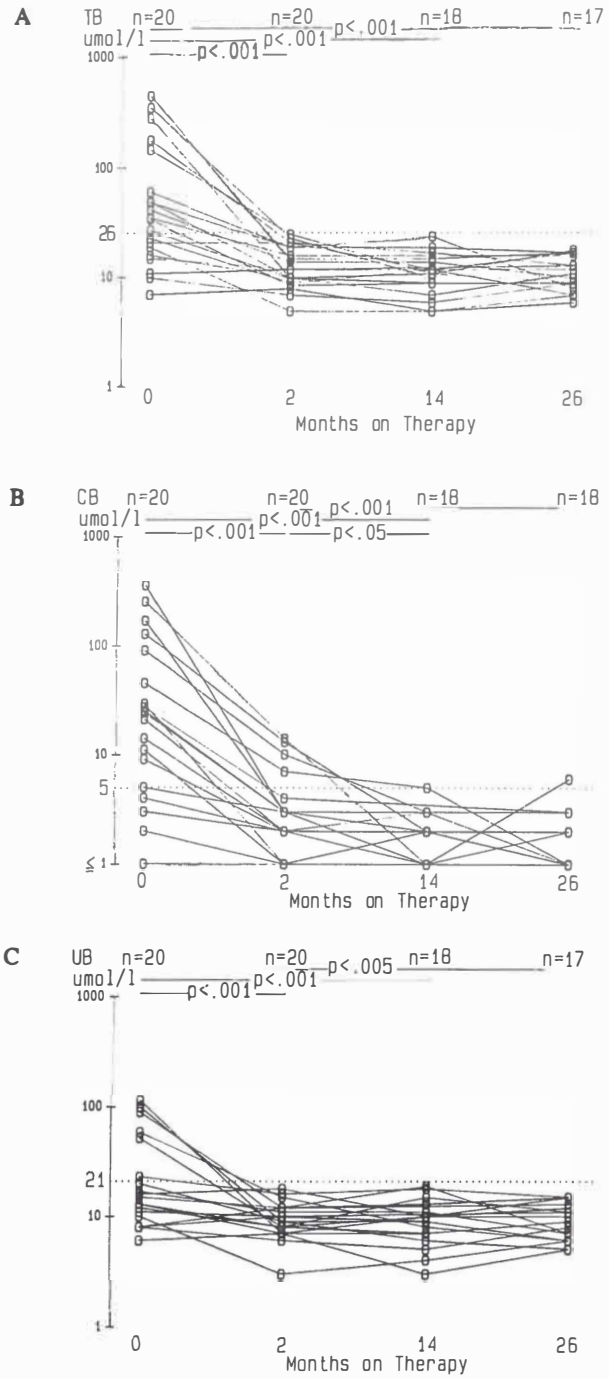


Figure 7.1.2 A) total, B) conjugated and C) unconjugated and bilirubin in 'autoimmune' CAH and changes during immunosuppression. Dotted lines indicate upper reference limits.

Gammaglobulin levels (n=17) were elevated at the start with a median value of 28 mg/l, falling to median 13.5 at two months (p<.005), and stabilization thereafter, with median values of 15 and 12 at 14 and 26 months respectively (figure 7.1.1C).
Direct serum bilirubin (CB) was elevated at commencement in 15 (70%) of the 21 patients, with a median value of 22.5 µg/l, which is 4.5 times the upper reference limit. Levels were reduced to median 2.5 µg/l after two months of therapy (p<.001), and to normal values in all patients after 14 months (median 2 µg/l) (p<.05), with stabilization during the next year (median 2) (figure 7.1.2B).
Indirect bilirubin (UB) was elevated in only 6 (30%) of the 21 patients, with a median of 14 µg/l initially, which is within the reference range. These values fell to 9 µg/l after two months, and stabilized during the next two years (median 9 at 14 and 26 months) (figure 7.1.2.C).
This resulted in a reduction in total bilirubin (TB) levels from median 34.5 µg/l at commencement to median 12 µg/l after two months (p<.001), with stabilization thereafter (median 11 at both 14 and 26 months) (figure 7.1.2A).

Correlations

For correlations the reader is referred to chapter 8.

DISCUSSION

With standardized immunosuppression routine serum liver biochemistry improved considerably in patients with previously untreated idiopathic 'autoimmune' IAI-CAH who had required such therapy.
During active disease the increase in serum conjugated bilirubin values was more pronounced and frequent than that of unconjugated bilirubin levels. This may indicate that the uptake of bilirubin by the hepatocytes is less disturbed than the excretion of conjugated bilirubin in IAI-CAH. Both normalized rapidly during therapy, even in the patient whose histology failed to improve.
Often values of AST and ALT did not normalize completely. It is well-known that serum ALT, gammaglobulin and bilirubin, using standard reference ranges, do not accurately predict histological activity.[506] Follow-up biopsies to monitor inflammatory activity are therefore needed as long as no better serological parameters of activity are available. In a biopsy of sufficient size,[507] sampling error for determination of inflammatory activity in CAH is less than 10%,[508] and intra-observer error less than 6%.[508] In other forms of liver disease the inter- and intra observer variability in grading the degree of piecemeal necrosis is greater than 50 and 40%, respectively.[509] In the next chapter we will therefore assess histology and relate this to serum biochemistry.

§7.2

SERUM SODIUM, DEPRESSED IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS, NORMALIZES DURING IMMUNOSUPPRESSIVE THERAPY

Relation to Histology, Synthesizing Capacity of the Liver and Routine Serum Biochemistry

SUMMARY

Although idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) usually reacts favorably to immunosuppressive therapy, it is unknown whether functional renal failure as the onset of hepatorenal syndrome (HRS) complicating IAI-CAH, is reversible without liver transplantation.

We studied serum sodium values (Na^+) in IAI-CAH before and during standardized immunosuppressive therapy with prednisolone and azathioprine. Na^+ in IAI-CAH (n=17) was lower than in healthy controls (n=105), and in 12 (70%) of the patients Na^+ was below the 99% lower limit of the reference range. Na^+ increased during therapy in all patients tested, from median 137 mmol/l at start to median 141 mmol/l after two months ($p<.01$), with a trend to further increase in the next year ($p=.09$), although the median remained 141 mmol/l, with finally normal values in all patients tested. The median Na^+ after two years was 141 mmol/l. Serum creatinin (Creat)increased from median 63 µmol/l at start of therapy to median 68 µmol/l after two months ($p<.05$), with medians of 72 and 62 µmol/l after 14 and 26 months respectively. Blood urea nitrogen (BUN) values increased from median 4.2 mmol/l at start to median 5.1 mmol/l after 14 months of therapy (<.001). These increases in Creat and BUN paralleled those in serum parameters of hepatic protein synthesizing capacity.

In untreated IAI-CAH Na^+ was negatively correlated to ALT (R=-.46) and gammaglobulin values (R=-.67). BUN positively correlated with antithrombin III values (R=.56).

In conclusion, serum sodium in IAI-CAH is below normal, probably as a result of reduced free water clearance, representing the onset of HRS. In IAI-CAH reacting to immunosuppression, this condition is reversible.

INTRODUCTION

Hepatorenal syndrome (HRS) is a condition associated with end-stage liver disease. The onset of this condition is usually termed 'functional renal failure' in liver disease. The kidneys from patients with HRS are normal, the function of these kidneys recovers if

they are used for transplantation,[510] and renal function usually normalizes after succesful liver transplantation in patients with HRS,[511,512] indicating the functional nature of the renal disorder in HRS. We recently showed that in patients with idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) reacting favorably to immunosuppressive therapy, hepatic protein-synthesizing capacity (HPSC) can normalize despite the presence of cirrhosis.[513] Whether this therapy can reverse HRS accompanying IAI-CAH is unknown.
Hyponatraemia in HRS is a consequence of reduced free water clearance (FWC) and it is a constant finding in patients with a FWC of 1 ml/min or less, while it is often considered an infrequent finding if patients excrete free water above this level.[514] However, if one determines Na^+ in a healthy population the 99% reference range is small (between 138 and 146 mmol/l), and Na^+ in a heterogenous population of patients with various liver diseases is lower than normal, even in the absence of cirrhosis.[515]

In the current study we investigated serum sodium values (Na^+) in patients with idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) and no signs of renal disease before and during standardized immunosuppressive therapy with prednisolone and azathioprine. Values were related to histology, serum creatinin, blood urea nitrogen, alanine aminotransferase, gammaglobulin, and parameters of protein synthesizing capacity.

MATERIALS AND METHODS

Patients

17 patients with IAI-CAH, 5 men and 12 women, all requiring immunosuppressive therapy according to accepted criteria,[516] were studied to evaluate therapy effects on serum sodium (Na^+), creatinin (Creat) and urea nitrogen (BUN). Their median age was 52 years (range 17-63), and median duration of disease 189 days (range 0-1841). Cirrhosis was present in 16 of these patients as assessed by laparoscopy and liver biopsy. All were subsequently treated with standardized immunosuppression: oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (14 patients) or two years (6 patients) of therapy with 10 mg prednisolone and 50 mg azathioprine daily. Serial routine liver biopsies and sera were obtained at the start and at two months, one and two years of therapy.

Liver histology

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[517] In contrast to the original system,[518] fibrosis/cirhhosis is not included in the HAS. The HAS is expressed as a numerical score (0-18), and is the summation of portal (score 0-4), periportal (score 0-10) and lobular (score 0-4) inflammation and necrosis.

Serum biochemistry

In the routine protocol sera Na^+, Creat, BUN, Alanine aminotransferase (ALT) (upper reference limit 30 U/l), and total protein (TP) were determined automatically (Technicon SMA-C) in blood taken between 7 and 10 A.M. The summation of gammaglobulin (GG) was determined as a percentage of TP by electrophoresis, and values were calculated by multiplication with TP (upper reference limit 20 g/l). Parameters of hepatic protein synthesizing capacity were determined as described in chapter 9.

Statistical analysis

Statistical analysis was performed with analysis of variance (ANOVA) with repeated measurements, the Mann-Whitney U test, the Wilcoxon matched pairs test and Spearman's rank correlation where appropriate, with $p<0.05$ as the level of significance.

RESULTS

Na^+ in IAI-CAH (n=17) was lower than in healthy controls (n=105), and in 12 (70%) of the patients Na^+ was below the 99% lower limit of the reference range. Na^+ increased during therapy in all patients tested, from median 137 mmol/l at start to median 141 mmol/l after two months ($p<.01$), with a trend to further increases in the next year to median 141 mmol/l ($p=.09$), with finally normal values in all patients. The median Na^+ after two years was 141 mmol/l (figure 7.2.1A).

Serum creatinin increased from median 63 µmol/l at start to median 68 µmol/l after two months ($p<.05$), with medians of 72 and 62 µmol/l after 14 and 26 months respectively (figure 7.2.1B).

Blood urea nitrogen values increased from median 4.2 mmol/l at start to median 5.1 mmol/l after 14 months of therapy ($p<.001$) (figure 7.2.1C).

For results of serum- and plasmabiochemistry and histology the reader is referred to chapter 7.1, 8, 9, 10 and 11.

In untreated IAI-CAH, Na^+ was negatively correlated to ALT ($R=-.46$) and gammaglobulin values ($R=-.67$). BUN positively correlated with antithrombin III values ($R=.56$). No other correlations were detected.

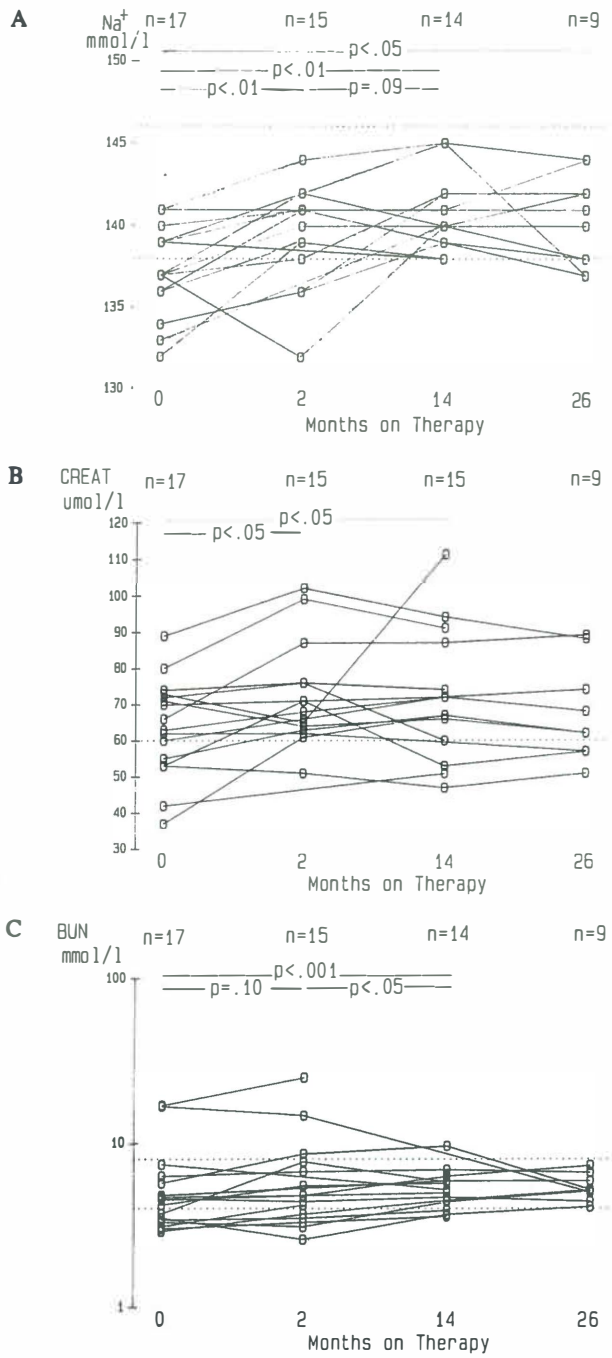


Figure 7.2.1 A) Serum sodium, B) creatinin and C) blood urea nitrogen in 'autoimmune' CAH and changes during immunosuppression. Lower (A,C) and higher (A,B,C) dotted lines are the lower and higher 99% limits of the reference range.

DISCUSSION

In the current study, we demonstrated that serum sodium levels in IAI-CAH with cirrhosis are below the 99% reference range for a healthy controlpopulation and that these serum Na^+levels all normalized with standardized immunosuppressive therapy with prednisolone and azathioprine. There was an inverse relation of serum Na^+ levels with serum ALT and GG levels.
Low Creat and BUN in untreated IAI-CAH probably indicate malnutrition and impaired hepatic protein synthesis and the increase in Creat and BUN with therapy probably indicate an ameliorated state of nutrition and protein synthesis.

Usually reference ranges for serum sodium (Na^+) are chosen with wide margins, but the use of more strict criteria based on a healthy reference population allows detection of hyponatraemia in many unselected liver patients and IAI-CAH patients. In the absence of other overt signs of hepatorenal failure, low Na^+ levels in IAI-CAH may reflect the almost subclinical onset of impaired free water clearance, marking the onset of HRS. The current knowledge of pathophysiology of ascites and functional renal failure in cirrhosis has been reviewed recently.[519,520] Hyponatraemia in HRS is a consequence of reduced free water clearance (FWC),[514] which, in part appears due to peripheral vasodilatation with an increase in antidiuretic hormone (ADH),[521-523] which is insufficiently counteracted by an increased renal prostaglandin E_2 (PGE_2) production.[524] This concept is supported by reduction of free water clearance by administration of nonsteroidal antiinflammatory drugs that inhibit renal PGE_2 production.[525]
The normalization of Na^+ values in IAI-CAH without LT but with immunosuppressive therapy, probably indicates that repair and restoration of functional hepatic capacity can reverse HRS. This confirms again the functional nature of the renal disorder in HRS. Furthermore it indicates that not only liver transplantation, but also immunosuppressive therapy in IAI-CAH is able to reverse functional renal failure or maybe even more advanced HRS in corticosteroid responsive patients. The initial response to such therapy an important determinant in both early[526] and longterm[527] prognosis in this disease.
Currently, we are investigating the effect of immunosuppression in IAI-CAH on serum ADH values.

In conclusion, serum sodium in IAI-CAH is below normal, probably as a result of reduced free water clearance, representing the onset of functional hepatorenal impairment. In IAI-CAH reacting to immunosuppression, this condition is, at least in its early stages, reversible without liver transplantation.
Further studies, including measurements of urine Na^+, are warranted.

§7.3

ROUTINE HAEMATOLOGICAL PARAMETERS IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS, AND CHANGES DURING IMMUNOSUPPRESSION

SUMMARY

We looked at routine haematological parameters in IAI-CAH, and changes in these parameters during fixed-dose immunosuppression with prednisolone and azathioprine. This was done to evaluate the likelihood of induction of cytopenia by azathioprine.
Leucocytes increased from median $5.3x10^9$ at the start to $7.8x10^9$ at two months ($p<.001$), with stabilization thereafter at median $7.35x10^9$ at one year and $6.3x10^9$ at two years. (Friedman 12.30, $p<.01$).
Thrombocytes did not change overall (Friedman ANOVA), although there was an increase from median $151x10^3$ at start to median $173x10^3$ at 2 months ($p<.05$). Values at 14 and 26 months (median 158 and 162) did not differ from start values, although values at 26 months tended to be higher ($p=.06$).
Haemoglobin increased from median 128 g/l at start to 136 g/l after 2 months ($p<.05$), further increased to median 145 g/l at 14 months ($p<.01$), but slightly decreased in the second year of therapy to median 140 g/l ($p<.05$). Hb at one and two years was higher than at start ($p<.001$ and $p<.01$ respectively). (Friedman 15.73, $p<.001$)).
Haematocrit increased between two months (median 39.7 %) and 14 months (median 41.9 %) ($p<.005$). Values at 14 and 26 months (median 40.9) were higher than those at start (median 37.7 %), and Ht at 26 months was higher than after 2 months ($p<.05$). (Friedman 16.23, $p<.001$).
Mean cell volume of erythrocytes only tended to increase from median 93.0 fl at start to median 97.2 fl at two months ($p=.06$), but no significant changes were observed (medians at 14 and 26 months were 92.8 fl and 94.8 fl respectively) (Friedman 5.10, $p=.165$).
The present study shows that standardized immunosuppressive therapy with prednisolone and azathioprine in a fixed dose combination schedule in 'autoimmune' CAH can result in improval of routine haematological parameters.

INTRODUCTION

Hypersplenism associated with cirrhosis and portal hypertension in chronic active hepatitis (CAH) is able to induce pancytopaenia.[528] Therapy with azathioprine may further reduce the number of blood cells. If cytopaenia is already marked, it has been

recommended to withhold azathioprine and to administer only corticosteroids as a therapy for idiopathic 'autoimmune' (IAI-) CAH.[529]
To assess the justification for fear of induction of cytopaenia by azathioprine in IAI-CAH, we looked at routine haematological parameters in IAI-CAH, and changes in these parameters during fixed dose immunosuppression with prednisolone and azathioprine.

MATERIALS AND METHODS

Patients

17 patients with IAI-CAH, 5 men and 12 women, all requiring immunosuppressive therapy according to accepted criteria,[530] were studied. Their median age was 52 years (range 17-63), and median duration of disease 189 days (range 0-1841). Cirrhosis was present in 16 of these patients as assessed by laparoscopy and liver biopsy. All were subsequently treated with standardized immunosuppression: oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (14 patients) or two years 6 patients) of therapy with 10 mg prednisolone and 50 mg azathioprine daily. Serial routine liver biopsies and sera were obtained at the start and at two months, one and two years of therapy.
We evaluated therapy effects on the number of peripheral leucocytes (LEU), thrombocytes (THR), haemoglobin content (Hb) of red blood cells, haematocrit (Ht) and mean cell volume (MCV) of erythrocytes.

Liver histology

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[531] In contrast to the original system,[532] fibrosis/cirhhosis is not included in the HAS. The HAS is expressed as a numerical score (0-18), and is the summation of portal (score 0-4), periportal (score 010) and lobular (score 0-4) inflammation and necrosis.

Haematology

LEU, THR, Hb, Ht and MCV were determined in blood (taken between 7 and 10 A.M.) by Coulter counter.

Statistical analysis

Statistical analysis was performed with the Wilcoxon's matched pairs tests with $p<0.05$ as the level of signifcance.

RESULTS

Results for histology and routine serum biochemistry are shown in chapters 7.1, 7.2 and 8. LEU increased from median $5.3x10^9$ at start to $7.8x10^9$ at two months ($p<.001$), with stabilization thereafter at median $7.35x10^9$ at one and $6.3x10^9$ at two years. (Friedman 12.30, $p<.01$) (figure 7.3.1A).
THR did not change overall (Friedman ANOVA), although there was an increase from median $151x10^3$ at start to median $173x10^3$ at 2 months ($p<.05$). In one patient THR dropped initially, then stabilized after 1 year. Another patient returned to baseline THR after an initial increase. Values at 14 and 26 months (median 158 and 162) did not differ from start values, although values at 26 months tended to be higher ($p=.06$) (figure 7.3.1B).

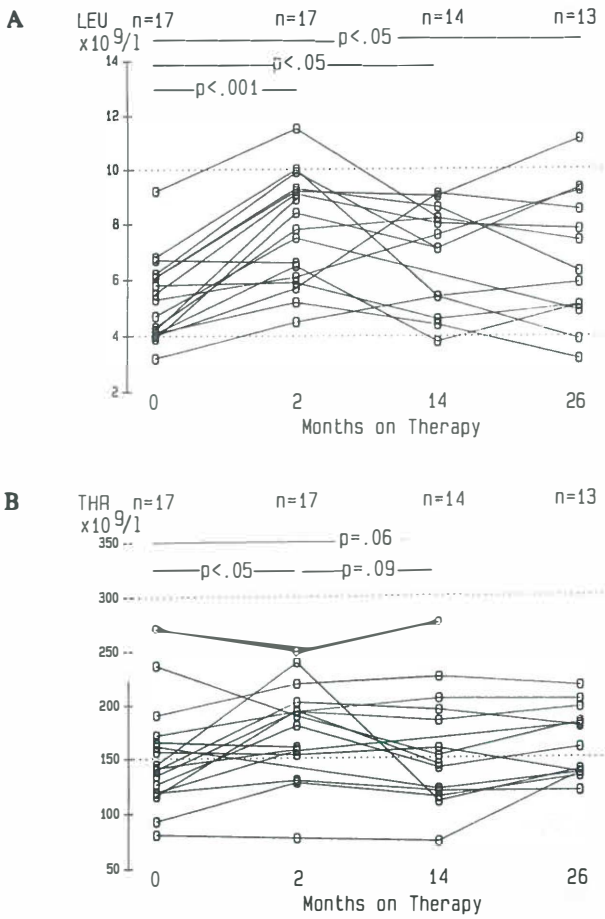
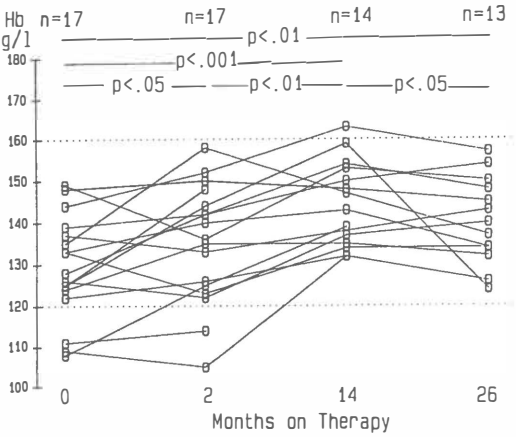


Figure 7.3.1 Numbers of A) leucocytes and B) thrombocytes C) Haemoglobin content and D) haematocrit in peripheral blood in 'autoimmune' CAH before and during immunosuppression. Dotted lines indicate upper and lower limits of the reference range.

C



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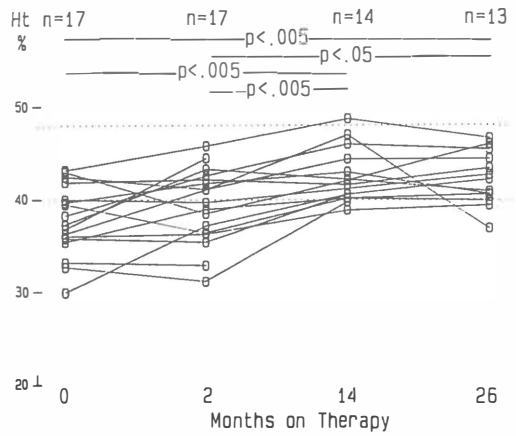


Hb increased from median 128 g/l at start to 136 g/l after 2 months (p<.05), further increased to median 145 g/l at 14 months (p<.01), but slightly decreased in the second year of therapy to median 140 g/l (p<.05). Hb at one and two years was higher than at start (p<.001 and p<.01 respectively). (Friedman 15.73, p<.001) (figure 7.3.1C).

Ht increased between two months (median 39.7 %) and 14 months (median 41.9 %) (p<.005). Values at 14 and 26 months (median 40.9) were higher than those at start (median 37.7 %), and Ht at 26 months was higher than after 2 months (p<.05). (Friedman 16.23, p<.001) (figure 7.3.1D).

MCV only tended to increase from median 93.0 fl at start to median 97.2 fl at two months (p=.06), but no significant changes were observed (medians at 14 and 26 months were 92.8 fl and 94.8 fl respectively) (Friedman 5.10, p=.165).

DISCUSSION

The present study shows that standardized immunosuppressive therapy with prednisolone and azathioprine in a fixed dose combination schedule in 'autoimmune' CAH can result in improval of routine haematological parameters. This contrasts with the common fear that azathioprine might induce cytopaenia, either through a depressive effect on the bonemarrow or by induction of liverfibrosis/cirrhosis causing worsening of portal hypertension and hypersplenism.

The peripheral cytopenia that is encountered in hypersplenic states can mainly be ascribed to an increased reversible pooling of blood cells in the enlarged spleen. These cells are in dynamic equilibrium with circulating cells and not permanently sequestered. For instance, the splenic platelet pool will only be dependent on two factors: the splenic blood flow on the one hand and intrasplenic transit time on the other. The splenic blood flow is dependent on spleen size and adrenergic activity.[533]

The observed beneficial effect of immunosuppressive therapy on leucocytes, Hb, Ht and, at least, the absence of a deleterious effect on the number of thrombocytes, may be due to recruitment of cells from the spleen. This may follow reduction in splenic blood flow and spleen size, when portal hypertension diminishes in relation to a reduction of hepatic inflammation.

Steroid administration has been shown to initially (first 8-12 hours) reduce the number of uncommitted circulating pluripotential haematopoietic cells (CFU-GEMM).[534,535] An initial depletion also equally affects all subclasses of lymphocytes.[536] A rise in CFU-GEMM occurs 24-36 hours after administration of steroids to levels above pre-treatment levels. Steroids reset the normal diurnal variation in numbers of blood cells. Initial high neutrophil counts drop 36 hours post steroid administration, and return to baseline values at about 60 hours. The number of monocytes drops after steroids, but then normalizes without rebound. Committed precursor levels (erythroid burst-forming units (BFU-E) and granulocyte/monocyte colony-forming units (CFU-GM)) showed diurnal variations similar to CFU-GEMM.[534,535] Unlike these studies, where one dose of steroids was administered, steroid therapy is continued in patients with IAI-CAH, so that the latter effect may prevail. This could be an additional explanation for the improvement in peripheral blood cell counts after commencement of immunosuppressive therapy in IAI-CAH.

Hb may also rise through enhancement of hepatic protein synthesizing capacity.[537] No variceal bleedings were observed before or during the treatment period in these patients, although minor gastrointestinal blood loss before therapy cannot be completely ruled out.

Addition of azathioprine to the therapeutic regimen can allow lower doses of prednisolone in order to achieve the same therapeutic results with lower rates of adverse effects. We feel that mild and moderate cytopaenia should no longer be considered as an absolute contraindication to the use of azathioprine. In severe cytopaenia in a patient

with IAI-CAH it might be wise, however, to start induction-therapy with prednisolone alone and to evaluate the possibility (dependent on improval of routine haematology) to add azathioprine after e.g. one or two months. In all patients it is, of course, advisable to monitor blood cell counts frequently especially in the first weeks of immunosuppressive therapy for IAI-CAH, especially if azathioprine is included in such therapy. This is particularly important to identify the occasional patient susceptible to azathioprine-induced cytopaenia.[537a-537d]

Peripheral blood cell counts will improve in the majority of patients with IAI-CAH during therapy with prednisolone and azathioprine.

CHAPTER 8

SERIAL HISTOLOGY AND ROUTINE LIVER BIOCHEMISTRY IN STANDARDIZED IMMUNOSUPPRESSIVE THERAPY OF IDIOPATHIC 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS.

SUMMARY

We analyzed histology and routine liver biochemistry from 21 patients with idiopathic autoimmune chronic active hepatitis (CAH) before and during standardized immunosuppression. Portal (P), periportal (PP) and lobular (L) inflammation were scored, and the summation of these produced a histological activity score (HAS) (0-18). A Histological Fibrosis/Cirrhosis Score (HFS) (0-4) was produced, and the numbers of four cell types scored in a 0-3 scale. Values before and changes during therapy were correlated to each other and with routine serum liver biochemistry.

At two months, 67 percent of the patients had already improved to a HAS≤3. A HAS≤1 ('complete histological remission') was achieved in 80 percent of the patients and 1<HAS≤3 ('partial histological remission') in 10 percent of the patients within two years. Periportal and lobular inflammation had disappeared in all of these 19 (90%) out of 21 patients in remission.

Continuing decrease of portal and periportal inflammatory activity with disappearance of plasma cells and decrease in numbers of lymphocytes was observed during the two years of immunosuppressive therapy.

HFS was high (median score 3) and did not change during therapy.

ALT , AST and gammaglobulin (GG) (medians 337 U/l, 359 U/l, and 28 g/l at start respectively) improved considerably (median 20 U/l, 20 U/l and 12 g/l after two years), while conjugated bilirubin (median 22.5 µmol/l) normalized within two months to median 2.5 µmol/l. Median unconjugated bilirubin was normal (14 µmol/l), but declined to 9 µmol/l in the first two months of therapy.

In untreated IAI-CAH the HAS, PP and number of plasma cells were weakly correlated to ALT (R=.41, .41, and 48 respectively) and did not correlate to the other serum biochemical parameters. L correlated only with GG (R=.50). ALT, AST, GG, CB and TB did correlate to the number of (peri)portal lymphocytes (R=.51, .52, .63, .43, and 43). Correlations among histological parameters and among serum biochemical parameters are shown as well.

In conclusion, results from the current study indicate that standardized immunosuppression with a fixed dose schedule was able to improve histology in 95%, with induction of 'complete histological remission' (HAS≤1) in 80% and 'partial histological

remission' (HAS≤3, but >1) in 10% of the patients with moderate to severe 'autoimmune' CAH. We propose that HAS≤3 is an objective of therapy, while a HAS≤1 and the absence of plasma cells in the biopsy may be required before an attempt at discontinuation of therapy can be successful. However, determination of satisfactory endpoints for treatment and establishing optimal guidelines for discontinuation of therapy remains a nagging clinical problem.

INTRODUCTION

Idiopathic 'autoimmune' chronic active hepatitis (CAH) usually reacts favorably to immunosuppressive therapy,[538] and immunosuppression has been shown to enhance survival in this type of CAH.[539-543] Remission has been induced in 60% of patients within 2.5 years of treatment in the Mayo Clinic studies.[541,544] A combination of fixed dose prednisolone and azathioprine daily proved superior to fixed dose, titrad or alternate day therapy with prednisolone alone, with regard to efficacy and adverse effects of therapy.[539] Azathioprine alone cannot induce remission in CAH,[539] but can maintain remission without prednisolone in a selected group of patients.[545] The descriptive nature of conventional systems for scoring histological activity of CAH[546,547] hampered the systematic study of changes in histology during therapy. Recently a numerical scoring system for asymptomatic CAH was introduced,[548] and a modified 'Histological Activity Score' correlated well with the conventional way of scoring activity in CAH.[549]

To assess the effects of a combination of fixed dose prednisolone and azathioprine on histology and routine liver tests, we analysed protocol liver biopsies and biochemistry at 0,2,14 and 26 months of such standardized immunosuppression in 21 patients with symptomatic 'autoimmune' CAH.

MATERIALS AND METHODS

Patients

We studied 21 untreated patients with idiopathic 'autoimmune' chronic active hepatitis (CAH) -3 of them with antibodies to hepatitis B surface antigen: 4 men and 17 women. These patients required immunosuppressive therapy according to accepted criteria.[539] Their median age was 52 years (range 16-71), and median duration of disease 75 days (range 0 months to 3 years). All were subsequently treated with oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (2 patients) or two years (19 patients) of therapy with 10 mg prednisolone and 50 mg azathioprine daily. Serial routine liver biopsies and sera were studied at the start and at two months, one and two years of therapy. One biopsy at 2 months, one at 14 months and 6 at 26 months were missing, although biochemistry was available.

Histology

Routine liver biopsies were obtained by using the Menghini technique. After fixation with formalin, biopsies were embedded in paraffin. Four micron sections were stained with hematoxylin-eosin, periodic acid-Schiff with previous diastase digestion, Perl's iron stain, Gomori's reticulin stain and Azan collagen stain. The biopsies were reviewed under code without knowledge of the patient's identity or previous histological diagnosis (by J.G. and B.v.H.). The numerical scoring system as described was used.[548] This system uses four histological categories: I)*periportal* inflammation (PP) (piecemeal necrosis) with or without bridging necrosis; II) intra*lobular* degeneration and focal necrosis (L); III) *portal* inflammation (P); and IV) fibrosis/cirrhosis. A numerical score was assigned ranging from 0 (no alteration) to 10 for category I, and from 0 to 4 for categories II to IV. As the score for inflammation (Categories I to III) and the *"Histologic Fibrosis/cirrhosis Score"* (HFS) (Category IV) might vary in opposite directions during follow-up, the four categories were monitored separately in the individual patient, and the summation of categories I, II, and III was termed *"Histological Activity Score" (HAS).*[549] In doing so, the combined score for piecemeal necrosis and bridging necrosis weighed more heavily than the separate scores for the other three categories. We showed that this system provides more accurate information of histological activity than conventional scoring does, while it correlates well with conventional grading of CAH.[549] 'Partial histological remission' was defined as HAS≤3, and HAS≤1 was termed 'complete histological remission'.

In addition, the composition of the inflammatory infiltrate was studied by semiquantitative grading of the numbers of lymphocytes, plasma cells, eosinophils and polymorphonuclear leucocytes in a 0-3 scale, before and during immunosuppression.

Serum biochemistry

Sera were obtained on the same day on which the liver biopsies were performed. Alanine aminotransferase (ALT) and aspartate aminotransferase (upper reference limit 30 U/l), total and conjugated bilirubin (UB and CB, with upper reference limits of 26 and 5 µmol/l respectively), and total protein (TP)(g/l) were determined automatically (Technicon SMA-C). Unconjugated bilirubin (UB) values were calculated by subtracting CB from TB (upper reference value 21 µg/l). The summation of gammaglobulin (GG) was determined as a percentage of TP by electrophoresis, and values were calculated by multiplication with TP (upper reference limit 20 g/l).

Statistical analysis

Non-parametric statistical analysis was performed with Friedman's analysis of variance (ANOVA), Wilcoxon's matched pairs test (p-values shown in figures), and Mann-Whitney U test where appropriate, with $p<0.05$ as the level of significance.

RESULTS

Histology

- Fibrosis/cirrhosis:

As shown in figure 8.1, initial scores (0-4) of hepatic fibrosis/cirrhosis (HFS) were high (median 3), and did not change during the 26 months of immunosuppressive therapy (median 3.5, 3, and 3 at 2, 14 and 26 months respectively) (p=.938 in ANOVA).

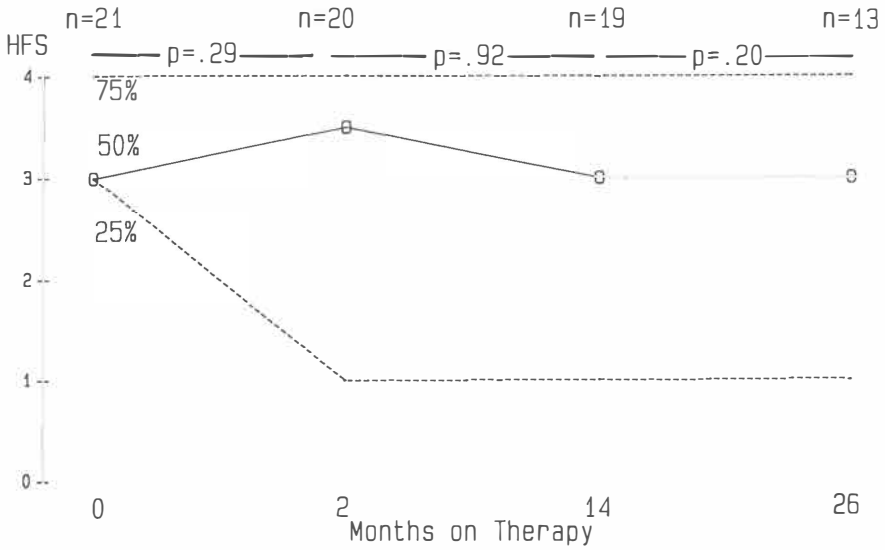


Figure 8.1 Histological Fibrosis Score (HFS) in 'autoimmune' CAH before and during immunosuppressive therapy.

- Inflammation/necrosis:

Results and the sequence of changes are shown in figures 8.2A-C and figure 8.3. Portal inflammation scores (0-4) (P) declined from median 3 at start of therapy to median 1 within the first 14 months of therapy (p<.05), and stabilized thereafter. Median values at 2 months and two years of therapy were both 1.

Periportal inflammation scores (0-10) (PP) fell from median 5 at start of therapy to median 0.5 at two months of therapy (p<.001), with further reduction to median 0 after 26 months (p<.05). The median PP score at 14 months was also 0.

Lobular inflammation scores (0-4) (L) were quite low at the start of therapy (median score 1). Lobular inflammation almost disappeared within two months of therapy (median 0) (p<.005), with a trend to even further reduction in the next two years (median 0 at both 14 and 26 months)(p=.09).

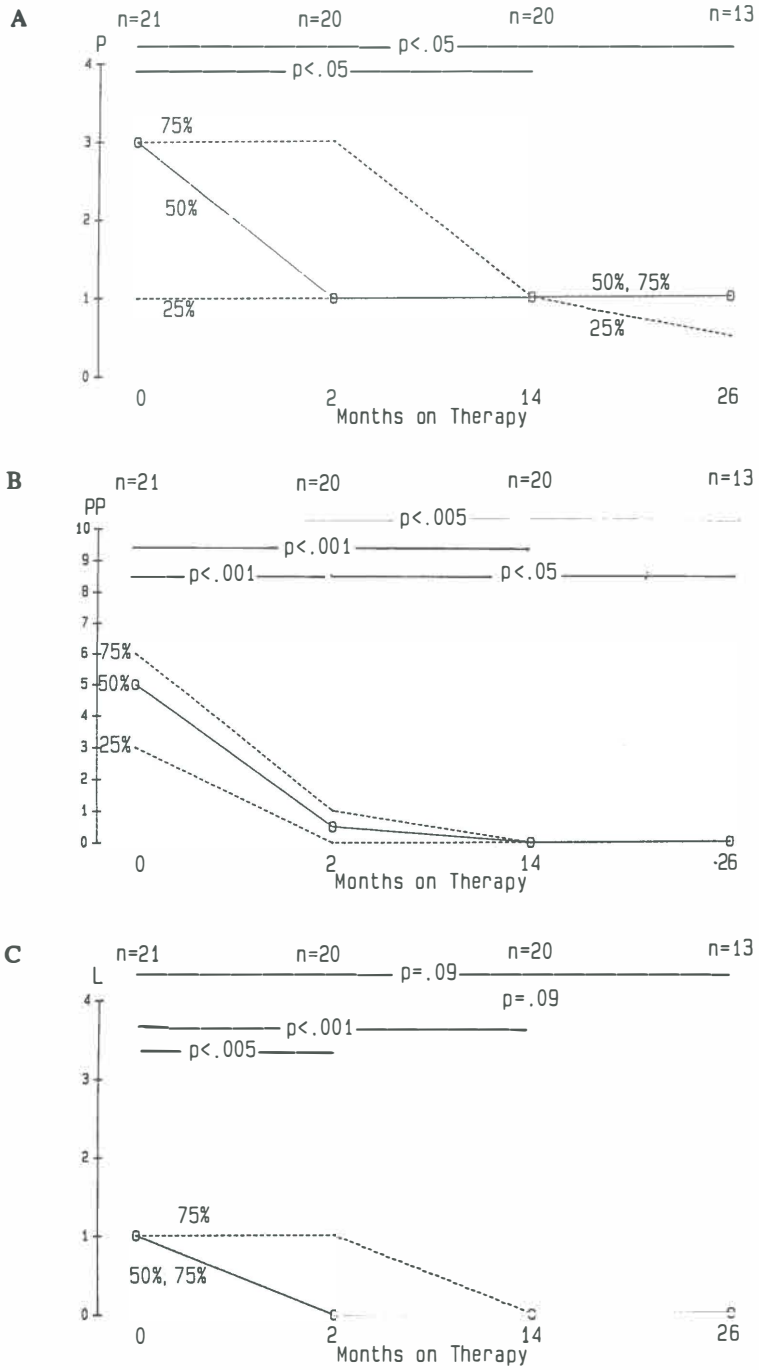


Figure 8.2 Scores of A) portal (P), B) periportal (PP) and C) lobular (L) inflammation in 'autoimmune' CAH before and during immunosuppressive therapy.

As shown in figure 8.3, the 'Histological Activity Score' (HAS), which is the summation of P, PP and L, fell from median 9 at start of therapy to median 3 after two months ($p<.001$). There was further more gradual improvement over the next two years of therapy with median values of 1 both at 14 and 26 months ($p<.05$). Eventually 17 (80%) of the 21 patients were in complete histological remission (HAS≤1), and 2 (10%) in partial histological remission (HAS≤3). CAH remained active in two (10%) of these patients during therapy: HAS only declined from 12 at start to 10 after 14 months in one of these two patients, while it declined from 10 at start to 4 after two years in the other patient. One of the patients with HAS≤1 at two months relapsed (HAS 9) at one year during unaltered immunosuppression, but reentered complete histological remission during the second year of unchanged therapy. After a HAS≤1 was reached, no other patients showed an increase in HAS to scores above 1 during this therapy. Mild to moderate cholestasis was only present in two patients with severe CAH with impaired synthetic function. This cholestasis disappeared within two months of immunosuppressive therapy.

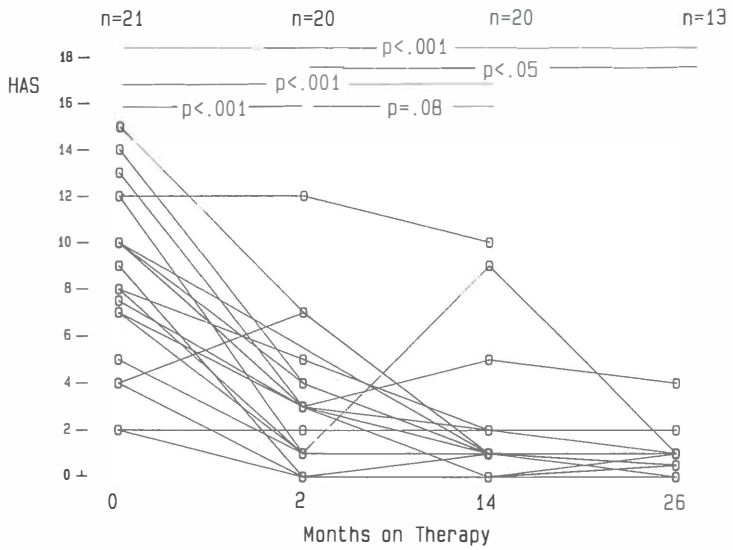


Figure 8.3 Histological Activity Score (=P+PP+L) in 'autoimmune' CAH before and during immunosuppressive therapy.

-Composition of the inflammatory infiltrate:

With respect to the composition of the inflammatory infiltrate, the results of scoring four different cell types in the inflammatory infiltrate of the liver on a 0-3 scale are shown in figure 8.4.

Lymphocytes dominated the infiltrate, followed by plasma cells, especially in the biopsies prior to therapy. During immunosuppression, the few granulocytes (eosinophils

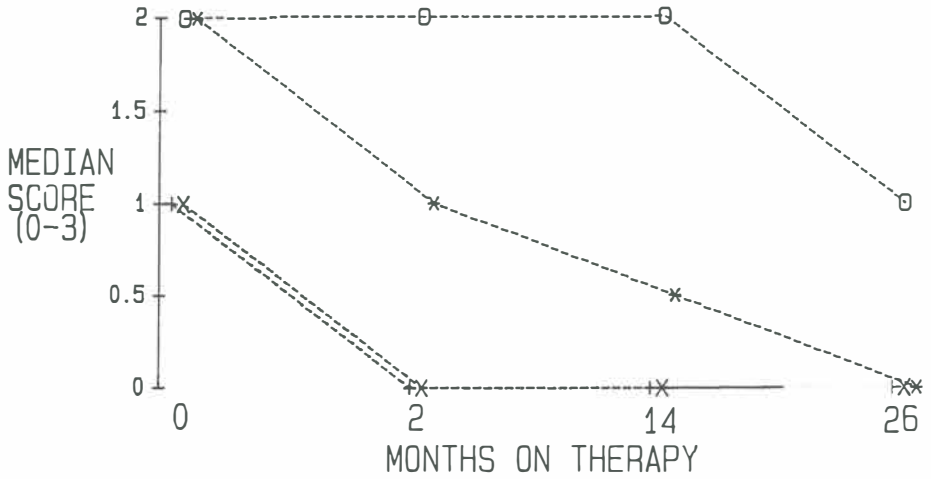


Figure 8.4 Composition of the inflammatory infiltrate in 'autoimmune' CAH before and during immunosuppressive therapy. Median scores are shown. o: lymphocytes *: plasma cells +: polymorphonuclear leucocytes x: eosinophils

and polymorphonuclear leucocytes) disappeared first, and the reduction in the number of plasma cells was more rapid than the reduction in the number of lymphocytes. After 26 months of therapy, portal lymphocytes remained.

Lymphocytes were invariably present at start (median 2), with a gradual decline over all 26 months of therapy ($p<.005$), especially in the first 14 months. Median scores of the number of lymphocytes were 2 at two and 1 at 14 months ($p<.05$), and 1 at 26 months ($p=.50$), when these were virtually the only inflammatory cells remaining.

The median score of plasma cells at start was 2, with a rapid decline in the first two months to median 0.5 ($p<.01$), stabilization in the next year of therapy at median 0 ($p=.80$), and a trend towards further improvement in the second year of therapy in some patients ($p=.07$), with a median score of 0 after two years.

Few polymorphonuclear leucocytes were present in the infiltrate before therapy (median score 1). They were reduced in number in the first two months of therapy (median score 0) ($p<.01$), and remained absent in the next two years of therapy (medians 0) ($p=.78$ and $p=.59$ respectively).

The few eosinophils (median score 0) at start disappeared in the first two months (median score 0 at 2 months) ($p<.001$) and did not reappear (median 0 at 14 and 26 months) ($p=.18$ and .32 respectively).

Serum biochemistry

For these results the reader is referred to chapter 7. For ALT the results are also shown in figure 8.5.

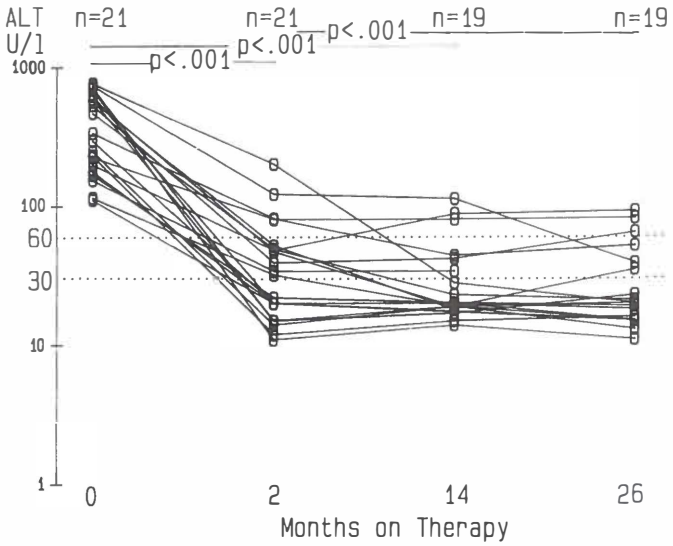


Figure 8.5 ALT in 'autoimmune' CAH before and during immunosuppressive therapy. Dotted lines indicate upper reference limit (N) and 2 N.

Correlations

ALT-, AST-, bilirubin-, and gammaglobulin values were related to each other and to parameters of liver histology in the 21 patients before institution of therapy. In untreated IAI-CAH the correlations between histological parameters, between serum biochemical parameters, and between histological and biochemical parameters are shown in tables 8.1, 8.2 and 8.3 respectively. No other correlations were detected.

Table 8.1. Correlations among histological parameters in untreated IAI-CAH.

		PP	L	lympho	pmn	eo	plasma
HAS	R	.92	.77	.62	.54		.56
	p	5	4	2	1		1
P	R				.37		
	p				0		
PP	R		.64	.66	.49		.45
	p		3	3	1		0
L	R			.55	.39		.60
	p			1	0		2
lympho	R					.45	.46
	p					1	1

R=Spearman's rank correlation
p-values: 0:<.10, 1:<.05, 2:<.01, 3:<.005, 4:<.001, 5:<.0005, 6:<.0001.
Abbreviations: (see also text). Lympho=lymphocytes; pmn=polymorphonuclear leucocytes; eo=eosinophils; plasma=plasma cells.

Table 8.2. Correlations between histological and serum biochemical parameters in untreated IAI-CAH.

		ALT	AST	GG	CB	TB
HAS	R	.41				
	p	0				
PP	R	.41				
	p	0				
L	R			.50		
	p			1		
lympho	R	.51	.52	.63	.43	.43
	p	1	1	2	0	0
plasma	R	.48				
	p	1				

R=Spearman's rank correlation
p-values: 0:<.10, 1:<.05, 2:<.01, 3:<.005, 4:<.001, 5:<.0005, 6:<.0001.
For abbreviations: see text and table 8.1.

Table 8.3. Correlations among serum biochemical parameters in untreated IAI-CAH.

		ALT	CB	UB	TB
AST	R	.82	.80	.66	.81
	p	5	4	3	4
ALT	R		.58	.51	.60
	p		1	1	2
CB	R			.84	.99
	p			5	6
UB	R				.89
	p				5

R=Spearman's rank correlation
p-values: 0:<.10, 1:<.05, 2:<.01, 3:<.005, 4:<.001, 5:<.0005, 6:<.0001.
Abbreviations: see text.

DISCUSSION

With standardized immunosuppression complete (HAS≤1), partial histological remission (HAS≤3) was achieved in 90% of patients with previously untreated idiopathic 'autoimmune' CAH requiring such therapy.
Our results therefore confirm the benefits of standardized immunosuppressive therapy in this category of CAH, the anti-HBs positive patients included.[538] However, a close follow-up is mandatory, since the current study also confirms that some patients are - partially- resistant to immunosuppressive therapy,[550] as illustrated by the two patients with no or only partial improvement in histology.
On light microscopy, the inflammatory infiltrate consisted predominantly of cells with the morphology of lymphocytes and plasma cells (IgG-type). Eggink recently showed that the former are predominantly CD8-positive cytotoxic/suppressor cells or killer/natural killer (OKM1,2+ and CD1-,CD3-) cells.[551]
Histology continued to improve during the 26 month period of immunosuppression, with progressive reduction of inflammation due to disappearance of the many plasma cells and of the few granulocytes, and with reduction in the number of lymphocytes. This observation supports the policy of continued immunosuppression for two years before considering a change in therapy.

It is well known that relapse rates can be high, especially after discontinuation of therapy.[544,552] Investigators from the Mayo Clinic observed relapse rates of 30% in patients who reverted to normal liver biopsies, but 80% if residual portal hepatitis was present at the time of drug withdrawal[553,554]. We therefore distinguished complete (HAS≤1) from partial (HAS≤3) histological remission. Interestingly, in 80% of the patients, such a complete histological remission was achieved with the standardized therapeutic regimen. HAS≤1 may be one of the requirements that have to be fulfilled before attempting to discontinue therapy. Although this is speculative, another requirement may be the absence of plasma cells in the liver biopsy, since their presence might reflect the potential for antibody-dependent cellular cytotoxicity, which plays a role in 'autoimmune' CAH.[555]

CAH is defined principally by its histological activity rather than by structural changes, such as fibrosis and cirrhosis. In the current study, histological scores of cirrhosis/ fibrosis (HFS) did not differ between 0 and 2, 2 and 12, and 12 and 24 months of immunosuppression. This could be the result of the decrease in inflammation and hence fibrogenesis after the institution of immunosuppression. Another explanation may be the considerable degree of fibrosis/cirrhosis in most patients before immunosuppression -this was confirmed laparoscopically, as biopsies show a considerable sampling error in this respect.[556]

During active disease, the increase in serum conjugated bilirubin values was more pronounced and frequent than that of unconjugated bilirubin levels. This may indicate that the uptake of bilirubin by the hepatocytes is less disturbed than the excretion of conjugated bilirubin in CAH. Both normalized rapidly during therapy, even in the patient whose histology failed to improve.
In accordance with the literature,[557] serum ALT, gammaglobulin and bilirubin, using standard reference ranges, did not accurately predict histological activity (table 8.2). This may be partly due to differences in long-time after the start of immunosuppression. Follow-up biopsies to monitor inflammatory activity are therefore needed as long as no better serological parameters of activity are available. In a biopsy of sufficient size,[558] sampling error for determination of inflammatory activity in CAH is less than 10%,[559] and intra-observer error less than 6%,[559] while in other forms of liver disease the inter- and intra-observer variability in grading the degree of piecemeal necrosis is greater than 50% and 40%, respectively.[560]

The patients in this study underwent a control biopsy after 2 months of standardized therapy, with subsequent biopsies after one and two years of therapy. As long as no non-invasive means for assessing histological activity have been established, it may be advisable to continue induction-therapy with 15 mg prednisolone and 75 mg azathioprine for at least two months, with a control liver biopsy after two months of therapy. The two month period is appropriate, since major reductions in inflammatory activity

take place in the first two months of therapy in responding patients. This allows therapy failure to be identified.
If after two months, the HAS is still more than 3 (despite improvement), one might continue induction-therapy for another two months, with a further biopsy at the end. If, however, there is worsening despite therapy, especially if there is a ≥67 percent increase in serum bilirubin and/or AST (ALT) level, the first step is usually to double the dose to 30 mg prednisolone in conjunction with 150 mg azathioprine daily.[550]
Further liver biopsies in patients with a HAS≤3 at two months can probably be limited to cases of major worsening of serum biochemistry, or before attempting to discontinue therapy. This is justified, since relapse during unchanged therapy was unusual after induction of remission.
Many of these control liver biopsies may become superfluous if new serological markers of disease-activity can be found.

In conclusion, results from the current study indicate that standardized immunosuppression with a fixed dose schedule improved histology in 95%, with induction of 'complete histological remission' (HAS≤1) in 80% and 'partial histological remission' (HAS≤3, but >1) in 10% of the patients with moderate to severe 'autoimmune' CAH. We propose that HAS≤3 is an objective of therapy, while a HAS≤1 and the absence of plasma cells in the biopsy may be required before an attempt at discontinuation of therapy can be successful. However, determination of satisfactory endpoints for treatment and establishing of optimal guidelines for discontinuation of therapy remains a nagging clinical problem.[561]

CHAPTER 9

REPAIR AND NORMALIZATION OF HEPATIC FUNCTIONAL CAPACITY OF PROTEIN SYNTHESIS, DESPITE PRESENCE OF CIRRHOSIS IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

SUMMARY

We evaluated the effect of immunosuppressive therapy on serum parameters of hepatic protein synthesizing capacity in 'autoimmune' chronic active hepatitis (CAH) in relation to histological activity of disease. Serum levels of albumin (ALB) were abnormal in 71% , of cholinesterase (CHE) in 76%, and antithrombin III (AT III) in 82%. Prothrombin time (PT) was abnormal in 42% and activated partial thromboplastin time (APTT) in 74% of the patients before therapy. All parameters improved during the first 14 months of therapy and normalized in all patients during two years of standardized immunosuppressive therapy with prednisolone and azathioprine. This was despite the presence of advanced fibrosis/cirrhosis in 20/21 of the patients at the start. CHE levels even increased during the second year of therapy.

Hepatic protein synthesizing capacity normalized in all patients with 'autoimmune' CAH in the course of two years of immunosuppressive therapy. CHE appears to be the most useful parameter for the routine follow-up of hepatic protein synthesizing capacity in CAH, while the other parameters can provide additional information. Normalization of hepatic protein synthesizing capacity is an important endpoint of therapy. Its normalization can take two years, probably reflecting ongoing repair and regeneration. This supports our current practice of continuing standardized immunosuppression for two years before considering an attempt to withdraw therapy.

INTRODUCTION

In HBsAg-negative chronic active hepatitis (CAH) protein synthesizing capacity of the liver can be severely impaired, as reflected in a low serum albumin (ALB) and a prolonged prothrombin time (PT),[562-565] especially if cirrhosis is present.[566] Hypo-albuminaemia and a prolonged PT were recognized with similar frequency in patients with and without extreme (>1000 IU/l) aminotransferase elevation.[567] Patients who sustain remission cannot be discerned from those who relapse after first treatment on the basis of serum albumin values.[568]

Serum cholinesterase (CHE) is a glycoprotein with a molecular weight of 350 kD, predominantly derived from pseudocholinesterase, which is exclusively synthesized in

the liver.[569,570] Its hypothesized function is to prevent inhibition of acetyl-cholinesterase.[571-573] Reduced serum concentrations of CHE have been observed frequently in patients with CAH[574] and in other forms of hepatocellular damage,[575,576] almost paralleling reductions in albumin,[577,578] while a normal CHE virtually excludes severe impairment of liver function.[579] A sudden drop in CHE can precede fulminant hepatic failure with hepatic coma in acute and chronic hepatitis.[574] The preoperative serum CHE level correlates with outcome of portocaval surgery in liver disease,[576] and monitoring of CHE serum levels proved useful in the follow-up after liver transplantation.[578,580] CHE levels, like the prothrombin index, correlated highly with the Child-Turcotte score,[581] and are of prognostic significance.[582,583]

Antithrombin III (ATIII) is a glycoprotein, a protease inhibitor, exclusively synthesized in the liver. Its levels, like those of CHE, are prognostic factors for blood loss during liver transplantation,[584] and ATIII levels have been shown to be reduced in liver cirrhosis in parallel to serum albumin values.[585-589] These reductions in ATIII levels contribute to an increased fibrinogen turnover, which might reflect low-grade intravascular coagulation.[590]

Early death in untreated CAH is usually caused by liver failure, while late mortality is more frequently due to complications of cirrhosis.[591] In idiopathic 'autoimmune' CAH, immunosuppressive therapy markedly enhances remission rates and survival, [592-594] although cirrhosis is frequently present at the time of diagnosis.[595] Indeed, the presence of cirrhosis before the institution of therapy has been shown to adversely affect the response to treatment[596] and the likelihood of survival without hepatic failure,[594] although the development of cirrhosis during immunosuppression does not affect survival.[597] The conclusion is that the gain in survival appears predominantly to be the result of improved liver function. Our knowledge about changes in liver function during treatment of 'autoimmune' CAH is limited, however.

Elevated serum bile acid levels (SBA) in CAH were reported to normalize with achievement of remission,[598] and SBA levels appeared to correlate with tests such as bromosulfophtalein, and indocyanine green and the aminopyrine breath test.[599,600] However, the value of these tests in assessing liver function in clinical practice remains to be established.

With regard to the hepatic protein synthesizing function, Soloway *et al.* showed that an abnormal PT occurs in about 40% of the CAH-patients before institution of therapy with prednisone and azathioprine, with normalization within three months of therapy. Albumin levels were reduced in 80% of these patients, with normal values in half of the 14 patients after one year of therapy.[592]

The purpose of the current study was to evaluate the effect of standardized immunosuppressive therapy on serum levels of albumin (ALB), cholinesterase (CHE) and antithrombin III (AT III), plasma prothrombin time (PT) and activated partial thromboplastin time (APTT), as parameters of hepatic protein synthesizing capacity in patients with 'autoimmune' CAH.

MATERIALS AND METHODS

Patients

We studied 21 untreated patients with idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) - 3 of them with antibodies to hepatitis B surface antigen: 4 men and 17 women. These patients required immunosuppressive therapy according to accepted criteria.[592] Their median age was 52 years (range 16-71), and median duration of disease 75 days (range 0 months to 3 years). All were subsequently treated with oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (2 patients) or two years (19 patients) of therapy with 10 mg prednisolone and 50 mg azathioprine daily. Serial routine liver biopsies and serum and plasma biochemistry were studied at the start and at two months, one and two years of therapy. One biopsy after 2 months, one at 14 months and 6 at 26 months were missing, although biochemistry was available. Two of the patients received a sublingual vitamin K analogon (10 mg daily) before and during the first three weeks of therapy, and analysis of start values of these two patients were performed after two and three weeks respectively of such therapy.

Liver histology

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[601] In contrast to the original system,[602] fibrosis/cirrhosis is not included in the HAS. The HAS is expressed as a numerical score (0-18), and is the summation of portal (score 0-4), periportal (score 0-10) and lobular (score 0-4) inflammation and necrosis.

Serum and plasma biochemistry

Serum and plasma samples were obtained on the same day on which the liver biopsies were performed. Alanine aminotransferase (ALT) (upper reference limit 30 U/l), total and conjugated bilirubin (UB and CB, with upper reference limits of 26 and 5 µmol/l respectively), and albumin (ALB)(lower reference value 40 g/l) were determined automatically (Technicon sequential multiple analyzer plus computer [SMA-C]). Unconjugated bilirubin (UB) values were calculated by subtracting CB from TB (upper reference value for UB was 21 µg/l).

Prothrombin time (PT) (upper reference limit 13 seconds), and activated partial thromboplastin time (APTT) (upper reference limit 13 seconds) were determined in plasma by Coagalyzer (Sherwood) using automated APTT (General Diagnostics) and Activated Thromboplastin (Dale) reagents, while antithrombin III (AT III) (lower reference limit 80%), was measured in plasma using a chromogenic substrate (S-2238 Kabri) as described.[603]

The serum cholinesterase concentration (CHE) was determined by measuring its catalytic activity, using acetylthiocholine iodide as a substrate[604] in a commercially available kinetic test (Boehringer Mannheim GmbH). When not determined immediately, stored (-20°C) and coded sera were used. Repeated freezing and thawing does not influence results.[605] Results of this assay are comparable to those of a more recently described assay.[606,607] For the reference values of CHE, we used the sera of 77 controls (41 women and 36 men) with apparently normal health: the lower reference limit of CHE is 1600 U/l for women and 1800 U/l for men.[605] We also compared values obtained before and during immunosuppression in patients to CHE values in 68 sex-matched controls out of this reference population.

Apart from giving absolute results, for reasons of comparison, values of ALB, CHE and AT III are also expressed as a percentage of the lower limit of the normal range. PT and APTT are always expressed as percentages of the upper limit of the normal range of standard control sera determined on the same day.

Statistical analysis

Non-parametric statistical analysis was performed with Friedman's analysis of variance (ANOVA), Wilcoxon's matched pairs test, Mann-Whitney U test and Spearman's rank correlation where appropriate, with $p<0.05$ as level of significance.

RESULTS

All parameters under investigation, except HFS, improved during immunosuppressive therapy (ANOVA $p<.01$).

Liver histology

As shown in chapter 8, initial scores (0-4) of hepatic fibrosis/cirrhosis (HFS) were high (median 3), and did not change during the 26 months of immunosuppressive therapy (median 3.5, 3, and 3 at 2, 14 and 26 months respectively) ($p=.938$ in ANOVA) (figure 9.1).

As shown in figure 9.2, the histologic activity diminished significantly from median 9 to 3 within 2 months ($p<.001$), with further improvement to median 1 in the next two years of therapy ($p<.05$), and stabilization thereafter. 19 (90%) of the patients achieved a HAS≤3, while 80% achieved a HAS≤1. Regarding histology, one patient was therapy-resistant, while a persisting HAS=10, and another patient improved to HAS=4.

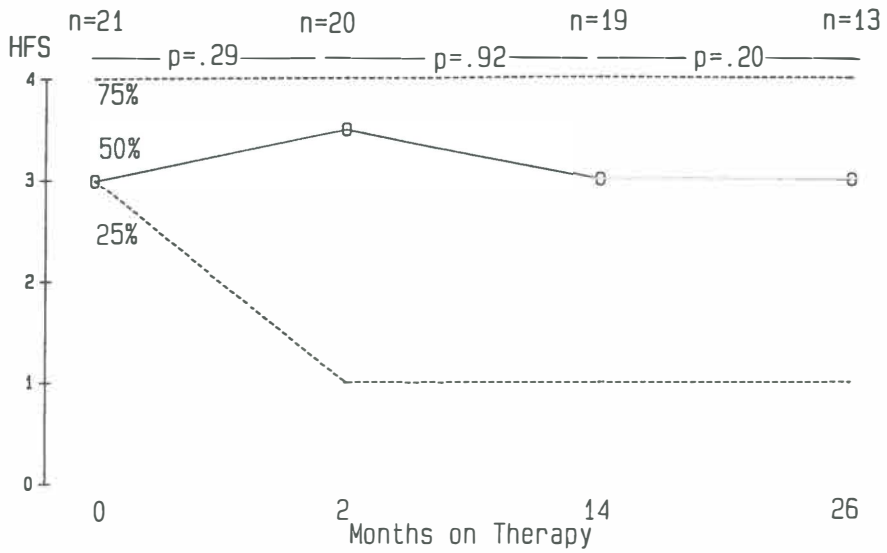


Figure 9.1 HFS in IAI-CAH before and during immunosuppression.

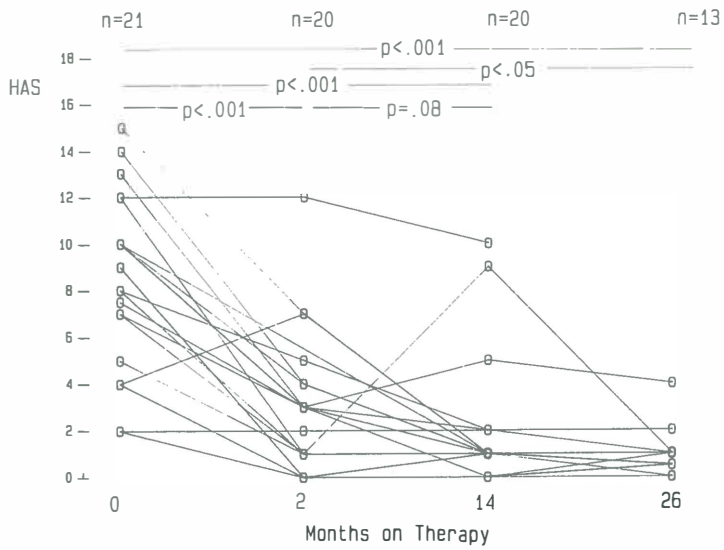


Figure 9.2 HAS in 'autoimmune' CAH before and during immunosuppression.

Serum and plasma biochemistry

For results of routine biochemistry the reader is referred to chapter 7.1. Results for ALT and UB are shown in figure 9.3A and 9.3B respectively.

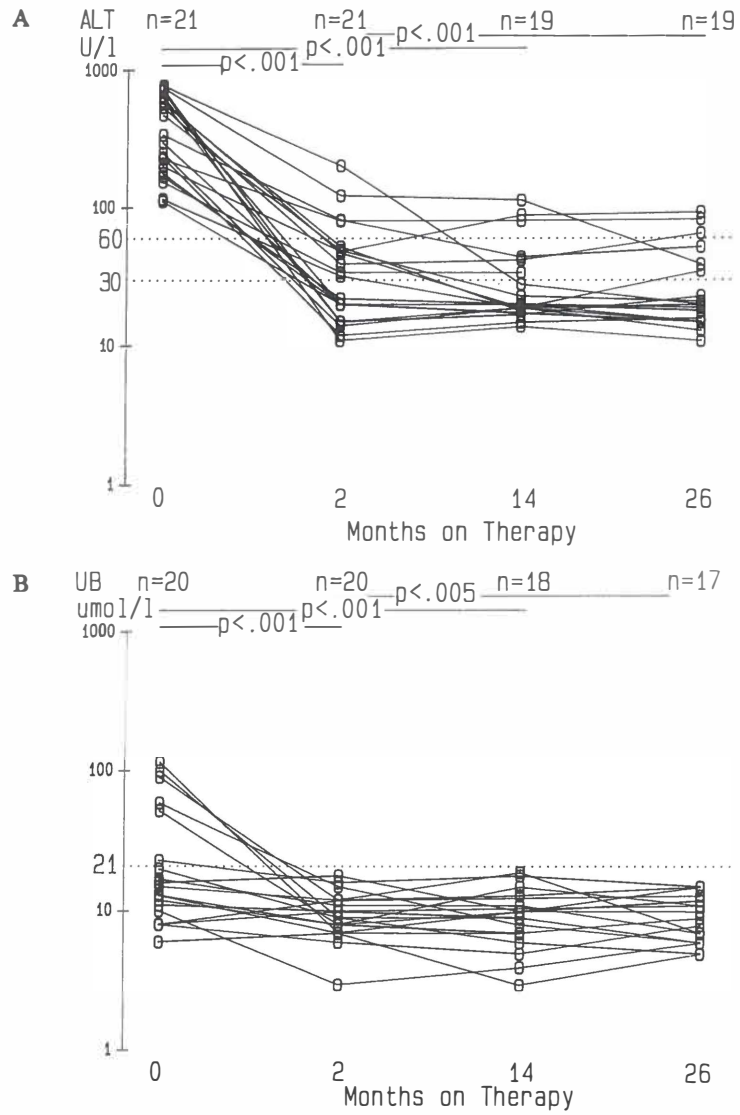


Figure 9.3 A) ALT and B) UB in 'autoimmune' CAH before and during immunosuppression.

ALB serum levels increased from median 32 to 37 g/l after the first two months ($p<.05$), to median 42 g/l after the next year ($p<.001$), stabilizing at 42 g/l after the second year ($p=.18$). Normal values were found in all but three patients (with near-nomal values of 38, 38 and 39 g/l) at the end of this study (figure 9.4A).
CHE serum levels increased from median 1185 to 1698 U/l after the first two months ($p<.01$) and to median 2323 U/l after the next year ($p<.001$). Levels increased to 2514 U/l after the second year ($p<.05$), with normal values in all patients within two years of therapy when compared to the 77 controls (figure 9.4B).

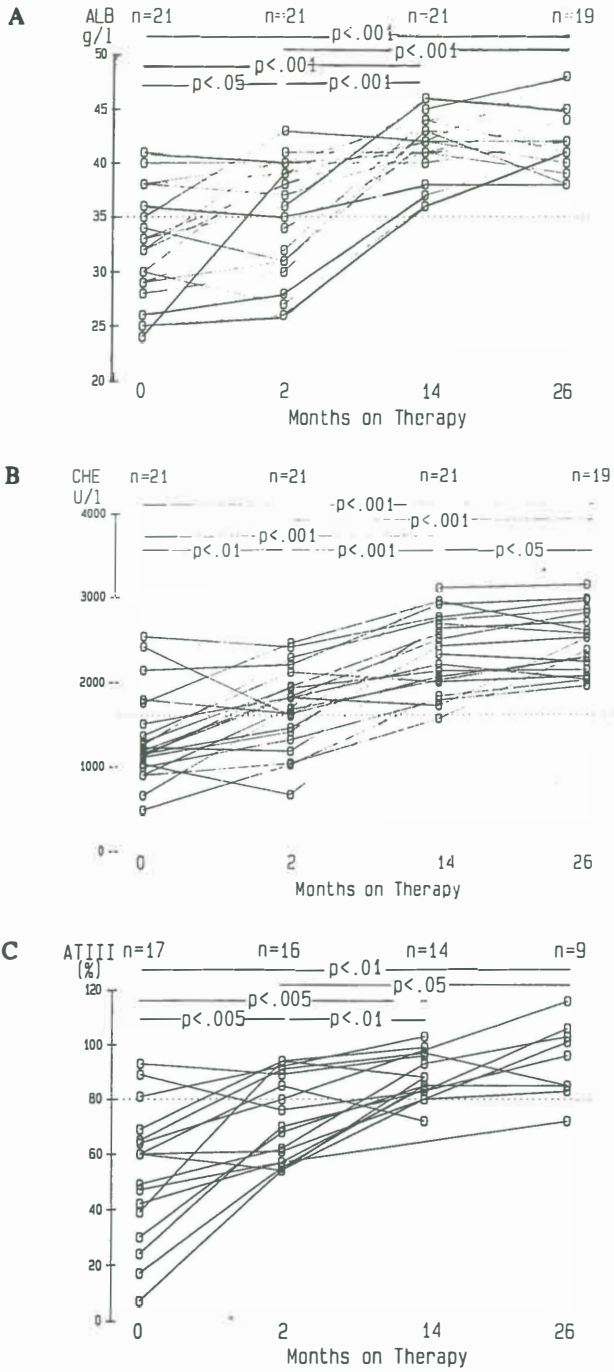


Figure 9.4 A) ALB, B) CHE and C) ATIII in 'autoimmune' CAH before and during immunosuppression. Dotted line indicates lower limit of reference range.

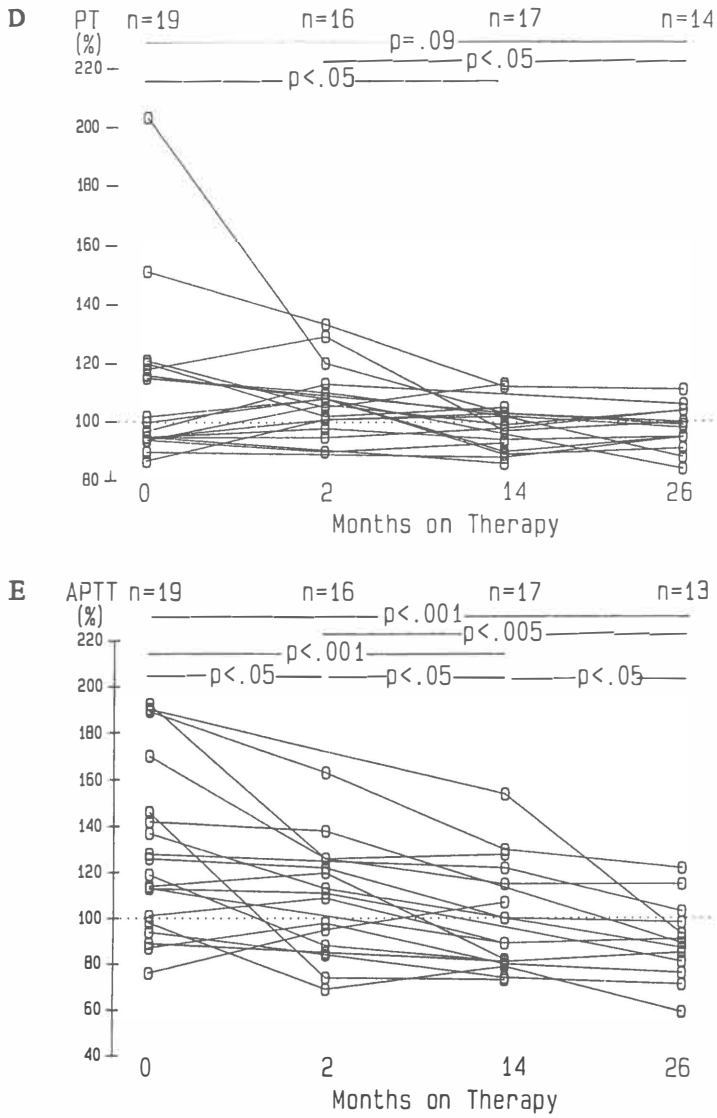


Figure 9.4 D) PT and E) APTT in 'autoimmune' CAH before and during immunosuppression. Dotted line indicates upper limit of reference range.

Compared to the 68 sex-matched controls CHE values in the IAI-CAH patients were below reference values at the start of therapy ($p<.0001$), after 2 months ($p<.0001$), and after one year ($p<.01$). However, CHE values did not differ significantly from reference values at two years of immunosuppression, although values were on the lower side of the reference range ($p=.06$).

ATIII plasma levels (n=17) increased from median 60% at the start to 69% after the first two months (p<.005), to median 87% after the next year (p<.01),and a median value of 96% after the second year (p=.23). Normal values were found in 16 (94%) out of 17 patients we could evaluate for this parameter within two years of therapy, while one patient had improved from an ATIII of 42% at start to 72% after two years (figure 9.4C).
There is a trend towards worsening of PT after the institution of therapy (p=.07), with a peak between 15 and 30 days after start. PT improved from median 100% at start to median 98% during the first 14 months (p<.05), and from median 105.5 at two months to median 98.5 at 26 months (p<.05). However, there were no major changes between values at start and after two years (p=.14), and Friedman ANOVA did not show changes during therapy either (figure 9.4D).
The APTT fell from median 119% to 110% after two months (p<.05), with further improvement in the next year to median 89% (p<.05), and a trend to further change in the next year (median 91%) (p=.06) (figure 9.4E).

Correlations

All parameters were related to each other and to parameters of liver histology.
In untreated IAI-CAH, the correlations between histological parameters, and between serum and plasma biochemical parameters, and between biochemical parameters is shown in tables 9.1 and 9.2 respectively. No other correlations were detected.

Table 9.1 Correlations in untreated IAI-CAH between parameters of serum/plasma biochemistry and histology (Spearman rank)(p<0.05).

	ALT	UB	CB	TB	ALB	CHE	ATIII	PT	APTT
P									
PP	.41								
L								.51	
HAS	.41								
HFS									.56

Table 9.2 Correlations in untreated IAI-CAH between parameters of serum/plasma biochemistry (Spearman rank)($p<0.05$). (open spaces indicate lack of significant correlation)

	AST	ALT	UB	CB	TB	ALB	CHE	ATIII	PT
AST		.82	.66	.80	.81		-.54		
ALT	.82		.51	.58	.60				
UB	.66	.51		.84	.89	-.42	-.31	-.54	
CB	.80	.58	.84		.99	-.62	-.56	-.56	
TB	.81	.60	.89	.99		-.58	-.55	-.56	
ALB			-.42	-.62	-.58		.74	.78	-.57
CHE	-.54		-.31	-.56	-.55	.74		.65	
ATIII			-.54	-.56	-.56	.78	.65		-.81
PT						-.57		-.81	
APTT				.44	.42	-.70	-.53	-.75	.55

DISCUSSION

Despite the presence of advanced stages of fibrosis/cirrhosis in all of these CAH-patients, standardized immunosuppression was not only able to induce remission, but induced restoration of functional hepatic protein synthesizing capacity. In this regard the results of such therapy in these patients are comparable to those obtained with liver transplantation.[578]

Prothrombin time (PT) tends to worsen immediately after institution of therapy, then improves (data not shown). This may be due to direct inhibitory effect of corticosteroids on synthesis of some clotting factors. Alternatively, there may be an imbalance among clotting factors during restoration of protein synthesis with, for instance, the disappearance of the stimulation of synthesis of coagulation factors like fibrinogen which may be present during the acute phase response. Glucocorticosteroids also have a direct effect on some coagulation tests, resulting in an increase in PT and ATIII, and a decrease in APTT.[607a] A relative shortage of vitamin K immediately after improvement of protein synthesis may also play a role. However, this would probably have affected the APTT more than PT. It may be worth considering routinely adding sublingual vitamin K (e.g. 10 mg daily) to the medication in the first two months of therapy.

The synthesis of ALB can be inhibited by several cytokines like interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumor necrosis factor α (TNFα).[41-45] Experiments with primary cultures of human hepatocytes and human hepatoma cells showed that Il-1ß, IL-6 and TNF-α inhibit liver synthesis of albumin at the transcriptional level in an

additive manner, with IL-6 as the principal inhibitor,[610,611] although monocytes can also produce inhibitory activity to IL-1ß.[613,614]

The normalization of hepatic functional protein-synthesizing capacity in severe CAH usually takes about two years, and therefore appears to be predominantly the result of repair and regeneration of liver tissue. Because CHE, like albumin,[615] is a negative acute phase reactant, the increment (particularly initially) in CHE levels during therapy may also result from a decline in titres of e.g. IL-1ß, IL-6 and TNFα. The same applies to the coagulation proteins involved in the APTT. Glucocorticoids can also selectively inhibit the transcription of the IL-1ß gene and decrease the stability of IL-1ß mRNA.[616] Furthermore, the rise in ALB, CHE and coagulation proteins may partly result from a direct stimulation of protein synthesis by corticosteroids, as is also observed in hepatocyte cultures.[615]

Determination of the PT appeared to be of limited value in CAH. On the other hand, CHE continues to improve, and APTT tends to improve, even during the second year of immunosuppressive therapy, while the other parameters of protein synthesis stabilize during this second year. This may indicate that CHE is a very sensitive parameter of protein synthesis, and is therefore ideal for the follow-up of hepatic protein synthesizing capacity in CAH.

There are well known conditions with elevation or highnormal levels of CHE, such as fatty liver, functional hyperbilirubinaemia, diabetes mellitus, hyperlipidaemia, nephrotic syndrome, protein losing enteropathy, and sometimes in thyrotoxicosis. These elevated serum levels can also be present in patients with familial hypercholinesterasaemia.[569] In the current study, no such influences were detected. Serum bilirubin levels, if elevated at all, normalized within two months, excluding an influence on CHE levels from the second month of therapy onwards. No elevation of CHE during remission from CAH was detected, not even in the one patient with corticosteroidinduced diabetes mellitus after two months of therapy. A lowering influence of oral contraceptives on CHE levels,[617,618] AT III (as much as 60% of its normal value),[619] and albumin[617] was described in healthy women. We did not detect such an influence on CHE values.[605] Since patients with CAH are usually advised not to use such medication, this is not a common problem when interpreting ATIII levels. Higher CHE levels during pregnancy were reported from the third month until six weeks after delivery.[572]

Serum albumin levels can be influenced by many other factors, as recently decribed.[620,621] High levels of albumin synthesis can be observed in the presence of ascites. In these patients, the serum albumin level is usually not a reliable index of the hepatic albumin synthesizing capacity. In non-ascitic cirrhotic subjects, however, a depressed serum albumin reflects decreased hepatic protein synthesis.[622]

Malnutrition is common in patients with severe liver disease. This can cause serum concentrations of albumin to be low.[622] Unpublished observations have found parallel changes in CHE and AT III. The improval of nutritional status during immunosuppressive therapy can also account for part of the increase in hepatic protein synthesis.

CHE levels vary considerably between individuals, but in any one person the level

remains remarkably constant from the third month after birth onwards, so that slight variations, even in the 'normal' area, may indicate changes in liver function[572,580]. This, combined with its short plasma half-life of 10 days, and with the ease, low cost and rapidity of the assay make CHE an essential parameter in the follow-up of CAH. The other parameters of protein synthesis provide complemetary information, and may be determined at larger intervals during follow-up of CAH.

To conclude, the protein synthesizing capacity of the liver of patients with 'autoimmune' CAH can normalize despite the presence of advanced fibrosis/cirrhosis. In this respect, the standardized immunosuppression mentioned yields results equivalent to those of a succesful liver transplantation. This is in accordance with the knowledge that patients with 'autoimmune' CAH do not usually require liver transplantation if therapy failure (especially if combined with endogenous hepatic encephalopathy, severe hepato-renal syndrome with ascites or recurrent variceal hemorrhage) does not complicate the disease.[623,624] Follow-up of hepatic protein synthesizing capacity in CAH is of major importance. CHE appeared to be very useful for routine follow-up determinations. Normalization of protein synthesizing capacity is an important endpoint of immunosuppressive therapy in 'autoimmune' CAH. It may require two years of therapy to achieve this goal, indicating that repair and restoration of liver function may take this long, but can eventually be achieved in all patients. This supports our current practice of continuing standardized immunosuppression for two years before considering an attempt to withdraw therapy.

CHAPTER 10

ACUTE PHASE REACTANTS, C-REACTIVE PROTEIN AND SERUM AMYLOID A, IN CHRONIC ACTIVE HEPATITIS

Relation to Histology and Synthesizing Capacity of the Liver and Influence of Immunosuppressive Therapy.

ABSTRACT

The liver is the major site of production of acute phase proteins. We studied the acute phase proteins C-reactive protein (CRP) and serum amyloid A (SAA) in patients with active chronic inflammation of the liver, the main organ producing CRP and SAA. In 81 untreated patients with histologically proven chronic active hepatitis (CAH), the CRP serum levels were only elevated slightly in 54 (67%) and SAA in only 12 (15%) patients. The CRP values were higher than the basal values of healthy controls ($p<.0001$); this was true for all CAH patients irrespective of aetiology. SAA values in CAH did not differ from basal control values. CRP serum levels correlated with periportal ($R=.39$, $p<.05$) and overall ($R=.40$, $p<.05$) inflammation, but not to portal or lobular inflammation ($n=29$). SAA values ,however, were correlated to serum cholinesterase (CHE) levels ($R=0.61$, $p<.005$), but not to inflammation ($n=29$). The effects of prednisolone and azathioprine on CRP and SAA in relation to CHE and histology were evaluated in 21 patients during two years of standardized treatment. Histologic activity as measured by the Histological Activity Score diminished significantly from median 9 to 3 within 2 months ($p<.001$), with further improvement to median 1 in the next two years of therapy ($p<.05$), and stabilization thereafter. CHE serum levels increased from median 1185 to 1698 U/l after the first two months ($p<.01$) and to median 2323 U/l after the next year ($p<.001$), and 2514 U/l after the second year ($p<.05$). In all patients, CHE values normalized within two years of therapy. CRP levels, median 7.1 mg/l at start, fell to normal (median 0.9 mg/l) within two months ($p<0.0005$), and stabilized thereafter. SAA increased from median 1.1 mg/l before therapy to 2.1 mg/l after one year of therapy ($p<0.05$), with stabilization thereafter.
To conclude, CRP and SAA responses appear to be markedly depressed in CAH. CRP did reflect histological inflammation to a minor degree, while SAA did not. Impairment of hepatic capacity to synthesize proteins apparently does affect SAA more severely than CRP. Immunosuppressive therapy resulted in a histology of 'CAH in remission' and normal CHE levels, thus leading to a downward regulation of the CRP-response, and a slight increase in SAA values.

INTRODUCTION

Monitoring the acute phase response (APR) is widely accepted as a valuable clinical tool in evaluating the activity of acute and chronic inflammatory diseases. The onset, magnitude and duration of the response varies considerably among the various acute phase proteins. Notably C-reactive protein (CRP) and serum amyloid A (SAA) can increase several hundredfold, thereby exceeding all other acute phase plasma proteins .[625,626]

CRP is synthesized exclusively in the liver,[627] although one report presents evidence for synthesis and secretion by cultured human T-lymphocytes.[628] The function of CRP may be bactericidal, by causing bacterial capsular swelling, promoting agglutination and complement fixation, and enhancing the phagocytosing capacity of neutrophils.[625] Degradation of CRP by lysosomes of neutrophils results in tuftsin-like peptides modulating the function of the neutrophils.[629,630]

The major site of SAA production is the liver,[631] although SAA-specific mRNAs have also been detected in a variety of extrahepatic tissues.[632-634] Although there is some speculation, the function of SAA remains unclear. An immunosuppressive role in the level of the T-lymphocyte macrophage interaction has been suggested,[635] while others showed that the SAA-HDL_3 complex binds to neutrophils in vitro and may modulate the function of these cells.[636,637] About 4-18 hours after tissue injury, both SAA and CRP plasma levels start to rise, reaching a peak value after 24-72 hours, with a subsequent rapid fall when the provoking stimulus has disappeared.[625,638,639] In chronic inflammatory conditions like rheumatoid arthritis and Crohn's disease, however, SAA and CRP levels remain elevated and fluctuate with disease activity.[625]

Although CRP measurements are very practical for routine use, considerable differences may exist in the response of CRP and SAA among diseases.[640] In some diseases SAA may be a more sensitive -although perhaps less specific- index of disease activity than CRP.[641-643]

A complicated situation exists in hepatitis, for here the major producing organ of acute phase proteins is affected. Most data deal with acute hepatitis, where the acute phase response (APR) appears to be depressed.[645-647] Low levels of haptoglobulin and increased serum levels of α_2-macroglobulin have been descibed in chronic hepatitis.[648,649] Elevated CRP levels were reported in only 0-24%,[649-651] and increased SAA values were found in about 35% of patients with CAH.[651]

Serum cholinesterase (CHE) is a direct marker for hepatic protein synthesizing capacity. In patients with hepatocellular damage, CHE is frequently reduced, almost paralleling reductions in albumin, while a normal CHE virtually excludes severe impairment of liver function.[652-656]

In order that we may investigate CRP and SAA responses in CAH, we studied these acute phase reactants in patients with clinical, biochemical and histologic features of CAH, but with differences in aetiology (n=80). In 23 of these untreated patients with

idiopathic 'autoimmune' CAH without anti-HBs, and 5 with anti-HBs, the overall grade of histologic activity and the degree of inflammation in different zones of the liver were related to the serum levels of both CRP and SAA. Both acute phase proteins were also related to serum CHE. In 20 of these CAH patients, the effect of two years of immunosuppression was evaluated by serial study of histology, CHE, CRP and SAA.

MATERIALS AND METHODS

Patients

We studied 81 untreated patients (32 men and 49 women) with histological, clinical and biochemical features of chronic active hepatitis (CAH), with a median age of 49 years (range 11 to 81), and aetiologies as shown (table 10.1).

Table 10.1. CAH-patients: Aetiology.

Aetiology of CAH		all	fig 10.2*	fig 10.3**
IAI, anti-HBs negative		36	24	18
IAI, anti-HBs positive		6	5	3
HBsAg-positive		19		
Hepatitis C (non-A,non-B)		5		
CAH/PBC 'overlap'		4		
CAH/PSC 'overlap'		1		
AAT-CAH		1		
'CAH' due to Wilson's disease		1		
drug-induced 'CAH'	-oxyphenisatin	5		
	-methyldopa	3		
Total		81	29	21

ad *) fig 10.2 denotes patients where CRP and SAA are related to HAS and CHE, as shown in figure 10.2A-D.

ad **) fig 10.3 denotes standardized treated patients with serum samples and biopsies at 0,2 and 12 and/or 24 months, as shown in figure 10.3A-D.

CAH/PBC : 'overlap' with primary biliary cirrhosis ('cholestatic CAH').

CAH/PSC : 'overlap' with primary sclerosing cholangitis.

AAT-'CAH': 'CAH' due to α1-antitrypsin deficiency.

IAI: idiopathic 'autoimmune'.

In 29 out of these patients with idiopathic 'autoimmune' CAH, 5 of them with anti-HBs (5 men and 23 women, median age 53 years, range 11-81), localization and degree of inflammation and fibrosis/cirrhosis in coded routine liver biopsies were numerically

scored, and serum cholinesterase (CHE), was measured. Both were compared with CRP and SAA.

21 out of these 29 patients, 4 men and 17 women, requiring immunosuppressive therapy according to accepted criteria,[657] were studied to evaluate therapy effects. Their median age was 52 years (range 16-71), and median duration of disease 75 days (range 0 weeks to 3 years). All were subsequently treated with oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (14 patients) or two years (6 patients) of therapy with 10 resp. 50 mg daily. Serial routine liver biopsies and sera (CHE, CRP and SAA) were studied at the start and at two months, one and two years of therapy. Two patients had a follow-up of 14 months.

Liver histology

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[658] In contrast to the original system,[659] fibrosis/cirhosis is not included in the HAS. The HAS is expressed as a numerical score (0-18), and is the summation of portal (score 0-4), periportal (score 0-10) and lobular (score 0-4) inflammation and necrosis.

Serology

All serum samples were immediately frozen and stored (-20°C) until analysis. Freezing and thawing did not affect the results of CRP, SAA and CHE,[660,661] and this is one of the reasons why we preferred determination of CHE to that of albumin. Basal control values for CRP and SAA were determined in sera from a group of 50 apparently healthy persons. For the reference values of CHE, we used the sera of 41 women and 36 men with apparently normal health.[661]

Cholinesterase (CHE) concentration was determined by measuring its catalytic activity, using acetylthiocholine iodide as a substrate in a commercially available kinetic test (Boehringer Mannheim GmbH).[662] The lower reference limit of CHE is 1600 U/l for women and 1800 U/l for men.[661]

C-reactive protein (CRP) was quantified by a sandwich ELISA.[663] The intra-assay coefficient of variation (CV) was 4.9% and the inter-assay CV was 5.1%. The basal control values provided a median and mean value of 0.5 mg/l, with a 95 percent upper limit of 2.0 mg/l.

Serum amyloid A (SAA) was quantified by a recently developed sandwich ELISA, with monoclonal anti-human-SAA as a second antibody.[660] The intra-assay CV was 6.0% and the inter-assay CV was 13%. The basal control values provided a median value of 0.7 mg/l, and a mean value of 0.8 mg/l, with a 95% upper limit of 2.6 mg/l.

Statistical analysis

Statistical analysis was performed with analysis of variance (ANOVA) with repeated measurements, the MannWhitney U test, the Wilcoxon matched pairs test and Spearman's rank correlation where appropriate, with $p<0.05$ as the level of significance.

RESULTS

CRP and SAA in untreated CAH in relation to aetiology

In 81 untreated patients with CAH, the CRP serum levels were only slightly elevated in 54 (67%) and SAA in only 12 (15%) of the patients.

Figure 10.1A shows the CRP values and figure 10.1B those of SAA for the different

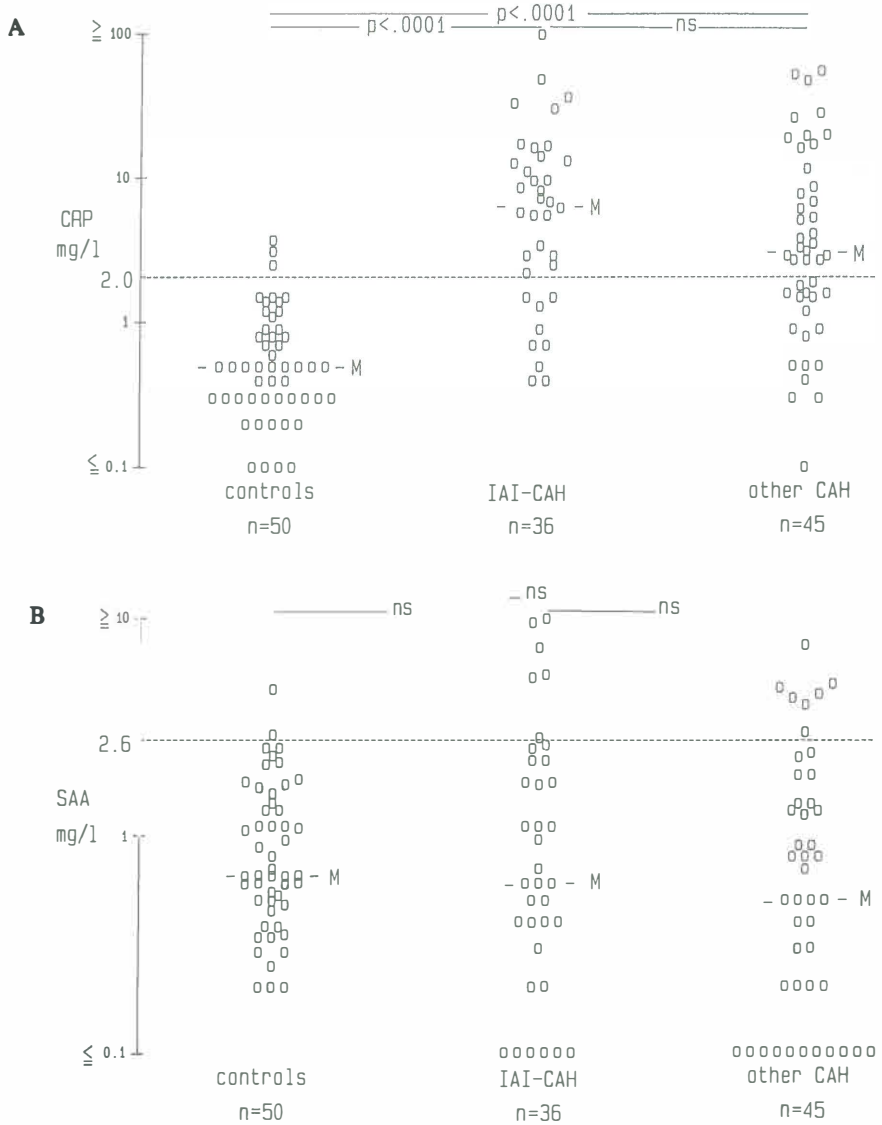


Figure 10.1 A) CRP and B) SAA in CAH and controls. Dotted lines indicate upper reference limits.
M=median. N=upper reference limit.

aetiologic groups. The CRP values in idiopathic 'autoimmune' CAH (IAI-CAH) without anti-HBs (median 6.6 mg/l) and CAH of other aetiology (median 2.9 mg/l) were higher than the basal control values (median 0.5 mg/l) ($p<0.0001$). SAA levels in both CAH groups (medians 0.7 and 0.5 mg/l), however, were not higher than the control values (median 0.7 mg/l) ($p=0.88$ and $p=0.44$).
The CRP-values in patients with anti-HBs negative IAI-CAH did not differ from those of the other CAH patients ($p=0.16$). The SAA values in these patients with IAI-CAH also did not differ from those in the other CAH patients ($p=0.44$).

CRP and SAA in relation to histologic activity and CHE

In 29 of these patients, correlations were made between CRP, SAA and CHE levels and the grade of inflammation in different localizations of the liver (portal, periportal, lobular, and the summation of these), as shown in table 10.2. The only correlations found were between CRP and overall, especially periportal, inflammation ($R=.40$, $p<.05$ and $R=.39$, $p<0.05$ respectively), and between SAA and CHE ($R=.61$, $p<0.005$) as shown in figures 10.2A-D.

Table 10.2. CRP, SAA and CHE versus Histological Activity in CAH

	CRP		SAA		CHE	
	R	p<	R	p<	R	p<
portal (P)	.24	.20	.25	.19	-.05	.78
periportal(PP)	**.39**	**.05**	.09	.65	-.06	.75
lobular (L)	.25	.19	.21	.28	.09	.65
HAS(=P+PP+L)	**.40**	**.05**	.06	.76	**-.10**	**.62**
CHE	-.06	.75	**.61**	**.005**		
SAA	.22	.24				

R= Spearman rank correlation. n=29 p= level of significance ns=not significant

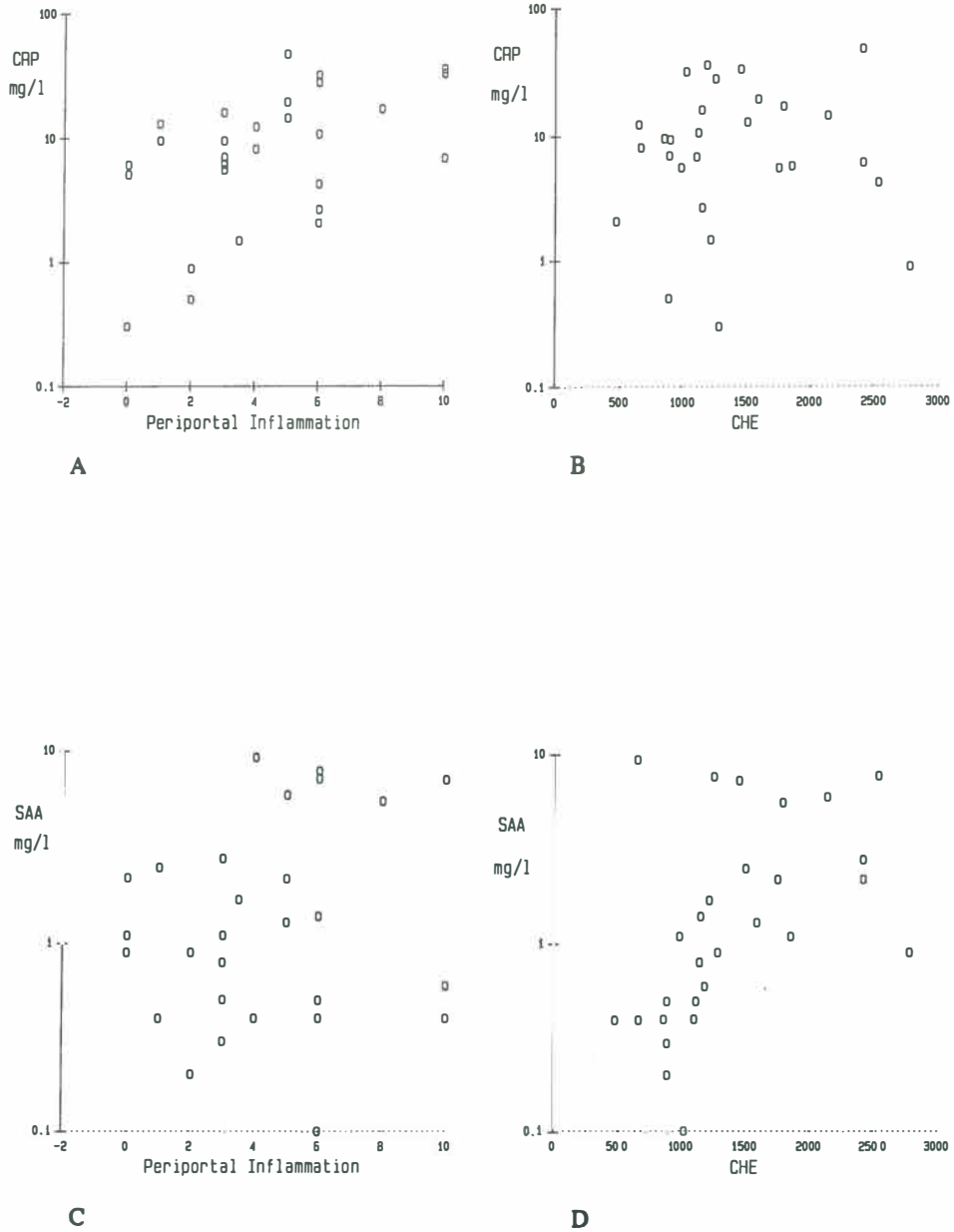


Figure 10.2A-D. CRP and SAA versus periportal inflammation and CHE in CAH.

Effects of standardized immunosuppressive therapy

21 patients (18 IAI-CAH without anti-HBs, 3 anti-HBs positive) maintained on standardized immunosuppressive therapy were fully evaluated with regard to CHE, SAA, and CRP levels and a liver biopsy at the start, after two months and after one and/or two years of therapy.

As shown in figure 10.3A, the HAS diminished from median 9 to median 3 within 2 months of standardized therapy ($p<.001$) and a further improvement in the next two years of therapy to median HAS=1 ($p<.05$). Reductions in periportal ($p<.001$) and lobular ($p<.005$) inflammation were observed in the first two months of therapy, with a further decrease in periportal and a trend to improvement in lobular inflammation in the next two years ($p<.05$ and $p=.09$ respectively). A gradual reduction in portal inflammation occurred in the first 14 months of therapy ($p<.05$).[656] A histology similar to chronic persistent hepatitis ('CAH in histological remission') usually remained, while periportal and lobular inflammation had diminished and (almost) disappeared in patients with HAS≤3.

As shown in figure 10.3B, CHE steadily increased during standardized immunosuppression,[656] from a median value of 1185 U/l at start to 1698 U/l after two months ($p<.01$), to 2323 U/l at one year ($p<.001$) and stabilization at median 2514 U/l after two years ($p=.36$). At the start of therapy, 5 (25%) patients had a normal CHE, while CHE became normal in all 21 patients within two years of therapy.

The CRP values fell from a median value of 7.1 mg/l at the start to 0.9 mg/l at two months ($p<.0005$) and stabilization at a median level of 1.3 mg/l at one ($p=0.11$) and 1.2 mg/l at two years of therapy ($p=1.0$), as shown in figure 10.3C. At the start of therapy, the CRP values were higher than basal control values ($p<.0001$). Compared to basal control values, CRP values did not differ at two months ($p=.24$) and 14 months ($p=.11$), and became slightly higher after two years of therapy ($p<.05$).

SAA values at start (median 1.1 mg/l) and after two months of therapy (median 2.4 mg/l) did not differ ($p=.25$), but there was a slight increase between SAA values at the start and after one year of therapy (median 2.1 mg/l) ($p<.05$), with no change in the second year (median 2.4 mg/l) ($p=.16$) (figure 10.3D). SAA values did not differ from basal control values ($p=0.08$), but they became higher than these values at two months, one and two years of therapy ($p<0.0005$).

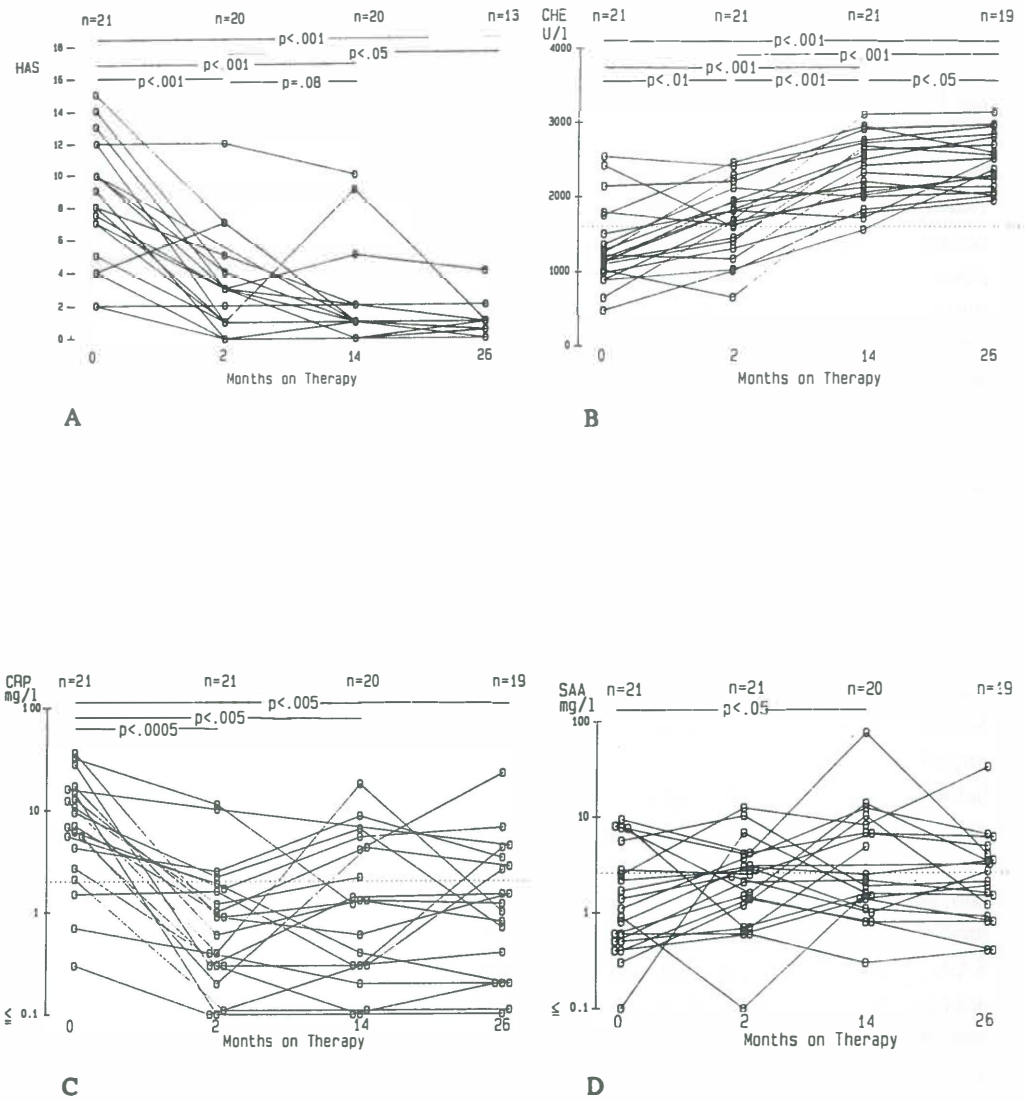


Figure 10.3 A) HAS, B) CHE, C) CRP and D) SAA in CAH before and during standardized immunosuppression. Dotted lines indicate upper (CRP, SAA) or lower (CHE) limits of reference range.

DISCUSSION

Evaluation of the acute phase response in liver disease is complicated by the fact that the liver is the major producing organ of most acute phase proteins (APP), including CRP and SAA.

No differences in CRP serum values in relation to the aetiology of CAH were observed. During more active disease, serum CRP values rise. However, the correlation with periportal hepatitis and piecemeal necrosis is weak and insufficient for clinical purposes. Although CRP acts as an APP in CAH, values lie in a range about two to three times lower than in a randomly selected group of patients with Crohn's disease or rheumatoid arthritis.[660] During immunosuppressive therapy, CRP values normalize in parallel to the decrease in HAS.

The response of SAA is remarkable in contrast to CRP. In untreated CAH, SAA levels do not differ from those in the controls, and there is no correlation with histologic activity.

The normal SAA in CAH may be due to impaired protein synthesis as the result of impaired liver function, as reflected in serum CHE levels. It has been demonstrated that the capacity of the liver to produce most acute phase proteins is well retained, even when the hepatic synthetic capacity is rapidly declining.[646] However, our results show that SAA is an exception to this rule and its reaction in CAH resembles those of serum amyloid P-component (SAP) and haptoglobin.[649,664,665]

Several explanations are possible for the observed profiles in CAH. Apart from the impaired liver function as a cause of an unresponsiveness of SAA, the difference between CRP- and SAA response could also result from different preferential areas of production for CRP and SAA, and therefore a different reaction of local protein synthesis to local, (peri)portal production of cytokines. CRP production starts in zone 1 and shifts towards zone 3 during the APR.[627] Studies regarding the site of SAA production in the liver are not conclusive.[666-668]

SAA is associated with high density lipoprotein (HDL_3).[669] Since both bound and unbound SAA are measured in our ELISA, the fact that HDL_3 is a negative acute phase reactant[670] is unlikely to influence results.

The synthesis of CRP and SAA can be stimulated by several cytokines like interleukin-1ß (IL-1ß),[671,672] interleukin-6 (IL-6)[673-676] and tumor necrosis factor α (TNFα).[677] Experiments with primary cultures of human hepatocytes and human hepatoma cells showed that Il-1ß, IL-6 and TNF-α stimulate liver synthesis of SAA and CRP at the transcriptional level in an additive manner, with IL-6 as the principal stimulator.[674-676] However, monocytes may also produce substances that inhibit SAA synthesis,[678] and they can produce inhibitory activity to IL-1ß.[679,680] The degree of stimulation of synthesis can lead to different levels among various APP's as the result of stimulation by one or more of these cytokines. However, the difference in CRP and SAA responses in CAH cannot be entirely explained by these differences in stimulation.

The normalization of hepatic functional protein synthesizing capacity (CHE) in severe CAH usually takes about two years, and therefore appears to be predominantly the result of repair and regeneration of liver tissue. Because CHE, like albumin,[674] is a negative acute phase reactant, the increment in CHE levels may also be the result of a decline in titres of e.g. IL-1ß, IL-6 and TNFα. Glucocorticoids can also selectively inhibit the transcription of the IL-1ß gene and decrease the stability of IL-1ß mRNA.[681] Furthermore, the rise in CHE and SAA may partly result from a direct stimulation of protein synthesis by corticosteroids, as is also observed in hepatocyte cultures.[675,682]

In conclusion, our results show that in chronic active hepatitis of all aetiologies studied, the acute phase response is depressed with regard to CRP and especially to SAA. SAA serum levels are usually not elevated in CAH, CRP is elevated, but not to a degree that reflects the high degree of inflammation in a voluminous organ like the liver. SAA is correlated with the serum cholinesterase, while CRP is not. CRP modestly reflected the degree of histological (periportal) inflammation, while SAA did not at all. Impairment of the liver capacity to synthesize proteins probably affected SAA more severely than CRP, resulting in this different behaviour of both proteins, as summarized in table 10.3.

Table 10.3. Mechanisms responsible for responses of CRP and SAA in CAH.

	CRP	SAA
Local inflammation	↑↑↑	↑↑
Depressed protein synthesis	↓	↓↓
Net result	↑↑	N

Effects of immunosuppression on CRP and SAA in CAH.

	CRP	SAA
Decline in inflammation	↓↓↓	↓↓
Increased protein synthesis	↑	↑↑↑
Direct effect of steroids	↓?	N ?
Net result	↓↓ (↓?)	↑/N

↑ = increase ↓ = decrease (number of arrows denotes degree of increase/decrease).
N=no change.

CHAPTER 11

SERUM N-TERMINAL PROPEPTIDE OF COLLAGEN TYPE III IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

Relation to Histology and Functional Capacity of the Liver and Effects of Standardized Immunosuppression.

SUMMARY

The serum N-terminal propeptide of collagen type III (PIIINP) predominantly reflects deposition of collagen type III in the liver. We studied this in untreated idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) (n=25, 5 anti-HBS positive), during complete histological remission under immunosuppressive therapy (n=23). In 21 patients, the effects of standardized therapy were related to histology and serum cholinesterase, reflecting liver function.

PIIINP was measured with a new rapid equilibrium type of PIIINP assay which is based on the human propeptide. We found that this correlated very well (R=.96) with the standard assay which is based on bovine PIIINP.

PIIINP was positively correlated to periportal inflammation (R=.45), but not to portal or lobular inflammation and necrosis, resulting in a correlation between PIIINP and overall histologic activity of R=.43. Very low PIIINP values (<2.5 μg/l) were related to histological remission, and very high values (≥18 μg/l) correlated to CAH. However, for values in between, serum PIIINP measurements could not replace a liver biopsy for assessment of histological activity. Static measurement of histological fibrosis/cirrhosis did not correlate with serum PIIINP levels. Despite a negative correlation between PIIINP and CHE in untreated CAH (R= -.62) and during therapy induced remission (R= -.44), the influence of liver functional capacity on PIIINP appeared minimal.

To conclude, elevated serum PIIINP levels in IAI-CAH reflect increased deposition of collagen type III in the liver, and are correlated with periportal inflammation, while impairment of liver function only leads to a slight increase in PIIINP. Although standardized immunosuppression led to normalization of PIIINP in half of the patients, PIIINP remained slightly elevated in the other 50%, despite induction of histological remission and normalization of CHE. This may (partly) explain ongoing formation of cirrhosis during therapy, as has been observed in some studies.

INTRODUCTION

Idiopathic 'autoimmune' chronic active hepatitis (CAH) usually reacts favorably to immunosuppressive therapy immunosuppression was shown to enhance survival in this type of CAH[683-687]. Fibrosis and formation of cirrhosis are sequelae of untreated CAH, and can even develop during therapy intended to suppress inflammation[687,688]. About 70% of liver collagens consist of collagen types I and III [689]. Collagen type III is predominant in early fibrosis; in late cirrhosis, type I, "hard collagen" prevails [689]. Type III collagen has a more rapid turnover than type I collagen [690]. The C- and the N-terminal procollagen-III-peptide (PIIINP) are split from the procollagen-III molecules before extracellular assemblage into fibers in the extracellular matrix. Generally, the amount of free PIIINP that circulates stochiometrically reflects deposition of collagen-III.[691-693]

Some of the serum PIIINP is derived from PIIINP which was retained in the deposited fibers.[694,695] Decreased degradation of PIIINP due to absent liver function can account for a rise in serum PIIINP in the anhepatic situation during liver transplantation[696]. However, it is unknown whether a decrease in hepatic functional capacity in itself, as can occur in CAH, can affect serum PIIINP levels.

Some studies indicated that serum PIIINP parallels the activity of inflammation in acute hepatitis, and returns to normal along with transaminases and bilirubin.[697] In alcoholic hepatitis, PIIINP correlated well with periportal and intralobular inflammation[698-702]. Several studies suggested a relationship between activity of CAH and PIIINP[703-706], although this could be masked in children with CAH by elevation of PIIINP due to growth[707]. Elevation of PIIINP in serum of patients with viral hepatitis persisting for more than 6.5 months, correlated with chronicity[697,708]. Therapy with corticosteroids can lower an elevated PIIINP in CAH.[704,709-712]

Recently it has been shown that PIIINP changed in accordance with other liver tests and normalized when remission of CAH had been achieved[713,714].
A recent study on liver transplantation in pigs indicated that PIIINP serum levels can rise in the anhepatic situation[696]. Serum cholinesterase (CHE) is a direct marker for hepatic protein synthesizing capacity. In patients with hepatocellular damage, CHE is frequently reduced, almost paralleling reductions in albumin, while a normal CHE virtually excludes severe impairment of liver function.[715-719]

In the present study, we analysed the serum PIIINP levels in relation to histology and liver function in untreated idiopathic 'autoimmune' CAH before and during two years of standardized immunosuppression, and we related these to histology and CHE.

MATERIALS AND METHODS

Patients

We studied 25 untreated patients (4 men and 21 women) with histological, clinical and biochemical features of chronic active hepatitis (CAH). They had a median age of 53 years (range 16 to 81) and a median duration of disease (between the tested serum sample and the diagnostic biopsy) of 72 days (range -21 days to + 3 years). 20 of these patients had idiopathic 'autoimmune' CAH, and 5 had circulating anti-HBs antibodies.
In these untreated CAH patients, localization and degree of inflammation and fibrosis/cirrhosis in coded routine liver biopsies were numerically scored, and serum cholinesterase (CHE), reflecting the hepatic protein synthesizing capacity, was measured. Both were compared with PIIINP. In 23 of the 25 patients, PIIINP was related to CHE on 44 occasions during complete histological remission (HAS≤1) under therapy with prednisolone (5-15 mg), in 21 patients in combination with azathioprine (50-75 mg).

21 out of these untreated CAH patients (3 of them with antiHBs: 4 men and 17 women), requiring immunosuppressive therapy according to accepted criteria [50], were studied to evaluate therapy-effects. Their median age was 52 years (range 16-71), and median duration of disease 75 days (range -21 days to +3 years). All were subsequently treated with oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (14 patients) or two years (6 patients) of therapy with 10 resp. 50 mg daily. Serial routine liver biopsies and sera (CHE, PIIINP) were studied at the start and at two months, one and two years of therapy. 16 of the patients were followed up for 26 months, the other 5 patients for 14 months. One biopsy at 2 months and one at 14 months was missing. One serum measured at 14 months failed in a patient followed up for 26 months.

Liver histology

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[719a] In contrast to the original system,[719b] fibrosis/cirhhosis is not included in the HAS, but is presented as a separate histologic fibrosis score (HFS) ranging from 0 to 4. The HAS is expressed as a numerical score (0-18), and is an addition of portal (score 0-4), periportal (score 0-10) and lobular (score 0-4) inflammation and necrosis.

Serum cholinesterase

Cholinesterase (CHE) concentration was determined by measuring its catalytic activity, using acetylthiocholine iodide as a substrate in a commercially available kinetic test (Boehringer Mannheim GmbH).[720] For the reference values of CHE we used the sera of 41 women and 36 men with apparently normal health. The lower reference limit of CHE is 1600 U/l for women and 1800 U/l for men.[721]

Serum PIIINP

A new radioimmunoassay (RIA) developed by Risteli and Risteli[722] for the determination of PIIINP was applied. This rapid equilibrium type of assay is based on the human propeptide and is not sensitive to smaller degradation products[721]. Therefore, in this new PIIINP-assay no serial dilutions of the sera and no 50% intercept method for calculating the results are necessary, since the standards and the serum samples give parallel inhibition curves. This is unlike the PIIINP RIA-gnost® (Behring).[722,723,724] This solves the most important problems that have been inherent in the determination of PIIINP[725]. Results of serum PIIINP obtained with the new equilibrium-RIA, based on the human propeptide, showed excellent correlation ($r=.96$, $r^2=.92$) with those of the 'RIA-gnost' in CAH-sera (see chapter 4) and showed similar changes during immunosuppression. The intra-assay variation was 4.1%. Sera had been frozen and stored (-20°C) until use. Storage of sera at -20°C [713], and bilirubin- and prednisolone-levels [704,727] in serum do not affect results of PIIINPdetermination. PIIINP can be elevated in renal insufficiency[728]. One patient had a 40% lowered glomerular filtration rate due to membrano-proliferative glomerulonephritis, but PIIINP values in this patient paralleled those of the other CAH patients. The age of all patients was above 16 years, allowing normal reference values for adults (upper limit 4.2 µg/l)[722] to be applied.

Statistical analysis

Statistical analysis was performed with Friedman's analysis of variance (ANOVA) with repeated measurements, MannWhitney's U test, Wilcoxon's matched pairs test, and Spearman's rank correlation test where appropriate, with $p<0.05$ as level of significance.

RESULTS

During untreated CAH

The correlations in 25 untreated patients, between PIIINP on one side, and CHE and histology on the other side, are shown in table 11.1. PIIINP was positively correlated to periportal ($R=.45$), but not to portal or lobular inflammation and necrosis, resulting in a correlation between PIIINP and HAS of $R=.43$. Static measurement of histological fibrosis/cirrhosis did not correlate with serum PIIINP levels. A negative correlation between PIIINP and CHE was detected ($R= -.62$) (figure 11.1A).

During histological remission under immunosuppression

On 44 occasions in 23 out of the 25 patients, we related PIIINP to CHE during complete histological remission ($HAS\leq1$) under immunosuppressive therapy. There was no correlation between PIIINP and histological inflammation/necrosis or fibrosis/cirrhosis. However, a negative correlation still existed between PIIINP and CHE ($R=-.44$, $p<0.01$) during histological remission, although the PIIINP values that went with the lower CHE values were usually still below 6 µg/l (figure 11.1B), indicating that impaired hepatic functional capacity leads to only minimal increases in serum PIIINP values.

Table 11.1. PIIINP, CHE and histology in untreated CAH (n=25). (Spearman's rank correlation).

Correlation with PIIINP		R	p
Histology Inflammation/necrosis:			
portal	(P)	.10	.13
periportal	(PP)	.45	<.05
lobular	(L)	.28	.15
HAS	(=P+PP+L)	.43	<.05
Fibrosis/cirrhosis	(HFS)	.19	.34
CHE		-.62	<.005

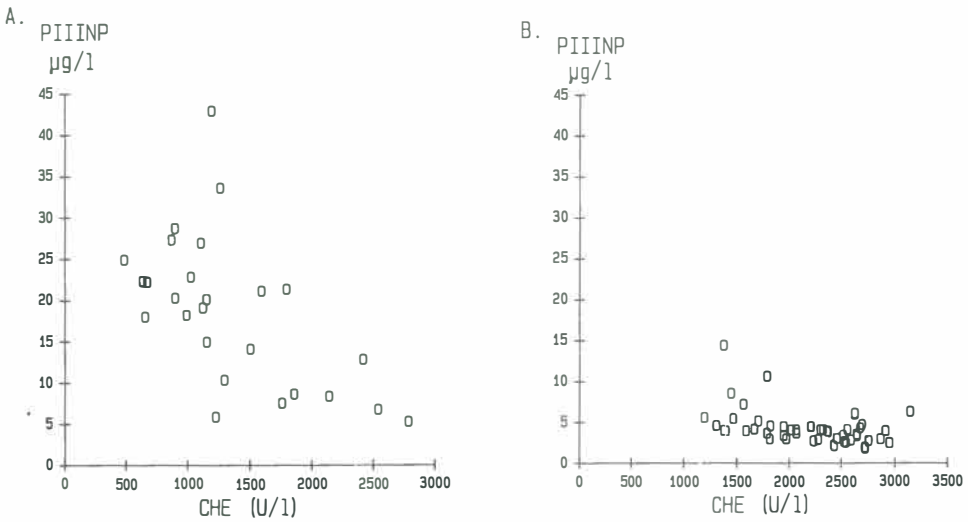
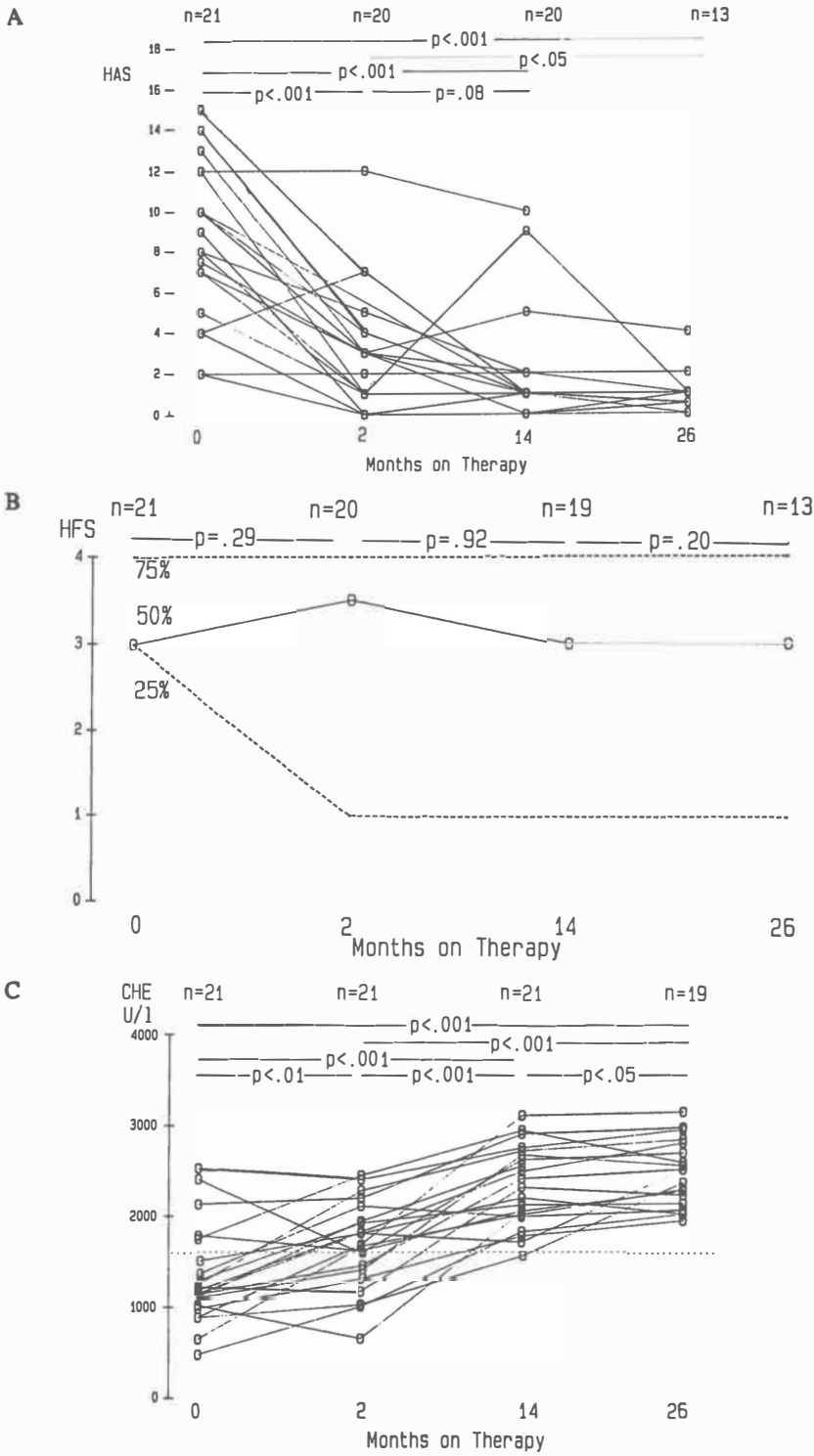


Figure 11.1. PIIINP versus CHE (A) in untreated IAI-CAH and (B) during therapy-induced histological remission (with HAS≤1).

Effects of standardized immunosuppressive therapy

It was possible to evaluate 21 patients maintained on standardized immunosuppressive therapy with regard to PIIINP- and CHE levels and a liver biopsy at the start, after two months and after one and/or two years of therapy. As shown in figure 11.2A, the HAS diminished from median 9 to median 3 within 2 months of standardized therapy ($p<0.001$), with a trend to further improvement in the next year of therapy to median HAS=1 ($p=0.08$), and stabilization thereafter (median HAS=1 at two years)($p=0.295$). In the first two months of therapy, reduction in the periportal inflammation score (0-4) from median 5 to a median score of 0.5 ($p<0.001$) took place, while the lobular


A
n=21
n=20
n=20
n=13
p<.001
p<.05
p<.001
p<.001
p=.08
HAS
18
16
14
12
10
8
6
4
2
0
0
2
14
26
Months on Therapy
B
n=21
n=20
n=19
n=13
HFS
p=.29
p=.92
p=.20
75%
50%
25%
4
3
2
1
0
0
2
14
26
Months on Therapy
C
CHE
U/l
n=21
n=21
n=21
n=19
p<.001
p<.001
p<.001
p<.01
p<.001
p<.05
4000
3000
2000
1000
0
0
2
14
26
Months on Therapy

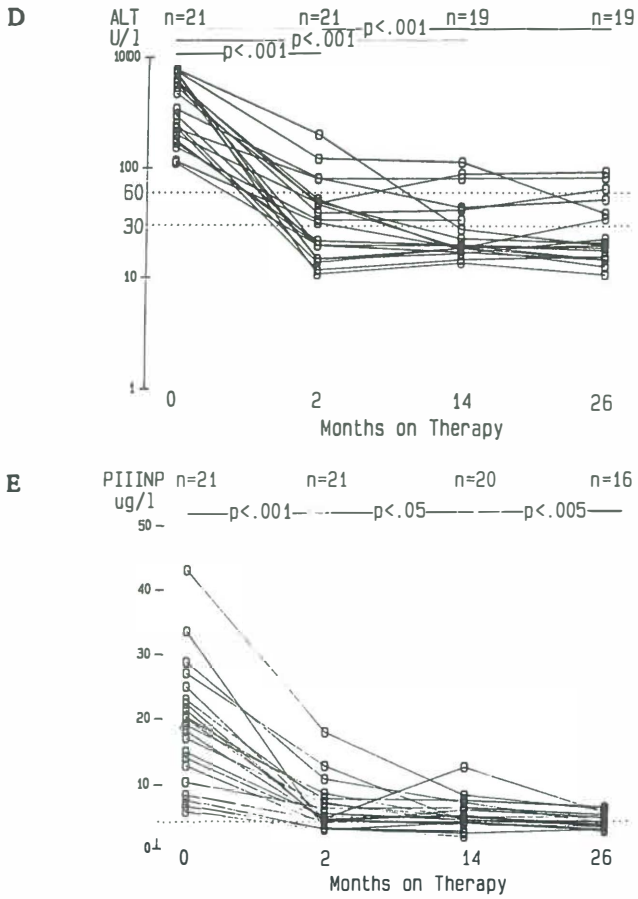


Figure 11.2. A) HAS, B) HFS, C) CHE, D) ALT and E) PIIINP in 'autoimmune' CAH before and during immunosuppressive therapy (n=21).
Dotted lines indicate upper (ALT, PIIINP) or lower (CHE) limits of reference range.

inflammation score (0-4) was reduced from median 1 to a median score of 0 ($p<0.005$), with further reduction and trend to reduction in the next two years of therapy (median scores of periportal and lobular inflammation at one and two years: 0)($p<0.05$ and $p=0.09$ respectively). Portal inflammation scores diminished in the initial 14 months of therapy from median 3 to median 1 ($p<0.05$), with stabilization thereafter. A histology similar to chronic persistent hepatitis ('CAH in histological remission') usually remained (median scores of portal inflammation were 1 at one and two years), while periportal and lobular inflammation had diminished and (almost) disappeared. The fibrosis/cirrhosis score (HFS) (0-4) was high at the start (median 3) and did not change during therapy (median 3.5, 3 and 3 at two months, one and two years) ($p=.29$, $p=.92$, $p=.20$ respectively) (figure 11.2B). These data, together with data on routine serum biochemistry have been shown in chapters 7 and 8.

CHE steadily increased during standardized immunosuppression, from a median value of 1185 U/l at the start to 1698 U/l after two months ($p<0.01$), to 2323 U/l at one year ($p<0.001$) and to median 2514 U/l after two years ($p<0.05$). At the start of therapy 5(25%) patients had a normal CHE, while CHE was normal in all 17 patients with one or two years of observation during therapy. This was frequently observed, despite the presence of cirrhosis as the HFS indicated. This is shown in figure 11.2C. For more details on serum biochemistry (including ALT and CHE) data shown in figure 11.2D) the reader is referred to chapters 7,8 and 9.

PIIINP values fell from a median value of 19.1 µg/l at start to 4.7 µg/l at two months ($p<0.001$) with a further reduction to median 4.6 µg/l at one ($p<0.05$) and 4.1 µg/l at two years of therapy (p0.005), as shown in figure 11.2E. HAS or PIIINP values before therapy (data not shown) did not differ between patients with HAS>3 at 14 or 26 months and those patients with HAS≤3 at both 14 and 26 months.

DISCUSSION

Fibrogenesis is closely associated with activity of CAH. The mechanisms underlying fibrogenesis in the liver are not completely clear. The increased production of collagen leading to hepatic fibrosis is the result of either increased net production of collagen per cell, or increased number of collagen-producing cells. The current knowledge of the many levels known at which this process is regulated was recently reviewed[729]. It has been suspected that cytokines such as interleukin-1, which is present in high levels during inflammation, stimulate Kupffer cells to induce fibrogenesis in fat-storing cells, myofibroblasts, and parenchymal cells in the liver. Kuppfer cell stimulation is augmented by fibroblast-activating and fibroblast-growth factors released from T-cells in the inflammatory infiltrate[730-734]. Tumor necrosis factor-alpha (TNF-α) and a release of transformation growth factor ß-1 (TGF-ß1) derived from Kupffer cells and Ito-cells, may play a role in stimulation of fibrogenesis[735-738]. In vitro experiments show that TGF-ß1 gene expression is inhibited by dexamethason.[738] This is probably one of the mechanisms whereby corticosteroids inhibit collagen synthesis in the liver, in addition to reducing inflammation.

Although some studies[700,701,704] suggest the contrary, the current study shows that PIIINP levels do not quantitatively reflect static measurement of hepatic fibrosis. As shown, histological scores of cirrhosis/fibrosis (HFS) were high and did not change during immunosuppressive therapy. An explanation may be the considerable degree of fibrosis/cirrhosis present in most of the patients before immunosuppression, and a decrease of inflammation, hence fibrogenesis, after the institution of immunosuppression. In our results, correlation of static measurements of histological fibrosis/cirrhosis (HFS) with PIIINP was weak, as could be expected, since PIIINP predominantly

reflects the dynamics of deposition of collagen III in the liver in CAH. This weak correlation probably results from the fact that patients with the most active deposition of collagen III will usually have the most advanced degree of cirrhosis. It has been demonstrated before, that PIIINP values are less affected than AST and ALT by the presence or absence of cirrhosis[739,740] and necrosis [704,741,742].
The correlation between PIIINP and periportal inflammation found in the current study appears insufficient to replace a liver biopsy for the assessment of histological inflammation in the liver in all cases. Many explanations may exist for this weak correlation:
Firstly, levels of cytokines, degree of fibrogenesis, and deposition of collagen may fluctuate quite rapidly, while the mounting or disappearance of a hepatic inflammatory infiltrate takes more time.
Secondly, a reduction in degradation of PIIINP may exist during severe CAH, when the liver function is impaired, as will be discussed.
Only high and very low PIIINP levels accurately reflect histological CAH or remission respectively. In some of these cases serum PIIINP levels might replace a liver biopsy. Serum PIIINP may be a useful parameter during follow-up of CAH. Especially as PIIINP predominantly reflects deposition of collagen type III in the liver in CAH, which fluctuates with immunological state of activation of the disease. Similarly, PIIINP levels during 'CAH in remission' in patients with cirrhosis are usually within a much smaller range than, for instance, those of aminotransferases.

Therefore, a slight elevation of serum PIIINP titres (up to 6 µg/l) in patients with some reduction in serum CHE levels does not necessarily reflect increased deposition of collagen type III in the liver, but may be due to impaired liver function. However, during histological remission in CAH patients with an impaired hepatic functional capacity, PIIINP titres above 6 µg/l probably indicate increased deposition of collagen type III. In severe CAH the hepatic functional capacity is usually impaired. In such a situation increased synthesis and deposition of collagen in the liver excist, but some of the elevation of PIIINP levels must be due to decreased breakdown of PIIINP in the liver[696].

Results of the present study indicate that, although PIIINP levels changed in concert with standard liver tests and were abnormal in severe CAH, PIIINP levels frequently did not completely normalize when remission of disease had been achieved after two years of standardized immunosuppression in CAH. This is in contradiction with a recent study, which showed that PIIINP values not only changed in accordance with other liver tests, but also normalized when remission of CAH had been achieved[713,714]. In that study, however, patients were selected to have either active severe disease, or complete remission.

Although we found that PIIINP levels can be slightly influenced by a reduction in liver function, even in the virtual absence of inflammation (HAS≤1), CHE normalized in all

patients receiving standardized immunosuppression. Therefore, the persistent, usually slight, elevation of PIIINP levels despite induction of histological remission probably reflects increased deposition of collagen type III in the liver of these patients.

This process, together with the increased levels of PIIINP during the induction of remission, may account for the observed development of fibrosis. In addition, fibrosis in combination with regeneration may lead to cirrhosis in many patients, during therapy of IAI-CAH.

Normalization of serum PIIINP and hepatic functional capacity could be important endpoints of therapy in IAI-CAH. However, it is questionable whether it is always necessary to prevent development of cirrhosis in all patients, although in the absence of cirrhosis there is probably a lower risk of development of hepatocellular carcinoma. Presence of fibrosis/cirrhosis at presentation is a frequent finding in IAI-CAH[708,743], and patients who develop cirrhosis during therapy have a 5-year survival after the development of cirrhosis that is similar to that of patients who do not develop this complication[687,688,744]. Furthermore, the risks of increased dosages of immunosuppressive medication may outweigh the potential benefits of lowering the rate of deposition of collagen III in many patients. In the near future, additional antifibrotic therapies will probably become available.[731, 747] This may prove useful as an additional therapy in some CAH patients, e.g. those with partial α_1-antitrypsin deficiency -phenotype MZ- where PIIINP remained markedly elevated despite the induction of histological remission (our unpublished observation).

In conclusion, serum PIIINP levels are elevated in CAH and show a continuous decline during two years of immunosuppressive therapy, with eventual normalization in about half of the patients. Serum PIIINP levels are not only related to periportal inflammation, but are also negatively correlated to serum CHE levels. However, in the absence of histological inflammation this influence of hepatic functional capacity on PIIINP levels is weak. The persistent, usually slight, elevation of PIIINP levels, despite induction of histological remission and normalized hepatic functional capacity in half of the IAI-CAH patients, probably reflects increased deposition of collagen type III in the liver of these patients, leading to progression of fibrosis/cirrhosis.
This may (partly) explain ongoing formation of cirrhosis during immunosuppressive therapy for IAI-CAH, as has been observed in the quoted studies.[687,688]

CHAPTER 12

THE SERUM N-TERMINAL PROPEPTIDE OF COLLAGEN TYPE III AS A PREDICTOR OF RELAPSE IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS

SUMMARY

We studied the serum N-terminal propeptide of collagen type III (PIIINP) and the alanine aminotransferase (ALT) in serial sera of 16 histologically documented relapses of idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH). The relapses occurred during tapering of immunosuppressive therapy in 9 patients with previous histological and biochemical remission.

PIIINP was above the upper reference limit (>N) before ALT was >3N ($p<0.005$) or >2N ($p<0.005$). However, the time from 'diagnostic moment' (i.e. the moment the value lies obove the upper reference limit) until peak-ALT did not differ if the upper reference limit for both PIIINP and ALT was defined as 1.25 times the 'baseline value'; despite these very strict criteria, still some patients showed elevation of PIIINP preceding that of ALT. ALT>1.25 times 'baseline value' occurred before ALT>90 or ALT>60 ($p<0.001$), but the moment that PIIINP was >N equalled the moment that PIIINP was >1.25 times 'baseline value'. Nevertheless, if we chose either PIIINP>N or PIIINP>1.25 times 'baseline value' as the 'diagnostic moment' (whichever of the two exceeded the limit first), the moment one of these two limits was exceeded preceded ALT>1.25 times 'baseline value'($p<0.05$), it preceded PIIINP>N ($p<0.005$), and it precceded PIIINP>1.25 times 'baseline value' ($p<0.001$) as limits.

We conclude that PIIINP is useful in early detection of relapse in IAI-CAH. Unlike ALT, when using PIIINP no individual reference ranges have to be used.

INTRODUCTION

Remission from CAH is usually defined as inflammation and necrosis confined to the portal tracts, serum aspartate aminotransferase (AST) less than twice the upper limit of normal, normalization of serum bilirubin and gammaglobulin, and absence of clinical symptoms[748,749]. Relapse with a histologic evidence of moderate to severe CAH has been reported to be invariably present during a threefold or greater elevation of the serum AST level with return of symptoms [748-750].

Relapse occurs in 49-87 percent of patients within 6 months after remission - during or after discontinuation of treatment. The majority of patients who experience relapse do

so within the first 3 months after termination of treatment,[749,751,752] one quarter of the patients having no clinical symptoms.[749]
Despite the widespread use of AST and gammaglobulin (GG) values during follow-up of CAH, biochemical values of AST and GG are unreliable predictors of morphologic activity: negative predictive values are only 44% and 33% for AST and GG respectively[753], while a two-fold or greater elevation in AST does not always identify histological CAH. 19% of normal AST determinations can occur in association with CAH after treatment.[753]

Although initial studies appeared to indicate that serum concentrations of bile acids[754,755] or amino acids[756-759] would provide a more sensitive indication of disease activity in CAH, subsequent publications proved that these parameters cannot replace a liver biopsy in the assessment of inflammatory activity, because they are influenced by many other variables.[760-762] In a biopsy of sufficient size[762,763], sampling error for determination of inflammatory activity in CAH is less than 10%.[764] Unlike other types of liver disease, intra-observer error is 6-7 percent in chronic hepatitis[764,765]. From these data it follows that at present a liver biopsy is required, not only for diagnosis, but also for monitoring inflammatory activity[766,767]. Although it is a minor procedure, a liver biopsy is still an invasive procedure with its own, albeit low, morbidity and mortality.[768] This precludes its use for frequent routine monitoring of activity of disease. Therefore, the search for a non-invasive parameter of histological inflammation and an early detector of relapse during and after diminishing therapy, continues.

Fibrosis and formation of cirrhosis are sequelae of untreated CAH, and can even develop during therapy intended to suppress inflammation[769,770]. About 70% of liver collagens consist of collagen types I and III [771]. Collagen type III is predominant in early fibrosis; in late cirrhosis, type I, "hard collagen" prevails [771]. Type III collagen has a more rapid turnover than type I collagen [772]. The C- and the N-terminal procollagen-III-peptide (PIIINP) are split from the procollagen-III molecules before extracellular assemblage into fibers in the extracellular matrix. Generally, the amount of free PIIINP that circulates stochiometrically reflects deposition of collagen-III.[773-775] Some of the serum PIIINP is derived from PIIINP which was retained in the deposited fibers.[776,777] Some studies indicated that serum PIIINP parallels the activity of inflammation in acute hepatitis, and returns to normal with transaminases and bilirubin [778], and that in alcoholic hepatitis PIIINP correlated well with periportal and intralobular inflammation[779-783]. Several studies suggested a relationship between activity of CAH and PIIINP[784-787], although this could be masked in children with CAH by elevated PIIINP due to growth[788]. Persistent elevation for more than 6.5 months of PIIINP in the serum of patients with viral hepatitis correlated with chronicity[778,789]. Therapy with corticosteroids can lower an elevated PIIINP in CAH[785,790-793], and recently it was shown that PIIINP changed in accordance with other liver test and normalized when remission of CAH had been achieved.[794,795] However, we found that normalization often is incomplete and requires prolonged therapy (Chapter 11), which might facilitate the

reported[796,797] development of fibrosis/cirrhosis during immunosuppressive therapy for IAI-CAH. A recent study on liver transplantation in pigs indicated that PIIINP serum levels can rise in the anhepatic situation[798]. However, only severe liver function abnormalities influence serum PIIINP levels significantly (Chapter 11).

This study observed serum N-terminal propeptide of collagen type III (PIIINP) and the alanine aminotransferase (ALT) in serial sera of 16 histologically documented relapses of idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH). These relapses occurred during withdrawal of immunosuppressive therapy in 9 patients with previous histological, clinical and biochemical remission.

MATERIALS AND METHODS

Patients

Serial sera from 16 IAI-CAH relapses in 9 patients were studied from the time of remission at the point where immunosuppression was diminished, until peak-ALT during subsequent relapse of IAI-CAH. At least two years earlier, before immunosuppression, all patients had fulfilled accepted criteria for the diagnosis and immunosuppressive treatment of IAI-CAH.[799] Histological (Histological Activity Score -HAS- ≤3), clinical and biochemical remission had been confirmed before reducing maintenance immunosuppressive therapy. During the 16 histologically documented relapses (HAS>3), the serum ALT was above three times normal (>3N). In part of the patients relapse was also documented by a live biopsy. At the peak-ALT, immunosuppression was increased, resulting in return to histological, clinical and biochemical remission in all patients.

Routine liver biochemistry

Determination of alanine aminotransferase (ALT) (upper reference limit 30 U/l) was performed automatically (Technicon SMA-C) on the day of serum sampling.

The upper reference limit for remission was set as an ALT level twice (2N) or three times (3N) that for a healthy population, or 1.25 times the 'baseline value' at start of therapy withdrawal (ALT=1.25 x B). The 'diagnostic moment'of relapse was defined as that time when values became elevated above this upper reference limit, and remained elevated until the peak-ALT.

PIIINP

A new radioimmunoassay (RIA) , developed by Risteli and Risteli was applied for the determination of PIIINP.[800] This rapid equilibrium type of assay is based on the human propeptide and is not sensitive to smaller degradation products[801]. Therefore, no serial dilutions of the sera and no 50% intercept method for calculating the results are necessary in this new PIIINP-assay, since the standards and the serum samples give

parallel inhibition curves. This is unlike the PIIINP RIA-gnost® (Behring).[802,803] This solves the most important problems that were inherent in the determination of PIIINP until now[804]. Results of serum PIIINP obtained with the new equilibrium-RIA, based on the human propeptide, showed excellent correlation ($r=.96$, $r^2=.92$) with those of the 'RIA-gnost' in CAH-sera (see chapter 4) and showed similar changes during immunosuppression (data not shown). The intra-assay variation was 4.1%.
Sera had been frozen and stored (-20°C) until use. Storage of sera at -20°C [805], and bilirubin- and prednisolone-levels [806,807] in serum do not affect results of PIIINP determination. Creatinine clearances were normal (PIIINP can be elevated in renal insufficiency[807]). The age of all patients was above 16 years, so the normal reference values for adults (upper limit 4.2 µg/l)[808] could be applied.

The upper reference limit for ALT was set at 2N, 3N or 1.25 times the 'baseline value' (1.25 x B). For PIIINP N or 1.25 x B was considered the upper reference limit. 'Baseline value' indicates the ALT or PIIINP at the start of tapering of immunosuppression. The 'diagnostic moment' is defined as the moment when values are elevated above the upper reference limit, and remain elevated until the peak-ALT.

Statistical analysis

Statistical analysis was performed with Wilcoxon's matched pairs test and Chi-square test where appropriate with $p<0.05$ as the level of significance.

RESULTS

Figure 12.1 shows PIIINP and ALT at the commencement of therapy withdrawal and during the 16 relapses at peak-ALT. Both significantly increase during progression of relapse ($p<0.01$).
In figure 12.2, median ALT and PIIINP are shown from start of therapy withdrawal until peak-ALT for 10 relapses. PIIINP was above the upper reference limit (N) before ALT >3N ($p<0.005$) and before ALT>2N ($p<0.005$). However, the time from diagnostic moment until peak-ALT did not differ, if the upper reference limit for both PIIINP and ALT was defined as 1.25 x B. Despite these strict criteria elevation of PIIINP preceded that of ALT in some patients. ALT>1.25 x B occurred before ALT>90 or ALT>60 ($p<0.001$), with no difference evident between using ALT>2N and ALT>3N as a limit. The moment that PIIINP was >N coincided with PIIINP>1.25 x B. Nevertheless, if we chose PIIINP>N and/or PIIINP>1.25 x B as the 'diagnostic moment', this preceded ALT>1.25 x B ($p<0.05$). It also preceded PIIINP>N ($p<0.005$) and PIIINP>1.25 x B ($p<0.001$) alone (figure 12.3).

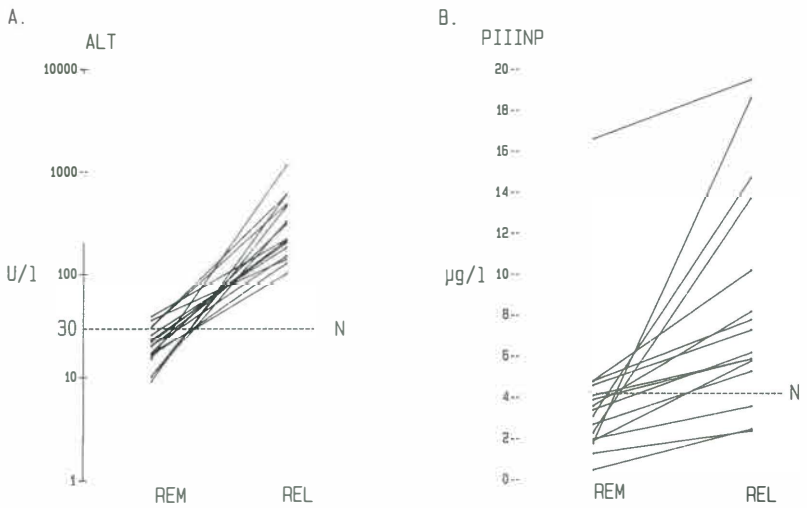


Figure 12.1. A) PIIINP and B) ALT during remission (REN) and relapse (REL) in idiopathic 'autoimmune' CAH. N=upper reference limit.

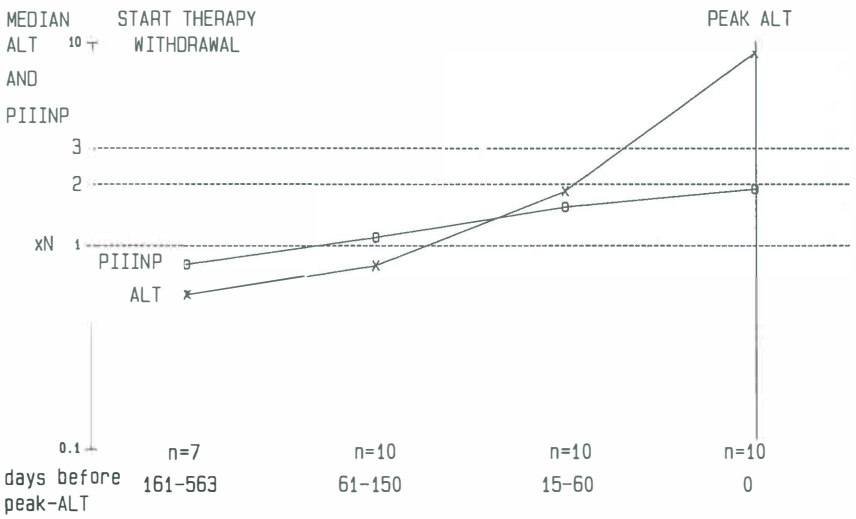


Figure 12.2. PIIINP and ALT before and during progression of relapse of idiopathic 'autoimmune' CAH during therapy withdrawal. N=the upper limit of the reference range: N=4.2 for PIIINP, and N=30 for ALT.

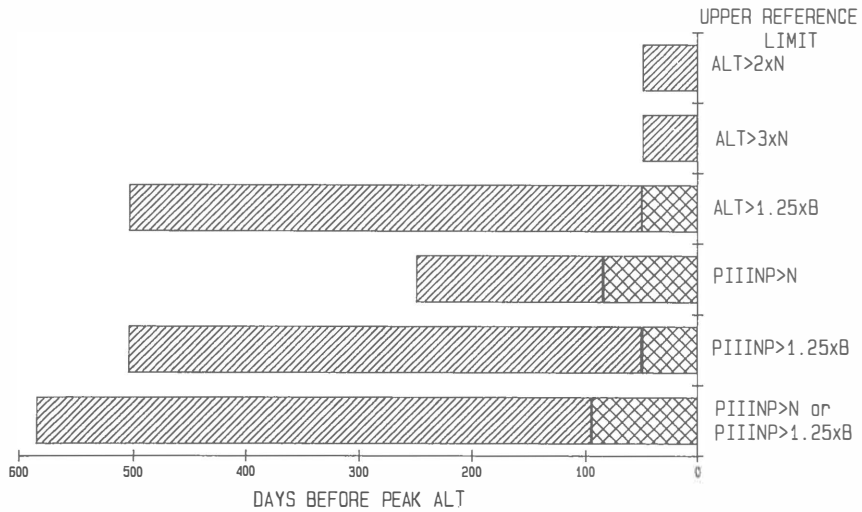


Figure 12.3. Number of days from 'diagnostic moment' until peak-ALT. (N=upper reference limit, B= baseline value at start of therapy withdrawal).
Single-hatched bars: 50% above median number of days.
Cross-hatched bars: 50% below median number of days.

DISCUSSION

From the current study, it follows that serum PIIINP is superior to serum ALT in serological detection of relapse in IAI-CAH, and that results largely depend on the chosen upper reference limit. Clearly, this limit should be set at a point where positive and negative predictive values are highest. In accordance with the mentioned serological definitions of relapse and remission in CAH, we chose 3N and 2N as upper reference limits for ALT. In doing so, it was clear that elevation of PIIINP preceded that of ALT. The positive predictive value of ALT could be improved by setting the upper reference limit at 1.25 x B. However, our research confirms that ALT and AST are unreliable predictors of morphologic activity in CAH. Even using a cut off point of 2N or 3N, the number of false positive diagnoses of relapse will undoubtedly increase if the upper reference limit is lowered to 1.25 x B. The combination of PIIINP>N and/or PIIINP>1.25 x B proved superior to all other possibilities mentioned. It also became clear from several relapses in the same patients in this study, that the elevation of PIIINP systematically precedes that of ALT more noticeably in some patients than in others.

We recently demonstrated that PIIINP can remain elevated despite induction of remission (HAS≤3). Fibrogenesis is probably directly stimulated by e.g. tumor necrosis factor-alpha and a release of transformation growth factor ß-1 derived from Kupffer

cells and Ito-cells.[808a-808d] Elevated serum ALT is the result of damage to hepatocytes resulting from the buildup of an inflammatory infiltrate, following immunological induction. Since such a buildup takes time, this could explain the earlier rise in PIIINP when compared to ALT. Moreover, serum ALT levels can be influenced by the degree of cirrhosis, while PIIINP is not; instead, PIIINP predominantly reflects the dynamic process of deposition of collagen III.
It may be better not to reduce immunosuppression in patients with elevated PIIINP. Such patients may even need additional therapy in order to prevent formation or progression of cirrhosis.

While such points require further elucidation, it is clear from this study that serum PIIINP is useful in early detection of relapse in IAI-CAH.

Section 4

Humoral immunology in chronic active hepatitis

'Autoimmune" chronic active hepatitis

CHAPTER 13

ANTIBODIES AGAINST 'LIVER-SPECIFIC MEMBRANE LIPOPROTEIN' (LSP) IN RELATION TO HISTOLOGY IN 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS, BEFORE AND DURING STANDARDIZED IMMUNOSUPPRESSION.

SUMMARY

Anti-LSP titres have been shown to correlate closely with the severity of histological damage in untreated patients with idiopathic 'autoimmune' chronic active hepatitis (IAICAH).

To further study the relationship between activity of disease and presence of anti-LSP antibodies in CAH, we assessed the presence of anti-LSP in relation to histological activity score -HAS (0-18)- in 'autoimmune' CAH, before and during two years of standardized immunosuppression.

Before therapy, all 21 patients tested had detectable antiLSP antibodies (titre median 150, range 50 to 750). In untreated IAI-CAH, there was no correlation between anti-LSP titres on the one side and HAS or periportal inflammation on the other side. Anti-LSP titres were, however, correlated to alanine aminotransferase (ALT) levels ($R=.78$, $p<.005$). At two months of therapy, anti-LSP was detectable in 15 (71%) of the 21 patients ($p<0.001$). After one and two years of standard therapy 6/21 (29%) ($p<0.01$) and 6/16 (38%) ($p=0.28$) patients respectively had detectable anti-LSP. During therapy, anti-LSP titres decreased in all patients under investigation. After one year of therapy, no changes from detectable to undetectable anti-LSP were observed. In the first two months of therapy the decrease in anti-LSP titres paralleled the decrease in HAS. Patients most frequently had a HAS≤1 and no detectable anti-LSP antibodies after one year of therapy. If HAS>1, anti-LSP was invariably detectable. Presence of anti-LSP, however, did not always indicate a HAS>1.

The results of the present study demonstrate that high titres of anti-LSP were invariably present in patients with IAI-CAH requiring immunosuppressive therapy according to accepted criteria. Despite the induction of histological remission, anti-LSP remained detectable in about half of these patients, although the titres declined. This finding probably indicates ongoing 'immunological activity' despite induction of histological remission.

INTRODUCTION

In IAI-CAH, there is considerable evidence that cytotoxic reactions of the antibody dependent (ADCC-)type are directed at hepatocyte plasma membrane-derived epitopes in the macromolecular, lipid-associated complex called 'liver specific antigen' (LSP).[809,810] In IAI-CAH, there is a defect in the LSP-specific suppressor T cell function.[811,812] Recent studies indicate that the basal defect probably lies in the inducers of these suppressor cells.[813]

Anti-LSP titres have been shown to correlate closely with the severity of histological damage in untreated patients with autoimmune chronic active hepatitis IAI-CAH.[814,815] One study reports that presence or re-appearance of anti-LSP during treatment withdrawal in patients who are apparently in complete remission invariably predicts relapse in IAI-CAH. It was suggested that regular determination of anti-LSP could provide a sensitive method for achieving optimum control in IAI-CAH.[816]

To study further the relationship between activity of disease and presence of anti-LSP antibodies in IAI-CAH, we assessed the presence of anti-LSP in relation to histology in 'autoimmune' CAH, before and during two years of standardized immunosuppression.

MATERIALS AND METHODS

Patients

We studied 21 untreated IAI-CAH patients (3 of them with anti-HBs, 4 men and 17 women), requiring immunosuppressive therapy according to accepted criteria,[817] to evaluate therapy effects. Their median age was 52 years (range 16 to 71), and median duration of disease 75 days (range -21 days to +3 years). All were subsequently treated with oral prednisolone 15 mg and azathioprine 75 mg daily for two months, followed by at least one year (14 patients) or two years (6 patients) of therapy with 10 resp. 50 mg daily. Serial routine liver biopsies and sera were studied at the start and at two months, one and two years of therapy. 16 of the patients were followed for 26 months, the other 5 patients for 14 months. One biopsy at 2 months and one at 14 months were missing. One serum at 14 months failed in a patient followed for 26 months.

Liver histology

Liver biopsies were processed and examined under code and assigned a Histological Activity Score (HAS), ranging from 0 to 18, which correlates well with conventional ways of scoring histology in CAH, as described.[818] In contrast to the original system,[819] fibrosis/cirhosis is not included in the HAS, but is presented as a separate histologic fibrosis score (HFS) ranging from 0 to 4. The HAS is expressed as a numerical score (0-18), and is an addition of portal (score 0-4), periportal (score 0-10) and lobular (score 0-4) inflammation and necrosis.

Routine liver biochemistry

Sera were obtained on the same day on which the liver biopsies were performed. Immediate determination of alanine aminotransferase (ALT) (upper reference limit 30 U/l) and total protein (TP) was performed automatically (Technicon SMA-C).

Anti-LSP

All serum samples were immediately frozen and stored (-20°C) until analysis. We found that freezing and thawing did not affect the results of anti-LSP determination.[820] Basal control values for anti-LSP were determined in sera from a group of 20 apparently healthy persons. Anti-LSP antibodies were measured in coded, heat-inactivated sera (30 min. at 56°C) with a staphylococcal protein A radioimmunoprecipitation assay, derived from the assay as described,[814] and modified as described.[820]

Statistical analysis

Statistical analysis was performed with Friedman's twotailed analysis of variance (ANOVA) with repeated measurements, Mann-Whitney's U test, Wilcoxon's matched pairs test and Spearman's rank correlation where appropriate, with $p<0.05$ as the level of significance.

RESULTS

Histology

As shown in figure 13.1A, the HAS diminished from median 9 to median 3 within 2 months of standardized therapy ($p<.001$) with a further improvement in the next two years of therapy to median HAS=1 ($p<.05$). Reductions in periportal ($p<.001$) and lobular ($p<.005$) inflammation were observed in the first two months of therapy, with a further decrease in periportal inflammation and a trend to improvement in lobular inflammation in the next two years ($p<.05$ and $p=.09$ respectively). A gradual reduction in portal inflammation occurred in the first 14 months of therapy ($p<.05$).[821] A histology similar to chronic persistent hepatitis ('CAH in histological remission') usually remained, while periportal and lobular inflammation had diminished and (almost) disappeared in patients with HAS≤3.

Serum biochemistry

Before therapy, ALT levels were markedly elevated in all patients (median 337 U/l). The levels declined during the first two months of therapy to median 32 U/l ($p<.001$), with stabilization thereafter at median 20 U/l, both at 14 and 26 months (figure 13.1B). Gammaglobulin levels ($n=17$) were elevated at start of therapy with a median value of 28 mg/l, falling to median 13.5 at two months ($p<.005$), and stabilizing thereafter, with median values of 15 and 12, at 14 and 26 months respectively (figure 13.1C).

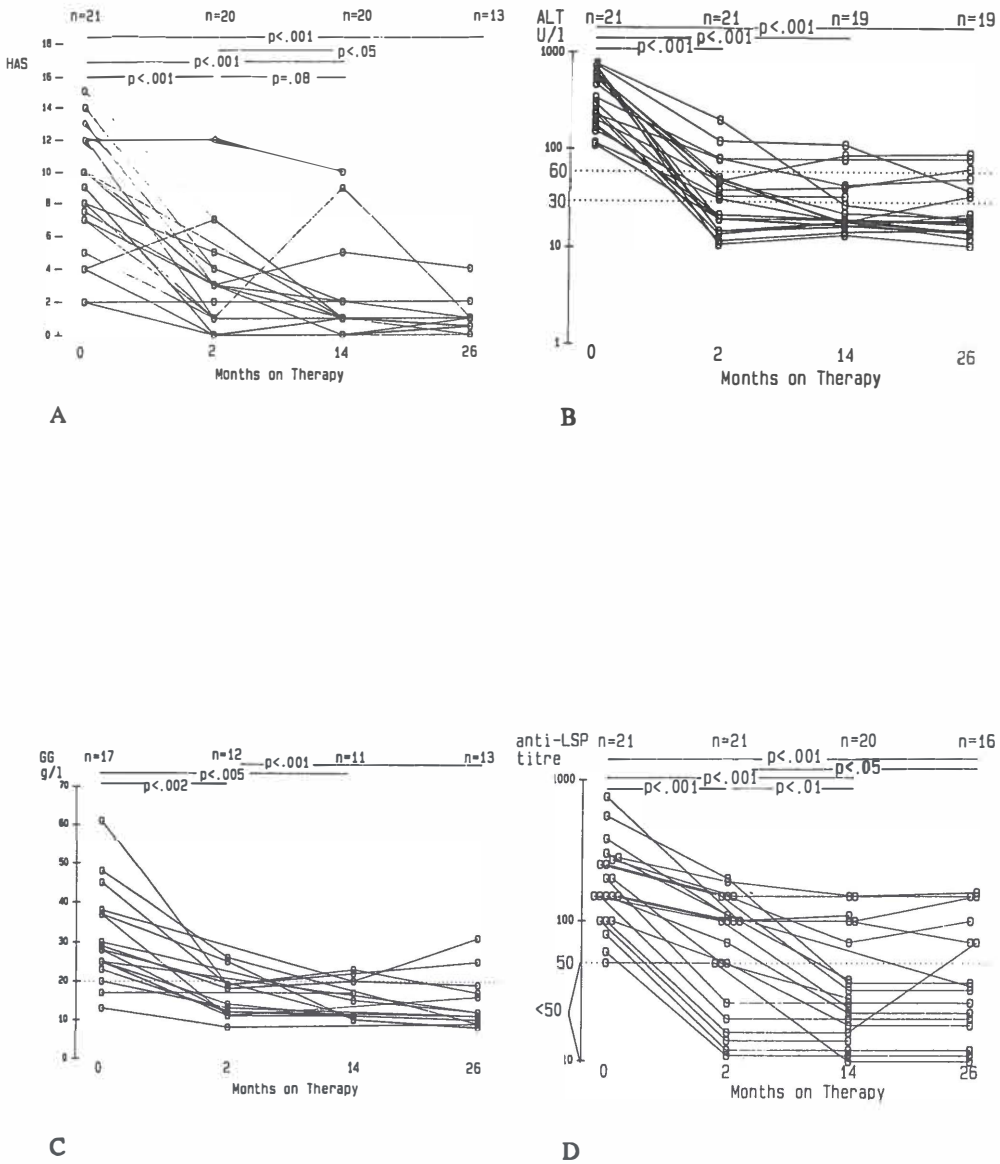


Figure 13.1. A) HAS, B) ALT, C) GG and D) anti-LSP in 'autoimmune' CAH before and during immunosuppressive therapy.
Upper reference limit N for anti-LSP is 1:50, for ALT 30 (2N=60).

Anti-LSP

Before therapy, all 21 patients tested had detectable antiLSP antibodies (titre median 150, range 50 to 750).

At two months of therapy, anti-LSP was detectable in 15 (71%) of the 21 patients ($p<0.001$). After one and two years of standard therapy, 6/21 patients (29%) ($p<0.01$) and 6/16 patients (38%) ($p=0.28$) respectively had detectable anti-LSP (figure 13.1D). During therapy, anti-LSP titres decreased in all patients under investigation. In all patients tested both after one and two years of therapy, no changes from detectable to undetectable anti-LSP were observed after one year. As indicated in figures 13.1A and 13.1D, for the first two months of therapy the decrease in anti-LSP titres paralleled the decrease in HAS. Patients most frequently had a HAS≤1 and no detectable antiLSP antibodies after one year of therapy. If HAS>1, anti-LSP was invariably detectable. Presence of anti-LSP, however, did not always indicate a HAS>1.

In untreated IAI-CAH, there was no correlation between anti-LSP titres on the one side and HAS or periportal inflammation on the other side. However, anti-LSP titres were correlated to alanine aminotransferase (ALT) levels ($R=.78$, $p<.005$). In both treated and

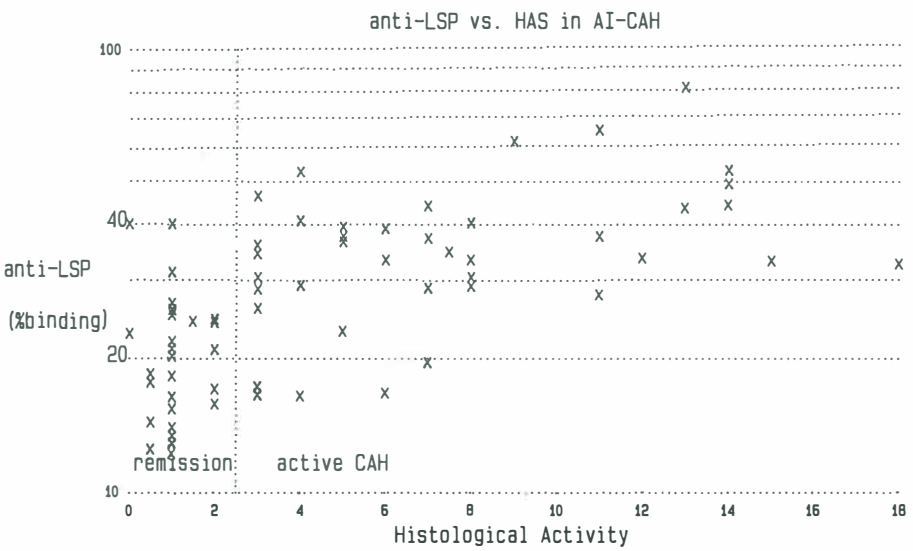


Figure 13.2. Correlation between anti-LSP binding percentages (radioimmunoassay) and HAS in treated and untreated IAI-CAH.

DISCUSSION

The results of the present study demonstrate that high titres of anti-LSP were invariably present in patients with IAI-CAH requiring immunosuppressive therapy according to accepted criteria. Despite the induction of histological remission, anti-LSP remained detectable in about half of these patients, although the titres declined. This finding probably indicates ongoing 'immunological activity', despite induction of histological remission. Our (uncontrolled) experience in some patients is, that higher levels of immunosuppressive drugs can sometimes suppress such a persistent manifestation of anti-LSP.
Corticosteroids improve the non-antigen specific T-suppressor cell functions in IAI-CAH, resulting in reversal of histological and biochemical manifestations of disease and sufficient to induce 'remission' according to currently accepted criteria.[822] However, corticosteroids have no effect on the genetically determined LSP-specific T-suppressor cell defect. It was previously postulated that this latter defect could allow continued stimulation of anti-LSP producing B lymphocytes. This obviously requires help of cytotoxic lymphocytes for maintenance of the antibody-dependent cellular cytotoxicity (ADCC) that plays an important role in the pathogenesis of IAI-CAH. The ADCC can probably be stopped, while the anti-LSP production still goes on at a lower level.[809-813,823,824]

The liver-specific, species cross-reactive hepatic asialoglycoprotein receptor (ASGR), also known as hepatic lectin, is an important constituent of LSP.[825] Anti-LSP antibodies appear to be predominantly directed at ASGR, although a role for anti-LSP directed at other antigenic specificities has not yet been excluded. In a recent study, anti-ASGR was found in 83% of patients with IAI-CAH.[826]

After one year of therapy, patients in remission most commonly had a HAS≤1 and no detectable anti-LSP. Therefore, we may assume that this combination reflects 'complete remission', and is an important endpoint of therapy in IAI-CAH. It is well known that relapse rates can be high, especially after discontinuation of therapy.[827,828] Investigators from the Mayo Clinic observed relapse rates of 30% in patients who reverted to normal liver biopsies, but 80% if residual portal hepatitis was present at the time of drug-withdrawal.[829,830] We therefore distinguished complete histological remission (HAS≤1) from partial histological remission (HAS≤3).[818] It is interesting to note that in 80% of the patients, such a complete histological remission was achieved with the standardized therapeutic regimen. HAS≤1 and negative anti-LSP may be mandatory requirements before attempting to discontinue therapy.

The finding that there was no change in levels of detectable anti-LSP after one year of standardized therapy, probably indicated that within one year of therapy the maximum immunosuppressive effect has been reached. Thus, further reduction in inflammatory

activity thereafter appears unlikely. Continuation of therapy may be warranted in most patients, however, to secure repair and full restoration of liver function, as has been shown in chapter 9.

CHAPTER 14

ANTIBODIES AGAINST 'LIVER SPECIFIC MEMBRANE LIPOPROTEIN' (LSP) AS AN EARLY PREDICTOR OF RELAPSE OF 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS DURING THERAPY WITHDRAWAL

SUMMARY

Relapse is frequent during or after withdrawal of immunosuppressive therapy in idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH). Serum aminotransferase levels, using standard reference ranges, insufficiently reflect histological activity, as do most other serological parameters. We determined serial alanineaminotransferase (ALT) and antibodies to the macromolecular, lipid-associated complex called 'liver specific membrane lipoprotein' (antiLSP), using a modified radioimmunoassay, in 12 relapses in 9 women during therapy withdrawal after histologically proven remission. *Results*: Anti-LSP was positive or became so in 11 (92%) out of the 12 cases of relapse during therapy withdrawal, while in 11 (92%) out of the 12 cases it had been undetectable at the start of reduction of therapy. In 2 cases, anti-LSP was positive at the start of reduction of therapy, and remained detectable when relapse occurred (with rising titres). In one patient, anti-LSP was not detected both before and during development of relapse. However, relapse was also not diagnosed using ALT values as a serological detector before the time of peak-ALT in this patient. In 9 (82%) of the 12 relapses, anti-LSP was negative at the start of diminishing therapy, while it was positive at the time of relapse (peak-ALT) (Chi-square 16.667, $p<.005$). In these nine cases, anti-LSP became positive before ALT>3N in 7 cases, at the same time as ALT>3N in 1 case, and later than ALT>3N in 1 case; for ALT>2N, these numbers were 6, 2, and 1; for ALT>1.25xB, it was 4, 2 and 3 respectively. For these 9 patients, the 'diagnostic moment' for detection of relapse was median 0 days before peak-ALT (range 0-49 days) with ALT>3N or ALT>2N as a criterium for relapse; for ALT>1.25xB, this was median 49 days (range 0-427); for presence of anti-LSP, it was median 133 (range 0-249) days before peak-ALT. Using anti-LSP, relapse was detected earlier than with the use of ALT>3N and ALT>2N as criteria ($p<0.005$ and $p<0.01$ respectively). With ALT>1.25xB as cut-off point, serological detection of relapse was also earlier than with ALT>3N or ALT>2N (both $p<0.05$) as diagnosticum. Between ALT>3N and ALT>2N there was no difference in timing of 'diagnostic moment'($p=0.63$). Neither was this different between anti-LSP and ALT>1.25xB ($p=0.54$).

The current study shows that anti-LSP was, or became, positive in 92% of the cases of relapse of idiopathic 'autoimmune' CAH that we studied. When compared to the current

serological criterium for relapse (ALT>3N), or even when compared to ALT>2N as a criterium, relapse was detected earlier using positivity of anti-LSP or ALT>1.25xB as a criterium.

INTRODUCTION

Remission from chronic active hepatitis (CAH) has been defined as inflammation and necrosis confined to the portal tracts, serum aspartate aminotransferase (AST) less than twice the upper limit of normal, normalization of serum bilirubin and gammaglobulin, and absence of clinical symptoms[831,832]. Relapse with histologic evidence of moderate to severe CAH has been reported to be invariably present during a threefold or greater elevation of serum aminotransferase levels, with return of symptoms.[831,832,833]

Relapse occurs in 49-87 percent of patients within 6 months after remission and during or after discontinuation of treatment. The majority of patients who experience relapse do so within the first 3 months after termination of treatment.[832,834,835] In one quarter of these patients there are no clinical symptoms when a relapse occurs.[832]

Despite the widespread use of AST and gammaglobulin (GG) values during follow-up of CAH, biochemical values of AST and GG are unreliable predictors of morphologic activity. Negative predictive values are only 44% and 33% for AST and GG respectively[832]. 19% of normal AST determinations can occur in association with CAH after treatment.[832].

Titres of antibodies against 'liver-specific membrane lipoproteins' (anti-LSP) have been shown to correlate closely with the severity of histological damage in untreated patients with idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH)[836,837] and can become negative in 60% of patients after two years of standardized immunosuppressive therapy.[838] McFarlane *et al.* demonstrated that presence/absence of anti-LSP predicted outcome of treatment withdrawal in IAI-CAH,[839] but these findings have never been confirmed. There is good reason for determining anti-LSP during therapy withdrawal, since considerable evidence exists that in CAH, cytotoxic reactions of the antibody dependent (ADCC-)type are directed at hepatocyte plasma membrane-derived epitopes in LSP.[840,841] In IAI-CAH, a defect in the LSP-specific suppressor T cell function exists.[842,843] Recent studies indicate that the basal defect probably lies in the inducers of these suppressor cells.[844]

In order to evaluate the clinical value of anti-LSP determinations, we studied serum anti-LSP in relation to ALT in 9 patients during 12 relapses of IAI-CAH, occurring during withdrawal of immunosuppressive therapy.

MATERIALS AND METHODS

Patients

Serial sera were studied from 12 randomly selected IAI-CAH relapses occurring during withdrawal of immunosuppression in 9 patients (all women, age median 51 -range 16 to 58 years). They were studied from histologically confirmed remission at the start of reduction of immunosuppression, until peak-ALT during subsequent relapse of CAH. At least two years earlier, all patients had fulfilled accepted criteria for diagnosis and immunosuppressive treatment of IAI-CAH[831]. Histological, biochemical and clinical remission had been confirmed before diminishing maintenance immunosuppressive therapy. Dosage of prednisolone and azathioprine at the start of therapy withdrawal and during relapse (peak-ALT) are shown in table 14.1. During the histologically documented relapses, the serum ALT was more than three times the upper reference limit (>3N). At the time of peak-ALT, immunosuppression was increased, resulting in a return to histological and biochemical remission in all patients.

Table 14.1. Dosage of prednisolone and azathioprine at start of diminishing therapy and at the time of peak-ALT during subsequent relapse.

patient	prednisolone (mg)		azathioprine (mg)	
	start	relapse	start	relapse
1	10	0	50	50
2	10	0	0	0
3	10	5	50	25
4	10	10	50	0
5	7.5	0	50	0
6	10	1	0	0
7	5	2	0	0
8	10	0	50	0
9	10	5	0	0
10	7.5	6.5	50	50
11	10	10	75	50
12	7.5	5	0	0

Routine liver biochemistry

Determination of alanine aminotransferase (ALT) (upper reference limit 30 U/l) was performed automatically (Technicon SMA-C) on the day of serum sampling. 2N, 3N, or 1.25 times the ' baseline value' at start of therapy withdrawal (ALT=1.25xB) was considered the upper reference limit for ALT. The 'diagnostic moment'of relapse was defined as the moment when values became elevated above the upper reference limit, and remained so until the peak-ALT.

Anti-LSP

All serum samples were immediately frozen and stored (-20°C) until analysis. We found that freezing and thawing did not affect the results of anti-LSP.[838] Basal control values for anti-LSP were determined in sera from a group of 20 apparently healthy persons, and binding percentages above mean+2SD of this group were considered positive results. Anti-LSP antibodies were measured in coded, heat-inactivated sera (30 min. at 56°C) with a staphylococcal protein A radioimmunoprecipitation assay, derived from the assay as described,[836] and modified as described.[838]

Statistical analysis

Statistical analysis was performed with Mann-Whitney U test and Chi-square test where appropriate, with $p<0.05$ as the level of significance.

RESULTS

ALT and anti-LSP titres as a function of time before peakALT are shown in figure 14.1. As shown in table 14.2, anti-LSP was positive or became so in 11 (92%) of the 12 cases of relapse during therapy withdrawal. In two cases, anti-LSP was positive at the start of diminishing therapy, and remained detectable when relapse developed (with rising titres). In one patient, anti-LSP was not detected either before or during development of relapse. However, relapse was also not diagnosed with ALT values as a serological

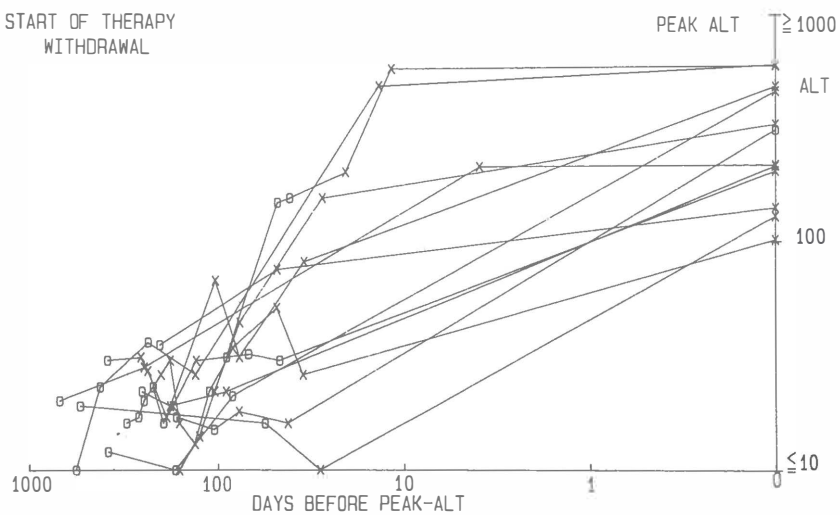


Figure 14.1. ALT and anti-LSP (x=positive, o=negative) from start of diminishing therapy (remission) to peak-ALT during subsequent relapse.

Table 14.2. Number of cases with and without anti-LSP during remission at start of diminishing immunosuppression and at the time of CAH relapse (peak ALT) (n=14).

RELAPSE			
anti-LSP	no anti-LSP	anti-LSP	TOTAL
anti-LSP	2	0	2
REMISSION			
no anti-LSP	9	1	10
TOTAL	11	1	12

Chi-square = 16.667 ($p<0.005$)

detector, before the time of peak-ALT in this patient. In 9 (82%) of the 12 relapses, anti-LSP was negative at the start of diminishing therapy, while it was positive at the time of relapse (peak-ALT) (Chi-square 16.667, $p<.005$).

As shown in table 14.3, in these nine cases, anti-LSP became positive before ALT>3N in 7 cases, at the same time as ALT>3N in 1 case, and later than ALT>3N in 1 case; for ALT>2N these numbers were 6, 2, and 1; for ALT>1.25xB it was 4, 2 and 3 respectively. For these 9 patients, the 'diagnostic moment' for detection of relapse was median 0 days before peak-ALT (range 0-49 days) with ALT>3N or ALT>2N as a criterium for relapse; for ALT>1.25xB, this was median 49 days (range 0-427); for presence of anti-LSP, it was median 133 (range 0-249) days before peak-ALT (table 14.3). Using anti-LSP, relapse was detected earlier than with the use of ALT>3N and ALT>2N as criteria ($p<0.005$ and $p<0.01$ respectively). With ALT>1.25xB as cut-off point, serological detection of relapse was also earlier than with ALT>3N or ALT>2N (both $p<0.05$) as diagnosticum. Between ALT>3N and ALT>2N there was no difference in timing of 'diagnostic moment'($p=0.63$). Neither was this different between presence of anti-LSP and ALT>1.25xB ($p=0.54$).

Table 14.3. Number of days between diagnostic moment and peak-ALT using different criteria for serological diagnosis relapse,in 14 cases of CAH-relapse during therapy withdrawal.

patient	ALT>3N	ALT>2N	ALT>1.25xB	anti-LSP+
1	0	0	0	181
2	14	14	427	133
3	0	0	132	175
4	0	0	0	28
5	49	49	49	21
6	0	0	203	203
7	0	49	49	49
8	0	0	84	0
9	4	4	4	249
median number of days.	0	0	49	133
patient				
10	0	0	0	*
11	0	105	105	**
12	28	28	126	**

N=upper reference limit. B='baseline value' of ALT at start of therapy withdrawal during histologically documented 'CAH in remisssion'.
*:anti-LSP negative both during remission and relapse. **:anti-LSP positive both during remission and relapse.

DISCUSSION

The current study shows that anti-LSP was or became positive in 92% of the cases of relapse of idiopathic 'autoimmune' CAH we studied. When compared to the current serological criterium for relapse (ALT>3N), or even when compared to ALT>2N as a criterium, relapse was detected earlier with positivity of anti-LSP or ALT>1.25xB as a criterium. In the only case where anti-LSP was and remained undetectable during therapy withdrawal, early diagnosis was not made with the help of ALT values either.
If ALT>1.25xB was used as the 'cut-off point', detection of relapse as early as with anti-LSP was possible. If we assume that the findings are correct in that anti-LSP is

pathogenetically involved in IAI-CAH, this early rise in ALT could mean that cell damage starts early after immunological activation of the disease occurs.
The sensitivity of a positive anti-LSP or ALT>1.25xB for detection of relapse is better than the current serological criterium of aminotransferase levels above three times normal. However, for determination of the specificity of a change from undetectable to detectable anti-LSP, or for a rise of ALT above 1.25 times individual baseline values during remission a prospective study with repeated liver biopsies during therapy withdrawal would be required. However such a study might have ethical drawbacks. Our additional experience also seems to indicate that patients without anti-LSP at the time of therapy withdrawal with remaining absence of anti-LSP thereafter tend to relapse less than patients with anti-LSP before or during therapy withdrawal.

Therefore, extrapolating the results of our study, we think it is safest not to diminish therapy in an IAI-CAH patient with detectable anti-LSP. In our opinion, other requirements to be fulfilled before reducing therapy (in a patient without complications) include the completion of at least two years of continuous immunosuppressive therapy in adequate dosage for at least two years, preferably concurrent with normalized synthetic capacity of the liver.[845] Determination of an individual 'baseline' ALT (B) in a patient without detectable anti-LSP at the time of a biopsy with 'CAH in remission' can help in follow-up during and after diminishing therapy. During this follow-up determination of anti-LSP (or ALT with ALT>1.25xB as cut-off) on a regular basis can enable early detection of relapse, requiring immediate reinstitution of the previous therapy.

CHAPTER 15

CLINICAL SIGNIFICANCE OF SUBGROUPS OF IDIOPATHIC 'AUTOIMMUNE' CHRONIC ACTIVE HEPATITIS, SEROLOGICALLY DEFINED BY PRESENCE/ABSENCE OF ANTI SOLUBLE LIVER ANTIGEN (SLA) AND OTHER AUTOANTIBODIES.

SUMMARY

A classification of idiopathic 'autoimmune' CAH, based on the presence or absence of autoantibodies against LKM-1, SLA, ANA, SMA and AMA was recently proposed (type 1= ANA-positive, 2=LKM-positive, 3= SLA-positive, 4= SMA-positive but ANA-negative, 5= unclassified: no autoantibodies or only AMA).
The aim of the present study was to investigate the clinical significance of such a classification in terms of survival, independent prognostic factors, and requirement for immunosuppressive therapy. We studied 79 patients with CAH, split into two overlapping groups: group I with IAI-CAH (n=69) and group II requiring standardized therapy with prednisolone and azathioprine (n=42, of which 32 had IAI-CAH). In addition, the prevalence of two AMA-subtypes and antibodies against liver membrane antigen (LMA) was evaluated.
The antibody profiles of IAI-CAH types 1,3 and 4 were frequently encountered in our group of Dutch patients with IAI-CAH, or other types of CAH and CAH-like liver disorders, while the aggressive type 2 was not seen among our patients.
Survival did not differ among patients with IAI-CAH type 1, 3, 4 or 'unclassified'. Similar results were obtained when this classification was applied to group II. Among these 'types' of CAH, no differences were found regarding the necessity to apply immunosuppressive therapy, although a subgroup of patients with IAI-CAH type 1 without SMA required immunosuppression less often than other patients.
We recently showed that immunosuppressed patients with IAI-CAH do better than those who do not fulfill treatment criteria and are left untreated, and that age, aetiology of CAH (especially presence or absence of anti-HBs), and the administration of standardized immunosuppression -versus other or no therapy- are independent risk factors for mortality in CAH. We now show that presence or absence of ANA, SMA, SLA, LMA, AMA are not independent prognostic variables in IAI-CAH or CAH of other aetiology, requiring immunosuppression.

To conclude, there is no apparent clinical reason for distinguishing, by presence or absence of ANA, SMA or SLA, more types of IAI-CAH than 'IAI-CAH type 2' and 'other IAI-CAH'. Pathogenetic differences between CAH with and without these autoantibodies cannot be excluded, however.

INTRODUCTION

Chronic active hepatitis (CAH) is a histologically and clinically defined syndrome of viral (hepatitis B, hepatitis C, or hepatitis D virus) or unknown aetiology (idiopathic 'autoimmune' CAH - IAI-CAH). Several 'CAH-related liver disorders' have been described with similar manifestations.[846-848] Although not pathognomonic for CAH or for one of its aetiologies, antinuclear antibodies (ANA) and anti smooth-muscle antibodies (SMA) -usually directed against actin-, antibodies directed at liver membrane antigen (LMA) or against epitopes in 'liver-specific membrane lipoprotein'(LSP) have been detected frequently and in high titres in IAI-CAH, while antimitochondrial antibodies (AMA) are infrequent in this disease. The outcome in patients requiring immunosuppressive therapy in IAI-CAH did not differ, however, between patients with 'classic' CAH with ANA or SMA, or those without these antibodies.[849]
Recently, apart from the 'classic (lupoid)' form of IAI-CAH, a second form of IAI-CAH was recognized with a more aggressive behaviour, worse prognosis, and frequently an acute (even fulminant) onset, requiring immunosuppressive treatment. Serologically, this 'autoimmune hepatitis type 2' (IAI-CAH type 2) was characterized by the presence of -previously described[850]- anti liver/kidney microsome antibodies of type 1 (anti-LKM1, directed at a 50 kD cytoskeletal protein in rough and smooth endoplasmatic reticulum) and the absence of antiactin, ANA, and AMA - although organ-specific autoantibodies, such as antithyroid antibodies, were often present.[851] It was found that an antibody to liver cytosol antigen(s) (anti-LC1) is a second marker of IAI-CAH type 2, while anti-LKM1 and anti-LC1 were not found in patients with other disorders.[852]
Manns *et al.* described autoantibodies to another soluble liver antigen (SLA) in 23 of 60 IAI-CAH patients (6 without detection of other autoantibodies), that were not detectable in viral CAH or other liver disorders.[853] It was proposed that the presence of SLA (with or without SMA, LMA or AMA) serologically defined a third type of IAI-CAH, responding well to immunosuppressive therapy. Furthermore, patients with SMA but without ANA might form a fourth subgroup where the necessity to institute therapy was more variable.[853] However, in a recent review of IAI-CAH, the significance of such a classification was questioned, and it was stated that more research in this field was needed.[854]

The aim of the present study was to investigate the clinical significance of a classification of IAI-CAH based on the presence or absence of SLA, anti-LKM1, ANA, SMA, and/or AMA in terms of patient survival, recognition of independent prognostic factors and requirement for immunosuppressive therapy. In addition, the prevalance of two AMA subtypes and anti liver membrane antibody (LMA) was evaluated.

MATERIALS AND METHODS

Patients

We studied 79 patients with histological and clinical features of CAH,[848] referred between 1970 and 1988, and divided into two overlapping groups (table 15.1).

Table 15.1. Patients and aetiology of CAH or 'CAH-related liver disorders'.

Chronic active hepatitis	(CAH)	group I	group II
1-idiopathic 'autoimmune'	(IAI-CAH)	69	32
2-anti-HBs positive	(antiHBs CAH)		5
3-hepatitis C (non-A,non-B)	(HCV-CAH)		1
'CAH-related liver disease'	('CAH')		
4-'overlap CAH/small-duct PSC'	(CAH/PSC)		1
5-oxyphenisatin induced CAH	(oxy-CAH)		1
6-α_1-antitrypsin deficiency	(A1AT-CAH)		2
total number of patients		69	42

In group I, consisting of 69 consecutive IAI-CAH patients, presently known causes of CAH and related liver disease (like viral CAH, α_1-antitrypsin deficiency, Wilson's disease, or drug-induced CAH) had been excluded.

Group II was formed by 32 out of the 69 patients with IAI-CAH from group I, and 10 other patients with CAH and related disorders of other aetiology (table 15.1). In group II, the common feature was that all patients required immunosuppressive therapy according to accepted criteria,[855,856] and were subsequently treated with standardized immunosuppression (prednisolone(Pred) 15 mg and azathioprine(Aza) 75 mg for two months, followed by 10 mg Pred and 50 mg Aza).

Methods

Coded sera, stored at -20°C, were inactivated by heating at 56°C for 30 minutes, then tested. Positive and negative control sera were added to all assays.

SLA was determined by radioimmunoassay (RIA) according to the blocking principle, as described.[853] Positive/negative ratios above 2.1 were regarded as diagnostic.

The AMA subtype antibodies M2 and Mx were determined by RIA.[857]

LKM-1 was determined by RIA according to a similar procedure[858] as well as by indirect immunofluorescence on cryostat rat kidney sections.

SMA and AMA, were also determined by indirect immunofluorescence on cryostat rat kidney sections.
LMA was detected by immunofluorescence on viable rabbit hepatocytes, as described.[859]

Analysis of survival

Data on survival had been retrieved from patient records, public registry offices, or family practitioners and specialists currently seeing the patients. We analyzed the interval between diagnosis of CAH by liver biopsy and November 1988, or until death or loss to follow-up. The probability of survival was calculated according to the Kaplan-Meier product limit method.[860]

Statistical analysis

Where appropriate, statistical analysis was performed with Chi-square analysis with $p<0.05$ as level of significance. Differences between subsets of patients in the analysis of survival were analyzed statistically by means of the log-rank test with $p<0.05$ as level of significance.[861] The prognostic significance of type of IAI-CAH as determined by presence or absence of ANA, SMA, SLA or LKM_1, as recently described, was determined by adding these variables to those we recently used for determining long-term prognostic variables in CAH.[862] Recalculation was performed using Cox's proportional hazards estimation of independent risk factors.[863]

RESULTS

The autoantibody profiles for SLA, anti-LKM1, ANA, SMA and AMA in patient groups I and II are shown in tables 15.2 and 15.3 respectively. Anti-LKM1, serologically defining IAI-CAH type 2, was not present in any of these patients.

As shown in table 15.2, the 'classic' type 1 IAI-CAH pattern, with detectable ANA with or without SMA, was found in 33 (48%) out of the 69 patients of group I, IAI-CAH type 3 (with SLA) was diagnosed in 6 (9%), and IAI-CAH type 4 (SMA but no ANA) in 17 (25%) of these patients. 13 (19%) out of the 69 patients remained unclassified when using this system and may be referred to as having 'cryptogenic' CAH. 45 (65%) out of the 69 IAI-CAH patients required some form of immunosuppressive treatment: such treatment was administered in 19 (58%) out of the 33 'type 1' patients, in five (83%) out of the six patients with type 3 IAI-CAH (SLA positive), in 15 (79%) out of the 19 patients with IAI-CAH type 4 (SMA detectable, but no ANA), and in 6 (46%) out of the 13 unclassified IAI-CAH patients. Observed occurrence of patients requiring immunosuppression did not differ from expected frequencies for any of the types of IAI-CAH, when compared to the whole group of IAI-CAH patients. Inclusion or exclusion of patients who were only given therapy during exacerbations did not affect

Table 15.2. Autoantibody profiles and therapy of 65 patients with IAI-CAH (group I).

IAI-CAH type:	LKM-1	SLA	ANA	SMA	AMA	Therapy 1 n	2 n	3 n	4 n	5 n	total n
1	-	-	+	+	-	9	3	2	7	3	
1	-	-	+	-	-	1	0	0	7	1	33
3	-	+	+	+	-	2	1	0	0	0	
3	-	+	-	+	-	1	0	0	1	0	
3	-	+	+	+	+	0	0	0	0	1	6
4	-	-	-	+	-	9	2	2	2	2	17
x	-	-	+	-	+	0	0	0	5	0	
x	-	-	-	-	+	0	0	0	0	1	
x	-	-	-	-	-	3	1	0	2	1	13
Total number of patients:						25	7	4	24	9	69

ad therapy: 1=Standardized therapy with prednisolone and azathioprine without preceding immunosuppression, 2= as 1, but with preceding immunosuppression, 3=non-standardized therapy with prednisolone and azathioprine, 4=untreated, 5=prednisolone during exacerbations alone.
x=unclassified (also called: type 5).
n denotes number of patients with this autoantibody pattern and this therapy.

Table 15.3. Number of patients in group I requiring immunosuppressive therapy; there was no significant difference between 'IAI-CAH types', although fewer patients in the subgroup of type 1 IAI-CAH without SMA required such therapy.

IAI-CAH type	patients on immunosuppression continuous		of any kind	
1	15/32	(47%)*	19/32	(59%)**
3	4/6	(67%)	5/6	(83%)
4	13/17	(76%)	15/17	(88%)
x	4/13	(31%)	6/13	(46%)
Total	36/69	(52%)	45/69	(65%)

ad *) 1/7 patients without SMA (Chi-square=11.2, p<0.005)
ad **)2/7 patients without SMA (Chi-square= 6.3, p<0.05)

results. The only exception was that the subgroup of patients with IAI-CAH 'type 1' with ANA but without SMA, required therapy significantly less often than the other patients (table 15.3).

As indicated in table 15.4, in group II, consisting of 42 patients requiring standardized immunosuppressive therapy according to accepted criteria, 16 patients had the autoantibody profile of IAI-CAH type 1; 12 of these patients had IAI-CAH without anti-HBs, while in 4 of these 16 CAH-patients antibodies to hepatitis B surface antigen (anti-HBs) were detected.

Table 15.4. Autoantibody-profiles in 42 patients in group II (CAH requiring immunosuppression).

type:	LKM-1	SLA	ANA	SMA	AMA	aetiology	Number of patients n	total n
1	-	-	+	+	-	1	12	
1	-	-	+	+	-	2	4	16
3	-	+	+	+	-	1	3	
3	-	+	-	+	-	1	1	4
4	-	-	-	+	-	1	11	
4	-	-	-	+	-	4	1	
4	-	-	-	+	-	5	1	
4	-	-	-	+	-	6	2	15
x	-	-	+	-	-	1	1	
x	-	-	-	-	-	1	4	
x	-	-	-	-	-	2	1	
x	-	-	-	-	-	3	1	7

Aetiology: numbers indicating aetiology of CAH correspond with those used in table 1.
Type denotes antibody profiles as described for IAI-CAH types 1-4.
x=unclassified (also called:type 5).

All 4 patients with SLA (the autoantibody pattern of IAI-CAH type 3) requiring standardized therapy had IAI-CAH without anti-HBs, and therefore also belonged to group I described above. SLA was not detected in the ten patients of group II with CAH or 'CAH-related liver disease' of other aetiologies.
The antibody profile of IAI-CAH type 4 (only SMA) was detected in 15 (36%) out of the 42 patients; 11 had IAI-CAH without anti-HBs, 1 an overlap syndrome between

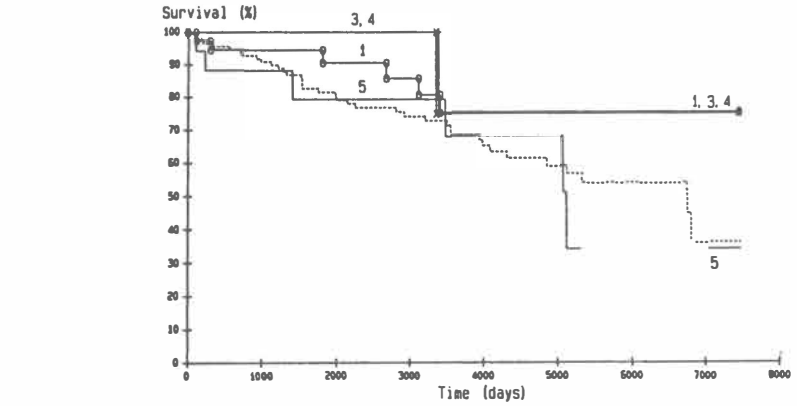
CAH and 'small-duct' primary sclerosing cholangitis (CAH/PSC), two patients had partial MZ-phenotype α_1-antitrypsin deficiency and one patient had been abusing oxyphenisatin before and during immunosuppressive therapy.
Seven patients in group II remained unclassified, when using the classification designed for IAI-CAH: one patient (2%) with IAI-CAH had only ANA, and in six patients (14%) of the 42 patients, none of the antibodies under investigation were detectable: 4 of these 6 patients had IAI-CAH, in one patient anti-HBs was detectable, and in one patient the onset of CAH had -in retrospect- followed a blood-transfusion, suggesting CAH due to hepatitis C.

The AMA-subtype antibodies against M2 and Mx were detected in none of the patients of group II (tested before therapy). In group II, LMA was detected in 7 (25%) out of 28 IAI-CAH patients tested. All of these patients required standardized immunosuppressive therapy. Out of these 7 patients, 6 (86%) had IAI-CAH type 1 (5 patients with both ANA and SMA, one with ANA but without SMA), and one patient had IAI-CAH type 4 (only SMA positive).

As shown in previous chapters for IAI-CAH, standardized immunosuppressive therapy improved the histological activity score, alanine aminotransferase (ALT) and bilirubin, and hepatic protein synthesizing capacity, reflected in serum cholinesterase. Analysis of variance of results of this therapy in group II revealed no difference between treated patients with antibody profiles of IAI-CAH types 1,3 and 4. However, in three patients out of the group with antibody profile type 4, serum cholinesterase showed less improvement after two years of therapy ($p<0.05$). In these patients, however, this was related to aetiology of CAH: two patients had partial MZ-phenotype α_1-antitrypsin deficiency and one patient had been abusing oxyphenisatin during therapy.

Although ANA and SMA frequently became undetectable after institution of immunosuppressive therapy, titres of SLA diminished, but with the antibody remaining detectable during immunosuppression. No new ANA or SMA was detected in patients tested serially before and during immunosuppression (data not shown).

Analysis of survival showed no differences in the log-rank test among patients with autoantibody patterns of type 1,3, 4 or the unclassified group ('type 5'), neither in group I (figure 15.1), nor in group II.
When presence/absence of ANA, SMA, SLA or AMA, were added to the variables we had recently evaluated for their long-term prognosis of survival in CAH,[862] recalculation showed that presence or absence of none of these antibodies was detected as an independent risk factor in the Cox's proportional hazards analysis.



Number of patients at risk	IAI-subgrouped				
1	37	24	15	15	15
3	6	6	4	4	4
4	9	9	4	4	4
5	17	10	7	3	3

Figure 15.1. Probability of survival (Kaplan-Meier) of patients with IAI-CAH (group I). 1= ANA-positive, 3= SLA-positive, 4= SMA-positive but ANA-negative, 5= unclassified (no autoantibodies or only AMA). Dashed line: probability of survival in all patients. No patients with IAI-CAH type 2 were detected. Probability of survival between groups 1, 3, 4 and 5 did not differ significantly.

DISCUSSION

Recently, an aggressive IAI-CAH type 2 was described with a worse prognosis than other types of IAI-CAH, characterized by the presence of antibodies against LKM-1 and/or LC1.[851,852] Further subdivision of IAI-CAH into four subtypes based on presence or absence of ANA, SMA, LKM-1 and SLA was proposed after detection of autoantibodies against SLA in a subgroup of patients with IAI-CAH,[853] but the clinical significance of such a classification was unknown. Using the proposed classification, the present study shows that outcome in terms of survival does not differ among patient groups with IAI-CAH type 1, 3, 4 or 'unclassified'. Similar results were obtained when this classification was applied to patients with CAH of various aetiologies who had fulfilled requirements for standardized immunosuppressive therapy. No differences among these 'types' of CAH were found regarding the necessity to apply immunosuppressive therapy, although a subgroup of patients with IAI-CAH type 1 without SMA required immunosuppression less often than other patients.

The antibody profiles of IAI-CAH types 1,3 and 4 were frequently encountered in our group of Dutch patients with IAI-CAH or other types of CAH and CAH-like liver disorders, while the aggressive type 2 was not seen among our patients.

We recently showed that immunosuppressed patients with IAI-CAH do better than those who do not fulfill treatment criteria and are left untreated. We also showed that age, aetiology of CAH (especially presence or absence of anti-HBs), and the administration of standardized immunosuppression versus other therapy or no therapy are independent risk factors for mortality in CAH.[862] We have now demonstrated that presence or absence of ANA, SMA, SLA or AMA are not independent prognostic variables in either IAI-CAH, or CAH requiring immunosuppressive therapy.

The question remains as to whether these antibodies have a pathogenetic role, or are related to different aetiologies of IAI-CAH, and can justify classification on such a basis.

Recently, it has been shown that antibodies against LMA are not specifically reactive to polypeptides or glycolipids,[864] and are directed against submembraneous constituents of the hepatocyte, i.e. cytoskeletal components. This means that LMA might be directed at neoantigens, presented on the cell membrane in reaction to CAH, questioning their pathogenetic role.[865] Presence of ANA and SMA in CAH are generally also regarded as such epiphenomena.

There is considerable evidence, however, confirming initial suggestions for a pathogenetic role of autoantibodies directed against 'liver-specific membrane lipoprotein' (LSP),[866-868] especially for an important constituent of LSP, the asialoglycoprotein receptor, also known as 'hepatic lectin':[869,870] An LSP-specific T-suppressor lymphocyte defect probably remains present when the aspecific T-suppressor defect in IAI-CAH is reversed by corticosteroids,[871,872] and a defect in the inducers T-lymphocytes of these suppressor cells probably plays an important role in 'classic' IAI-CAH, and possibly IAI-CAH generally.[873]

It has been shown that antibodies to SLA and LSP are not cross-reactive.[853] This, of course does not exclude a possible pathogenetic role of antibodies against SLA, for instance in antibody dependent cellular cytotoxicity, in a subgroup of patients with IAI-CAH. Neither is such a pathogenetic role for SLA excluded by the cytoplasmic nature of the antigens, since cytoplasmic antigens can be present at the surface of the cell membrane.[874]

Anti-LKM-1 has been extensively studied.[875-876] Recently Manns *et al.* cloned the LKM-1 gene,[877] and part of the gene showed homology with part of the genetic code of the Epstein-Barr virus(EBV) (Manns: personal communication). At present, it is unknown if this homology has an aetiological implication, although it is tempting to speculate that EBV might trigger autoimmunity in anti-LKM1 positive CAH. Other evidence for a viral 'trigger' of IAI-CAH is related to the measles virus. Antibodies to this virus are frequent in IAI-CAH,[878,879] and the persisting measles virus genome was detected in a high percentage of IAI-CAH patients, in contrast to a control group.[880]

Antiviral antibodies to the cytomegalovirus (CMV) are also frequently detected in CAH,[881,882] but we recently demonstrated that an aetiological role in IAI-CAH is unlikely, at least by molecular mimicry between CMV-induced antigens and epitopes in LSP.[883]

In conclusion, other studies indicate that the prognosis of patients with IAI-CAH type 2 is worse than that of patients without anti-LKM-1 or anti-LC1 antibodies. However, the present study indicates that there is no apparent clinical reason for distinguishing subtypes of IAI-CAH based on presence or absence of ANA, SMA or SLA. There might however be pathogenetic considerations for such classifications. Cloning of autoantigens such as SLA may provide more insight into the possible pathogenetic importance of presence of antibodies against other autoantigens than those represented in LSP.

Chronic active hepatitis of various aetiologies

CHAPTER 16

ANTI-CARDIOLIPIN ANTIBODIES IN CHRONIC ACTIVE HEPATITIS.

Prevalence in Subgroups and Lack of Correlation with Anti-DNA Antibodies or Extrahepatic Disease.

SUMMARY

We assessed the prevalence, clinical associations and interrelations of anti-cardiolipin antibodies (ACA), anti-DNA antibodies and anti-nuclear antibodies (ANA) in 176 patients with histologically proven chronic active hepatitis (CAH) of various aetiologies.
ACA were present in 30 (17%) of all 176 patients, and in 17 (26%) of the patients with idiopathic or 'autoimmune' CAH (IAI-CAH). ANA were present in 57 (32%) of the 176 CAH-patients, and were in most cases (n=52) of the homogeneous type. In 2 out of 3 ANA-positive patients with 'cholestatic CAH', anticentromere antibodies were detected. Anti-'nuclear lamina' antibodies (ANLA) were present in 96 (55%) of the patients and were equally distributed over the several subgroups.
Anti-DNA as assessed by immunofluorescence with Crithidia Luciliae as a substrate, were absent in 98% of the 176 CAH-patients. In 2 idiopathic or 'autoimmune'-CAH patients without anti-HBs, and one patient with 'anti-HBs positive CAH' these antibodies were detectable. By ELISA, 10% of IAI-CAH patients were positive for IgG-anti-DNA and 18% for IgM-class anti-DNA.
No clear relationship was detected in CAH between ACA and thrombosis or habitual abortion. Neither with regard to ANA, nor to anti-DNA or ACA could a clear relationship be detected with associated extrahepatic autoimmune disease.

INTRODUCTION

Anti-phospholipid antibodies (anti-PL) have been detected in infectious and autoimmune diseases since the introduction of the Wasserman reaction.[884] The finding of a biological false positive syphilis test serology (BFP-STS),[885] which is considered to be primarily an IgM response,[886] was noted in 16% of patients with systemic lupus erythematosus (SLE) ,[887] and in several other diseases such as rheumatoid arthritis,[888] autoimmune thyroiditis,[888] and chronic active hepatitis.[889-891] In the 1950's, several reports of an (in vitro) coagulation inhibitor in association with the BFP-STS led to the

demonstration of the anti-PL antibody nature of the 'lupus anticoagulant' (LAC), which in some cases could be absorbed by cardiolipin and Kahn antigen as used in syphilis test reactions.[892]

Recently, sensitive assays have been developed to quantify these anti-cardiolipin antibodies (ACA).[893,894] The association between a BFP-STS and the presence of LAC, and between the presence of ACA and LAC has been demonstrated.[892,893,895-897] IgG-ACA have been detected in the sera of 19-82% of patients with SLE,[893,894,898-901] and also in 33-50% of patients with rheumatic disease,[899,901] 28% of patients with psoriatic arthritis,[899] 71% of patients with syphilis,[901] 61% of patients with malaria.[901] It has also been demonstrated in ANA-positive Felty's syndrome, essential type II mixed cryoglobulinaemia, mixed connective tissue disease, systemic vasculitis, primary sicca syndrome,[893,899,901] and even with a prevalence of 52% in an elderly normal population.[902]

A relationship between both ACA and LAC with thrombosis,[893,903-906] habitual abortion,[907-911] and thrombopaenia[895,897] has been suggested in several studies in highly selected groups of (mostly) SLE patients.[912]

Cross-reactivity between ACA and anti-DNA antibodies in SLE may exist[913-916] and may be more frequent with single stranded DNA than with double stranded DNA.[917] The epitopes involved in this cross-reactivity appear to be the phosphodiester-linked phosphate groups present in all polynucleotides and in cardiolipin.[918,919] Smeenk *et al.* have found that low-avidity serum anti-DNA antibodies cross-react with cardiolipin, while high-avidity antibodies do not.[920]

In chronic active hepatitis (CAH), anti-DNA antibodies have been recognized since the earlier descriptions of the disease, usually assessed by the Farr assay.[921-925] However, no data are available on the prevalence of ACA, its relationship with ANA and anti-DNA, and its possible clinical significance in CAH.

To assess the prevalence and clinical associations of ACA in a disease different from SLE, we studied ACA and its relation to ANA and anti-DNA as assessed by two different methods in a well-defined population of 176 consecutive patients with histologically proven chronic active hepatitis of various aetiology. We evaluated whether the presence of ACA was associated with extrahepatic and/or thrombotic disease in this group of patients.

MATERIALS AND METHODS

Patients

We studied 176 consecutive patients with a histology of CAH,[926,927] referred to the division of Hepatology of the University Hospital of Groningen between 1969 and 1988 and belonging to several subgroups (table 16.1). Patients were classified according to

the (presumed) aetiology of CAH into CAH with circulating hepatitis B surface antigen (HBsAg-CAH); CAH with circulating antibodies to HBsAg but without HBsAg (anti-HBs-CAH); idiopathic 'autoimmune' CAH (IAI-CAH) without anti-HBs; 'non-A,non-B' CAH without HBsAg or anti-HBs but related to blood-transfusion or intravenous drug-abuse - probably related to the hepatitis C virus in most of these patients (HCV-CAH)-; CAH related to α1-antitrypsin deficiency (A1AT-CAH) (all patients underwent phenotyping); and "other CAH". The group with "other CAH" consisted of the CAH-related disorders: 'overlap' between CAH and primary biliary cirrhosis (CAH/PBC); 'overlap' between CAH and primary sclerosing cholangitis (CAH/PSC); drug-induced CAH (DI-CAH); and CAH related to Wilson's disease (Wilson-CAH). Age, sex and classification of the patients are shown in table 1. Sera from 32 apparently healthy adults served as controls.

Methods

Patient records were reviewed according to a protocol for the presence of venous or arterial thrombosis, habitual abortion and extrahepatic autoimmune disease. Sera obtained during active hepatitis at the time of the diagnostic biopsy had been frozen at -20°C and were thawed at room temperature (without heat inactivation) for determination of ACA, anti-DNA, and ANA. Positive and negative controls were added to all measurement series. All sera were tested for ACA, ANA and anti-DNA (immunofluorescence). Testing for anti-DNA of the IgG and the IgM class by enzyme-linked immunosorbent assay (ELISA) was performed in all ACA positive sera, and in all sera from patients with idiopathic or 'autoimmune' (IAI-)CAH without anti-HBs. In addition, ANA-positive IAI-CAH sera were tested for the presence of antibodies to extractable nuclear antigens (ENA).

Anti-cardiolipin ELISA

ACA were quantified by ELISA according to the descriptions of Loizou *et al.*[894] The upper reference limit, defined as the 95th percentile of the control population, was 22.5 Units (median 5 U) (figure 16.1).

Anti-DNA ELISA and immunofluorescence

An immunofluorescence assay for the detection of anti-DNA antibodies, using the kinetoplast of Crithidia Luciliae as a substrate, was performed as described.[928] As a conjugate, FITC-labelled anti-total IgG (Sigma) was used. Sera were tested in doubling dilutions from 1:10 through to 1:360. For anti-DNA by immunofluorescence, a positive fluorescence in a dilution of 1:40 or more was considered a positive result.

Where anti-DNA were also assessed by ELISA, we used calf thymus DNA (Sigma) as a substrate (after precoating with protamine sulphate). Horse radish peroxidase labelled goat anti-human IgG or IgM (Kallestad) was used as a conjugate. The upper reference limit for anti-DNA antibodies by ELISA, defined as the 95th percentile of the control population, was 10 Units.

ANA immunofluorescence

ANA fluorescence with cultured human fibroblasts as a substrate, was performed as described.[929] Sera were tested in doubling dilutions from 1:10 through to 1:360. A positive fluorescence in a dilution of 1:40 or more was considered a positive result.

Antibodies against extractable nuclear antigens (a-ENA)

Determination of a-ENA was performed by counter immunoelectrophoresis using an extract of rabbit thymus acetone powder (Pel-Freeze) (for SS-B (La), nRNP and Sm) and an extract of human spleen (for SS-A (Ro)) as antigenic sources, together with the corresponding reference sera (CDC, Atlanta, USA).

Statistical analysis

Statistical analysis was performed with the Mann-Whitney U test, and Spearman's rank correlation where appropriate, with $p<0.05$ as the level of significance.

RESULTS

Patients

The ages of the patients with oxy-CAH were higher (median 61 years) than in all other groups ($p<.005$-$p<.05$). Ages between other groups did not differ (table 16.1).

ACA

The prevalence of ACA in the several subgroups is shown in table 16.2. In 30 of 176 CAH-patients (17%), ACA were detected during active disease (figure 16.1). There was no significant difference between subgroups of CAH regarding positivity for ACA. Although the median values (8.0-13.0 U) were within the reference range for all CAH groups, ACA values in all groups were higher than reference ACA-levels (median 3.9 U) ($p<.05$-$p<.0001$).

ANA

57/176 (32%) patients tested had anti-nuclear antibodies during active CAH (figure 16.2). ANA-titres in IAI-CAH (median 40)($p<.0001$), in anti-HBs CAH (median 40) ($p<.005$), and in 'other' CAH (median 5) ($p<.01$), were higher than in HBsAg-CAH (median 0). ANA titres in anti-HBs CAH were higher than those in NANB-CAH (median 0) ($p<.05$). In 53/59 (90%) positive cases, ANA were of the homogeneous type. A discretely speckled fluorescence pattern was seen in 2 IAI-CAH patients and 2 CAH/PBC patients (1 of these in combination with a faint homogeneous fluorescence). A coarsely speckled pattern, was observed in one patient with IAI-CAH. A ring-like fluorescence of the nuclear lamina was observed in 55% of the patients, with equal distribution over the subgroups (table 16.2).

Table 16.1. CAH patients grouped according to aetiology.

Aetiology*	Number of patients	Sex M: F	Age (years) median	range
IAI, no anti-HBs	65	11: 54	51	11-78
HbsAg	49	36: 13	42	18-74
IAI, with anti-HBs	13	3: 10	46	17-81
HCV	10	4: 6	37	18-77
DI	17	3: 14	61	24-78
other	22	6: 16	34	15-72
Total	176	63:113	46	11-81

IAI = idiopathic or 'autoimmune' CAH
HBsAg = CAH with circulating hepatitis B surface antigen (HBsAg) Anti-HBs CAH= CAH with circulating antibodies to HbsAg
HCV= CAH due to parenteral non-A,non-B hepatitis (probably due to hepatitis C virus)
DI = drug-induced 'CAH' due to: oxyphenisatin(12), α-methyl-dopa(3), nitrofurantoin(1), diclofenac(1).
Other CAH:
9x CAH/PBC= 'cholestatic CAH' or 'mixed form'CAH/PBC
5x CAH/PSC= 'small-duct' PSC and/or CAH 3x Wilson-CAH= CAH associated with Wilson's disease
5x A1AT-CAH= CAH related to α_1-antitrypsin deficiency

(PBC= primary biliary cirrhosis) (PSC= primary sclerosing cholangitis)

Table 16.2. Prevalence of several autoantibodies in CAH subgroups.

aetiology	patients (n)	ANA (n,%)	ANLA (n,%)	ACA (n,%)	anti-DNA(IF) (n,%)
IAI-CAH	65	36(55)*	34(52)	17(26)	2(3)&
HBsAg CAH	49	4 (8)	26(53)	5(10)	0
anti-HBs CAH	13	7(54)	8(62)	0	1(8)
HCV-CAH	10	0	6(60)	1(10)	0
DI-CAH	17	4(24)	6(35)	3(18)	0
CAH/PBC	9	3(33)#	7(78)	1(11)	0
CAH/PSC	5	1(20)	3(60)	3(60)	0
Wilson-CAH	3	0	2(67)	0	0
AAT-CAH	5	2(40)	4(80)	0	0
Total	176	57(32)	96(55)	30(17)	3(1.7)

numbers denote number of patients with positive results (percentages between brackets).
ad *) 2 sera discretely speckled, 1 serum coarsely speckled + homogeneous.
ad #) 1 serum discretely speckled, 1 serum discretely speckled + homogeneous.
All other ANA-fluorescence patterns were homogeneous.
ad &) 6/61(10%) IgG anti-DNA by ELISA, and 14/61(23%) IgM anti-DNA by ELISA.

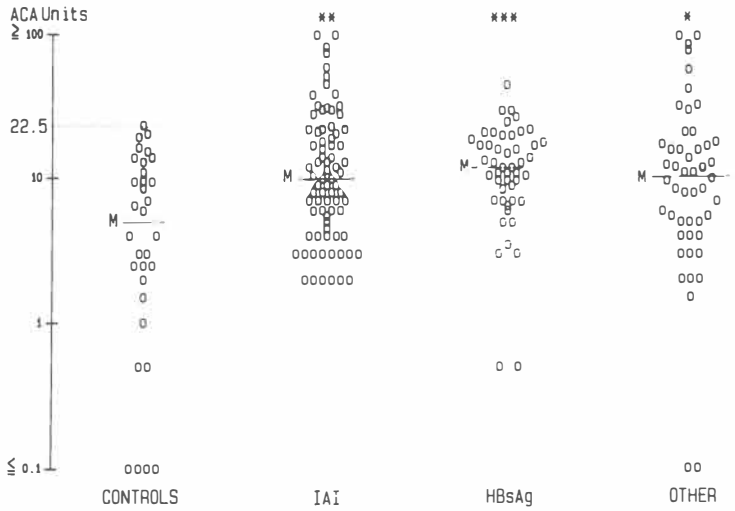


Figure 16.1. Anti-cardiolipin antibodies (ELISA) in CAH (n=176) and controls (n=32). M= median. Dotted line=95% upper reference limit (22.5U).
IAI=IAI-CAH (with or without anti-HBs) (n=78), HBsAg=HBsAg-positive CAH (n=49), other=other types of CAH, as shown in table 16.1 (n=49).
*:p<0.05, **:p<0.005, ***:p<0.001 versus controls.

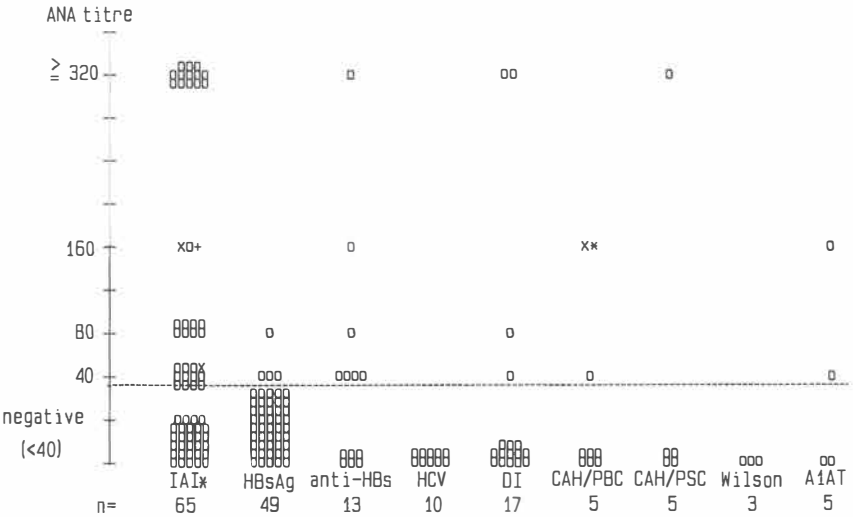


Figure 16.2. Anti-nuclear antibodies in CAH (n=176). Fluorescence pattern: Δ=homogeneous, *=homogeneous+discretely speckled, x=discretely speckled, +=coarsely speckled. IAI*=IAI-CAH without anti-HBs.
For further abbreviations: see table 16.1.

ENA

No antibodies to ENA were detected in sera from the ANA-positive IAI-CAH patients.

Anti-DNA

Only 3/176 patients showed anti-DNA antibodies by immunofluorescence with the kinetoplast of Crithidia Luciliae as a substrate, as shown in table 16.2. All three patients (2 with IAI-CAH, 1 with anti-HBs CAH) had a homogeneous ANA fluorescence pattern with a titre ≥320. ACA, ANA and anti-DNA (both immunofluorescence and ELISA) of these 3 patients are shown in table 16.3.

Table 16.3. Antibody titres in CAH with positive anti-DNA(IF).

Patient number	anti-DNA(IF) Crithidia (titre)	anti-DNA ELISA(IgG) (Units)	anti-DNA ELISA(IgM) (Units)	ACA(IgG) ELISA (Units)	ANA (IF) (titre)	CAH-subgroup
1	160	3	19	6	640	IAI
2	320	9	2	19	640	IAI
3	160	15	52	7	640	anti-HBs
normal	<10	<10	<10	<22.5	<40	

By ELISA, anti-DNA antibodies of the IgG-class were detected in 6/61 (10%) IAI-CAH patients , and anti-DNA antibodies of the IgM-class in 11/61 (18%) IAI-CAH patients, as shown in figure 16.3. In 3 of these patients, IgG and IgM anti-DNA were both present (table 16.4).

The total prevalence of anti-DNA antibodies was 17(28%) out of 61 patients with IAI-CAH, as determined by ELISA.

Table 16.4. ACA and anti-DNA (ELISA) in IAI-CAH (n=61)

		IgG anti-DNA		IgM anti-DNA	
		+	-	+	-
ACA	+	2	13	5	10
	-	4	42	9	37

numbers denote number of patients.

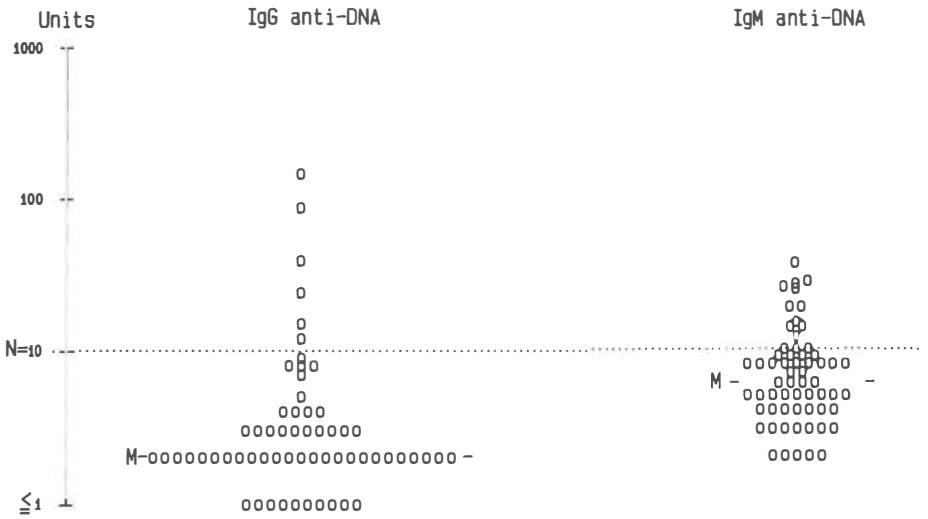


Figure 16.3. Anti-DNA antibodies (ELISA) in IAI-CAH (n=61).

Serological relation between ACA, ANA, and anti-DNA

In the IAI-CAH patients, co-occurrence of anti-DNA IgG with ACA was rare, while co-occurrence of ACA with anti-DNA IgM was present in only 5/61 IAI-CAH patients tested (table 16.4). None of 9 ACA-positive patients with CAH of other aetiologies had detectable anti-DNA IgG and IgM anti-DNA was found by ELISA in only one of these patients. A weak correlation was present between the titres of ACA and that of ANA ($R=.19$, $p<.05$).

Correlations with extrahepatic disease

One patient had had thrombosis just before the start of standardized therapy. No ACA were present when tested on several occasions before and during therapy with anticoagulants and immunosuppressive medication.

14 (25%) of 55 women in the child-bearing age (17 to 45 years of age) had detectable ACA. In our retrospective analysis, no cases of habitual abortion were detected. From two patients who had had a spontaneous abortion (one patient before and one patient after the time of diagnosis of CAH), one had ACA while the other lacked this autoantibody. Two patients (one with and one without ACA) had each delivered three healthy children during remission from CAH during maintainance therapy consisting of 10 mg prednisolone and 50 mg azathioprine daily; the patient without ACA experienced one spontaneous abortion after the second uncomplicated delivery of a healthy baby.

Table 16.5. Associated (autoimmune) disease in 176 patients with CAH.

symptoms	n=	number of patients with ACA	ANA	anti-DNA(IF)
Venous thrombosis	1	0	0	0
Recurrent abortion (out of 55 women)	0			
Spontaneous abortion (only once)	2	1	0	0
Hemolytic anaemia	3	1	0	0
Arthritis	17	5	9	1
Pleuritis	1	1	1	1
Pericarditis	2	1	1	1
Hashimoto's thyroiditis with hypothyroidism	2	0	1	0
Goiter (hypothyroidism after thyroidectomy)	1	1	0	0
Diabetes mellitus	6	2	2	0
Bronchial asthma	7	1	2	0
Interstitial pulmonary fibrosis	1	0	0	0
Raynaud's phenomenon	1	1	0	0
Oesophageal hypomotility	1	1	1	0
Transient lymphadenopathy	2	1	0	0
Relapsing iridocyclitis	1	0	0	0
Ulcerative colitis	2	2	0	0
Achlorhydria	1	0	1	1
Chronic gastritis	2	0	1	0
Glomerulonephritis				
-Membranoproliferative	1	0	1	0
-Focal	1	0	1	1
Proteinuria, cause unknown	3	0	1	1
Pancreatic insufficiency	1	0	1	1
Malabsorption	1	0	1	1
Polyneuritis + tremors	1	0	1	0
Vitiligo				
Stiff painful muscles (myositis?)	3	0	1	0
Arteritis	1	0	1	0
Allergic vasculitis	1	0	1	0
Sjögren's syndrome	1	0	1	0
Alzheimer's disease	1	0	1	0
TOTAL	69	18	31	8

One or more extrahepatic (autoimmune) disease manifestations were detected in association with CAH 69 times in 47 (27%) out of the 176 patients, as shown in table 16.5. No clear associations between associated (autoimmune) disease and the presence of ANA, ACA or anti-DNA (ELISA) could be detected, although two of the three patients with anti-DNA (as detected by the Crithidia assay) had associated autoimmune disease (table 16.5).

DISCUSSION

Prevalence

The present study indicates that ACA is not infrequent in CAH. Cross-reactivity between ACA and anti-DNA in CAH appears to be limited, and no relation of ACA with thrombotic events, recurrent abortion or associated extra-hepatic autoimmune diseases became apparent.

Anti-double-stranded DNA antibodies were absent in 98% of our 176 CAH patients as assessed by indirect immunofluorescence with Crithidia Luciliae as a substrate, while these antibodies were detected more frequently by ELISA. Others, however, have reported a higher prevalence of anti-DNA positivity in CAH by the Crithidia assay;[931,932] however, most of these cases of CAH were drug-related. Our findings support the suggestion from other studies[933,934,935] that anti-DNA, as detected by the Crithidia assay, allows an immunological distinction between CAH and SLE.

ANA were detected frequently in our CAH patients, the highest frequency being found in IAI-CAH. The fluorescence pattern was usually of the homogeneous type, in accordance with the literature.[936] ANA in autoimmune liver disease can be directed to a nuclear protein of 54 kD, to a nuclear matrix protein of 150 kD, a U1-RNP protein of 70 kD, and to a nuclear 25 kD doublet.[937] ANA against histones, as frequently present in PBC, are rare in hepatitis B virus-associated CAH.[938] Characterization of these antigens is still incomplete.

A ring-like perinuclear lamina fluorescence pattern was detected in 55% of our CAH patients. Such a pattern has recently been described both in acute hepatitis, granulomatous hepatitis, CAH and PBC. The antibodies responsible for the ring-like fluorescence in CAH and acute hepatitis appeared to be predominantly directed at lamins A and C of 70 and 60 kD (anti-nuclear lamina antibodies, or ANLA),[939,940] and not at the nuclear envelope-associated proteins with a molecular weight of approximately 200 kD, which is specific for PBC.[940-943]

The absence of antibodies to ENA in our IAI-CAH patients, as assessed by counter immunoelectrophoresis, is in agreement with the absence of anti-SS-B (La) in CAH,[933] when detected by immunodiffusion. However, Kurki *et al.* found anti-SS-B (La) in 38%

of CAH patients when using a sensitive ELISA.[933] Presence of anti-SS-B would be in agreement with the concept that the sicca syndrome associated with CAH is of the primary type, but the sicca syndrome is of PBC of the secondary type.[944] We detected the sicca syndrome in only one patient in our population. This could explain why we could not find anti-SS-B (La). Using sensitive methods of detection, Penner also found that anti-La antibodies (50 kD) and circulating immune complexes containing histones (especially anti-H1) were invariably present in patients with IAI-CAH associated with the sicca syndrome. IAI-CAH associated with membranous glomerulonephritis has been described to be associated with U1-RNP-containing immune complexes.[945] Sjoegren's syndrome in PBC can also be associated with sensitisation to salivary antigens and deposition of Ro/anti-Ro immune complexes.[946]

Serological correlations

As has already been mentioned, low-avidity anti-DNA antibodies cross-react extensively with cardiolipin, while high-avidity antibodies do not.[920] Our study indicates that cross-reactivity between ACA and anti-DNA detected by immunofluorescence is limited in CAH. In most patients, anti-DNA antibodies detected by ELISA remained undetected in the immunofluorescence assay with Crithidia Luciliae as a substrate. This may be due to the fact that the latter assay detects predominantly high avidity anti-DNA, while the ELISA probably binds also anti-DNA of low-avidity. This indicates that low-avidity anti-DNA antibodies can occur in CAH, while high-avidity anti-DNA is very uncommon.

Clinical correlations

Despite the limitations of a retrospective study, our findings confirm the frequent occurrence of multisystem involvement in CAH and its lack of association with ANA,[947] while no correlations with ACA could be detected.

We observed a relatively high prevalence of a discretely speckled ANA-fluorescence in cholestatic CAH ('CAH/PBC'). In ANA-positive PBC-patients,[948-950] antibodies to a chromatin-associated centromere-protein of 19.5 kD, at present known to be part of protein(s) with a larger molecular weight, are associated with the presence of one or more features of the CREST-syndrome (calcinosis, Raynaud's phenomenon, oesofageal hypomotility, sclerodactyly, telangiectasia),[935,949-952] the sicca syndrome9[53,954] and kerato-conjunctivitis sicca.[955,956] Despite the presence of a fluorescence pattern consistent with the presence of anti-centromere antibodies in CAH/PBC 'overlap' patients, no signs of the CREST syndrome or the sicca syndrome were detected in our 'CAH/PBC overlap' patients. However, no 'anti-centromere fluorescence pattern' was detected in patients with such features.

As mentioned, the correlation between ACA and thrombotic events or habitual abortion has been detected predominantly in highly selected groups of patients, usually with SLE. In an unselected population of patients, such correlations may be less clear. For instance, in an unselected population of patients with recurrent foetal loss Petri *et al.* found no association with the presence of LAC and ACA.[956] In our CAH patients, no correlation between the presence of ACA and thrombosis or habitual abortion could be detected. Unlike SLE, in CAH the occurrence of spontaneous abortion (10 percent) is not higher than expected, although a higher than expected rate of perinatal fetal loss and obstetric complications occurs.[957] ACA may be an epiphenomenon delineating a subgroup of patients at risk for habitual abortion and thrombosis in some diseases, -for instance SLE-, but not in other diseases such as CAH.

To conclude, ACA were present in 17% (in IAI-CAH 26%) of patients with CAH. Anti-DNA of high avidity is uncommon in CAH, while low avidity anti-DNA can be present. Detection of anti-DNA by indirect immunofluorescence on the kinetoplast of Crithidia Luciliae, favors a diagnosis of SLE instead of CAH. Cross-reactions between ACA and anti-DNA appear to be limited in CAH. ANA are frequently present and usually of the homogeneous type, except in CAH/PBC 'overlap syndrome' where a discrete-speckled ANA-pattern is more common. A ring-like anti nuclear lamina fluorescence pattern was frequent, probably reflecting the presence of anti-lamin A and C antibodies. No relationship between ACA and thrombosis or habitual abortion was detected. Neither were relationships detected with frequently occurring associated (autoimmune) disease.

CHAPTER 17

HUMORAL RESPONSES AGAINST DEFINED CYTOMEGALOVIRUS ANTIGENS AND 'LIVER-SPECIFIC MEMBRANE LIPOPROTEIN' IN CHRONIC ACTIVE HEPATITIS

Lack of evidence for aetiologic involvement of cytomegalovirus by molecular mimicry.

SUMMARY

There is evidence that antibodies to 'liver-specific membrane lipoprotein' (anti-LSP) are aetiologically involved in idiopathic or 'autoimmune'(IAI) chronic active hepatitis (CAH), but the trigger for development of IAI-CAH remains unknown. The prevalence of anti-cytomegalovirus antibodies (aCMV) in CAH has been reported to be higher than in the normal population. We tested the hypothesis that CMV is aetiologically involved in IAI-CAH.

Methods: Sera taken during active disease from 178 patients with CAH (82 IAI, 48 HBsAg-positive, 48 'other' CAH) were stored at -20°C and then tested by ELISA for aCMV of both IgG- and IgM-class to late antigen (LA). 34 IAI-CAH patients were also tested for presence of anti-LSP by radioimmunoassay (RIA), and the effect of preabsorption of anti-LSP positive IAI-CAH serum with LA was tested. 16 IAI-CAH sera - 4 anti-LSP negative but anti-LA-IgG-positive (group I), 6 anti-LSP-positive but anti-LA-negative (group II), and 6 positive for both anti-LSP and anti-LA-IgG (group III)- were tested for presence of defined anti-CMV-membrane antigens with a molecular weight of 58 kD (58-MA) and CMV-immediate early antigen of 67 kD (67-IEA), by newly developed sandwich ELISA's using monoclonal antibodies.

Results: 122 (69%) of the 178 patients had anti-LA-IgG (more in IAI-CAH and HBsAg-positive CAH than in 'other CAH'). 69 (39%) had anti-LA-IgM (lower in IAI-CAH than in HBsAg-CAH or 'other' CAH). Age- and sex-matched prevalence of IgG-class aCMV in CAH was higher than reported in 1731 healthy blood donors (HBD) in a recent German study, but similar to prevalence in 1251 HBD in a recent Swedish study. Anti-LA-IgG titres were higher in IAI-CAH and HBsAg-positive CAH than in 'other CAH'. 22/34 IAI-CAH patients tested had both anti-LA-IgG and anti-LSP, 2/34 had none of these antibodies, 8/34 had anti-LSP but no anti-LA-IgG, 2/34 had no anti-LSP but had anti-LA-IgG. Preabsorption of anti-LSP positive IAI-CAH serum with LA before RIA did not lower anti-LSP titres. In group I, 4/4 patients had a67-IEA and 3/4 had a58-MA. None of the 6 patients in group II, but all in group III had a58-MA or a67-IEA.

Conclusions: Prevalence of anti-CMV-LA IgG in CAH is similar to or higher than in HBD. Prevalence and titres are higher in IAI-CAH and HBsAg-positive CAH than in 'other' CAH. There is no correlation between presence/absence of anti-LA-IgG and anti-LSP, despite the fact that both are often present. It is unlikely that there is an aetiological role for CMV in inducing autoimmunity in IAI-CAH by molecular mimicry between LSP and defined CMV-induced membrane antigens.

INTRODUCTION

Chronic active hepatitis (CAH) is characterized by a chronic portal and periportal inflammation of the liver, with piecemeal necrosis and peripolesis,[958] and with many aetiologies resulting in the same morphology. Frequently, however, no aetiology can be detected, and in these patients the CAH is termed 'idiopathic' or 'cryptogenic'. Since many of these patients display autoantibodies, hypergammaglobulinaemia, and other characteristics attributed to autoimmunity, idiopathic CAH in such patients is termed idiopathic 'autoimmune' CAH (IAI-CAH). It is this group of patients that usually reacts favorably to immunosuppressive therapy.[959] More specific treatment modalities, however, await elucidation of aetiological factors.

Besides the development of autoantibodies, increased antibody titres to exogenous antigens were reported, notably bacterial and viral.[960,961] High antibody titres can occur against gut-derived bacteria such as E. Coli, Bacteroides, and Salmonella.[960,962] The production of antibodies to Haemophilus influenzae B -which usually targets the respiratory tract- is, however, seldom increased in patients with acute and chronic liver disease. If increased, it is due to a non-specific hyperreactive state.[960] With regard to antiviral antibodies in CAH, elevated antibody titres were described for measles, rubella[963-966] and cytomegalovirus (CMV),[966] while no increased antibody titres against Coxsackie B virus were detected.[965] Elevated antibody titres to CMV early antigen[967] were also described in oxyphenisatin-induced CAH,[968] and in acute hepatitis B.[969] A higher prevalence of antibody titres to adenovirus was also found in acute hepatitis.[970]

The presence of antiviral antibodies was found to be uncommon in chronic persistent hepatitis, and in cirrhosis without hepatitis.[963,971] In a patient with CAH, significantly rising antibody titres to rubella and measles virus were described, following the onset of liver disease.[965] Anti-measles antibodies in CAH can belong to the IgM-class of antibodies,[972] suggesting persistent production of measles virus antigens.

In contrast to the situation in the immunocompromised host (ICH),[973] hepatitis -especially of the chronic type- due to CMV in the non-ICH is less common. Histological and immunological differences between these two situations exist.[974] Before intranuclear early and late antigens are induced by CMV-infection, the cell surface already expresses CMV-specific membrane neoantigens, CMV-MA.[975] CMV-MA may contribute to early recognition and elimination of infected cells by cell-mediated immunity. Analysis of serological responses against defined CMV-antigens is now feasible by antigen capture ELISA's using monoclonal antibodies.[976,977]

There is considerable evidence that in CAH, cytotoxic reactions of the antibody dependent (ADCC-)type are directed at hepatocyte plasma membrane-derived epitopes in the macromolecular, lipid-associated complex called 'liver specific antigen' (LSP).[978,979] The liver-specific, species cross-reactive hepatic receptor for desialylated glycoproteins, the asialoglycoprotein receptor (ASGR), also known as hepatic lectin, is an important constituent of LSP.[980] Anti-LSP antibodies appear to be predominantly directed at the ASGR, although a role for anti-LSP directed at other antigenic sites is not yet excluded. Anti-LSP is usually present in IAI-CAH patients requiring immunosuppressive therapy according to accepted criteria, and anti-ASGR is found in most of these anti-LSP positive CAH patients.[981] It has also been shown that anti-ASGR antibodies preferentially coat periportal hepatocytes in the antegradely and retrogradely perfused rat liver.[982] An LSP-specific T-suppressor lymphocyte defect remains present when the aspecific T-suppressor defect in IAI-CAH is reversed by corticosteroids.[983,984] A defect in the inducer T-lymphocytes of these suppressor cells probably plays an important role in IAI-CAH.[985] The triggers for the induction of autoimmunity remain unknown, however.

It is not clear whether the increased antibody titres in CAH to viral and bacterial antigens are epiphenomena, or whether these agents are involved in the aetiology of IAI-CAH.
Another autoimmune disease, diabetes mellitus type I, recently appeared to be associated with the cytomegalovirus. CMV genome was found in the lymphocytes of 13/59 (22%) of diabetic patients, in contrast to 1/38 (2.6%) of the control subjects, and there was a strong correlation between CMV genome and presence of islet cell autoantibodies.[986]

To further investigate the relationship between CAH and CMV, we quantified antibodies of the IgG and IgM class to CMV late antigen (LA) in a population of 178 CAH patients with various aetiologies. This was to compare qualitative and quantitative differences between IAI-CAH patients and patients with CAH of known aetiology, with respect to these antibodies. The prevalence of anti-CMV antibodies was compared to studies of healthy blood donors. In the IAI-CAH patients we investigated the relationship between the presence of anti-LSP, anti-CMV-LA, anti-CMV-specific immediate early antigen with a molecular weight of 67 kiloDalton (67-IEA) and anti-CMV-specific membrane antigen of 58 kD (58-MA). The ability of CMV-LA antigens to inhibit binding of IAI-CAH serum containing anti-LSP in the radioimmunoassay for detection of anti-LSP was tested for, in order that we may study a possible molecular mimicry between CMV-encoded and auto-antigens. Such a mimicry might provide a clue to the induction of IAI-CAH.

MATERIALS AND METHODS

Patients

From a series of 178 consecutive patients with histologically confirmed CAH, referred to the division of Hepatology of the University Hospital Groningen between 1969 and 1988, sera had been frozen and stored (-20°C) until use. 82 patients had IAI- CAH (12 with anti-HBs), 48 had hepatitis B-surface antigen positive CAH (HbsAg CAH), and 48 had 'other' CAH (table 17.1).

Table 17.1. CAH patients stratified on aetiologies

AETIOLOGY OF CAH			n=	TOTAL
'AUTOIMMUNE'	-idiopathic	(IAI-CAH)	70	
	-anti-HBs positive	(anti-HBs CAH)	12	82
-HBsAg POSITIVE		(HBsAg CAH)	48	48
-OTHER				
-hepatitis C ('non-A,non-B') CAH		(HCV-CAH)	11	
-'cholestatic CAH'		(CAH/PBC)	9	
-'small-duct' PSC and/or CAH		(CAH/PSC)	5	
-drug-induced 'CAH'*		(DI-CAH)	16	
-Wilson's disease 'CAH		'(Wilson-CAH)	3	
-α_1-antitrypsin deficiency		(A1AT-CAH)	3	
-IAI-CAH in multicystic liver		(cyst-CAH)	1	
				48
Total				178

n denotes number of patients in each group.
ad *) Drug-induced cases were due to: oxyphenisatin(12), α-methyl-dopa(3), nitrofurantoin(1), diclofenac(1).

Sera from all 178 patients were investigated for the presence of antibodies to CMV- late antigen (CMV-LA) of the IgG and IgM classes. Assessment for anti-LSP was carried out in 34 untreated patients with IAI-CAH (4 patients with circulating antibodies to hepatitis B surface antigen (anti-HBs CAH), and 30 patients without such antibodies). We tested for inhibition of anti-LSP binding by LA-antigen. Newly developed sandwich ELISA's for detection of anti CMV 67-IEA and anti CMV 58-MA antibodies were performed on sera from 16 IAI-CAH patients: 6 with anti-LSP but no anti-LA IgG, 4 with anti-LA IgG but no anti-LSP, and 6 with both antibodies.

To provide enough age-matched controls with which to compare prevalence of anti CMV antibodies in CAH, we compared prevalence of anti CMV antibodies in our patient groups with that in North European healthy blood donors from two recently published studies that used similar sensitive detection techniques.[987,988]

Anti-LSP radioimmunoassay

We slightly modified the original methods for radiolabelling LSP and performing the radioimmunoassay (RIA)[989], and the assay was performed as described.[990] Positive and negative control sera were added to all runs.

Anti-CMV-LA ELISA

IgG and IgM antibodies to CMV-LA were determined by enzyme-linked immunosorbent assay (ELISA) as described.[991] CMV and control antigens used as a substrate were prepared from fibroblasts, harvested 6-7 days after CMV infection. As controls in each assay, and for standardization between different assays, a CMV antibody-positive reference serum and a CMV-antibody negative reference serum pool were always included. All sera were tested at several dilutions. The difference in concentration of antibodies in the tested serum and the CMV antibody-positive reference serum was determined essentially by the so-called effective dose method.[991a] The concentration of antibodies to CMV-induced antigens was expressed as a percentage of the CMV antibody-positive reference sample, which was designated as 100%, after logit transformation of the sigmoidal titration curve into a linear response curve. Then these percentages were converted into arbitrary units, and a concentration of more than 1 U/ml was considered to be a positive result.

Inhibition experiments

Inhibition of anti-LSP binding by LA-antigen was tested by adding LA in three doubling dilutions (1:50, 1:100, 1:200), diluted with the 0.1% BSA-borate-EDTA buffer used in the anti-LSP RIA, to an IAI-CAH serum containing both anti-LSP and anti-CMV-LA of the IgG class, and to another IAI-CAH serum containg anti-LSP but not anti-CMV LA antibodies. As controls in the same run, the anti-LSP titre was determined in these two sera in the same dilution, but without adding LA. This was done in buffer with LA in the three doubling dilutions mentioned before, and in buffer with control antigen (uninfected fibroblast material) in a 1:50 dilution, which is used in the anti-CMV-LA ELISA. Other anti-LSP positive and negative reference sera were included in the same run.

Antigen capture ELISA for 67-IEA and 58-MA

Newly developed monoclonal antibody based antigen capture immunoassays,[992] were used for detection of anti-CMV antibodies against the CMV-specific immediate early antigen with a molecular weight of 67 kiloDalton (67-IEA) and the CMV-specific membrane antigen of 58 kD (58-MA). The cell-free human CMV strain AD-169,

prepared as decribed,[991] was used to infect monolayers of human embryonic fibroblasts. Antigens from late-infected fibroblasts (CMV-antigen, 4-5 days post infection) were prepared by detergent extraction and purified with low- and high-speed centrifuging. Monoclonal antibodies against these antigens were raised and isolated from mouse ascites using standard ammonium sulphate precipitation and DEAE chromatography techniques. Monoclonal antibody C10 reacted with a CMV-antigen present in the nucleus of CMV infected cells from about 1h post-infection,[992a] and in both nucleus and cytoplasmic granules of late-infected cells. Another monoclonal antibody (C40), also prepared with these techniques, was reactive with a CMV glycoprotein which has homology with herpes simplex glycoprotein B (58-MA) (unpublished results, courtesy of M.P. Cranage, Cambridge; Cranage *et al.*, 1986).[993] C40 is reactive with the perinuclear region and the plasma membranes of late infected cells. These monoclonal antibodies were used for coating the ELISA plates (Greiner) in Na_2CO_3 pH 9.6 for 40 hrs at 4°C. After incubating with either CMV- or control antigen, diluted 1:75 in incubation buffer (10 mM Tris-HCl pH 8.0, 0.3 M NaCl, 1% bovine serum albumin (BSA) and 0.05% Tween 20) each serum was tested in several dilutions with starting from 1:100 in duplicate. After coating, and between incubations (45 min at 20°C), plates were washed five times with 'washing buffer' (10 mM Tris-HCl pH 8.0, 0.15 M NaCl and 0.05% Tween 20) using an automatic washing device (Titertek). Peroxidase labelled conjugate (goat anti-human IgG, Kallestad) was added in a dilution of 1:100 in incubation buffer containing 1% normal mouse serum. After the final wash, the plates were treated with a substrate solution (ortho-phenylene-diamine dihydrochloride (Kodak), 0.2 mg/ml in 50 mM phosphate buffer pH 5.6 containing 0.006% H_2SO_4. Extinctions were measured at 492 nm using a Titertek Multiscan. The antigen-specific extinction was calculated for each serum dilution by subtracting the optical density (OD) of the control antigen from the OD of the CMV-LA. The amount of antibody present in a serum to antigens captured in the assay, was expressed as a percentage of the positive reference serum, as described for anti CMV-LA antibodies (see above). It was established that the apparent molecular weights of the captured antigens were similar to the apparent molecular weights obtained in immunoprecipitation experiments with C10 and C40 monoclonal antibodies.

Statistical analysis

Statistical analysis was performed with the Mann Whitney U test, Chi-square test and Spearman rank correlation where appropriate, and with $p<0.05$ as the level of significance. All analyses were performed in duplo, with a Pearson correlation coefficient between both results of >0.97.

RESULTS

Anti-CMV-LA IgG and IgM ELISA

IgG class antibodies to CMV-LA were detected in 122 (69%) of the 178 CAH patients. The prevalence of anti-CMV-LA-IgG in IAI-CAH and HBsAg-CAH was higher than in 'other' CAH (Chi-square=23.111, p<0.0001) (table 17.2), and the titres were higher in IAI-CAH and HBsAg-CAH than in 'other' CAH (figure 17.1A).

Table 17.2. Prevalence of anti-CMV-LA antibodies in CAH. (n=178)

AETIOLOGY OF CAH	Anti-CMV-LA IgG-class		antibodies of IgM-class	
'Autoimmune' CAH	61/82	(74%)	26/82	(32%)**
HBsAg-CAH	35/48	(73%)	22/48	(46%)
'Other' CAH	26/48	(54%)*	21/48	(44%)
Total	122/178	(69%)	69/178	(39%)

ad *) Chi-square=23.111, p<0.0001
ad **) Chi-square= 5.992, p<0.02

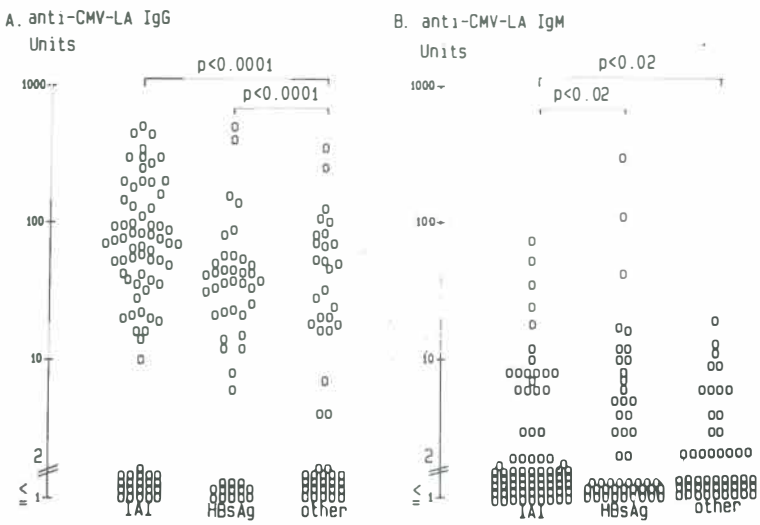


Figure 17.1. Anti-CMV-LA of A) IgG class and B) IgM class in IAI-CAH (n=82), HBsAg-positive CAH (n=48), and 'other' CAH (n=48).

Table 17.3. Prevalence of anti-CMV-LA-IgG A) in 'autoimmune' CAH (n=82) compared to anti-CMV antibodies of age-matched healthy blood donors, B) in all CAH patients (n=178).

A) 'Autoimmune' CAH (n=82)

Age	Observed	Expected based on reference	
		988	987
1-9	0/0	0/0	0/0
10-20	5/13	7/13	2/13
21-25	3/3	2/3	1/3
26-30	5/5	3/5	2/5
31-35	3/5	4/5	2/5
36-40	5/6	5/6	3/6
41-45	1/4	3/4	2/4
46-50	7/7	6/7	3/7
51-55	8/12	10/12	8/12
56-60	10/11	10/11	6/11
61-65	6/7	6/7	4/7
≥66	8/9	9/9	5/9
Total	61/82	65/82 *	38/82 **

Observed versus expected frequences:
ad *) Chi-square= 1.187 , p=0.2753
ad **) Chi-square=25.944 , p<0.0001

B) All CAH patients (n=178).

	Observed	Expected based on reference	
		988	987
Totall	22/178	131/178 #	66/178 ##

Observed versus expected frequences:
ad #) Chi-square= 2.342 , p=0.1218
ad ##) Chi-square=75.515 , p<0.0001

IgM-class antibodies to CMV-LA were detected in 69 (39%) of the 178 CAH patients, usually in low titres, as shown in figure 17.1B. There was no difference in titres of anti-CMV-LA-IgM among IAI-CAH, HBsAg-CAH or 'other' CAH, but the prevalence of anti-CMV-LA-IgM was lower in IAI-CAH than in HBsAg-CAH or 'other' CAH (Chi-square=5.992, p<0.02) (table 17.2).

Prevalence of IgG-class anti-CMV-LA among our 178 patients with CAH was higher than expected when compared to the prevalence of anti-CMV antibodies in 1731 healthy German blood donors (Chi-square=75.515, p<0.0001). However, it was similar to prevalence in 1251 Swedish healthy blood donors (Chi-square=2.342, p=0.1218). Also, when IAI-CAH or HBsAg-CAH were considered separately, the observed number of patients with anti-CMV-LA-IgG was higher than expected based on the German study, but not different from expected values when based on the Swedish study of healthy blood donors. When matching our CAH patients for age with the blood donors from both studies, similar results were obtained (table 17.3 shows the results for IAI-CAH).

Anti-LSP in relation to anti-CMV-LA antibodies

Out of the 34 IAI-CAH patients tested for both antibodies, 22 had both anti-LSP and anti-LA-IgG, 2 had none of these antibodies, 8 had anti-LSP but no anti-CMV-LA-IgG, and 2 had anti-CMV-LA-IgG but no anti-LSP. All 4 anti-HBs positive patients of this group had anti-LSP and 3 of them had anti-CMV-LA of the IgG-class (table 17.4). With (Chi-square=0.926, p=0.338) or without (Chi-square=0.78, p=0.381) these anti-HBs positive patients, presence/absence of one or the other antibody did not predict presence/absence of the other (table 17.4), although the group of patients with both antibodies was bigger than the other groups (Chi-square=31.412 or 23.552 respectively, both with p<0.001).

Table 17.4. Presence/absence of anti-LSP and anti-CMV-LA-IgG in 34 patients with 'autoimmune' CAH did not correlate.

	IAI or anti-HBs		IAI (no anti-HBs)	
	aLA	no aLA	aLA	no aLA
aLSP	22	8	18	7
no aLSP	2	2	2	2
Chi-square=	0.926		0.78	
p =	0.338		0.381	

numbers denote number of patients.

All of the anti-LSP positive patients, and the one anti-HBs-positive but anti-LSP negative patient, fulfilled requirements for immunosuppressive therapy. In contrast, the anti-LSP negative and anti-HBs negative IAI-CAH patients did not.

Inhibition experiment

Addition of LA-antigen to an anti-LSP positive, anti-LA IgG positive serum did not inhibit binding of anti-LSP in the anti-LSP RIA. The same finding applied to addition of LA to an anti-LSP positive, but anti-LA IgG negative serum (table 17.5).

Table 17.5. No inhibition of anti-LSP binding with LSP by CMV-LA in idiopathic 'autoimmune' CAH.

sample	aLA	aLSP	added LA-antigen	anti-LSP titre
patient 1	+	+	none	100
serum	+	+	1: 50	100
	+	+	1:100	100
	+	+	1:200	100
patient 2	-	+	none	200
serum	-	+	1: 50	200
	-	+	1:100	150
	-	+	1:200	200
buffer	-	-	1: 50	neg
	-	-	1:100	neg
	-	-	1:200	neg
buffer	-	-	control antigen 1:50	neg

aLSP denotes anti-LSP antibodies.
aLA denotes anti-CMV antibodies of IgG-class to late antigen.

67-IEA and 58-MA sandwich ELISA

To test for coexpression of two defined CMV-induced antigens on the one hand (immediate early antigen of 67 kD molecular weight, and membrane antigen of 58 kD) and epitopes of LSP on the other hand, we tested 16 sera from patients during untreated IAI-CAH with anti-LSP and/or anti-CMV-LA IgG. Antibodies against 67-IEA were present in all 10 anti-CMV-LA-IgG-positive sera. Anti-58-MA was present in all sera with both anti-LA and anti-LSP, and in three of the four anti-LA IgG positive but anti-LSP negative sera. Anti-67-IEA or anti-58-MA were not present in any of the 6 anti-LSP-positive but anti-CMV-LA-negative sera (table 17.6).

The conclusion from these experiments is that there are no arguments for expression of CMV membrane antigens as an inducer for development of anti-LSP, neither by coexpression of antigens nor by molecular mimicry.

Table 17.6. Antibodies against LSP and defined CMV antigens in 16 patients with IAI-CAH.

	Patient	aLSP	aLA-IgG	aLA-IgM	a67-IEA	a58-MA
	1	neg	82	1	212	10
aLA-IgG +	2	neg	69	1	60	45
aLSP -	3	neg	62	1	109	26
	4	neg	55	1	131	neg
	5	250	neg	neg	neg	neg
	6	200	neg	2	neg	neg
aLA-IgG -	7	550	neg	neg	neg	neg
aLSP +	8	150	neg	neg	neg	neg
	9	100	neg	neg	neg	neg
	10	100	neg	neg	neg	neg
	11	50	95	5	65	20
	12	100	300	neg	570	30
aLA-IgG +	13	200	180	1	366	28
aLSP +	14	100	300	9	229	181
	15	150	500	1	985	191
	16	150	145	1	309	115

aLSP denotes anti-LSP antibodies.
aLA denotes anti-CMV antibodies to late antigen (IgG or IgM class).
a67-IEA denotes antibodies against CMV immediate early antigen with a molecular weight of 67 kDalton.
a58-MA denotes antibodies against CMV induced membrane antigen with a molecular weight of 58 kDalton.

DISCUSSION

The results from the current study demonstrate that in idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH), induction of autoimmunity, either by coexpression of LSP with CMV membrane antigens or by molecular mimicry between these antigens, is highly unlikely. Anti-LSP did not cross-react with LA, 67-IEA or 58-MA, the main membrane antigens induced by CMV.

Anti-CMV-LA, anti-LSP, or both antibodies were frequently present in IAI-CAH, and in patients with CAH of other aetiology. Prevalence of anti-CMV-LA IgG in IAI-CAH and in HBsAg-CAH is similar to or higher than in healthy blood donors. Titres of IgG-class anti-CMV-LA were higher in IAI-CAH and HBsAg-CAH than in 'other' CAH. An

explanation for this might be that these patients had more active disease. For HBsAg-CAH and anti-HBs-positive IAI-CAH, another possibility is that these patients have a higher risk of becoming a CMV-carrier.

As demonstrated before, antibodies against LSP or its main antigen, the asialoglycoprotein receptor (ASGR) (=hepatic lectin),[981] are usually present in IAI-CAH patients requiring immunosuppressive therapy. Anti-LSP titres have been shown to correlate with the severity of histological damage in untreated patients with IAI-CAH. Changes in titres more or less parallel those in histological inflammation immediately after the institution of immunosuppressive therapy.[990]
Cytotoxic reactions of the ADCC-type are directed at antigenic sites of LSP in CAH and some other liver diseases: CD8+ lymphocytes and OKM+ (natural killer/killer -NK/K-) cells predominated the periportal area in IAI-CAH.[994] Lymphocytes from patients with a variety of acute and chronic liver diseases are able to 'kill' heterologous[994-999] or autologous[1000-1003] hepatocytes, or LSP-coated avian erythrocytes[1004] *in vitro*. These cytotoxic reactions are apparently directed at antigenic sites of LSP -cytotoxicity is blocked by addition of LSP[1003]-, with lymphocyte cytotoxicity predominantly mediated by a non-T cell population bearing receptors for complement.
In IAI-CAH, a genetically determined defect exists in T-cell inducers of antigen-specific T suppressor-cells[984,985]. It is possible that these patients are provoked into ADCC against LSP, or more specifically, the ASGR. Corticosteroids can improve the non-antigenspecific T-suppressor cell functions in AI-CAH, resulting in reversal of histological and biochemical manifestations of disease and sufficient to induce 'remission' according to currently accepted criteria. However, corticosteroids have no effect on the genetically determined LSP-specific T-suppressor cell defect.[983,984] This latter defect could allow for continued stimulation of anti-LSP producing B lymphocytes.
The high titres of IgG-class anti-CMV-LA in untreated IAI-CAH and HBsAg-CAH may be due to a state of polyclonal stimulation of B-lymphocytes as a result of the non-antigen specific T-suppressor cell defects. This is consistent with the literature, indicating that no increased anti-CMV antibody titres are detected in chronic persistent hepatitis.

Results from the present study do not exclude induction of LSP-autoantigens on the hepatocyte membrane and subsequent anti-LSP formation by CMV membrane antigens not tested for. However this is unlikely, since the antigens tested for are almost invariably present after infection with CMV. Therefore, in patients with anti-LSP but not anti-CMV-LA, such a mechanism would be less likely. The same applies to the hypothesis of diminished sequestration of CMV-derived antigens in the diseased liver, with subsequent antibody formation.[1005]

Recently, persistent measles virus genome was identified in the lymphocytes from 12/18 patients with IAI-CAH, in contrast to 1/45 controls.[1006] This finding correlated strongly with the presence of high antibody titres to measles. This finding raises the possibility

that measles virus may be involved in the aetiology and pathogenesis of IAI-CAH. However, there is no direct proof of aetiological involvement, and age-matched comparison with the healthy population may be required to validate these data. Other viruses might also play a role in IAI-CAH. Recently the autoantigen of the severe form of IAI-CAH , the liver-kidney microsomal antigen type 1, was cloned.[1007] It appeared to show homology with nucleic acid sequences of the Epstein Barr virus (Manns: personal communication).

From the present study, it follows that a role for CMV in inducing IAI-CAH is unlikely to take place by molecular mimicry or coexpression of epitopes in LSP and in CMV-induced membrane antigens. If CMV indeed plays an aetiological role in (some of) the patients with IAI-CAH, it might be by induction of other cell membrane antigens, for instance by inducing HLA class II antigens, or by encoding a glycoprotein homologous to MHC class-I antigens, as recently reported,[1008] making the cell a target for T-lymphocyte cytotoxicity. Further investigation is warranted into a possible aetiological role for CMV by such other mechanisms, and of the role of other environmental factors and their interplay with genetic susceptibility, in the induction of IAI-CAH.

GENERAL DISCUSSION

The aim of the present study was to analyze the progression of a small group of patients with idiopathic 'autoimmune' CAH (IAI-CAH), treated according to the same standardized protocol, against the background of a larger group of patients with CAH of various etiologies and referred in the same period of time. Guidelines were derived for monitoring this disease, with or without therapy.

The present study shows that:
Treatment of IAI-CAH according to a standardized protocol with fixed dose immunosuppressive therapy induces a rapid reduction in inflammation, apparent after two months, resulting in an excellent prognosis. It enables clinicians to limit the number of follow-up visits for patients fulfilling certain criteria. Serial monitoring of histological inflammation in CAH is facilitated by the help of a numerical scoring system for histological activity. An existing scoring system has been modified, and proved effective. From the current work, it also can be derived that histology is not only important for obtaining a diagnosis of CAH, but also for monitoring disease activity thereafter. Simple parameters of activity are required for this follow-up. Among such parameters, serum ALT (using individual reference ranges) and PIIINP appear to be the most useful.

Among circulating autoantibodies in IAI-CAH, at least antibodies to antigens in the 'liver-specific membrane lipoprotein' (LSP) (including the asialoglycoprotein receptor (ASGR)) appears to have pathogenetic importance. Relapse in IAI-CAH is invariably preceded by presence of anti-LSP antibodies, which means that this antibody can also have clinical significance in the guidance of therapy-withdrawal, along with PIIINP and ALT (using individual reference values).

Other autoantibodies have some relative significance in diagnosis of IAI-CAH, although most of these are probably epiphenomena. In autoimmune CAH, the incidence of anti-DNAis very low (unlike SLE) and, if present, is of low avidity. This may enable an immunological distinction from SLE, a disease that seldom co-exists with IAI-CAH. Until now, the clinician treating a patient with IAI-CAH has been most interested in parameters for activity of disease and inflammation.

However, the present work illustrates that, as the result of reduction/disappearance of inflammation, the hepatic protein synthesizing capacity can increase and normalize, even if cirrhosis is present. This applied, not only to more common parameters like serum albumin or cholinesterase, but also to SAA and haemoglobin. The significance is that during therapy of IAI-CAH (and possibly other kinds of liver disease), not only should inflammation or cholestasis be monitored, but also cholinesterase (and e.g. ATIII and albumin).

Furthermore, immunosuppressive therapy resulted in an increase in, and normalization of, the serum sodium concentration, indicating an apparently subclinical derangement of the hepatorenal axis in IAI-CAH which can be reversed with therapy. Until now, the literature has only described worsening of the hepatorenal syndrome during the course of several types of liver disease, only reversible by liver transplantation. This also applies to hepatic protein synthesizing capacity: although scattered data indicated restoration of serum albumin during therapy of CAH, the present study clearly demonstrates that normalization can occur despite the presence of cirrhosis, and that continuation of therapy is usually necessary beyond the point of reduction or disappearance of inflammation. The hallmark of therapy has been the suppression of inflammation. Although prednisolone and azathioprine can have considerable side effects, these were not severe or frequent. Some studies reported that azathioprine might enhance the formation of fibrosis and could induce cytopenia, but our data does not support these fears.

Future research

Most patients with IAI-CAH need immunosuppressive treatment for more than our standard period of two years. Further investigation is warranted into simple parameters to aid early identification or prediction of CAH relapse, and into factors that play a role in relapse and therapy-failure. The proposed role for especially PIIINP, ALT, CHE, and anti-LSP in monitoring IAI-CAH should be confirmed in other studies. Further study is justified in the field of hepatic protein synthesis and in water- and salt-homeostasis in CAH, and other diseases with a component of hepatitis. Research into the pathogenesis and etiology of IAI-CAH should be extended, to design more specific therapies.

Model

The cause of 'idiopathic autoimmune' CAH remains unknown. We closely examined hepatitis B and the cytomegalovirus. Patients with IAI-CAH who previously lost the hepatitis B virus and are anti-HBs positive can have the same favorable response to immunosuppressive therapy as other IAI-CAH patients. Induction of autoimmune CAH in these patients might be caused by persistence of LSP on the hepatocytes, induced by the previous HBV-infection, with antibody-formation and antibody-dependant cellular cytotoxicity. With regard to the cytomegalovirus (CMV), no relationship could be detected between presence of the virus and autoimmune CAH. Molecular mimicry between CMV-LA antigens and LSP-antigens was excluded. However, neither for HBV nor for CMV has an etiological relationship with autoimmune CAH been excluded in patients with circulating antibodies to these viruses. Although it is well known that HBV can induce formation of anti-LSP (which can persist after the disappearance of

IAI-CAH patients with antibodies to both CMV membrane antigens and LSP-antigens, expression of LSP with subsequent antibody formation may have been induced by CMV-infection of hepatocytes. In this thesis, the model which assumes genetic factors cooperating with environmental factors is supported. These environmental factors can be of viral, bacterial, chemical or other origin. Further research is warranted into viral, bacterial and other possible etiological factors. The inflammation which progressively results from the host/environment interaction has been followed by measurements relatively early (like anti-LSP) or late (serum albumin, ALT) in this progression. Inbetween lies the acute-phase reaction (CRP, SAA) and fibrogenesis (PIIINP). These parameters can be useful in monitoring the disease. The ideal parameter for monitoring reflects both the present and the past status of inflammation and hepatic functional reserve. By combining several of the mentioned parameters, like PIIINP and CHE, almost ideal monitoring is feasable. The behaviour of parameters and their application in monitoring of IAI-CAH require further investigation, especially the early indicators such as interleukins and circulating interleukin-receptors.

SUMMARY

This thesis describes the outcome -survival- of a large group of 186 consecutive patients with chronic active hepatitis of various etiologies, and describes in detail the progress of 21 patients from this group with 'autoimmune' chronic active hepatitis maintained on standardized immunosuppressive therapy.

Idiopathic 'autoimmune' chronic active hepatitis (IAI-CAH) is generally considered as a primarily hepatocytic liver disease of unknown cause, characterized by (episodes of) periportal and portal inflammation and piecemeal necrosis, with the potential to progress to cirrhosis or associated with cirrhosis. It lasts 6 months or longer.

The *introduction* briefly reviews the current knowledge on IAI-CAH. This is put into a historical perspective, definitions are summarized, clinical symptoms and differential diagnoses are mentioned, data on dismal prognosis in untreated disease and improved prognosis in treated IAI-CAH are reviewed. Treatment options are summarized, problems in the diagnosis and management of the disease are mentioned, and an overview regarding parameters of inflammation and liver function useful in monitoring IAI-CAH is provided. Furthermore, the present knowledge on pathogenesis and possible aetiologies of the disease is put in an immunological context. Untreated IAI-CAH has a high morbidity and mortality. Early mortality is typically due to liver failure, while late mortality usually results from the complications related to cirrhosis. Immunosuppressive therapy can reduce inflammation and enhance survival. Formation of cirrhosis can continue despite induction of 'remission' from CAH. Clinical, histological and biochemical findings must all be considered in concert for decisions concerning therapy. Data are scarce on early changes in inflammation and liver function during immunosuppressive therapy. No optimal tests for the follow-up of IAI-CAH are available. Histology forms the basis for the diagnosis of CAH, but may be insufficient and impractical for follow-up once therapy has been started. Standard laboratory blood-tests do not accurately reflect morphology. Until now, limited information was available regarding changes in hepatic functional capacity during immunosuppressive therapy of IAI-CAH. This warranted a search into parameters of activity and hepatic functional capacity in IAI-CAH.

Section 1 deals with patients and methods.

Chapter 1 provides data on the patients included in these studies.
Chapter 2 refers to the methods used in the studies.

In Chapter 3, we compared numerical histological scoring of activity with conventional pathological descriptions of treated and untreated CAH. The proposed histological activity score (HAS) correlated very well with conventional scores. In contrast to the original method of scoring, we propose to leave fibrosis/cirrhosis out of this score of

activity. Maximum HAS is 18. HAS≤1 denotes the (virtual) absence of inflammatory infiltrate. A HAS of 2 or 3 usually denotes a histology similar to chronic persistent hepatitis (CPH); 90% of biopsies with a histology similar to CPH when scored conventionally, and 36% of biopsies with minimal CAH (CAHmin.), showed a HAS≤3. We therefore termed 1<HAS≤3 "partial histological remission" and propose to reserve the term "complete histological remission" for HAS≤1. We conclude that HAS accurately reflected histological activity of CAH, and is more accurate than conventional descriptions for serial measurement of morphological activity of disease. Furthermore, it enables comparison with other parameters.

In Chapter 4, three radioimmunoassays for the determination of the serum N-terminal propeptide of collagen type III were compared in chronic active hepatitis: a new rapid equilibrium type of assay based on the human propeptide, developed by Risteli and Risteli, was compared with the PIIINP RIA-gnost® (Behring), and the 'PIIINP Fab assay®'(Behring), both based on a reference bovine PIIINP antigen (col 1-3) and antibodies raised in rabbits. The correlation between the human PIIINP RIA and the PIIINP RIA-gnost® was excellent, with less correlation with the 'PIIINP Fab assay®'. The new human assay is therefore as accurate as the standard PIIINP RIA-gnost® used thus far, but it is not sensitive to smaller degradation products. Therefore, unlike in the PIIINP RIA-gnost® (Behring), no serial dilutions of the sera and no 50% intercept method for calculating the results are necessary in this new PIIINP assay, since the standards and the serum samples give parallel inhibition curves. This solves the most important problems that have been inherent in the determination of PIIINP, and provides a rapid reliable test. We used this tests in studies on PIIINP that will follow.

In Chapter 5, the establishment of a modified radioimmunoassay for the determination of autoantibodies against 'liver-specific membrane lipoprotein (LSP)' is described. This modification of the original procedure yielded a rapid, reproducible and quantitative assay for determination of anti-LSP antibodies.

In section 2 Chapter 6, we performed an analysis of survival of all 186 consecutive patients with CAH of various aetiologies included in the current study. 85(±3) percent of all 186 patients with CAH survived 5 years, 70(±4) percent survived 10 years, 55%(±5) survived 15 years, and 40%(±10) survived 20 years after diagnosis. Ages between therapy groups and aetiological groups did not differ significantly. Cox's proportional hazards estimation identified increasing age, presence of antiHBs, and no therapy (patients receiving standard immunosuppression had the best prognosis) as independant risk factors. Sex, presence or absence of antinuclear antibodies, anti-smooth muscle antibodies, level of serum alanine aminotransferase or serum cholinesterase did not influence survival. Most causes of deaths registered in patients with CAH were not directly related to the liver disease. Remarkably, there was a high incidence of plasmocytoma.

In Section 3, several parameters of inflammation and liver function, their relations, and the changes during immunosuppressive therapy are evaluated in 21 patients with 'autoimmune' CAH.

In Chapter 7, routine liver biochemistry in IAI-CAH before and during standardized immunosuppression was serially analyzed. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gammaglobulin (GG) were elevated at the start and improved considerably to normal median values with therapy. However, ALT and AST remained elevated between one and three times the upper reference limit in about half of the patients. Conjugated bilirubin was elevated, then normalized within two months to normal values in all patients. Median unconjugated bilirubin was normal at the start, nevertheless values declined within the first two months of therapy.

Chapter 8 describes histology and its changes during immunosuppressive therapy (0, 2, 14 and 26 months). The high median HAS declined considerably within the first two months of therapy, with further improvement during the next two years of therapy. At two months 67 percent of the patients had already improved to a HAS≤3. The fixed-dose therapy schedule was able to improve histology in 95% of the patients. A HAS≤1 ('complete histological remission') was achieved in 80 percent of the patients and 1<HAS≤3 ('partial histological remission') in 10 percent of the patients within two years of treatment. Periportal and lobular inflammation had disappeared in all of these patients in remission. Continuing decrease in portal and periportal inflammatory activity, with disappearance of plasma cells and decrease in numbers of lymphocytes, was observed during the two years of immunosuppressive therapy. Usually, after two years only lymphocytes remained. HFS was high (median score 3) and did not change during therapy.

Chapter 9 shows that hepatic protein synthesizing capacity, impaired in the majority of the 21 AI-CAH patients, normalized in the course of two years in all patients under investigation, despite the presence of cirrhosis in all but one patient. This was reflected in increasing and normalizing serum albumin (ALB), (pseudo-)cholinesterase (CHE), plasma antithrombin III (AT III), and decreasing and normalizing activated partial thromboplastin time (APTT), usually already in the first 14 months of therapy. The median prothrombin time (PT) was slightly elevated but did not change during therapy, although there was a trend to a transient further increase into the abnormal range within the first four weeks of therapy. CHE, unlike ALB and AT III was still increasing within the second year of therapy. Hence, CHE may be the most sensitive of the parameters of hepatic protein synthesizing capacity under investigation.

Chapter 10 shows that the acute-phase response is depressed in CAH: serum values of C-reactive protein (CRP) are only slightly elevated during this inflammation of its producing organ, and these levels still correlated to periportal inflammation scores. In

contrast to CRP, serum amyloid precursor A protein (SAA) levels are normal in CAH, and slightly increase during therapy. SAA values are related to CHE: its levels reflect hepatic protein synthesizing capacity and not inflammation in CAH.

Chapter 11 describes that the serum N-terminal propeptide of collagen type III (PIIINP) is elevated in 'autoimmune' CAH. PIIINP values rapidly decrease in the first two months of therapy. PIIINP levels finally normalize, or nearly normalize, but this can take 14 months of therapy despite a more rapid improvement in histology. This delay in normalization, and the incompleteness of normalization in half of the patients, may clarify why cirrhosis can develop during immunosuppressive therapy. PIIINP is predominantly correlated to histological inflammation and to ALT, but its levels also show a weak inverse correlation to CHE, probably reflecting reduced degradation of PIIINP in the liver during severely impaired liver function.

In Chapter 12, serum ALT and PIIINP were studied in 16 CAH relapses occurring during therapy withdrawal in 9 patients. During remission, ALT was below twice the upper reference limit, and PIIINP was below the reference limit in all cases. Using two or three times the upper reference limit of ALT as 'cut-off' for diagnosis of CAH-relapse, as recommended in the literature, ALT only rose above this limit median one week before the peak-ALT. An increase of PIIINP above the reference limit occurred weeks to months before the peak-ALT was reached. Using ALT, it was only possible to detect relapse as early as with PIIINP if each patient served as his/her own control for ALT, with the (arbitrarily chosen) upper reference limit at 1.25 times the ALT value obtained during histological remission (HAS≤3 and preferably HAS≤1).

Section 4 describes some immunological abnormalities in chronic active hepatitis, and their clinical relevance. The results in Chapter 13 show that autoantibodies to 'liver-specific membrane lipoprotein (LSP) were invariably present in patients with 'autoimmune' CAH requiring standardized immunosuppressive therapy. Titres declined in all patients during such therapy. In the course of two years anti-LSP became undetectable in half of the patients, while it remained detectable in the other half. As shown by others, presence of anti-LSP probably indicates ongoing stimulation of B-lymphocytes as the result of a defect in inducers of LSP-specific T-suppressor lymphocytes.

In Chapter 14, serum anti-LSP antibodies in 12 CAH relapses occurring during therapy withdrawal in 9 patients were studied. At the start of therapy withdrawal, all patients were in histological remission, and in only one case anti-LSP was detectable. Weeks to months before the peak-ALT was reached, anti-LSP became detectable where previously undetectable (with one exception). A change from undetectable to detectable anti-LSP preceded relapse, and it preceded its detection by the conventional serological definition of relapse (ALT>3x the upper reference limit). It also preceded "non-

remission" based on ALT exceeding two times the upper reference limit. This supports the theory that in IAI-CAH anti-LSP should be undetectable before considering to withdraw immunosuppressive therapy.

Chapter 15 describes that autoantibodies to a soluble liver antigen (SLA) are present in about 10 percent of our population of patients with idiopathic 'autoimmune' CAH. No patients with anti liver-kidney-microsomal antibodies type 1 (anti-LKM1) -denoting a more severe type of IAI-CAH- were detected in this population. Therefore, our patients with IAI could be divided into three of the recently proposed four subtypes of IAI-CAH with the help of ANA, SMA and SLA. Survival in these three groups did not differ, and presence or absence of the antibodies mentioned were not independant prognostic factors. This questions the clinical significance of this classification of IAI-CAH in four subtypes. However, the groups may have different aetiologies of IAI-CAH.

Chapter 16 shows that anti-cardiolipin antibodies (ACA), as detected by ELISA, were not infrequent in CAH and 'CAH-related disorders' of various aetiologies, with the highest prevalence and titres in IAI-CAH. In contrast, anti-DNA antibodies, detected by immunofluorescence with the kinetoplast of Crithidia Luciliae as a substrate, were absent in 98 percent of CAH patients, allowing the possibility of an immunological distinction from SLE. The prevalence of anti-DNA measured by ELISA was higher than with the immunofluorescence mentioned, especially for anti-DNA antibodies of the IgM class. This difference may be due to a difference in avidity of the anti-DNA antibodies measured using both methods. From the results, it also followed that cross-reactivity between ACA and anti-DNA was limited in CAH and, if present, probably only applies to anti-DNA of low avidity.

Chapter 17 shows the high incidence and titres of IgG-class antibodies to cytomegalovirus-induced late antigens (CMV-LA) in IAI-CAH and HBsAg-positive CAH. Frequently, anti-CMV-LA and anti-LSP were both present in IAI-CAH. However, there was no significant correlation between presence of these antibodies. Binding of anti-LSP in the radioimmunoassay for anti-LSP was not inhibited by addition of CMV-LA. Antibodies to the defined CMV-induced antigens 'immediate early antigen (IEA)' of 67 kD and to 'membrane antigen (MA)' of 58 kD molecular weight were only positive in patients with anti-CMV-LA IgG, not in patients with anti-LSP but without anti-CMV-LA IgG. From these results, it can be deduced that an aetiological role of CMV in IAI-CAH by molecular mimicry between CMV-induced membrane antigens and LSP is highly unlikely. This does not exclude an aetiological role for CMV by means of other pathways.

SAMENVATTING

Chronisch actieve hepatitis (CAH) is een chronische ontsteking van de lever -6 maanden of langer-, waarbij met name de zogenaamde portale driehoekjes en omringende gebieden door ontstekingscellen 'aangevreten' worden ('piecemeal necrosis'). De ziekte kan snel ontstaan en in korte tijd fataal zijn, maar meestal is het een ontsteking die aktieve en minder aktieve perioden kent. Bij een aantal vormen van CAH is er een afweerreactie van het lichaam tegen de eigen lever gericht. Een dergelijke autoimmuunreactie kan ontstaan op basis van bekende (als hepatitis B virus) maar ook onbekende ('idiopathische') oorzaken. In het laatste geval spreken we van idiopathische CAH, welke veelal autoimmune kenmerken heeft (idiopatische 'autoimmune' CAH, IAI-CAH).

In dit proefschrift wordt een groep van 186 patiënten beschreven met, onder andere door leverbiopsie bewezen, CAH met verschillende onderliggende oorzaken. De langetermijn overleving van deze patienten, en factoren die daarbij een rol spelen, werden geanalyseerd. Uit deze groep wordt een kleine groep van 21 patiënten met 'autoimmune' CAH in detail beschreven; deze groep werd op gestandaardiseerde wijze behandeld met medicatie die immuunreacties onderdrukt (immunosuppressie).

In de inleiding wordt een kort overzicht van de huidige kennis omtrent IAI-CAH gegeven. De geschiedenis van onze kennis van deze aandoening, definities, klinische verschijnselen, uit te sluiten andere ziekten welke op IAI-CAH kunnen lijken, kennis omtrent slechte prognose zonder behandeling en verbetering van overlevingskansen met behandeling van IAI-CAH worden behandeld, mogelijke behandelingen samengevat, en een overzicht wordt verschaft van diverse parameters om ontsteking en leverfunctie te meten in IAI-CAH (van belang voor 'monitoring' van zowel ziekte als resultaat van behandeling). Voorts wordt de huidige kennis van pathogenese en mogelijke inducerende factoren die leiden tot IAI-CAH samengevat in immunologische context. Onbehandelde IAI-CAH leidt veelal tot morbiditeit en sterfte. Een vroeg overlijden is in het algemeen het gevolg van een falen van de lever om de normale functies uit te oefenen, terwijl late sterfte veelal het gevolg is van complicaties verbonden met het ontstaan van levercirrose door CAH. Immuunsuppressieve behandeling kan de chronische leverontsteking doen afnemen, en kan de overlevingskans aanmerkelijk verbeteren in IAI-CAH. Door oorzaken die tot nu toe onbekend waren kan het ontstaan van cirrose echter voortgaan, ondanks het induceren van 'CAH in remissie' met een dergelijke therapie. Klinische (klachten, symptomen), biochemische (bloedonderzoek), en histologische (leverbiopsie) gegevens moeten in combinatie beoordeeld worden om beslissingen omtrent therapie te kunnen nemen. Gegevens omtrent vroege veranderingen in ontsteking en leverfunctie na het starten van immunosuppressieve behandeling zijn schaars. Voor het vervolgen van deze patiënten zijn geen optimale testen beschikbaar. Een leverbiopsie vormt de basis voor de diagnose CAH, maar is onvoldoende en onpraktisch voor het vervolgen van een patiënt nadat therapie gestart is. De absolute uitkomsten van routine bloedonderzoek vormen geen nauwkeurige weerspiegeling van de morfologie van de lever. Tot nu

toe was slechts weinig bekend van veranderingen in functionele capaciteit van de lever onder invloed van immunosuppressieve therapie in IAI-CAH. Daarom was het gewenst parameters van ontstekingsactiviteit en leverfunctie in IAI-CAH te onderzoeken.

Sectie 1 behandelt patiënten en methoden.

Hoofdstuk 1 beschrijft de patiënten die in de diverse studies zijn opgenomen.
Hoofdstuk 2 refereert aan de methoden die in de diverse hoofdstukken zijn toegepast.
In Hoofdstuk 3 vergeleken we een scoringsmethode waarbij de ernst van de ontsteking en van de verbindweefseling/cirrose in de lever in getallen wordt uitgedrukt, met de klassieke pathologische beschrijvingen, zowel in behandelde als onbehandelde CAH. De eerste, numerieke, methode leverde voor wat betreft de ontsteking een histologische activiteitsscore (HAS) op van nul tot achttien. In tegenstelling tot de originele methode scoorden wij fibrose/cirrose apart. De HAS correleerde zeer goed met de conventionele scoringsmethode. HAS≤1 betekent (vrijwel) afwezigheid van ontstekingsinfiltraat. Een HAS van 2 of 3 houdt meestal een histologie als van chronisch persisterende hepatitis (CPH) in. 90% van de biopten met een histologie als van CPH en 36% van de biopten met 'minimal CAH (CAHmin.)' volgens de oude methode, had een HAS≤3. We stellen daarom voor 1<HAS≤3 'partiële histologische remissie' te noemen, en HAS≤1 met 'complete histologische remissie' aan te duiden. De conclusie luidt dat HAS de histologische activiteit van CAH goed weerspiegelt, op een meer precieze wijze dan met klassieke scoringsmethoden mogelijk is, en dat de HAS vergelijking van opeenvolgende leverbiopten vergemakkelijkt. Bovendien maakt het een statistisch verantwoorde vergelijking met andere parameters (bijvoorbeeld bloedonderzoek) mogelijk.

In Hoofdstuk 4 worden drie radioimmunoassays vergeleken waarmee het N-terminale propeptide van collageen type III (PIIINP) gemeten werd bij patiënten met CAH. Bij het 'afzetten' van bindweefsel in de lever komt een klein stukje van het 'procollageen III' in het bloed vrij, en er zijn aanwijzingen dat dit PIIINP voornamelijk de depositie van collageen type III in de lever weergeeft bij patiënten met CAH. Aangezien verbindweefseling en cirrosevorming in de lever complicaties van CAH zijn lijkt een dergelijke test van belang voor dynamische 'monitoring' van depositie van collageen III in de lever bij CAH. Een van de drie tests, de zogenaamde PIIINP RIA-gnost® (Behring), is de standaardtest. Helaas is deze test omslachtig en een berekening is vereist om te compenseren voor het feit dat kleinere degradatieprodukten ook gemeten worden (waardoor standaarden en te testen sera geen parallelle inhibitiecurves geven). Een nieuwe 'equilibrium-type' test is gebaseerd op het humane propeptide waardoor dit laatste probleem vervalt. De correlatie van resultaten van deze nieuwe 'humane PIIINP test' met genoemde standaardtest is uitstekend, terwijl de uitkomsten in de 'PIIINP Fab assay' slechts matig met beide andere tests correleerde. De humane PIIINP test geeft dus resultaten die vergelijkbaar zijn met de standaard PIIINP RIA-gnost®, maar heeft een aantal nadelen van de standaardtest niet. De humane PIIINP test werd dan ook in de latere studies gebruikt.

In Hoofdstuk 5 wordt onze modificatie van een radioimmunoassay voor het bepalen van autoantistoffen gericht tegen 'lever-specifiek membraan lipoproteine (LSP)' beschreven. Zowel labellingsmethode als de assay zelf werden gewijzigd. Dit leverde een snelle, reproduceerbare en kwantitatieve testmethode op voor de meting van anti-LSP.

In sectie 2, Hoofdstuk 6 verrichtten we een survival-analyse van alle 186 patiënten met CAH die in de huidige studie waren begrepen. Met behulp van de Kaplan-Meier product-limit methode werd de overleving van patiënten met CAH berekend voor alle patiënten, en uitgesplitst naar etiologie en therapie. Van de gehele groep van 186 patiënten was de 5-jaars overlevingskans na stellen van de diagnose 85(±3)%, terwijl deze kans voor 10 jaar 70(±4)% was, voor 15 jaar 55(±5)%, en voor 20 jaar 40(±10)%. De leeftijd van de patiënten in de verschillende etiologische- en therapie-groepen verschilde niet. Met behulp van de Cox's 'proportional hazards estimation' identificeerden wij hogere leeftijd, aanwezigheid van anti-HBs antistoffen in het bloed, het niet (kunnen) geven van standaard immunosuppressieve therapie en verhoogd serum alanine aminotransferase (ALT) als onafhankelijke risicofactoren voor overlijden, terwijl geslacht, aanwezigheid van antistoffen tegen kernantigenen (ANA) of gericht tegen gladde-spier antigenen (SMA), en serum cholinesterase (CHE) de kans om te overleven niet beïnvloedden. In de meeste gevallen waarin de oorzak van overlijden achterhaald kon worden, stond deze niet in direkt verband met de leverziekte. Wel was het opvallend dat meer patiënten dan verwacht de ziekte van Kahler (plasmocytoom) kregen.

In sectie 3 worden diverse parameters van ontsteking en leverfunctie apart en in relatie tot elkaar bestudeerd, en worden de veranderingen in deze parameters onder invloed van gestandaardiseerde immunosuppressieve behandeling geëvalueerd bij 21 patiënten met 'autoimmune' CAH.

In Hoofdstuk 7 wordt de routine 'lever-biochemie' in het bloed voor en tijdens deze behandeling bij deze 21 patiënten serieel vervolgd. Serum alanine aminotransferase (ALT), aspartaat aminotransferase (AST) en gamma globuline (GG) in onbehandelde IAI-CAH waren hoger dan de referentiewaarden, en deze parameters verbeterden aanzienlijk -met medianen binnen het referentiegebied- onder invloed van therapie, hoewel ALT en AST in ongeveer de helft van de patiënten tussen één en drie maal de bovenste grens van het referentiegebied bleven. Geconjugeerd bilirubine was verhoogd, maar normaliseerde in alle patiënten binnen twee maanden behandeling. Het mediane ongeconjugeerde bilirubine was normaal bij aanvang van therapie, maar de waarden daalden binnen het referentiegebied in de eerste twee maanden van behandeling.
Hoofdstuk 8 beschrijft de histologie voor en tijdens behandeling (0, 2, 14 en 26 maanden). De hoge mediane HAS (ontsteking in de lever, zie boven) aan het begin van de behandeling nam aanzienlijk af in de eerste twee maanden van behandelen, met verdere verbetering in de daaropvolgende twee jaar behandeling. Na twee maanden behandelen had tweederde van de patiënten al een HAS≤3. Uiteindelijk was het gebruikte behande-

lingsschema, met standaarddoses prednisolon en azathioprine, in staat de ontsteking in de leverbiopsie bij 95% van de patiënten terug te dringen. Hierbij werd in 80% van de patiënten een HAS≤1 ('complete histologische remissie') bereikt, en bij 10% een 'partiële histologische remissie' (1<HAS≤3) binnen twee jaar behandeling, waarbij periportale en lobulaire ontsteking in alle patiënten bij wie histologische remissie bereikt werd verdwenen was. Gedurende de twee jaar immuunsuppressieve medicatie werd een voortgaande afname van portale en periportale ontsteking (driehoekjes en daaromheen) in de leverbiopsie gezien, waarbij, in het algemeen, na twee jaar slechts lymfocyten resteerden. De histologische fibrose score (HFS) was hoog (mediaan 3) en veranderde niet tijdens therapie.

De gegevens in Hoofdstuk 9 tonen aan dat de capaciteit voor het aanmaken van nieuwe eiwitten, die te laag is bij de 21 patiënten met onbehandelde IAI-CAH, normaliseert in de loop van twee jaar immunosuppressie bij al deze patiënten, ondanks de aanwezigheid van cirrose bij 20 van de 21 patiënten (mede met laparoscopie vastgesteld). Dit bleek uit een toename in bloedspiegels van albumine (ALB), (pseudo-) cholinesterase (CHE), antithrombine III (ATIII), en een afname en normaliseren van de partiëel geactiveerde thromboplastine tijd (APTT), meestal reeds binnen de eerste 14 maanden van de behandeling. De mediane prothrombinetijd (PT) was licht verhoogd voor behandeling, maar veranderde niet tijdens therapie, hoewel er een trend tot passagière verslechtering was in de eerste vier weken van de behandeling. Aangezien CHE bleef stijgen in het tweede jaar therapie, terwijl dat niet gold voor ALB en ATIII, is CHE mogelijk de meest sensitieve van de onderzochte parameters voor capaciteit van eiwitsynthese door de lever.

In Hoofdstuk 10 blijkt dat de zogenaamde 'acute fase respons' verlaagd is bij patiënten met CAH: in het serum van patiënten met CAH zijn de waarden van het C-reactieve eiwit (CRP) slechts licht verhoogd tijdens deze ontsteking van het belangrijkste CRP-producerende orgaan. De serum CRP-waarden zijn wel gecorreleerd met periportale ontsteking in het leverbiopt. In tegenstelling tot CRP, zijn de serumspiegels van een ander 'acute fase eiwit', het 'serum amyloid precursor A eiwit' (SAA), normaal in CAH, en de SAA-spiegels nemen in geringe mate toe tijdens immunosuppressie. De SAA-waarden blijken met CHE (eiwitsynthese) gecorreleerd te zijn en niet met histologische parameters van ontsteking.

Hoofdstuk 11 beschrijft dat de serumwaarden van het N-terminale propeptide van collageen type III (PIIINP) abnormaal hoog zijn bij patiënten met IAI-CAH. PIIINP-spiegels worden normaal of bijna normaal onder invloed van gestandaardiseerde immunosuppressie, hoewel dit 14 maanden kan duren ondanks het feit dat de histologie al eerder verbeterd is. De traagheid waarmee de verbetering plaatsvindt -na een aanvankelijk snelle verbetering in de eerste twee maanden-, en het feit dat PIIINP niet geheel normaliseert in de helft van de patiënten, zijn mogelijk de verklaring voor het feit dat cirrose kan ontstaan tijdens immunosuppressieve therapie. Serum PIIINP spiegels zijn voorna-

melijk gecorreleerd met ontsteking in de leverbiopsie en met alanine aminotransferase (ALT), hoewel PIIINP ook een zwakke omgekeerde correlatie met CHE vertoont, hetgeen waarschijnlijk een afname van afbraak van PIIINP in de lever tijdens ernstig gestoorde leverfunctie weergeeft.

In Hoofdstuk 12 werden 16 situaties (bij 9 patiënten) waarbij IAI-CAH terugkeerde ('relapse') na verminderen van immuunsuppressie, terwijl tevoren de CAH 'in remissie' was, bestudeerd. Tijdens remissie was de ALT onder twee maal de bovenste grens van het referentiegebied, terwijl PIIINP in alle gevallen binnen het referentiegebied lag. Als twee of drie maal de bovengrens van het referentiegebied als grens werd genomen, waarboven van 'relapse' gesproken werd -zoals in de literatuur aangegeven-, dan kwam ALT pas boven deze grens vlak (een week) voor het maximum van de maximale ALT tijdens 'relapse'. Een stijging van PIIINP boven referentiewaarden echter, trad weken tot maanden voor de maximale ALT bij 'relapse' op. Als ALT gebruikt werd als indicator was een even vroege detectie van relapse mogelijk als met PIIINP indien iedere patiënt diende als zijn/haar eigen controle voor ALT waarden, en we tevens de (arbitrair gekozen) bovenste referentie-limiet op 1.25 maal de ALT-waarde tijdens histologische remissie (HAS≤3 en liefst HAS≤1) stelden.

Sectie 4 beschrijft enkele immunologische afwijkingen bij chronisch actieve hepatitis, en hun klinische relevantie.

In Hoofdstuk 13 laten we zien dat autoantistoffen tegen het zogenaamde 'lever-specifieke membraan lipoproteïne (LSP)' steeds aanwezig waren bij patiënten met 'autoimmuun' CAH die voldeden aan de criteria voor behandeling met gestandaardiseerde immunosuppressieve therapie. De titers van anti-LSP namen af tijdens een dergelijke behandeling, en in de loop van twee jaar behandelen werd anti-LSP in de helft van de patiënten 'niet detecteerbaar'. Zoals anderen aantoonden, geeft de aanwezigheid van anti-LSP waarschijnlijk een voortgaande stimulatie van B-lymfocyten weer die het gevolg is van een defect in de 'inducers' van LSP-specifieke 'T-suppressor' cellen, met andere woorden: LSP geeft waarschijnlijk de 'immunologische activiteit' van de ziekte weer.

In Hoofdstuk 14 worden anti-LSP antistoffen bestudeerd in 9 patiënten die tijdens behandeling in histologische remissie waren tijdens 12 situaties waarin CAH terugkeerde na vermindering van immunosuppressieve medicatie. De periode van begin van vermindering van medicatie tot maximale ALT tijdens 'relapse' werd bestudeerd. Aan het begin van de vermindering van therapie was anti-LSP in slecht één van de 12 gevallen aantoonbaar, terwijl dit tijdens de piek-ALT bij 'relapse' in 11 van de 12 gevallen (92%) het geval was. Weken tot maanden voor de piek-ALT was anti-LSP al te detecteren in de 11 gevallen waarin anti-LSP eerst niet aantoonbaar was. Een verandering van niet detecteerbaar zijn van anti-LSP naar detecteerbaar anti-LSP ging vooraf aan 'relapse' van CAH, en ging vooraf aan de diagnose daarvan op basis van huidige serologische defini-

ties van relapse (ALT boven drie maal de hoogste referentiewaarde), en ging ook vooraf aan het niet in remissie zijn gebaseerd op een ALT boven twee maal de hoogste referentiewaarde.

De gegevens in Hoofdstuk 15 laten zien dat autoantistoffen tegen een oplosbaar (soluble) lever antigen (SLA) aanwezig waren bij 10% van de geteste patiënten met IAI-CAH. Wij vonden géén anti lever-nier-microsomale antistoffen van type 1 (anti-LKM1) -geassocieerd met een ernstiger vorm van IAI-CAH- in deze groep. Derhalve konden onze IAI-CAH patiënten ingedeeld worden in drie van de vier recentelijk voorgestelde subtypes van IAI-CAH met behulp van ANA, SMA en SLA. Het bleek echter dat de overlevingskansen tussen deze groepen patiënten niet verschilde, en dat de aan- of afwezigheid van genoemde autoantistoffen geen onafhankelijke prognostische factor is. Het is dus de vraag of de recent voorgestelde classificatie van IAI-CAH zinvol is voor de kliniek. Het zou echter zo kunnen zijn dat de etiologie in deze groepen verschillend is.

Hoofdstuk 16 laat zien dat aanwezigheid van anti-cardiolipine antistoffen (ACA), bepaald met behulp van ELISA, geen zeldzaamheid is in CAH en 'CAH-related disorders' met verschillende etiologieën. De hoogste titers van ACA kwamen voor bij IAI-CAH. Daarentegen kwamen anti-DNA antistoffen, bepaald door middel van immunofluorescentie met de kinetoplast van Crithidia Luciliae als substraat, niet voor bij 98% van de patiënten met CAH. Dit zou gebruikt kunnen worden als hulp bij de differentiaal diagnose tussen CAH en systemische lupus erythematosus (SLE) in de zeldzame gevallen waar het onderscheid moeilijk is. De prevalentie van anti-DNA gemeten met een ELISA was hoger dan met voorgenoemde immunofluorescentie methode, vooral wat betreft anti-DNA van de IgM-klasse. Dit verschil kan het gevolg zijn van het feit dat deze twee tests anti-DNA van verschillende aviditeit meten. Het blijkt voorts dat kruisreacties tussen anti-DNA en ACA slechts in beperkte mate voorkomen in CAH, en, indien aanwezig, waarschijnlijk vooral anti-DNA van lage aviditeit betreffen.

In Hoofdstuk 17 wordt aangetoond dat er met name in IAI-CAH en HBsAg-CAH een hoge incidentie van door cytomegalovirus geïnduceerde late antigenen (CMV-LA) is. In IAI-CAH kwam zowel anti-CMV-LA als anti-LSP veelvuldig voor, maar het vóórkomen van deze antistoffen was niet gecorreleerd. Binding van anti-LSP in de RIA voor anti-LSP werd niet geremd door toevoeging van CMV-LA. Antistoffen tegen de goed gedefinieerde, door CMV geïnduceerde, antigenen 'immediate early antigen (IEA)', met een molecuulgewicht van 67 kiloDalton (kD), en 'membrane antigen (MA)' met een molecuulgewicht van 58 kD werden slechts gevonden bij die patiënten met IAI-CAH die anti-CMV-LA-IgG hadden. Bij patiënten met anti-LSP, maar zonder anti-CMV-LA-IgG, werden deze antistoffen niet gevonden. Hieruit volgt dat CMV geen etiologische rol speelt in IAI-CAH via 'molecular mimicry' (het op elkaar lijken) van door CMV geïnduceerde antigenen en antigenen in LSP. Dit sluit echter een oorzakelijke rol voor CMV via andere mechanismen niet uit.

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CHAPTER 17

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The author of this thesis was born on June 14, 1956 at Voorburg, The Netherlands.
In 1962 he moved to Arnhem, The Netherlands, where he attended highschool from 1968-1974 (gymnasium β, Thorbecke Lyceum). From 1974-1979 he attended medical school at the State University of Groningen, The Netherlands, where he also assisted in educational programs in obstetrics/gynaecology and did a special project in nephropathology. In 1979 he married Beryl Flikweert. After internships and a special project on lung disease in the Groningen University Hospital (Academisch Ziekenhuis Groningen) he obtained his M.D. degree in February 1982. January 1983 he entered a residency in internal medicine in the same hospital, which was completed in December 1987. He developed a special interest in hepatology, gastroenterology and immunology. During his residency he passed the American Visa Qualifying Exam (VQE or FMGEMS) in 1984. In august 1986 his interests in hepatology and immunology urged him to start research in chronic active hepatitis. As a specialist in internal medicine he worked in the Division of Hepatology of Groningen University Hospital, where he also continued research, resulting in this PhD-thesis. For this purpose he visited the Liver Unit in King's College Hospital, London (G.-B.) (director: Roger Williams) in 1986 to work in the laboratory and attend the liver clinical course. In 1987 he visited and worked in the laboratory of prof. dr. dr. K.-H. Meyer zum Büschenfelde and prof. dr. M. Manns, in the Clinic of the Johannes Gutenberg Universität in Mainz (F.R.G.).
Bart van Hoek is currently employed as a fellow in the advanced hepatology training program at Mayo Clinic, Rochester, Minnesota, U.S.A., Department of Gastroenterology and Internal Medicine. He is involved in immunological research in liver disease and is supported by the Netherlands Organization for Scientific Research. He is a member of the European Association for the Study of the Liver, The Dutch Society for Gastroenterology, The Dutch Society for Hepatology, the Flemish Society for Gastroenterology and the American Association for the Study of Liver Diseases.