**PhD értekezés**

# Bioinformatikai eljárások alkalmazása a molekuláris biológiában és az immunológiában


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**PTE-ÁOK Immunológiai és Biotechnológiai Intézet**

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# 1. Rövidítések jegyzéke

ACE: Automated cDNA Editor
AUSA: AUtomated Sequence Annotator
C++: C plusz plusz programozási nyelv (http://www.gnu.org/)
CS: citrát szintáz
cDNS: complementer DNS
DNS: dezoxi-ribo nuklein sav
ELISA: Enzyme Labeled Immuno Assay
GCC: GNU Compiler Collection (http://gcc.gnu.org/)
GenBank: Genome Data Bank
GNU: GNU is Not Linux (http://www.gnu.org/)
GST: glutation S transzferáz
GPL: General Public Licence (http://www.gnu.org/copyleft/gpl.html)
HBX: Hepatitisz B vírus X protein
HBxAg: Hepatitisz B vírus X antigén
HLA: Humán Leukocita Antigén
HPLC: High Pressure Liquid Cromatograph
HRPO: horse radish peroxidase (retek peroxidáz)
KLH: tengeri csiga hemocianin
MHC: Major Histocompatibility Complex
mRNS: messenger RNS
MS: Microsoft Inc. (http://www.microsoft.com)
NCBI: National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)
Ig: immunoglobulin
OD: optikai denzitás
PBS: phosphate buffered saline
PC: personal computer (személyi számítógép)
PCR: polimerase chain reaction (polimeráz láncreakció)
R: regressziós koefficiens
RNS: ribo nuklein sav
SD: standard deviáció
SPSS: Statistical Package for Social Sciences (http://www.spss.com)
SWRITER: Sheet WRITER
3D: három dimenzió(s)

# 2. Összefoglalás:

A bioinformatika az egyik legfiatalabb tudományág (Boguski, 1994a; van der Greef, 2004; Molenbergs, 2005). Kialakulása a biostatisztikai számítások és a molekulamodellek használatával több, mint fél évszázada kezdődött, ugyanakkor gyors fejlődését és önálló tudománnyá válását csak az elmúlt másfél évtizedben, a számítástechnika nagyarányú hardver- és szoftver-fejlesztései tették lehetővé (Attwood, 1999; Rhee, 2005). Napjainkra az *in silico* bioinformatika széles körben alkalmazott kutatási, kutatás-fejlesztési területté vált, mely egyenrangú partnere az *in vitro* és *in vivo* végzett kutatásoknak. A bioinformatikai módszerfejlesztésekkel párhuzamosan szükségessé vált ugyanakkor mind az adatbázis struktúrák, mind a predikciós technikák kritikai elemzése, az élő rendszereken mért adatokkal történő folyamatos összevetése (Bernstein, 1977; Attwood, 1999; Brusic, 2000; Doytchinova, 2002).

Munkám mind az adatfeldolgozásra, mind az adatbankok segítségével történő predikcióra kiterjedt. Mindkét témakör a biológiai kutatás szolgálata mellett felvetette a bioinformatikai módszerekkel kapott eredmények gyakorlati használhatóságának kérdését, ezért négy tématerületen végeztem vizsgálatokat. Az első időszakban a DNS szekvenálást megkönnyítő és az adatfeldolgozást segítő szoftverek fejlesztését tűztem ki célul. A mintafelvitelt és a mintakövetést biztonságossá tevő, ezzel a technikai hibákat csökkentő, a mérésbiztonságot növelő eszközt (SWriter program) sikerült kialakítanunk. A második témakörben kifejlesztett alkalmazások az EST szekvenálás eredményeinek feldolgozását és a nemzetközi adatbázisokba történő automatikus továbbítását tették lehetővé. Munkám harmadik szakaszában az immunológiai predikciós technikák kritikai elemzését tűztem ki célul. Elsők közt sikerült felhívnunk a figyelmet arra, hogy az immunogenitást előrejelző algoritmusok (az *in silico* és az *in vitro* mért immunszerológiai adatok összehasonlítása kapcsán) csak megfelelő speciális adatbázisokon történő finomítás után válhatnak a napi kutatási és kutatás-fejlesztési gyakorlatban valóban nagyhatású eszközzé. A negyedik témakörben általánosan használt biostatisztikai eljárások segítségével összeállított panel nagyszámú immunszerológiai mérési minta multiparaméteres elemzését tette lehetővé, olyan, új összefüggések feltárásával, melyek a klinikai prognózis megítélése szempontjából gyakorlati jelentőséggel bírnak.

Mind a négy témakörben munkánk a bioinformatika aktuálisan legfontosabb irányaihoz kapcsolódott és a tudományág gyors fejlődését bizonyítja, hogy a tézisek benyújtása előtt nyolc évvel kezdődött munka egyes elemeit ma már túlhaladta a fejlődés. Ugyanakkor mind a négy tématerületen - a nemzetközi publikációinktól függetlenül is – a gyakorlati alkalmazás bizonyította a kutatási fázis eredményességét.

# 3. Bevezetés:

Az alap és alkalmazott biológiai kutatás egyik leggyorsabban fejlődő területe a molekuláris biológia. A biológiailag aktív vegyületek finomszerkezetének kutatásában a genetikai módszerek alkalmazása, a hagyományos fiziko-kémiai szerkezetvizsgálatok, valamint a biokémiai és funkcionális módszerek ***együttes alkalmazása*** általánossá vált napjainkra. Az immunológiai eljárásokon alapuló mikroanalitika a molekuláris felszínek térképezésében új lehetőségeket nyitott meg a monoklonális ellenanyagok felfedezését követően (Geysen, 1985; Baxevanis, 1997; Nagy, 2002; Boguski, 2003; Pál, 2004).

**Bioinformatika:**

A **bioinformatikát** definiálhatjuk, mint a számítógépes módszerek kidolgozása és alkalmazása a biológiai információ kezelésére és elemzésére (Boguski, 1994a). Szélesebb körben érthetjük rajta bármilyen biológiai információ számítástechnikai eszközzel történő feldolgozását, szűkebb értelemben pedig, szekvencia adatok kezelését és elemzését (Niiler, 2000; Janssen, 2005). A bioinformatikai kutatások kilenc fő területre oszthatók (Attwood, 1999):

1. Biológiai adatbázisok létrehozása, analizálása, keresése
2. Molekulaszerkezet 3D modellezése
3. Molekulaszerkezeti és funkcionális predikció
4. Szekvencia összehasonlítás, illesztés
5. Filogenetikai számítások
6. Molekulainterakció vizsgálata
7. Génexpressziós elemzések
8. Biológiai kutatási folyamatok automatizálása
9. Biostatisztika

A biológiai adatbázisok kialakítása és a molekulamodellek megalkotása a XX. századi természettudományos kutatás, elsősorban a szerves kémia és biokémia, valamint a molekuláris biológia kirobbanó eredményeit használta fel, ugyanakkor a számítógépes adatfeldolgozás adta technikai háttér egy új dimenziót nyitott meg (de Grooth, 1999; van der Greef, 2004).

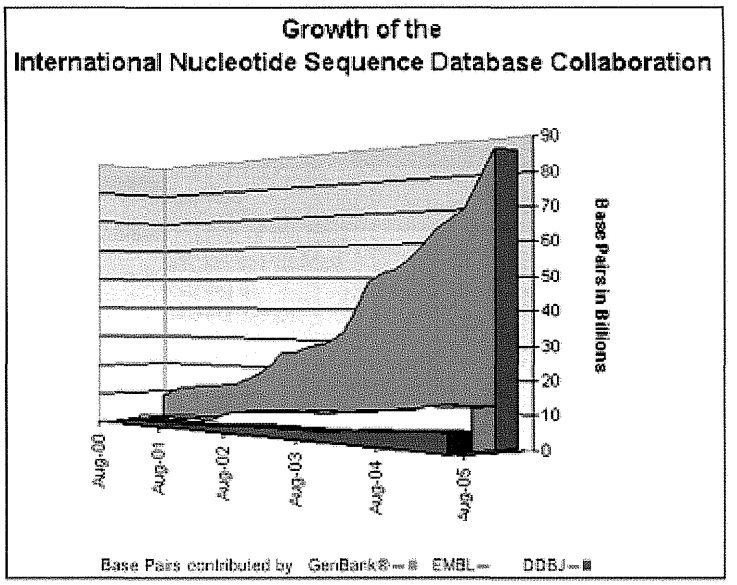
Az első teljesen ismert szekvenciájú fehérje, az inzulin, aminosav sorrendjének és szerkezetének megismerése után közel 5 év telt el az első enzim, a ribonukleáz szerkezetének megismeréséig (Ryle, 1955; Hirs, 1960). Ma több mint 400 000 fehérje szerkezete ismert. A nukleinsav szekvenciák megismerését a PCR és az arra alapuló szekvenálási technikák forradalmasították (Harper, 1994). A klónozás és „*shot-gun*" szekvenálás lehetővé tette nagyobb nukleinsavak (akár egész genomok) megismerését és nagy mennyiségű adatot szolgáltató szekvenáló pojektek beindítását (Koonin, 1998; Felsenfeld 1999; Andrade, 1999) (**1. Ábra**).

**1. Ábra:** Szekvencia és struktúra adatok elhelyezése az adatbázisokban. (Attwood, 1999)

Az 1980-as években a korszerű molekuláris biológiai módszerek hatására jelentősen megnőtt az irodalomban közzétett biológiai (szekvencia) információ. Ezen adatok egy központi adatbázisban való elhelyezésének előnyeit látva egyszerre párhuzamosan több munkacsoport is elkezdte összegyűjteni a rendelkezésre álló szekvencia információt (Barker, 1988; Mewes 1998; Attwood 1999). Napjainkban több mint 500 az *Internet*en elérhető adatbázisok száma (Brusic, 2000; Discala, 2000). Az adatbázisok integrálásával a keresési lehetőség és az információszerzés lehetősége jelentősen nőtt. Ma már számtalan más adatbázisokat integráló, keresztbe kapcsoló rendszer közül választhatunk (Bleasby, 1994; Henikoff, 1996; Etzold, 1996; Bairoch, 1998).

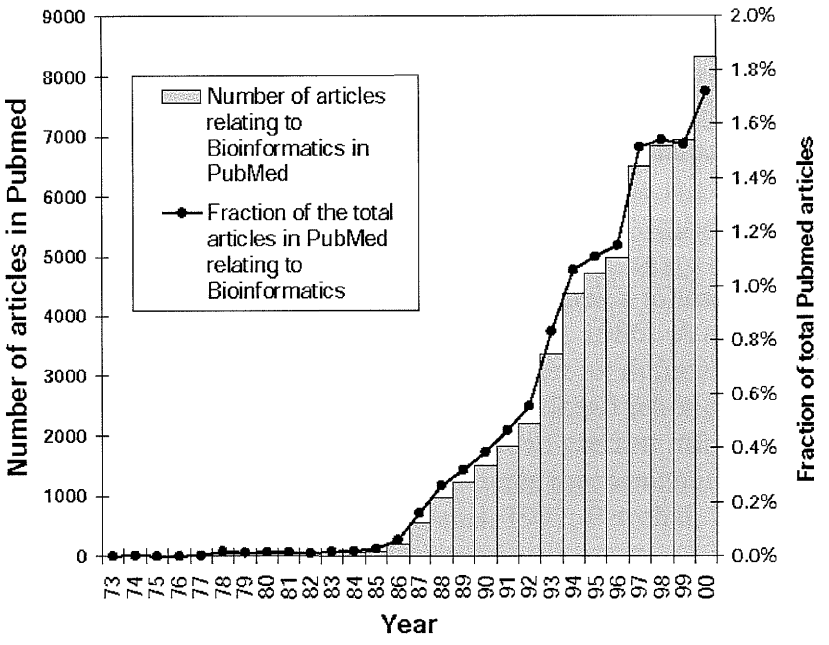
A számtalan, új biológiai információt jelentő szekvencia-adatnak a kutatás számára való megosztását már a nyolcvanas években igényelték a kutatók. Ekkor születtek meg az első biológiai adatbázisok (Johns, 1996). Ezek a biológiai adatbázisok szekvencia, szerkezeti, immunológiai, fiziológiai információkat tartalmaznak (Brusic, 2000). A biotechnológia és bioinformatika forradalmi fejlődése hatalmas adatmennyiséget eredményezett (Discala, 2000). A biológiai adatbázisokban elhelyezett szekvencia-adatok a **HUMAN GENOME PROJECT**nek és egyéb kutatási programoknak köszönhetően exponeciálisan nőnek (Bernstein, 1977; Boguski, 1994a) (**2. Ábra**).

Az adatok nagy része a kutatók számára szabadon elérhető a világhálón (Brusick, 2000; Discala, 2000). Az adatok gyors és komplex elérhetősége, feldolgozása, összehasonlítása és értékelése a modern bioinformatikai módszerek nélkül nem lenne megoldható. A bioinformatika lehetővé teszi laboratóriumi kísérletek megtervezését, esetleges eredményeinek megbecsülését, felgyorsítva a biológiai információk megszerzését (Boguski, 1994a; Boguski 1994b). A laboratóriumi folyamatok automatizálása meggyorsítja a nagy mennyiségű adatot termelő (pl.: szekvenáló) projektekből nyerhető információ prezentálását, feldolgozását (Andersson, 1998; Altman, 1998).




**2. Ábra.** Az adatbázisokban elhelyezett nukleotid szekvenciák száma. (Benson, 2005) http://www.ncbi.nlm.nih.gov/Genbank/index.html

Nem elegendő és nem minden esetben lehetséges a nagy érzékenységű mérési adatok hagyományos statisztikai számolása és interpretálása. Fontossá vált a kapott adatok feldolgozására számítógépes molekulamodellek létrehozása, ezeknek a különböző biokémiai reakciókban történő elemzése, a szerkezetváltozások és biológiai kölcsönhatások szimulációja és virtuális ábrázolása. Ezen problémákkal foglakozó új tudományt a bioinformatikát, a biotechnológia és a számítástechnika utóbbi tíz évben lezajlott rohamos fejlődése hívta életre. Az elmúlt években jelentősen nőtt a bioinformatikával foglalkozó publikációk száma, mely mára a PubMed-ben fellelhető új irodalom közel 2%-a (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) (Zlotogorski, 1990; Franklin, 1994; Niiler, 2000; Janssen, 2005) (**3. Ábra**).




**3. Ábra.** Bioinformatikai tárgyú cikkek aránya az irodalomban.
(Dr. Szilágyi András, ELTE TTK Biológiai Fizika Tanszék)
http://www.enzim.hu/~szia/bioinformatika/ea01/ea01.htm

Az elmúlt néhány évben kapott hangsúlyt a bioinformatikai módszerek orvosi alkalmazása, új területet nyitva az egyébként is gyorsan fejlődő orvostudományban (Lerner, 1994; Foote, 1998; Boguski, 2003; Boguski, 2004a; Imani, 2005).

A bioinformatikai és biotechnológiai módszerek jelentős fejlődése és a tervezett molekulák iránti igény általánossá válása lehetővé tette a kívánt biológiai hatásnak legjobban megfelelő, előre megtervezett molekulák széleskörű alkalmazását a biológiai kutatásban. A tervezett szerves molekulák célzott felhasználása és számítógépes szimulációja új utakat teremt az alapkutatásban és gyógyításban is. Lerövidíti, és olcsóbbá teszi a költséges laboratóriumi lépéseket (Geysen, 1985; Yu W, 1997; Liu, 1999; Andersson, 1998; Foote 1998). Az immunológiai alapkutatás egyik költséges és továbbra is időigényes része a különböző mono- és poliklonális ellenanyagok előállítása. Gyakran fontos, hogy ellenanyagaink molekula specifikusak legyenek. A növekvő igények gyakran csak a molekula egy részét, esetleg speciális funkcionális csoportjának felismerését követelik meg, mely megnehezíti a kívánt ellenanyagok előállítását. Napjainkban a számítástechnika gyors fejlődése és az ellenanyagokról, ellenanyag-antigén kapcsolatokról szerzett ismereteink kiszélesedése és elmélyülése lehetővé teszi a két technológia ötvözését, és bioinformatikai módszerek bevonását az epitópok meghatározásába (Doytchinova, 2002; Flower, 2002; De Groot, 2003).

A bioinformatika módszereinek ilyen hasznosítása számos előnnyel jár. A költséges és időigényes ellenanyag előállítás jelentősen gyorsabbá, olcsóbbá válhat az erőforrás igényes peptidekkel történő immunizálás kiváltásával, miközben új perspektívák nyílnak meg. Az epitópok számítógépes tervezése lehetővé teheti specifikus szintetikus vakcinák kifejlesztését és előállítását (pl.: diftéria, hepatitis, influenza, HIV ellenes szérumok), és gének meghatározását. Új fejezetet nyithat a tumor-terápiában és megelőzésben, speciális immunpeptidek kifejlesztésével, melyekkel immunizálva a szervezet a tumor ellen, vagy onkogének ellen immunválaszt generál (van Regenmortel 1986; Hopp, 1986; Pellequer, 1991; de Groth, 1999)

**Epitóp predikció:**

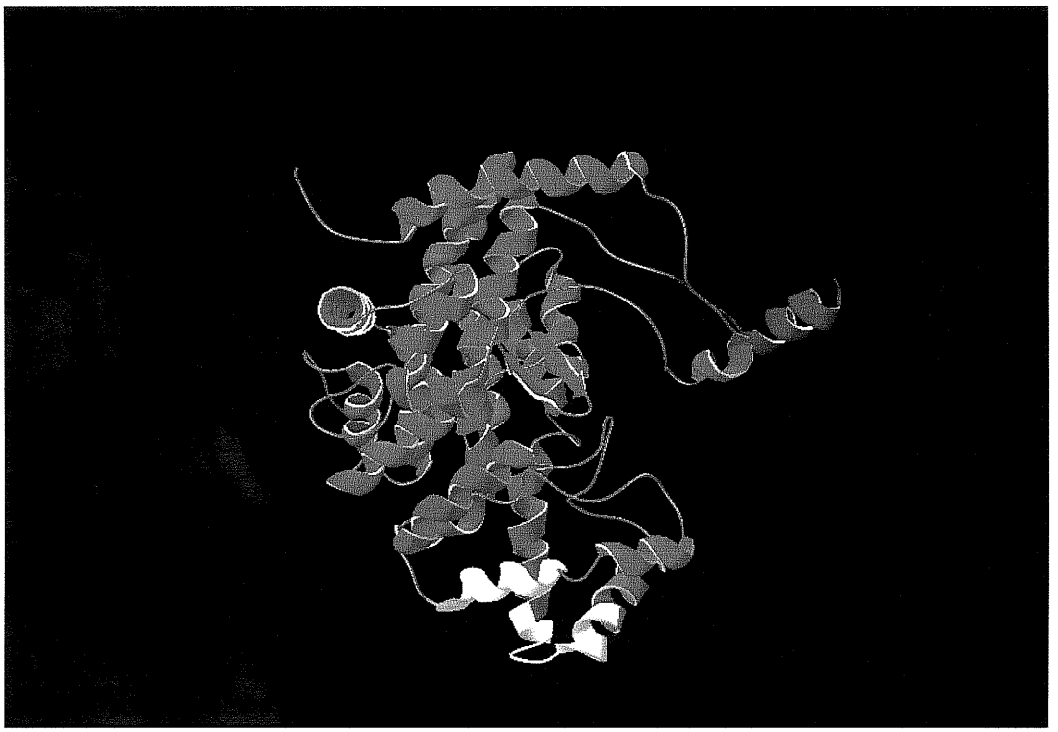
Az immunológiai felismerést befolyásoló molekuláris mikrokörnyezet, ezen belül az antigének alapvető fiziko-kémiai tulajdonságainak tanulmányozása napjaink immunológiai alapkutatásának egyik fontos kérdése. Az immunreaktivitást meghatározó elektrosztatikus és hidrofil/hidrofób régiók térbeli elhelyezkedése az antigén molekula felszínén mind az immunológiai felismerésben, mind a kiváltott effektor válaszban meghatározó szerepet játszik (van Regenmortel, 1997; Arnon, 1992; van Regenmortel, 1996). Egyre több adat utal fehérje és peptid antigének esetében arra, hogy az aminosav sorrendnek a töltéseloszlások meghatározásában van elsődleges szerepe (van Regenmortel, 1986). A monoklonális ellenanyag technika felfedezése teremtette meg a lehetőséget jól definiált modellvizsgálatok végzésére. A különböző, ismert biológiai aktivitással rendelkező fehérjék felszíni epitópjainak monoklonális ellenanyagokkal végzett térképezése kapcsán szerzett információk nagymértékben járultak hozzá az immunológiai felismerés mechanizmusának tisztázásához (Lerner, 1982; Lerner 1984; Stemler, 1990; Baxevanis, 1997; Petrohai, 2005; Nyárády, 2005).

**Epitóp**nak, vagy antigén determinánsnak nevezzük egy protein azon meghatározott részeit, melyekhez specifikus felismerő részeikkel az immunglobulinok kötődnek (Amit, 1986; van Regenmortel, 1987; Sheriff, 1987). Az epitópok relatív entitások, mivel komplementer paratópok szükségesek felismerésükhöz (Getzoff, 1987). Az epitópok ezért rendelkeznek antigén reaktivitással, képesek az immunglobulinokhoz kötődni, önmagukban is immunogenitással rendelkeznek, vagyis képesek immunválaszt kiváltani (un. anti-idiotípus immunválasz) (Chou, 1974; Geysen, 1987; Hudecz 1994).

A fehérjék felszínét tehát felfoghatjuk, mint egymást átfedő epitópok halmazát, melyen belül megkülönböztetünk kiemelt immundomináns és kevésbé reaktív epitópokat. Az epitópok számát definiálhatjuk a fehérje ellen termelhető, egymástól különböző monoklonális antitestek számával (Benjamin, 1984; Atassi, 1984).

Röntgen-krisztallográfiával megállapították, hogy az epitóp-paratróp kapcsolat egy kb. 20Å x 30Å területen történik a molekula felszínén (Barlow, 1986). Nem valószínű, hogy ebben a három-dimenziós epitóp-paratróp kapcsolatban elsősorban a polipeptidláncot felépítő, egymást követő aminosavak alkotta **lineáris epitóp**ok fordulnak elő (Geysen, 1988). Valószínűbb az úgynevezett **konformációs epitóp**ok előfordulása (Benjamin, 1984; Laver, 1990).

Immunológiai szempontból, mind a lineáris mind a konformációs epitópok fontosak (Atassi, 1978; Benjamin 1984). Immun-biotechnológiai szempontból a lineáris epitópoknak van fontosabb szerepe egyszerűbb kezelhetőségük miatt (Green, 1982; Getzoff, 1987; Laver, 1990). (**4. Ábra**)



**4. Ábra.** Lineáris és konformációs epitóp a CS felszínén. (A lineáris epitópóp pirossal, a konformációs epitóp sárgával jelölve.)

Az epitóp-paratróp kapcsolatok pontos meghatározására fizikai módszereket alkalmaznak a leggyakrabban (MNR és röntgen-krisztallográfia) (Amit, 1986; Sheriff, 1987; Garcia de la Torre, 1999). Az egyéb módszerek (tömegspektrometria, átfedő peptid könyvtárak) csak valamilyen közelítést tesznek lehetővé (Atassi, 1985; Sturniolo, 1999; Nagy, 2002; Czömpöly, 2003; Pál, 2003; Németh, 2004). A különböző immunbiológiai kautéláknak az ismerete feltétlenül szükséges, amikor epitópokat szeretnénk számítógéppel meghatározni.

A számítástechnika gyors fejlődése lehetővé tette a lineáris aminosav szekvencia ismeretében a fehérjék térbeli szerkezetének hozzávetőleges predikcióját, mely kulcsa az epitópok meghatározásának. Az eddig leírt epitóp predikciós módszerek a primer aminosav szekvencia ismeretében próbálnak a fehérje harmadlagos térbeli szerkezetére vagy egyéb attribútumára (másodlagos szerkezet, mobilitás, hidrofobicitás, hidrofilicitás stb.) következtetni (van Regenmortel, 1989; Laver, 1990; Stern, 1991).

Általánosságban, minden predikciós módszer első lépéseként az ismert háromdimenziós szerkezetű fehérjék molekuláris hőmérséklet, szerkezet és mobilitás statisztikai analízisével mind a 20 aminosavhoz egy-egy értéket rendelünk relatív tulajdonságuk alapján, így egy táblázatot hozunk létre.

Predikciós profil készítésekor az előbbiekben szerkesztett táblázatból minden pozicióban az ott helyet foglaló aminosavnak megfelelő értéket helyettesítjük be. Ezt követően egy 5-21 aminosav hosszúságú „ablakkal", mely lehetővé teszi, hogy ezt a néhány elemből álló peptidszakaszt a molekula többi részétől függetlenül vizsgáljuk. Az ablakot végigfuttatva a szekvencián a táblázatból kiolvasott értékek ablakban lévő átlagát kiszámoljuk, majd hozzárendeljük a szekvenciához, és grafikonon ábrázoljuk. Pl. hidrofilitási profil számolása esetén, a magasabb értékű régiók a hidrofil részeknek felelnek meg (Hopp, 1981; Regenmortel, 1989).

A profilban lévő kimagasló szélsőségek elkerülése végett a szokásos analizálás előtt a profil finomítása történik. Erre két módszer terjedt el: az egyikben, a régiókban lévő "fogakat" távolítják el, a másikban a változó értékeket egész számokkal helyettesítik. Az első finomítási módszer klasszikus megközelítse az ablakban lévő pozíciók súlyozása, mely legegyszerűbben *Karplus* és *Schulcz* szerint történik, egyszerűen az adott pozícióban lévő aminosav értékét a súlyszámmal megszorozzuk. *Van Regenmortel* e helyett egy Gauss-eloszlást használ (Karplus, 1985; Parker, 1986; van Regenmortel 1988).

A különböző profilok összehasonlíthatósága végett szokásos következő lépés az adatok normalizálása, ezt követően kerülhet sor az epitóp pozíciók meghatározására.

Az epitóp predikciós módszerek legtöbbje a primer szerkezetből következtet a másodlagos szerkezetre és az epitópokra az aminosavak valamely tulajdonságának analízisével (van Regenmortel, 1988; Stern 1991; Németh 2004). Vizsgálhatjuk, a fehérjét alkotó aminosavak hidrofobicitását, hidrofilitását, flexibilitást, az atomi mobilitást, az $\alpha$-helix és $\beta$-lemez szerkezeteket formáló hajlamot, akrofilitást és antigenitást, stb.. Ezen módszerek alkalmazásához, számtalan szoftvert fejlesztettek ki. Az egyszerűbbek egy módszert használnak (pl.: HYDRO), míg léteznek bonyolultabb rendszerek, melyekkel számtalan módszert ill. azok kombinációját különböző paraméterekkel kipróbálhatunk (pl.: Wisconsin GCG Package, PrediTop) (Pollard, 1990; Vihinen, 1993; Pellequer 1993; Womble, 2000).

A fehérjék másodlagos szerkezete az $\alpha$-helix és $\beta$-lemez ismétlődő struktúrák, melyet az aminosavak, a köztük lévő hidrogén kötések és az oldószer és oldalláncok közötti kölcsönhatások révén alakítanak ki. A fehérjeszerkezetek kb. 50%-a hajlatok míg másik 50%-a ismétlődő struktúrákban helyezkedik el (25% $\alpha$-helix , 25% $\beta$-lemez). A másodlagos szerkezet jelentős mértékben összefügg a fehérjék antigenitásával (Hopp, 1981; Garnier, 1990a; Garnier 1990b).

Az $\alpha$-helixek és $\beta$-lemezek hidrofóbok, és általában a fehérjék belsejében helyezkednek el, míg a flexibilis hajlatok és kanyarulatok a molekulák hidrofil felszínén.

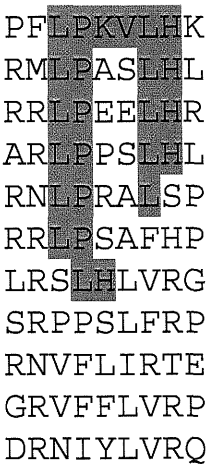
Ezeket a törvényszerűségeket írják le a *Chou-Fasman* és *Garnier* algoritmusok, melyek 55-70% sikerrel detektálják az epitópokat (Chou, 1974; Chou, 1978; Garnier, 1978; Taylor, 1984; Garnier, 1990a).

A fehérjék hidrofil részeinek felszínen való elhelyezkedését használja *Hopp és Wood* algoritmusa, *Kyte és Dolittle hydropatiás indexe* az oldalláncok vízhez való kötődésével von párhuzamot. *Parker, Guo és Hodges* az aminosavak a hidrofilitást HPLC-vel való vizsgálattal állapította meg (Hopp, 1981; Kyte, 1982; Parker, 1986).

*Hopp akrofilitási indexe*, a 49 ismert szerkezetű fehérjében az aminosavak relatív felszíni expozícióját vizsgálja (Hopp, 1993).

A nagyobb flexibilitású részek, felszínen való elhelyezkedését és így az immunválaszban való lehetséges részvételét mutatja meg *Karplus* és *Shultz*-féle *flexibilitási index* (Karpuls, 1985).

*Welling* 20 sokat tanulmányozott fehérje 606 epitópjában előforduló aminosavakat dolgozta fel statisztikailag (Welling, 1985). Vizsgálatában az epitópok minden pozíciója hasonló fontossággal bírt, amit eredményeinek használatakor figyelembe kell venni. Ismertek ugyanis ún. kitüntetett pozíciók melyek, megváltoztatása egy-egy epitópban megszünteti az antitest-antigénkötődést, ugyanakkor más pozíciókban történő aminosav változás nem befolyásolja a reakciót (**5. Ábra**).




**5. Ábra.** Anti-HBxAg monoklonális antitest által felismert epitópok (random fág könyvtár, (Pál, 2003). Szürke háttérrel a kitüntetett epitópok.

*Jameson és Wolf* antigenitási vizsgálatai azt bizonyítják, érdemes több különböző módszer együttes alkalmazása. Vizsgálataikban súlyozottan használtak öt módszert (Jameson, 1988).

Az epitóp meghatározást specifikusabbá tehetjük az érzékenység csökkenése nélkül, jelentősen növelve a találatok biztonságát, több különböző módszer párhuzamos használatával (Jameson, 1988; Hudecz, 1994).

Egy-egy módszer eredményessége jelentősen függ az alkalmazott módszertől, a vizsgált fehérjétől és a korábban alkalmazott, a módszer paramétereinek beállításához használt adatbázistól. Az epitóp felismerés pontosabb, ha a módszert az általunk vizsgált fehérjéhez hasonló kvalitású, funkciójú már vizsgált fehérjén tesztelt módszerrel analizáljuk. Így a statisztikai megfigyeléseinket a vizsgálandó szerkezethez hasonló mintán teszteljük. Ezekben az esetekben módszereink hibái kiegyenlíthetik egymást, jelentősen javítva az epitóp meghatározást.

Az epitóp predikció a bioinformatika önálló ága (Pongor, 1985; Boguski, 1994a; Németh, 2004). Segítségével jelentősen lerövidíthető az új ellenanyagok előállítása, miközben új perspektívák nyílnak meg az immunológia területén. Lehetővé válik, új ellenanyagok előállítása számtalan kórokozóval és betegséggel szemben (pl. hepatitisz, HIV, influenza, káriesz stb), leegyszerűsíthetjük kutatásunkat, könnyebben eredményhez juthatunk (de Groth, 1999; Guex 1999; Boguski, 2004a; Imani, 2005).

A számítógépes predikció új perspektívákat nyitott az immunbiológia területén is. A módszer korlátainak ismerete nélkül azoban megnő a kapott adatok téves éretelmezésének veszélye, hibás következtetésekre juthatunk, rosszabb esetben elérhetetlen célokat követünk (Foote, 1998; de Groth, 1999; van Regenmortel 1998).

Egyetlen predikciós módszer ismerete (egydimenziós – primer szerkezet - analízise) nem elég az érzékeny háromdimenziós információ kiszűréséhez, az epitóp meghatározásához (Mendez-Arias, 1990; Pellequer, 1991; Vihinen 1994; Peri, 2001). Az aminosavak kvalitásainak gyenge korrelációja a fehérjék szerkezetével csak rontja a helyzetet, mivel 20-30%-ban szekvenciájukban hasonló fehérjék hasonló harmadlagos konformációt vehetnek fel (van Regenmortel, 1989; Vihinen, 1994; Laskowski, 1997; Sonnhammer, 1998). Emellett,

hasonló szekvenciájú rövid peptidek gyakran különböző harmadlagos szerkezettel bírnak más, nem rokon fehérjeláncok alkotórészeként (Fauchere, 1983; van Regenmortel, 1988). Így, egy antigenitás szempontjából jól leírt minta alapján készült táblázat sem nyújt garanciát a sikerhez (van Regenmortel, 1989; Laver, 1990; Rötzschke, 1994). Megoldásként a különböző algoritmusok és táblázatok olyan kombinációja juttat minket a legjobb eredményhez, amelyben a vizsgált molekula harmadlagos szerkezetét is figyelembe vesszük (Jameson, 1988; van Regenmortel, 1989; Mendez-Arias, 1990; Makyustov, 1993; Hudecz, 1994). A különböző algoritmusok interpretálásával számtalan epitóp prediktáló program született. Ezen programok felhasználását és eredményességét limitálják az algoritmusok és azok adaptálásának hiányosságai (Pongor, 1985; Gribskov, 1986; Wolf, 1988; Mendez-Arias, 1990; Makyustov, 1993; Pellequer , 1993; Hammer, 1994; Womble, 2000; van Regenmortel, 1996).

Szükséges a jelenlegi algoritmusok tökéletesítése, új algoritmusok keresése és az eredmények minél nagyobb, antigenitás szempontjából jellemzett mintán való ellenőrzése és finomhangolása.

Az epitóp predikció eredményessége számtalan módszerrel vizsgálható. Legegyszerűbb az ismert antigenitású fehérjemintán az epitópok elhelyezkedésének összehasonlítása az algoritmusok által meghatározott fehérjerészletekkel és ezen adatok statisztikai analízise (Hopp, 1985; Jameson, 1988; van Regenmortel, 1988; Pellequer, 1991; Petrohai, 2005; Nyárády, 2005). A módszerek leírása hasonlóképpen egy adott antigenitásában ismert adatbázison való tesztelés eredménye legtöbbször. A módszerek ezért az „ideális"-hoz az eredeti molekulákhoz hasonló szekvenciák esetében hoznak biztos eredményt (Németh, 2004; Nyárády, 2005).

Az irodalomban leírt epitóp prediktáló módszerek hatékonysága kétféleképpen vizsgálható: jól karakterizált ellenanyagok epitóp felismerésének összehasonlítása a becsült szekvenciával, vagy becsült szakaszok szintetizálása és ezek kötődésének vizsgálata ellenanyaghoz (Hopp, 1985; van Regenmortel, 1986).

# 4. Kutatási témakörök és célkitűzések:

Munkám célja a molekuláris biológiai és immunológiai kutatáshoz kapcsolódó bioinformatikai módszerek fejlesztése, optimalizálása és felhasználási területük kritikai elemzése volt. Három fő területre koncentráltam:

1. DNS szekvenálás, laboratóriumi fázisát megkönnyítő szoftver fejlesztése,
2. DNS szekvenálás eredményeinek automatikus értékelését és adatbázisokba juttatását megkönnyítő szoftverek fejlesztése,
3. az *in silico* epitóp-predikció immunológiai használatának elemzése és validálása *in vitro* immunszerológiai mérésekkel
4. biostatisztikai módszerek kombinált alkalmazása biológiai mérésekből származó nagy adathalmazok belső összefüggéseinek feltárására.

## 4.1 EST szekvenálási eredmények automatikus kiértékelése

A célkitűzések:

1. a EST szekvenáláskor kapott eredményeket minőségileg kiértékeljük és a technikai hibákat kizárjuk;
2. kiválasszuk a további elemzésre alkalmas szekvenciákat;
3. hasonlóság keresést végezzünk standard adatbázisok felhasználásával;
4. a kapott eredmények annotálását és adatbázisokban történő elhelyezését automatikusan végezzük el.

A fenti munkák közvetlenül kapcsolódnak a molekuláris biológiai laboratóriumi technikákhoz. Gyakorlati felhasználásuk az operációs rendszerek megújításáig zavartalan volt. A további munkáim fehérje szintű vizsgálatokhoz kapcsolódnak.

## 4.2 Laboratóriumi DNS szekvenálást segítő szoftver fejlesztése

Célkitűzésünk a DNS szekvenálás laboratóriumi fázisát segítő szoftver fejlesztése volt.

1. A laboratóriumban használt nevezéktan alapján elkészíti a gélfájlt.
2. Segíti a minták gélre jutatásánál a mintatévesztés csökkenését.

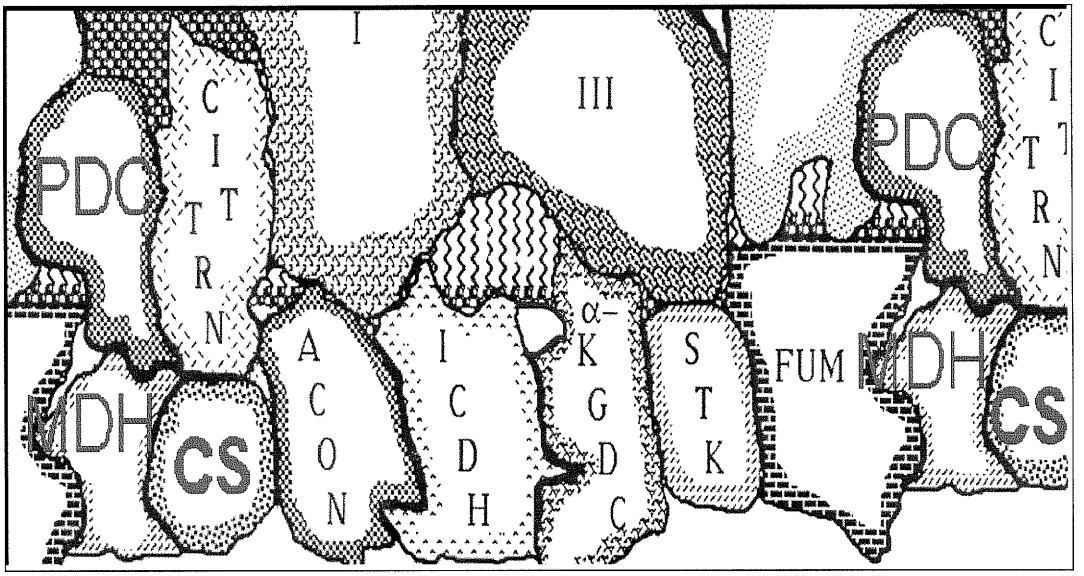
## 4.3 Immunglobulinok, B-sejt és T-sejt receptorok által felismert epitópok térképezése

A fő célkitűzésünk az volt, hogy a rendelkezésre álló predikciós módszerek lehető legszélesebb körét felhasználva, jól jellemzett immunológiai modelleken összehasonlító vizsgálatokat végezzünk.

### 4.3.1 *In silico* epitóp predikciós analízis

A munka első fázisát a kiválasztott modell molekulák immundomináns epitópjainak analízise jelentette. Monoklonális ellenanyagok által felismert eptiópok *in silico* predikcióját (a szabadon elérhető programok felhasználásával) és *in vitro* (immunszerológiai) meghatározását használtam modellként.

Két, immunbiológiai szempontból érdekes molekulamodellt vizsgáltunk. A hepatitisz B vírus X antigén (HBxAg) sajátos fiziko-kémiai tulajdonságainál fogva (45%-ban hidrofób aminosavak építik fel) mind elméleti immunpatológiai, mind gyakorlati immundiagnosztikai szempontból fokozottan érdekes (Miller, 1989; Murakami, 1999). A betegség lefolyása során a HBxAg egyes epitópjainak más-más kóroktani szerepe van (Kaneko, 1988; Kim, 1991; Kim, 1993; Murakami, 1999), így az egyes epitópokkal szembeni immunválasz vizsgálata prognosztikai jelentőséggel bír (Blumberg, 1997; Andrisani, 1999; Pál, 2001). Kihívást jelent az immunesszé-fejlesztésben is ez ellen a sajátos szerkezetű molekula ellen immunológiai reagenst (esetünkben monoklonális ellenanyagot) fejleszteni (Yen, 1996; Blumberg, 1997; Pál, 2001; Pál, 2003). Mindkét esetben a bioinformatikai elemzés szükségessége nyilvánvaló volt.




**6. Ábra.** Humán mitokondriális belsőmembrán CS elhelyezkedése.

A másik modell molekula, a mitokondriális belsőmembránban található citrát szintáz (CS) enzim evolúciós szempontból bír jelentőséggel: az egyik legősibb és nagyfokban konzervált fehérjestruktúra, ami a Szent-Györgyi-Krebs ciklusban történő biológiai energiatermelés kulcsenzime, a legtöbb baktériumban, és eukarióta genomban kódolva van (Krebs, 1935; Buckel, 1967) (**6. Ábra**). Prokarióta eredeténél fogva az eukarióta szervezetek immunrendszere számára „idegen"-ként jelentkezik, ennek ellenére vele szemben nagyon erős tolerancia alakult ki az evolúció során (Kamiike, 1989; Abbas, 1994). Az elmúlt években vált világossá, hogy ez a tolerancia egy aktív funkció, ami az immunológiai felismerés szintjén már jelentős különbségeket mutat a fiziológiás és a kóros biológiai folyamatokban (Clark, 1978-79; Spitzer, 1990; Schwartz, 2000; Aluvihare, 2004; Goodnow, 2005). Az elméletileg lehetséges epitópok bioinformatikai térképezése tehát ebben az esetben is jelentőséggel bír.

### 4.3.2 Az epitóp predikció effektivitása és validálása

Mindkét modellünk vizsgálatának lényegi elemét képezte a bioinformatikai (*in silico*) módszerekkel megjósolt epitópokkal szembeni valódi – *in vivo* kialakult – immunválasz mérése *in vitro* immunszerológiai módszerekkel, és a kapott eredmények összehasonlítása. A CS modell esetében emberi vérszérumokban a fiziológiásan és kóros körülmények közt termelődött specifikus ellenanyagok epitóptérképezésének adatait tudtuk az *in silico* predikcióval összevetni. A HBxAg esetében az antigénpredikció eredményeit anti-HBxAg monoklonális ellenanyagok epitópspecificitásának meghatározása során kapott adatokkal hasonlítottuk össze.

## 4.4 Biostatisztikai módszerek kombinált alkalmazása biológiai mérésekből származó nagy adathalmazok belső összefüggéseinek feltárására

Biológiai mintákon történő mérések kiértékelése sokszor jelent problémát. Különösen igaz ez olyan, nagyszámú mérésből származó adathalmazok esetében, ahol több paraméter egymásközti összefüggését vizsgáljuk. Különböző betegcsoportok vérszérumaiban mért anti-HBxAg ellenanyagok immunszerológiai vizsgálatát végeztük és a kapott eredményeket analizáltuk biostatisztikai módszerekkel, a belső korrelációk megállapítására alkalmas algoritmust hoztunk létre.

# 5. Anyagok és módszerek:

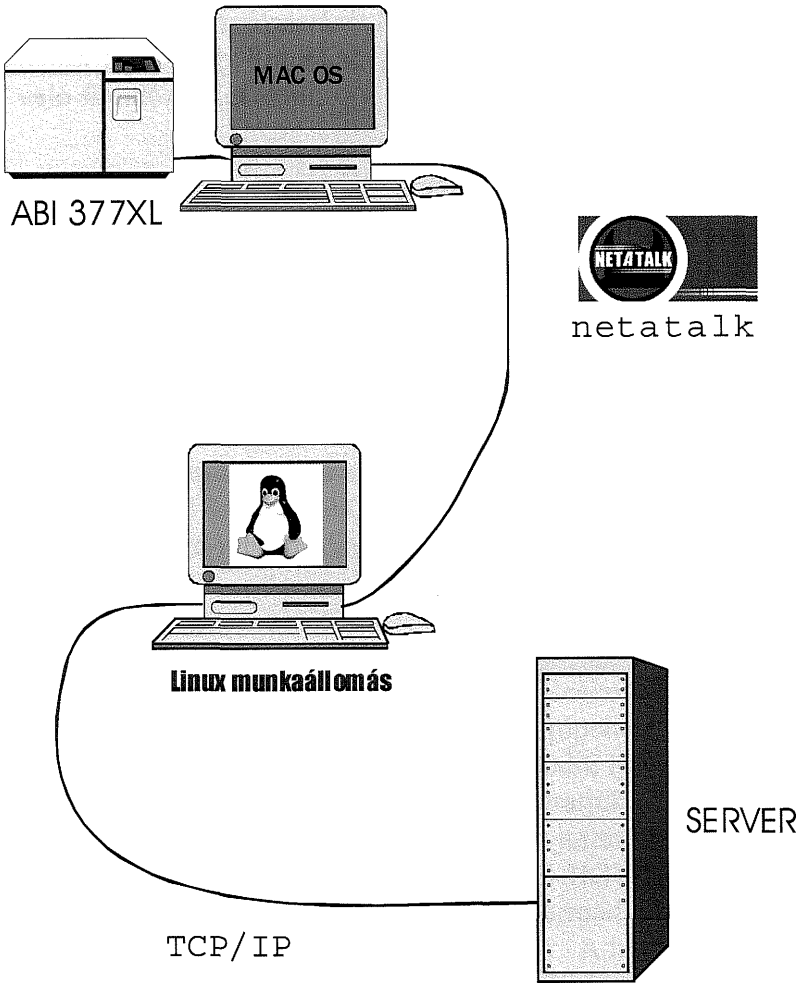
## 5.1 Módszerek a DNS szekvenálás eredményeinek automatikus értékelésére

**Minta szekvenciák generálása és EST szekvenálás:**

A referenciaként számon tartott *T. cruzii* CL Brenner klónból (Urmenyi, 1999) készítettünk cDNS könyvtárat. Az oligo(dT) szekvenciával normalizáltuk a poli(A+) RNS-t, hogy kiegyenlítsük az előforduló mRNS-ek számát. A cDNS könyvtárat transzformáltuk *E. coli* DH5-$\alpha$ törzsbe. Több mint 23000 klónt választottunk ki véletlenszerűen melyeket 384 lyukú mikrotitter lapra helyeztünk. Magas minőségű kettősszálú plazmid DNS-t tisztítottunk Wizard Plus SV miniperp DNS (Promega) vagy PERFECTprep-96 szettel (5 Prime-3 Prime Inc.) ABI® Prism 21M13 fluorescens (Perkin Elmer Cetus) és DYEnamic festékkel (Amersham Life Sciences) direkt jelölt T7 primerrel automata ciklus szekvenálást végeztünk. A mintákat ABI® 377XL szekvenátorokon analizáltuk.

**Szekvencia analizáló rendszer:**

Az ABI 377XL szekvenátor adatait a hozzá kapcsolt Apple Power Macintosh 8600-on (Operációsrendszer: MacOS 8.0; Apple Computer Inc. USA) dolgoztuk fel. A kapott kromatogram fájlokat a `netatalk` (AppleTalk Protocol Suite 1.4; http://netatalk.sourceforge.net) programmal IBM PC (RedHat Linux 4.2; `kernel 2.0.30`; http://www.redhat.com) és Sun Sparc Ultra 5 (Sun Solaris 2.6; Sun Microsystems Inc., USA; http://www.sun.com) számítógépekre másoltuk. Az adatok további feldolgozása és a szükséges szofteverek fejlesztése itt történt. A szkripteket `bash` (bourne again shell)-ben írtam. A szükséges saját alkalmazásokat C++-ban keszítettük és GNU GCC-vel (GNU Compiler Collection 2.7.2.3 (http://gcc.gnu.org); Free Software Foundation Inc., USA) fordítottuk le (**7. Ábra**).




**7. Ábra.** Szekvencia analizáló rendszer vázlata. Az ABI 377 szekvenátorhoz kötött Macintosch-ről, `netatalk` protokollal jut a szekvencia egy Linux munkaállomásra, innen TCP/IP protokollal történik a további adatátvitel.

**Base calling:**

A kromatogrammok analizálására a bázisok és szekvenálás minőségét a Phil Green *phred* programjával végeztük (http://www.phrap.org; Ewing B, 1998a; Ewing B, 1998b). Szekvenciák ellenőrzésére és vizualizálására a *consed* programot használtuk (http://www.phrap.org; Gordon, 1988).

**Homológia vizsgálatok:**

A cDNS szekvenciákat az alacsony komplexitású szekvenciáktól a *dust* és *seq* programokkal szűrtük ki (http://blast.wustl.edu; Wooton, 1993; Altschul 1994).

A szekvenált, feldolgozott EST-eket *blastcli* programmal (Altschul, 1997) automatizáltan kerestük a nemredundáns nukleotid GenBank szekvencia adatbázisban (http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html). A keresésre a *BLASTN* és *BLASTX* programokat használtuk. A paraméterek E=1e-5, E2=1e-5, B=10, H=0, V=10 voltak. Homológiavizsgálatot végeztünk a SWISPROT adatbázissal (Bairoch, 1997) szemben is a *BLASX* kliens verziójával. A paraméterek ebben az esetben E=1e-4, E2=1e-5, B=10, H=0, V=10 voltak.

Az eredmények automatikus kiválasztását *btab* programmal végeztük (http://thr.cit.nih.gov/sw.html#btab; Dubnick, 1992). A programot módosítanunk kellett, a Linux platformon való futtatás érdekében.

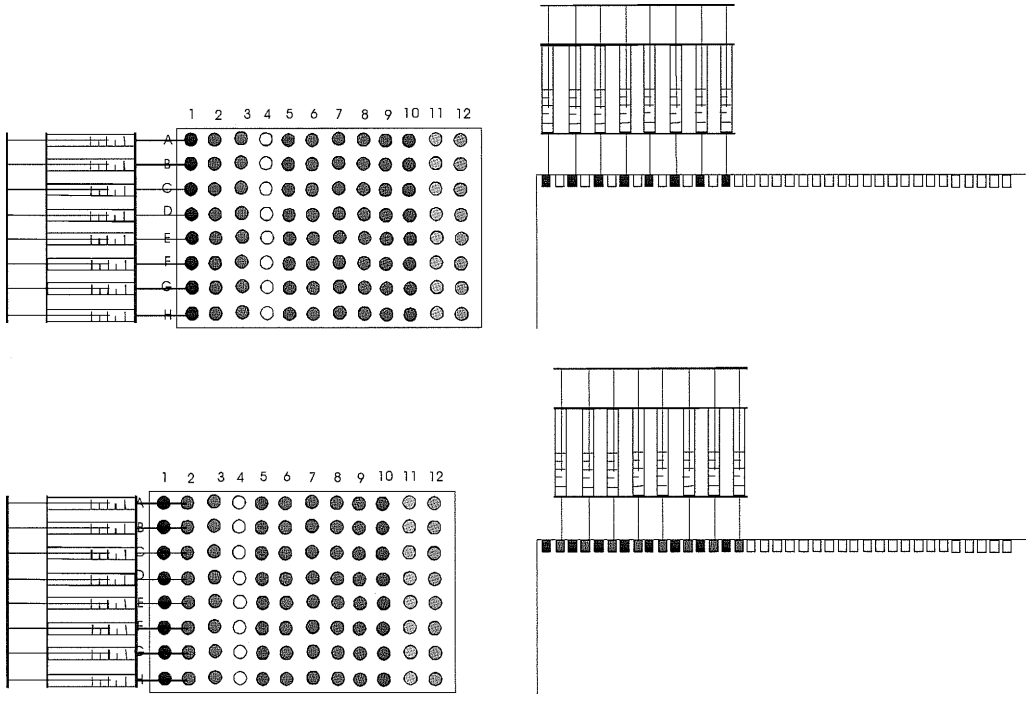
## 5.2 Laboratóriumi szekvenálást segítő szoftver fejlesztése

**Szekvencia analizáló rendszer:**

Szekvenálásra, Uppsalaban, az akkori Orvosi Genetikai Intézetben (jelenleg Genetikai és Patológiai Intézet) egy ABI Prism ® 377XL (Applied Biosystems Inc, USA) szekvenátort használtunk. A szekvenátor adatait a hozzá kapcsolt Macintosh Power PC-n (http://www.apple.com; MacOS 8.0; Apple Computer Inc., USA) dolgoztuk fel az ABI Prism® DataCollection Software-rel (Applied Biosystems Inc., USA).

**Minták gélre való juttatása:**

Szekvenálás előtt az előkészített, 96-lyukú mikrotitter tálcákon lévő reakciókat 8 csöves Hamilton pipettával juttattuk a 64-lyukú gélre. A Hamilton pipetta csövei és a gél lyukai között osztásbeli eltérés van, ezért a pipettával minden második lyukba juttathatunk mintát (**8. Ábra**). Először az 1. lemez 1. oszlopát (1a1 -1h1) mintát juttatjuk az 1. gél 1,3,5...15 oszlopára, majd a 1. lemez 2. oszlopát (1a2 – 1h2) mintákat juttatjuk a 1. gél 2,4,6...16 oszlopára. Ezután következik az 1. lemez 3 oszlopának (1a3-1h3) felhelyezése az 1. gél 17, 19, 21...31 oszlopára és így tovább. A 2. gélt az 1. lemez harmadik harmadán (1a9-1h12) és a 2. lemez első harmadán lévő mintákkal töltjük fel (2a1-2h4). A harmadik gélre a második lemez 5-12 oszlopának mintái kerülnek. Ez a felhelyezés figyelmet követel, hogy a minták összekeveredését elkerüljük.




**8. Ábra.** Hamilton pipetta és a minták gélre juttatása.

**Adatok átvitele MacOS és Unix operációs rendszerek között:**

A kapott kromatogram fájlokat a *netatalk-1.4* programmal IBM PC (http://www.redhat.com; RedHat Linux 4.2 – kernel 2.0.30; Red Hat Inc., USA) és Sun Sparc Ultra 5 (http://www.sun.com; Sun Solaris; Sun Microsystems Inc., USA) számítógépre másoltuk (l. **7. Ábra**).

**Programozás és fordítás:**

A scripteket `bash` (bourne again shell)-ben írtam. A programot C++-ban programoztam és GNU GCC-vel (http://gcc.gnu.org; Free Software Foundation Inc., USA) fordítottuk le.

## 5.3 Immunglobulinok által felismert epitópok térképezése

### 5.3.1 *In silico* epitóp térképezés

**Humán mitokondriális CS B-sejt epitóp predikció:**

Vizsgálatunkhoz a humán mitokondriális CS szekvenciát használtuk (EC 2.3.3.1) Gene Bank accession no. **PID O75390**, (Goldenthal, 1998)) melyet az NCBI fehérje szekvencia adatbázisból töltöttünk le (Benson, 1991; http://www.ncbi.nlm.nih.gov/Entrez/index.html). A lehetséges antigének meghatározása céljából a *Chou-Fasmann* másodlagos szerkezet (Chou, 1974; Chou, 1978; Prevelige, 1990) és az *Eisenberg*-féle hidrofobicitás (Eisenberg, 1990) meghatározásokat végeztünk. Mindkét esetben, egy általunk elkészített *MS Excel* táblázatban, 7 aminosavra kiterjedő ablakkal végeztük a meghatározásokat, a szerzők által használt eredeti mátrixokkal. A kis valószínűséggel hidrofób ($P_{hydrophobicity} < 0$) és nagy valószínűséggel β-csavar szerkezetet ($P_{\beta\text{-turn}} > 1$) mutató szekvenciákat választottuk ki. A *GCG Wisconsin Programcsomag*hoz (http://www.gcg.com; Genetics Computer Group Inc.,USA) tartozó *PepPlot*, *PeptideStructure* és *PlotStructure* alkalmazásokkal finomhangoltuk predikciónkat (Gribskov, 1986; Wolf, 1988; Womble, 2000). Kiszámítottuk a molekula felületen történő elhelyezkedés valószínűségét ($P_{surface} > 1$), a *Jameson-Wolf antigenitási indexet* ($P_{JW\ antigenity} > 1$) (Jameson, 1988), a *Hopp és Wood féle hidrofilicitási indexet* ($P_{HW\ hidrophilicity} > 1$) (Hopp, 1981; Dolz, 1994).

A Swiss-Model Automated Protein Modelling Server-en (http://www.embl-heidelberg.de/predictprotein) az *E. coli* CS (EC 4.1.3.7, Brookhaven Protein Data Bank No. 1K3P (Nguyen, 2001)) való hasonlóság alapján szerkesztett 3D humán CS szerkezetét letöltöttük (Rost, 1996; Peitsch, 1996; Guex, 1999; Schwede, 2000; Peitsch, 2002).

**Hepatitis B-vírus X-protein epitope predikció:**

A *Chou-Fasman* és *Garnier-Ousguthorpe-Robson* másodlagos szerkezet predikciós algoritmust ($P_{hydrophobicity} < 0$; $P_{\beta\text{-turn}} > 1$) (Chou, 1974; Garnier, 1978; Gibrat, 1987; Prevelige, 1990) és a *Jameson-Wolf* antigenitási indexet ($P_{JW\ antigenity} > 1$) (Jameson, 1988) használtuk a HBX epitóp meghatározásához a *Wisconsin GCG Programcsomag ProteinStructure* programjából (Genetics Computer Group Inc.,USA).

**T-sejt epitóp térképezés:**

T-sejt epitóp térképezést virtuális mátrix módszerrel végeztem, feltételezve, hogy kötés specifikus értékek hozzárendelésével és kombinálásával egy adott HLA szekvenciához történő hasonlóság alapján meghatározható a másik HLA zseb specifitása. A *TEPITOPE2000* (http://www.vaccinome.com/pages/597444/; Juergern Hammer, Vaccinome 1999) programmal (Hammer, 1994; Sturiniolo, 1999) a rendelkezésre álló mind a 25 HLA-II allélel vizsgáltuk a CS-t. (DRB1*0101; DRB1*0102; DRB1*0301; DRB*0401; DRB1*0402; DRB1*0404; DRB1*0410; DRB1*0421; DRB1*0701; DRB1*0801; DRB1*0802; DRB1*0804; DRB1*0806; DRB1*1101; DRB1*1104; DRB1*1106; DRB1*1107; DRB1*1305; DRB1*1307; DRB1*1311; DRB1*1321; DRB1*1501; DRB1*1502; DRB1*0101). A HLA kötési zsebeket egyenlően súlyoztuk. Egy százalékos határértéket használtam a nagy valószínűséggel epitóp szekvenciák azonosítására.

## 5.3.2 Epitóp predikció *in vivo* ellenőrzése

Rekombináns HBX (aminosav 1-154) glutathion S-transzferáz (GST) fehérjét készítettünk (Marczinovits, 1997). A korábban kifejlesztett rekombináns HBX elleni ellenanyagot (Pál, 2001) használtuk, mely a 3F6-G10 klón terméke. Az *E. coli* XL1-Blue törzset és az M13KO7 fágot a Stratagen (USA), a DH5-alfa törzset az Ivitrogen szállította. Nitrocelulóz mátrixot a Paharmacia Biotech-től (Svédország), a 96-lyukú polisztirén tálcákat a NUNC-tól (Franciaország) szereztük be. Az alkalikus foszfatáz (AP) kötött egér ellenes IgG antitesteket és retek-peroxidázt (HPRO) a Dako (Dánia) szállította. A Lusia-Bertani (LB) baktérium médiumot és minden egyéb a kísérletek során használt szert a Sigma-tól (Németország) vásároltuk.

**Vizsgált minták:**

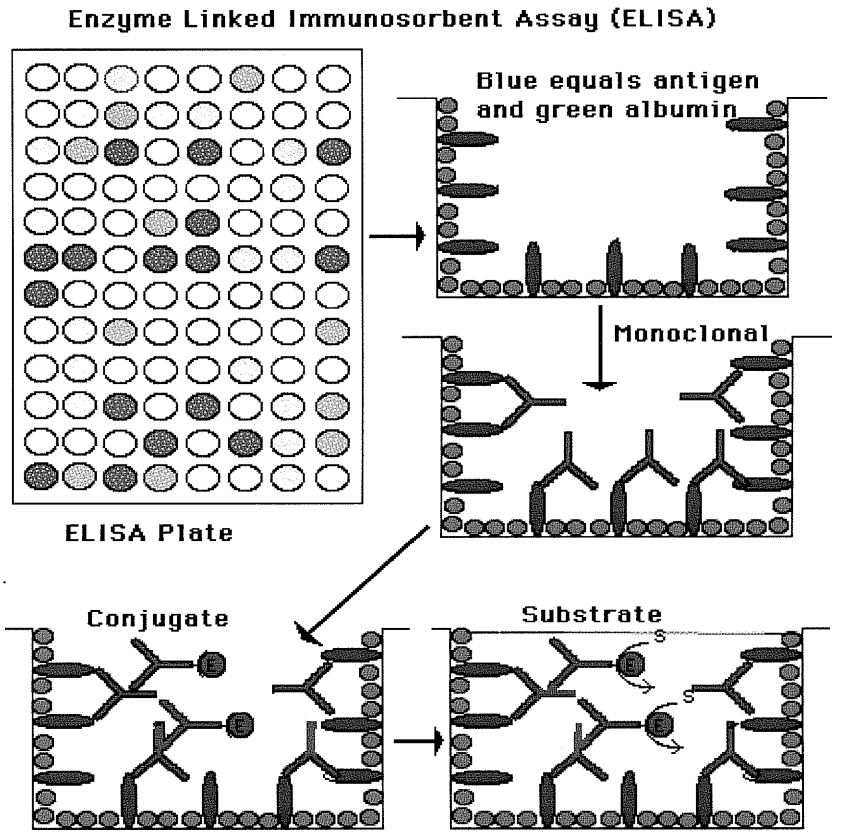
**a.) CS:** Háromszáz-harmincnégy egészséges embertől származó 5 éves periódus alatt gyűjtött szérumot (63 magyar, 51 brit és 176 finn véradó és 44 egészséges gyermek), valamint 326 jól dokumentált autoimmun betegtől származó és 52 szívtranszplantált beteg vérmintáit analizáltuk. A szérumokat -70°C-on tároltuk felhasználásig. A vizsgált csoportokban a kor és nem aránya megegyezett.

**b.) HBX:** PTE-ÁOK I. Belgyógyászati Klinikáján és a Johan Béla Nemzeti Epidemiológiai Központ Virológiai Osztályán gyűjtött és laboratóriumban HBV pozitivitását HBsAg és HBcAg specifikus ELISA-val ellenőrzött (Dialab, Magyarország). Tizennégy HBV fertőzött akut hepatitiszes, 80 krónikus hepatitiszben szenvedő, 14 tünetmentes HBV hordozó és 22 egészséges donor mintáit vizsgáltuk.

**Anti-CS autoantitestek immunszerológiai detektálása ELISA-val:**

Indirekt ELISA-t használtunk (**9. Ábra**) az anti-CS autoantitenstek kimutatásához: sertés szív CS-t (Sigma, USA) mint antigént inkubáltunk 12 órán át +4 °C-on, amit 60 perces inkubálás követett 37 °C-on 96-lyukú polisztirén tálcákon (NUNC, Franciaország). A nem specifikus kötések telítését követően (0,5% zselatin (Sigma), PBS (pH 7,3)), a mintákat háromszor mostuk 0,1% Tween 20 (Sigma, USA) PBS oldatával. A mintákat három példányban inkubáltuk 1:100 hígításban (mosófolyadék) 60 percig. Végül a mintákat anti-humán IgG-HRPO vagy anti-humán IgM HRPO másodlagos antitestekkel (Dako, Dánia) inkubáltuk 60 percen át. A színreakciót *o*-fenilén-diaminnal (Sigma, USA) hívtuk elő és

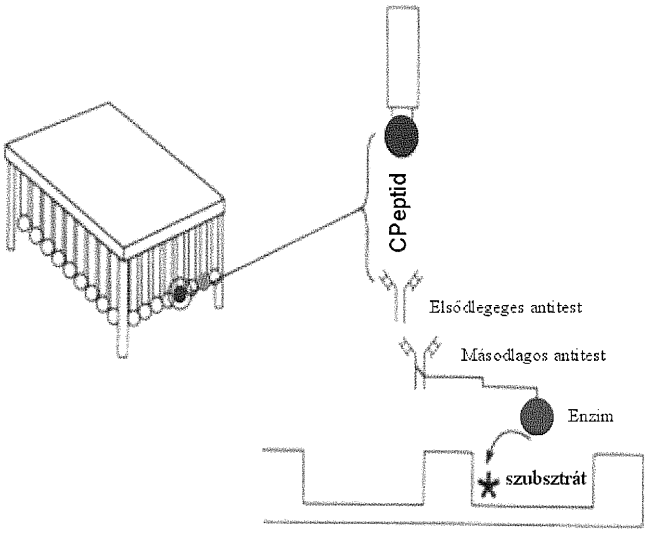
Dynatech MR7000 (USA) mikrofotométerrel mértük λ=492 nm (OD, optikai denzitás). Az eredményeket CS-t nem, de a többi összetevőt tartalmazó kontrollal hasonlítottuk össze.




**9. Ábra.** Az ELISA sémás ábrája.

## Tűhegy szintézis:

Negyven, egymást 5 aminosavval átfedő, dekapeptideket szintetizáltunk a 9 prediktált epitóp régiónak megfelelően. A szintézis β-Ala-Gly-el funkcionalizáltatott polietilén tűkön történt (Mimotopes, Australia) Fmoc/tBu reagenssel a *Geysen* módszere szerint (Geysen, 1984). Oldallánc védő csoportnak $^{t}$Bu (Thr, Ser, Tyr), O$^{t}$Bu (Asp, Glu), Trt (Cys, His, Gln, Asn), Pbf (Arg) és Boc (Lys, Trp) reagenseket használtunk. A Fmoc α-amino védőcsoportokat 20 %-os piperidine/DMF (v/v) oldattal távolítottuk el. A kötést DIC/HOBt módszerrel történt és bromofenol kékkel monitorizáltuk (Krchnák, 1988). A peptideket az *N*-terminálison acetiláltuk, az oldallánc védő csoportokat TFA – EDT – anizol 38:1:1 (v/v/v) oldattal távolítottuk el. A nem védett peptidek kovalens kötésben maradtak a tűhegyekhez. Kontrollként két peptid szintézise történt: PLAQGGGGGG és GLAQGGGGGG. A tűhegyszintézist sémásan a **10. Ábra** mutatja be.




**10. Ábra.** A tűhegyszintézis sémás ábrája.

## CS azonosság keresése:

Az antitest kötődését vizsgáló kísérleteink mellet a humán CS azonosságát vizsgáltuk az NCBI non-redundáns fehérje adatbázisában. Összehasonlításra a BLASTP2 (Altschul, 1997; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) programot használtuk (E = 20000; Matrix PAM30; Gap cost Existence: 9 Extension: 1; Word size = 2, az adatbázis nagysága 2004 Szeptemberében 688 443 072 volt). Az immumológiailag releváns régiók azonosságot mutattak bizonyítottan vagy feltételezhetően CS funkciójú fehérjékkel. Hasonlóságot CS-hoz nem köthető funkciójú szekvenciákkal nem találtunk.

## HBX szintetikus peptidek:

A HBX másodlagos szerkezet predikció alapján a Szegedi Tudományegyetem Kémiai intézetében szintetizáltattunk és tisztíttattunk HPLC-vel szintetikus peptideket.

A rekombináns tripszinnel emésztett (Kele, 1998) peptid fragmentumokat C18 reverzfázisú HPLC oszlopon (Nucleosil 5C18, 300 Å) 40 perces lineáris 20%-tól 60%-os koncentrációjú (0,1% trifluorecetsav 80%-os vizes acetonitril) oldatban 1ml/perc áramlással szeparáltuk. Az oszlopról lekerülő oldatot 215 nm-en vizsgáltuk, 1 ml frakciókban. A szeparált peptidek HBX ellenes monoklonális ellenanyaghoz való kötődését ELISA-val vizsgáltuk. A legnagyobb ELISA pozitivitású frakciókat TSQ7000-es tripla-quad tömegspektrométeren vizsgáltuk, nano-ES/MS/MS (Wilm, 1996).

## Monoklonális anti-HBxAg ellenanyagok előállítása:

Az immunizáláshoz 6-8 hetes, nőstény, beltenyésztett BALB/c egereket használunk. Az alapimmunizálását (intrakután) komplett Freund adjuvánssal, a megerősítő immunizálást (intrakután és intraperitoneálisan) inkomplett Freund adjuvánssal végezzük. Antigénként 20μg/200μl rekombináns HBx-GST-t, illetve a KLH-$HBx_{13-26}$ szintetikus peptidet használunk. Az utolsó emlékeztető oltás után a sejtfúziót az immunizált egér lépéből preparált limfocitákkal és Sp-2/0-Ag-14 nem szekretáló, $HGRT^-$ mielóma sejtekkel végezzük, polietilénglikol segítségével. A hibrideket 20% borjúsavó és HAT tartalmú DMEM tenyésztő

médiumon szelektáljuk. A kiválogatott monoklónokat ismételten klónozzuk az immunszerológiai (indirekt ELISA) tesztelések eredményei alapján. A legjobb klónokból, hibridóma sejtfermentorban ellenanyagot állítunk elő a további vizsgálatokhoz.

**Szerológiai vizsgálatok:**

**Simple binding ELISA:**

Az ELISA vizsgálatokat a következőképpen végeztük: a mikrotitrátor lemezhez egy éjszakán keresztül 4°C-on 2μg/ml HBxAg-GST-t, illetve GST-hez fuzionáltatott HBxAg fragmenseket, KLH-HBx13-26 aa., illetve 5μg/ml BPAG1 antigén konstrukciókat kötünk ki karbonát pufferben (pH 9,6). 0,2% Tween 20 tartalmú PBS-ben történő háromszori mosás után telítjük a lemezt 0.5% PBS-zselatinnal. Ezek után 37°C-on, 1 órán át inkubáljuk a szérumokkal (1/100), illetve a felülúszókkal. Háromszori mosás után tormaperoxidázzal jelölt anti-humán IgG/IgM ellenanyagokkal, illetve a felülúszó esetében anti-egér Ig-nal történő, 1 órás inkubáció következik 37°C -on. A mikrotiter lemez mosása után a reakciót o-fenilén-diaminnal hívjuk elő. Az OD értékeket 490 nm-en mérjük automata mikrofotométer segítségével.

**Sandwich ELISA:**

Mikrotitrátor lemezhez egy éjszakán keresztül 4°C-on 10μg/ml anti-HBxAg monoklonális ellenanyagot kötünk ki karbonát pufferben (pH 9,6). 0,2%. Tween 20 tartalmú PBS-ben történő háromszori mosás után telítjük a lemezt 0,5% PBS-zselatinnal. A standard sorban HBxAg-GST antigént tizenkét léptékű felező hígításban 1/100 higítású normál szérummal pH 2-es glicin pufferben inkubáljuk 56°C-on, 10 percig, majd 1M Tris-sel pH 8-9-re állítjuk be. A hepatitis B pozitív és az egészséges kontroll szérumokat 100-as higításban használjuk és hasonlóan kezeljük mint a standard sorhoz használt rekombináns antigént. Ezek után 37°C-on, 6 órás inkubáció után biotinált, második anti-HBxAg monoklonális ellenanyag 1 órás inkubációja következik, 37°C -on. A mikrotiter lemez mosása után streptavidin-peroxidáz reakciót o-fenilén-diaminnal hívjuk elő. Az OD értékeket 490 nm-en mérjük automata mikrofotométer segítségével.

**Anti-HBxAg epitópjának tömegspektrometriai meghatározása:**

Az antigén, gélben történő emésztését végezzük. Natív elektroforézis után a gél sávokat finoman szétvágjuk. A proteint dithiothreitollal redukáljuk és jodoacetamiddal állítjuk be az alkalikus kémhatást (pH 8,0). Hozzáadva a tripszint (1: 10 higításban) egy éjszakán keresztül 37°C-on inkubáljuk. A peptid-fragmentumokat a gélből kioldjuk (5% hangyansav, 50% metilaklohol, 45% víz). Az egyes peptidek elválasztást HPLC-vel, 4,0 x 250mm, Nucleosil 5C18 300 oszlopon végezzük (0,1% aqueous trifluoroacetic acid, és 0,1% trifluoroacetic acid in 80% MECN oldószerekben, 5-95% 40 min grádiens mellett, 1,0 ml/min átfolyási sebességgel, 215nm-en detektálva). A tömegspektrometriát nano-electrospray ionizálással (gold coated capillary) végezzük.

**Random peptid könyvtár szűrése:**

A fágok dúsítását az irodalomban korábban közöltek szerint (Parmley, 1988) végezzük. Egy petricsészét egy éjszakán át érzékenyítünk anti-HBX antitesttel (40 ug/ml az első, 4 ug/ml a második dúsítási ciklusban). 0,05% tweent tartalmazó PBS-el történt mosás, majd 3%

BSA-PBS-el való blokkolás és újabb mosás után, 1010 fágot (1%BSA-PBS-el blokkolva) adunk a lemezhez. 2 órás 37°C-on végzett inkubáció és tweent (0,05% az első, 0,5% a második ciklusban) tartalmazó PBS-el történt mosás után a fágokat eluáljuk (1mg/ml BSA , 0,1 M glycine pH 2,2 HCl), majd neutralizálást követően (2M Tris Base) XL1-Blue *E.coli*-t fertőzünk velük. M13KO7 helper fággal történt felülfertőzés után a fágokat kicsapjuk (16,7% PEG8000, 3,3 M NaCl) és egy második dúsítási ciklust kezdtünk. A második ciklus után a fágokkal fertőzött majd M13KO7 helper fággal felülfertőzött XL1-Blue *E.coli* telepeket nitrocellulóz membránra emeljük. A membránokat blokkolás után (TBS/0,05% Tween 20/5% tejpor) 2 órán át inkubáljuk anti-HBX antitesttel (1 ug/ml), majd 1 órán át alkalikus-foszfatáz konjugált egérelleni IgG másodlagos antitesttel. Az előhívást BCIP/NBT szubsztráttal végezzük. Így negyven pozitív klónt választunk ki, amelyeket ELISA-val tovább tesztelünk, oly módon, hogy a fágokat kötjük ki a lemezre. Az ELISA alapján tíz klónból DNS-t izolálunk, amit szekvenálunk.

**Átfedő GST-X fúziós peptidek kifejlesztése:**

pGEX vektorba klónozott teljes HBX szakaszt mintaként használva három primer párral PCR-t (95°C 3min; 30 ciklus: 95°C 45sec, 50°C 30 sec, 72°C 1 min; 72°C 10 min) végezünk. A reakció a következőket tartalmazta: 50 ng templát, 200 nM dNTP, 0,5 uM primer, 1x Pfu puffer, 1 U Pfu polimeráz. A tisztított termékeket BamHI majd EcoRI restrikciós enzimekkel emésztjük, pGEX vektorba kötjük, majd a kapott konstrukciókkal DH5α *E.coli*-t transzformálunk. Az olvasási keret helyességét mindhárom plazmidnál szekvenálással ellenőrizzük. Rekombináns GST-HBxAg fragmensek: 1-90aa., 77-116aa., 96-135aa.,116-142aa., 77-143aa. Az átfedő rekombináns peptideket anti-HBX ellenanyagokkal ELISA rendszerben teszteljük.

**Peptidszintézis:**

A kiválasztott X antigén és BPAG1 szekvenciákat a t-Boc módszert alkalmazva kémiailag szintetizáljuk, HPLC-vel tisztítjuk és tömegspektrometriai módszerrel ellenőrizzük. (A szintetikus HBxAg: 13-26aa, amit KLH hordozóhoz konjugálunk.)

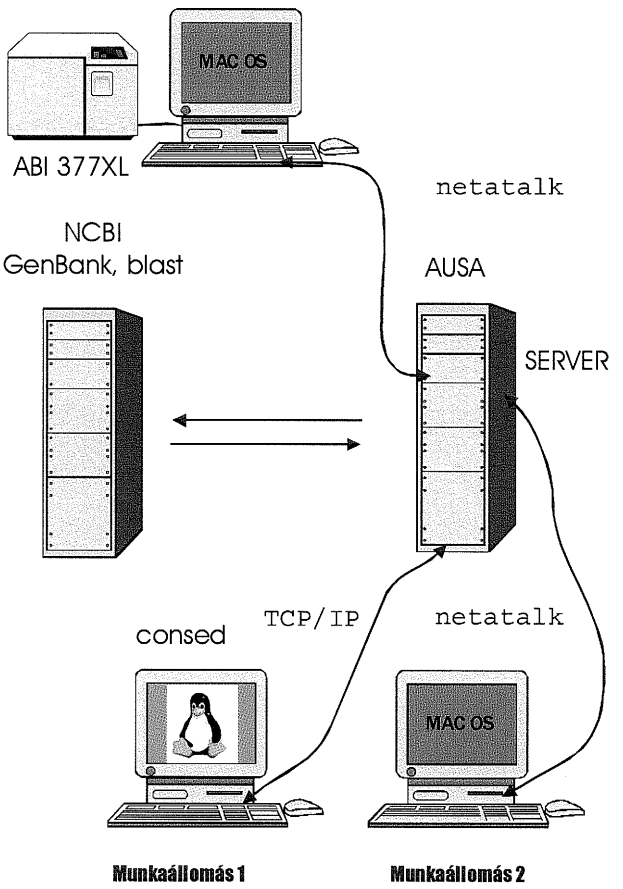
## 5.4 Biostatisztikai módszerek kombinált alkalmazása

A statisztikai analízist egy IBM PC kompatibilis személyi számítógépen futtatott SPSS 9.0 (SPSS Inc., 233 S Wacker Drive, Chicago, 60606, Ill., USA) programcsomaggal végeztük. Az ELISA eredményeket regresszió analízisét mind a három csoportban (akut, krónikus és hordozó) minden mintával minden minta ellen mátrixban elvégeztük (N, C, C1, C2, C3, X, X[14-16]). A 0,4-nél kisebb abszolút értékű regressziós koeficinenssel ($|R| > 0,4$) rendelkező és/vagy ahol a P érték 0,05-nél nagyobb párokat elvetettük. Az eredményeket táblázatban ábrázoltuk.

# 6. Eredmények:

## 6.1 *Automated Sequence Annotator* (*AUSA*)- DNS szekvenálás eredményeinek automatikus értékelése

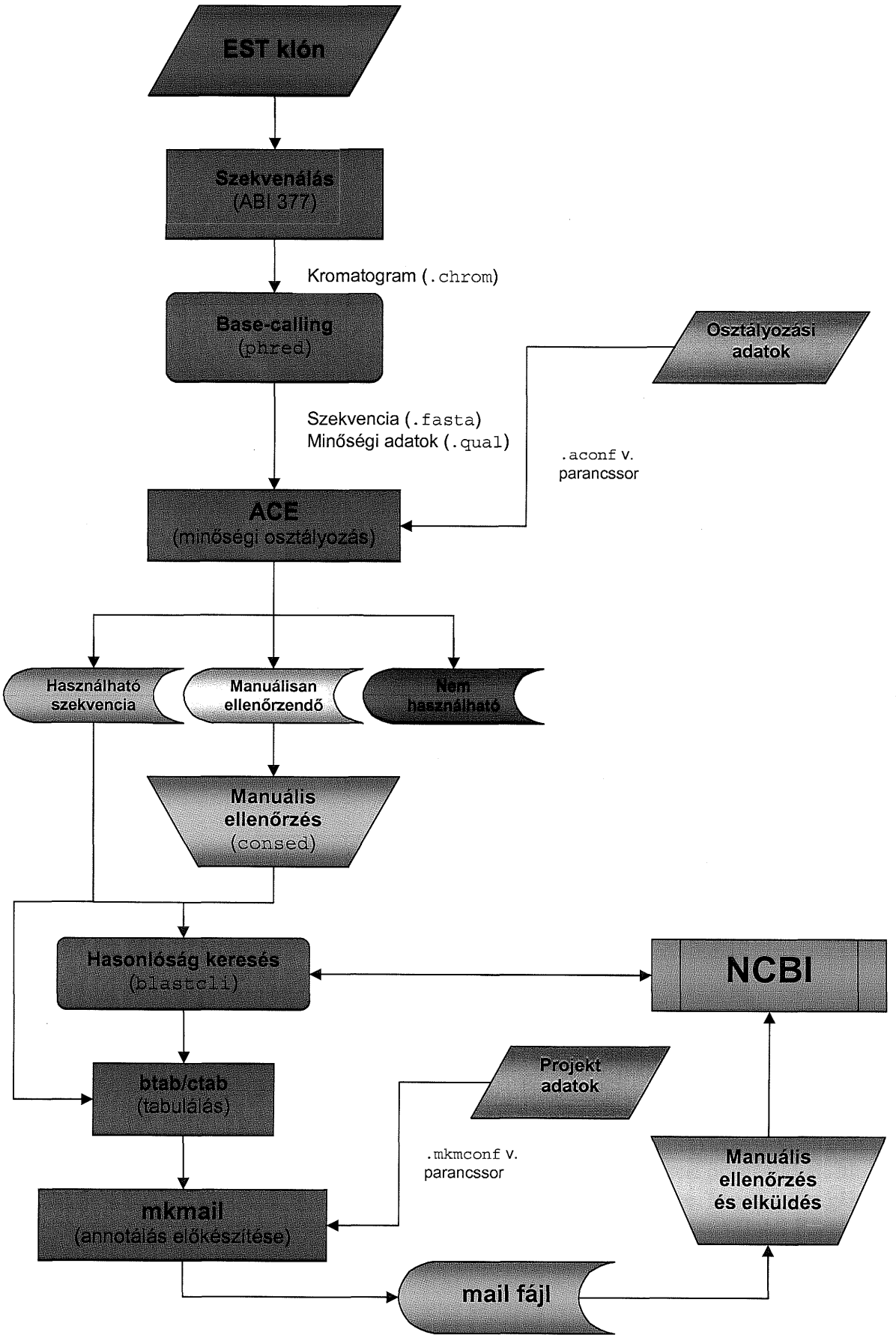
A hozzáférhető szoftverek segítségével, valamint saját szkriptjeinkkel és programjainkkal sikerült létrehozni egy automatikus rendszert. Ez a rendszer, emberi beavatkozás nélkül, a szekvenálás eredményeként kapott kromatogrammokat automatikusan, a szekvenátorra kötött Apple Power PC-ről Unix (Linux/Solaris) platformra juttatja, majd azt automatikusan szerkeszti, szűri és adatbázisokban hasonlóságot keres. Végül a szekvenciát és a hozzátartozó eredményeket, valamint az automatikusan fel nem dolgozható szakaszokat (pl: szekvenálási hiba, nem kielégítő minőség, rövidség) a felhasználónak elektronikus levélben elküldi (**11. Ábra**).




**11. Ábra.** Automatikus szekvencia feldolgozó rendszer sémás ábrája.

Szkriptünket, melyet *AUSA* (*AUtomated Sequence Annotator*) névre kereszteltünk, rendszeres, előre programozható időközönként indít el az operációs rendszer ún. `cron job` formájában. Az *AUSA* szkriptgyüjtemény, a kromatogrammokat egy kijelölt mappából, ahová

a felhasználó másolta az annotálni kívánt szekvenciák kromatogrammját, AppleTalk protokoll használatával a *netatalk* kapcsolaton keresztül Unix rendszerre másolja. Itt szkriptünk gondoskodott a kromatogram fájlok biztonsági másolásáról és az annotációjáról (**12. Ábra**).

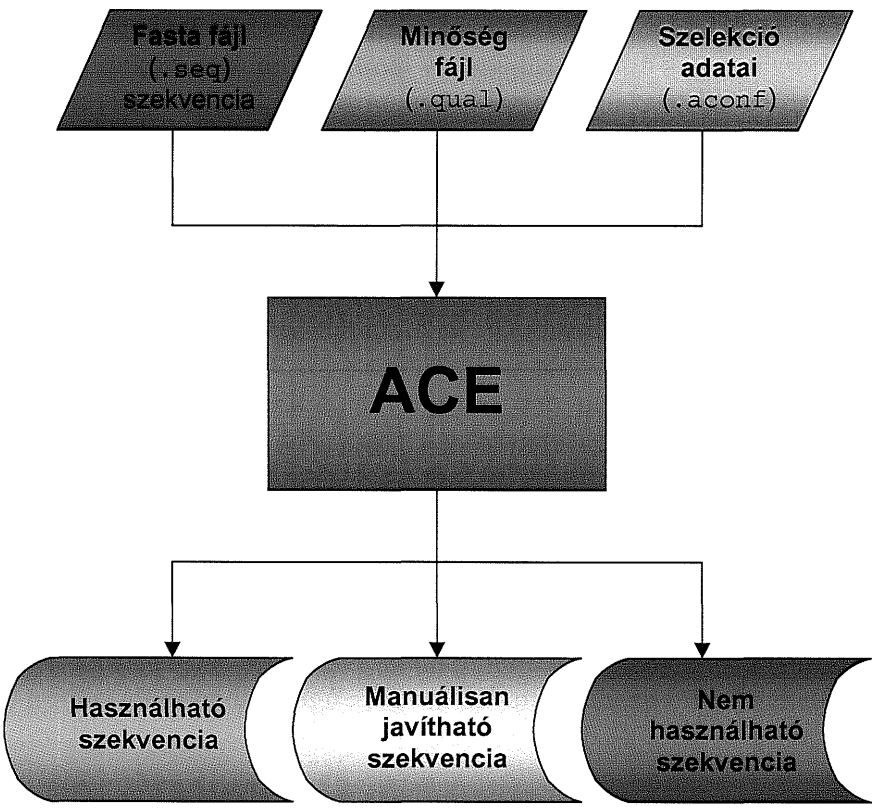



**12. Ábra.** AUSA (AUtomated Sequence Annotator) folyamatábrája.

Az *AUSA* először a *phred* base-caller futatásával meghatározta a kromatogrammokból (`.chr` kiterjesztés) a szekvenciát (`.seq`) és az adott szekvencia bázisainak minőségét (`.qual` ), vagyis a hiba valószínűségét. Ezt követően elindítja az *ACE* (*Automated CDNA Editor*) programot.

### 6.1.1 *Automated CDNA Editor (ACE)*

Az *ACE* tetszőleges minőségi paraméterek és primerek szerint képes egy adott szekvenált polinukleotidot szerkeszteni (**13. Ábra**). A program bemeneti adatai a nukleotidhoz tartozó szekvencia (`.seq` file), a bázisokhoz tartozó minőségi értékek (`.qual` fájl) és konfigurációs file (`.aconf` file).




**13. Ábra.** ACE (Automated CDNA Editor) folyamatábrája.

A program első lépésben egy helyi összerendezést (local alignmentet) végez az eredeti *Smith-Waterman* algoritmussal (Smith, 1981) az általunk megadott insert primerekkel. Itt megadható a találat, eltérés és hiányokhoz rendelt ún. gap-penalty értéke. Mivel a vizsgált szekvenciák rövidek, ezért a futási idő kisebb teljesítményű számítógépeken (Intel Pentium MMX 166MHz) sem jelent problémát.

Ezt követően az általunk beállított paraméter segítségével a *phed* programmal meghatározott minőségi értékeknek megfelelően (`.qual` fájl) eltávolítja az alacsony, nem

megfelelő minőségű részeket. Ennek az a szerepe, hogy az adatbázisokba beküldendő szekvenciák jó minőségűek legyenek.

Amennyiben a vizsgált szekvencia poli-A szakaszt tartalmaz, a program eltávolítja azt is.

*ACE* program kimenete egy az előbbieknek megfelelően „kurtított" szekvencia lesz, valamint egy pozitív egész szám (`integer`). Ez a szám adja meg, hogy sikerült-e minden lépésnek eleget tenni, vagy valamilyen hibával történt a program leállása. Ezt a kilépéskor visszaadott értéket használja a programot meghívó szkript a későbbiekben arra, hogy mi történjen az adott szekvenciával. A rövid, vagy nem megfelelő minőségű cDNS-eket külön könyvtárba mentjük, és a felhasználót e-mailen értesíti a szkript.

A megfelelő minőségű és hosszúságú szakaszok alacsony komplexitású szűrését ezután a *dust* és *seq* programok végzik. A *blastcli*, kliens meghívásával, a BLASTN és BLASTX programokkal távoli szerveren végzünk hasonlóság keresést.

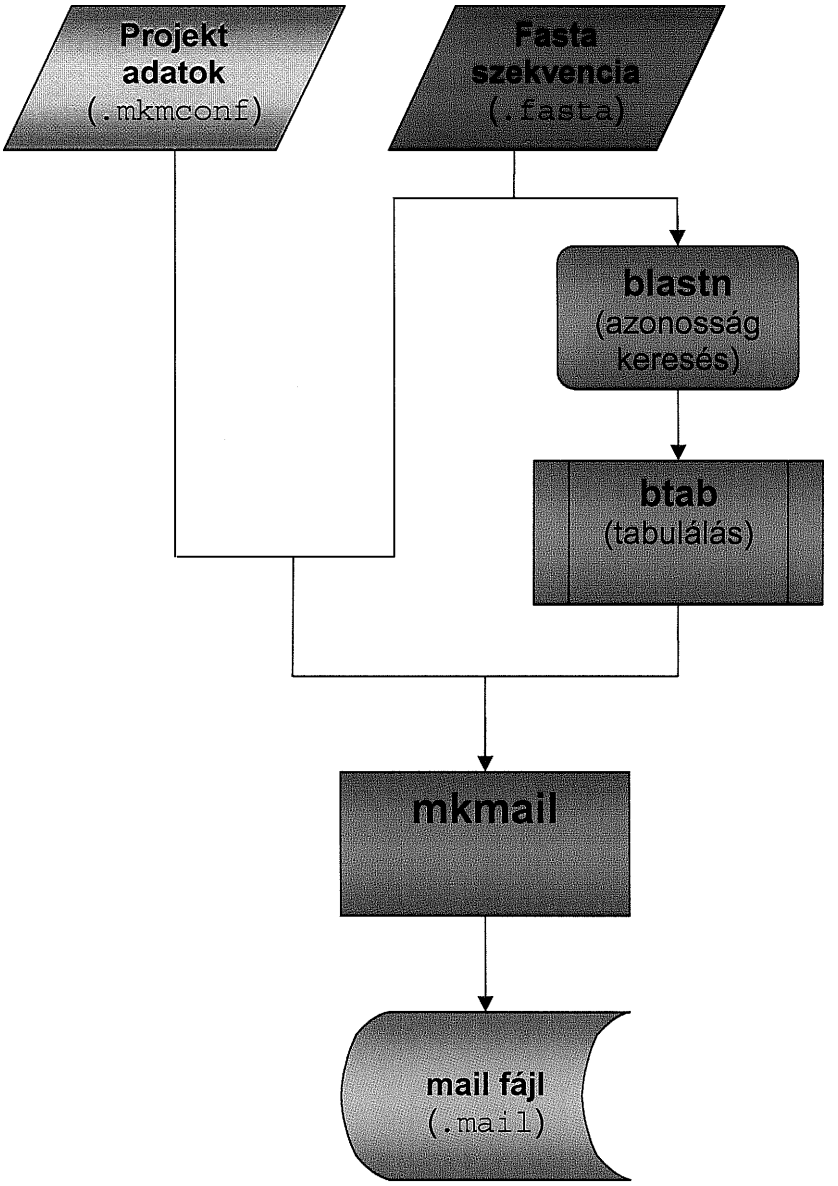
A keresési eredményeket a *btab* programmal szkriptünk számára olvasható programmá alakítjuk. Ezt az általunk írt *ctab* programmal vizsgáljuk. A *ctab* a parancssorban megadott paraméterek alapján visszaadja a kért számú és valószínűségű keresési eredményt (*BLAST* E és P érték).

Ezt követően kerül hívásra a *MAKEMAIL* program.

### 6.1.2 MAKEMAIL

A *MAKEMAIL* program (**14. Ábra**) kimenete egy elektronikus levélben elküldhető fájl, mely megfelel az NCBI ajánlásának a GENBANK-ba történő adat elhelyezésnek (http://www.ncbi.nlm.nih.gov/BankIt/help.html#gen_sub_info). A program bemeneti adatai egy konfigurációs fájl, vagy ennek hiányában a parancssori paraméterek, ahol a beküldéshez szükséges az adott projekt nem változó adatai megadhatóak. A szekvenciát és hozzá tartozó hasonlósági adatokat a program a szekvenciaadatokból (`.fasta`, fasta formátum) és *ctab/btab* formátumú fájlokból kapja.

A felhasználó a *MAKEMAIL* eredményét ellenőrizheti vagy beállítástól függően, kellő szigorú paraméterek esetén (*ACE* és *BLAST*) továbbíthatja automatikusan a GENBANK-ba. A nem megfelelő minőségű és hosszúságú szekvenciák manuálisan ellenőrizhetőek.




**14. Ábra.** MAKEMAIL program folyamatábrája.

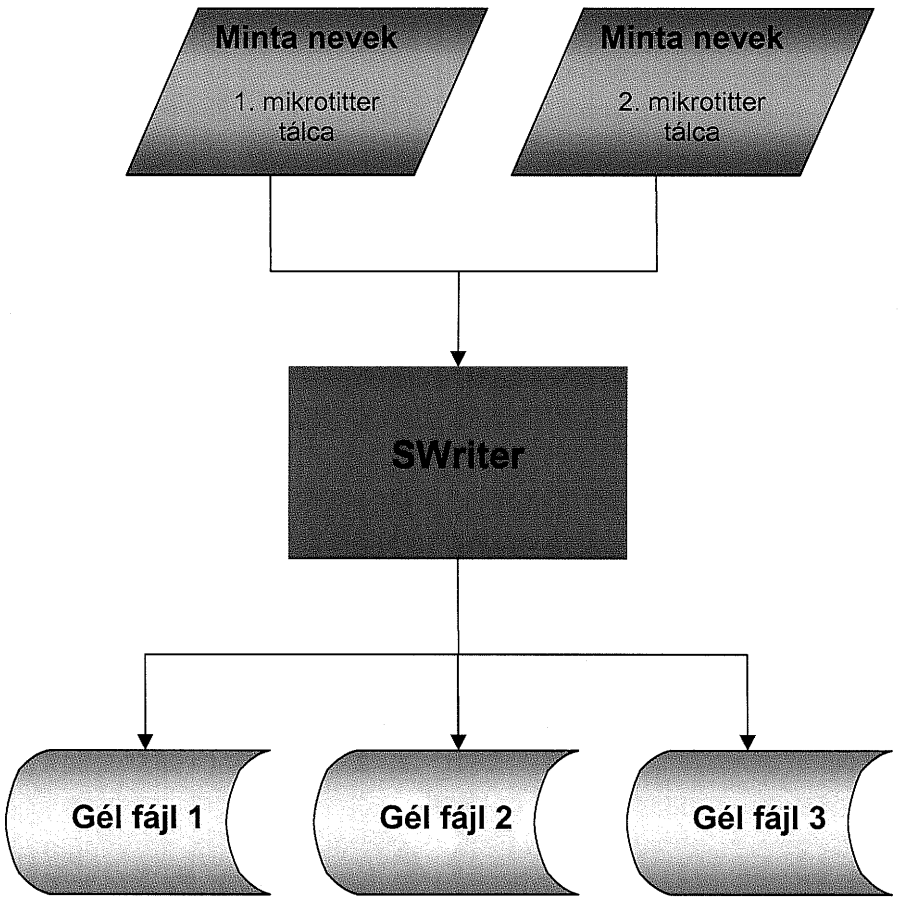
## 6.2 *SWriter (Sheet Writer)* – laboratóriumi munkát segítő program

Szekvenálás előtt, az előkészített, 96-lyukú mikrotitter tálcákon lévő reakciókat 8 csöves Hamilton pipettával jutattuk a 64-lyukú gélre, mely a minták összekeveredését eredményezheti. A Hamilton pipetta csövei, és gél lyukai között osztásbeli eltérés van, ezért a pipettával minden második lyukba juttathatunk mintát.

A munka eredménye a *SWriter* valamilyen Unix rendszeren (pl. Solaris, BSD, Linux) futtatható program lett. A program vagy a manuálisan beírt mintaneveket, vagy azokat egy előre elkészített fájlból beolvasva, vagy a laboratóriumban használt rendszernek megfelelően a 96-lyukú lemez nevének és abban lévő pozíciónak megfelelően generál 3 gél fájlt. Ezek a

fájlok használhatóak utána a szekvenálás futtatására, a szekvenátorhoz kapcsolt Power Macintosh megfelelő könyvtárba való másolása (és esetleges átnevezése) után (**15. Ábra**).

A program használatával kiküszöbölhetővé váltak és megszűntek az elnevezésből adódó hibák. A név és gélfájlok archíválásával valamint egységes nevezéktan használatával nyomon követhetővé vált a minták gélre helyezése és a szekvenálás.




**15. Ábra.** SWriter (Sheet Writer) program folyamatábrája.

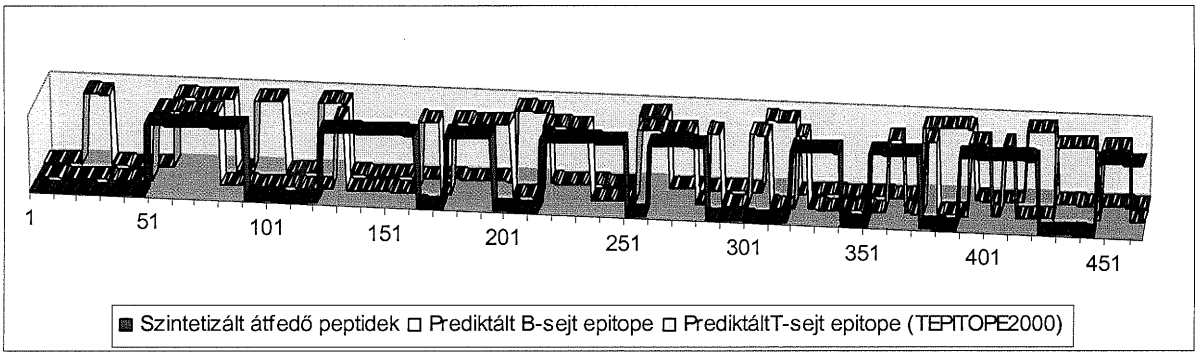
## 6.3 Immunglobulinok által felismert epitópok térképezése

### 6.3.1 *In silico* epitóp térképezés

Normál és patológiás körülmények között nyert antitestekkel végeztük el a teljes konzervatív mitokondriális belső-membrán fehérje, a CS enzim, epitóp térképezését. Összehasonlítottuk az *in silico* predikciós és *in vitro* immunszerológiai eredményeinket.

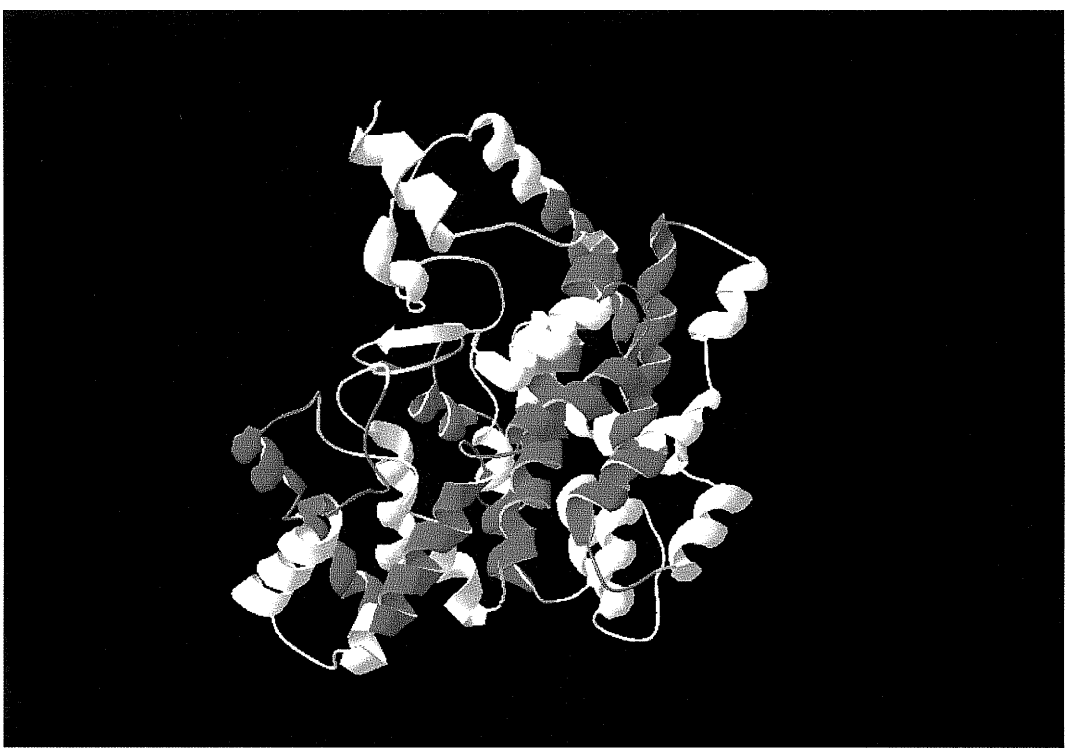
### 6.3.1.1 CS B-sejt epitóp predikció eredménye:

Feltételezve, hogy az epitópok a fehérjék hidrofil régióiban vagy azok közelében helyezkednek el, 15 nagy valószínűséggel $\beta$-csavart ($P_{\beta\text{-turn}} > 1$) és kevésbé hidrofil ($P_{\text{hydrophobicity}} < 0$) régiót választottunk ki. Ezek a régiók a CS molekula következő részét fedték le: 51-78, 85-91, 103-119, 127-132, 138-161, 176-195, 217-239, 244-250, 261-269, 281-285, 302-309, 320-331, 354-364, 390-398, 409-414, 448-463 (**16. Ábra**).




**16. Ábra**. Szintetizált átfedő peptidek és predektált B- és T-sejt epitópok

A 3D modell figyelembevételével, két szekvenciaszakaszt kizárhattunk, mert bár megfeleltek az előbbi kritériumoknak, a molekula belső felszínén helyezkedtek el. A 103-119 és 302-309 szegentumok a molekula belső részében az antitestek számára nehezen elérhetőn helyezkedtek el (**17. Ábra**).



**17. Ábra.** Szintetizált átfedő peptidek által lefedett régiók sárgával jelölve a humán CS felszínén.

A predikció és 3D szerkezet alapján 9 epitóp gyanús régiót különítettünk el: 51-90 (I), 124-163 (II), 176-195 (III), 216-250 (IV), 261-285 (V), 320-339 (VI), 354-373 (VII), 390-414 (VIII), és 447-466 (IX) (**18. Ábra**). Az eredmények immunszerológiai ellenőrzését tűhegy rendszerben vizsgáltuk, egymást 5 aminosavval átfedő peptidekkel (**I. Táblázat**).

**18. Ábra.** Humán CS a valószínű B-sejt epitópok sárgával jelölve.

**I. Táblázat.** Az *in silico* kiválasztott humán CS-t 5 aminosavval átfedő tűhegyen szintetizált dekapeptidek. Sárga háttér: prediktált epitópok. Zöld háttér: az eredeti szekvenciától a technikai kötöttségek miatt eltérően szintetizált aminosavak. *Dőlt betű:* jelöli a lehetséges HLA kötődésű helyeket.

I.
(51) *FRQQHGKTVVGQITVDMMYGGMRGMKGLVYETSVLDP*DEG (90)
FRQQHGKTVV KMRGMKGLVY
GKTVVGQITV EGLVYETSVL
GQITVDMMYG DTSVLDPDEG
GMMYGGMRGM

II.
(124) *VTGCIPT*EEQVSWLSKEWAKRAALPSHVVTMLDNFPTNLH (163)
VTGCIPTEEQ RAALPSHVVT
PTEEQVSWLS SHVVTMLDNF
VSWLSKEWAK MLDNFPTNLH
KEWAKRAALP

III.
(176) NSESNFAQAYARGISRTKYW (195)
NSESNFAQAY
FAQAYARGIS
ARGISRTKYW

IV.
(216) *IYRNLYWEGSGIGAIDSNLDW*SHNFTNMLGYTDHQ (250)
IYRNLYWEGS WSHNFTNMLG
YWEGSGIGAI TNMLGYTDHQ
GIGAIDSNLD
DSNLDWSHNF

V.
(261) *IHSDH*EGGNVSAHTSHLVGSALSDP (285)
IHSDHEGGNV
EGGNVSAHTS
SAHTSHLVGS
HLVGSALSDP

VI.
(320) GKDVSDEKLRDYIWNTLNSG (339)
GKDVSDEKLR
DEKLRDYIWN
DYIWNTLNSG

VII.
(354) DPRYTCQ*REFALKHLPNDPM* (373)
DPRYTCQREF
CQREFALKHL
ALKHLPNDPM

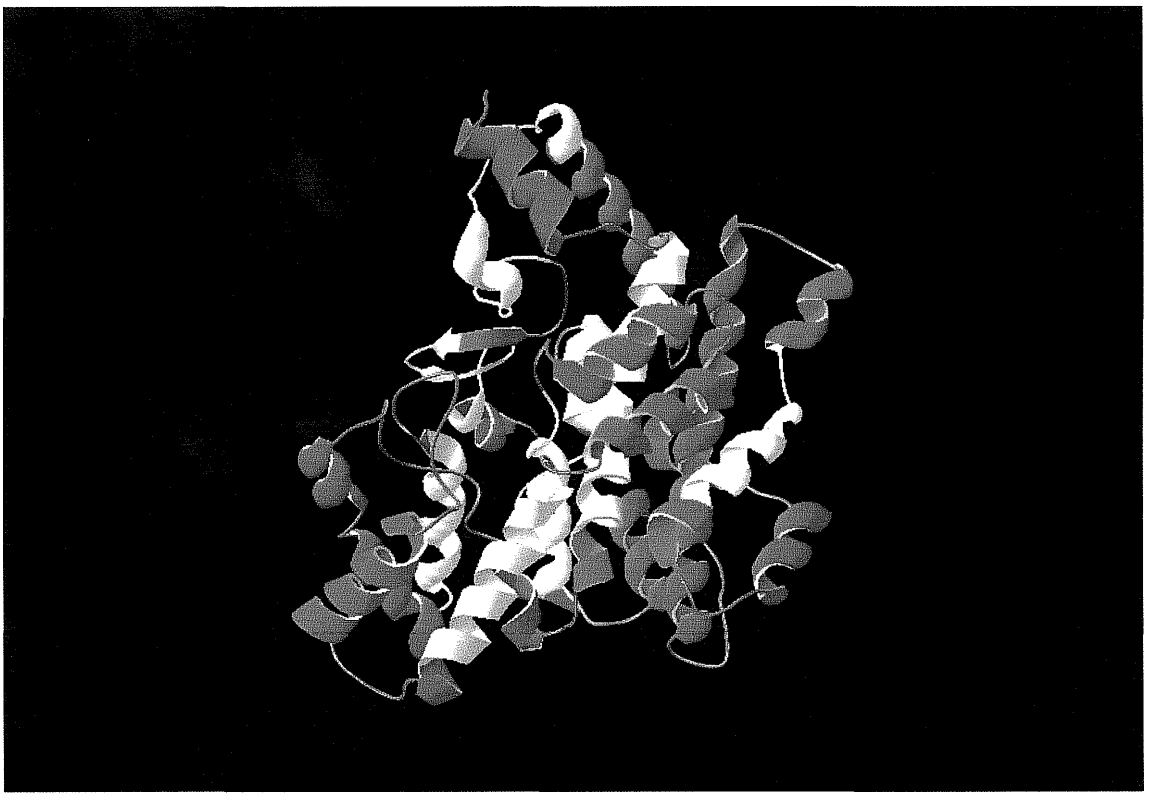
VIII.
(390) EQGKAKNPWPNVDAHSGVLLQYYGM (414)
EQGKAKNPWP
KNPWPNVDAH
NVDAHSGVLL
SGVLLQYYTV

IX.
(447) ERPKSMSTEGLMKFVDSKSG (466)
ERPKSMSTEG
MSTEGLMKFV
LMKFVDSKSG

### 6.3.1.2 CS T-sejt epitóp predikció eredménye:

A 18-29, 35-88, 92-115, 119-131, 139-165, 195-237, 254-266, 306-319, 361-393, és 406-446 aminosav szekvenciákat predektáltuk mint lehetséges T-sej epitópokat a virtuális mátrix módszerrel. Ezek a szekvenciák legalább az egyik virtuális HLA-hoz kötődést valószínűsítettek. A legalább 10 virtuális HLA hoz kötődő régiót nagy affinitású, erős epitóp

potenciállal rendelkező szekvenciának minősítettük. Kilenc erős T-sejt epitópot találtunk: (18-29, 57-83, 91-103, 119-131, 201-215, 254-266, 306-319, 373-393, 415-427). A 3D modellezés szerint két szakasz a CS molekula belsejében lévő α-helixen helyezkedik el (**19. Ábra**). A prediktált 9 epitópból 4 átfedést mutat a feltételezett B-sejt epitópokkal: 57-60, 57-79, 255-266, 306-319, és 415-427. A 306-319 szakasz a molekula belsejében helyezkedik el. Három T-sejt epitóp a prediktált B-sejt epitópok mellet, azok közvetlen közelében helyezkedett el (T|B: 91-103|103-119, 119-131|103-119, 415-427|409-414).

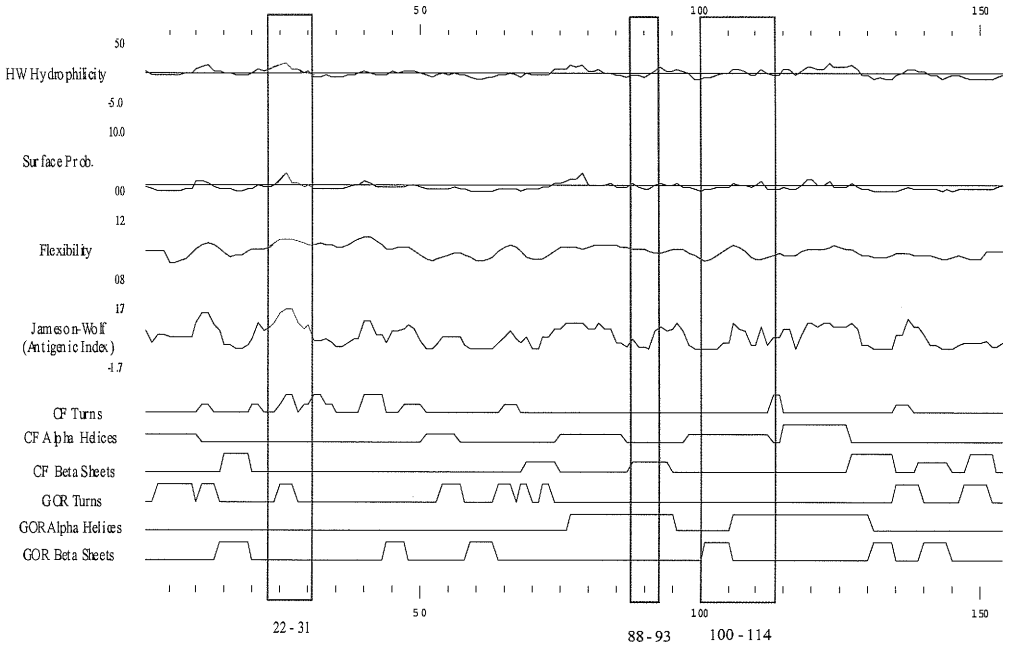


**19. Ábra.** Valószínűsíthető T-sejt epitópok a humán CS felszínén sárgával jelölve.

### 6.3.1.3 HBxAg B-sejt epitóp predikció eredménye:

A HBxAg 3D szerkezete még nem meghatározott. A fehérje 84 hidrofób aminosavat tartalmaz, ami a teljes molekula 54%-a. A molekula másodlagos szerkezetének *in silico* elemzése a *Cho-Fasman* és *Garnier-Osbourne-Robson* algoritmusokkal kanyart valószínűsített a molekula N-terminálisán és C-terminálisához közel eső részeken (Chou, 1974; Chou, 1978; Garnier, 1978). A 75-130 aminosavak közötti rész a predikció alapján α-hélix szerkezetet valószínűsít. β-lemez struktúra a molekula egészében valószínűsíthető. Glikolizációs helyek nem ismertek.

A *Jamson és Wolf* antigenicitási index alapján két szekvenciát (22-31 és 100-114) valószínűsítettünk epitópnak (Jamson, 1988). Az M13 filamentózus fág felszínen expresszáltatott random peptidfragmens könyvtár segítségével elvégeztük a monoklonális ellenanyag epitópspecificitásának meghatározását (Pál, 2003) és a kapott – több módszerrel kontrolált – eredmények nem támasztották alá az epitóp predikciót. Az elvégzett epitópmeghatározás a 88-93 aminósav szekvencia területén igazolta az ellenanyag felismerőhelyét. (**20. Ábra**)




**20. Ábra.** HBxAg B-sejt epitóp predikció eredménye a Wisconsin GCG programcsomaggal. Kékkel a prediktált de a monoklonális anti-HBXAg ellenes szérumunk által fel nem ismert epitópok (22-31; 100-114), pirossal a random peptid fág könyvtárral meghatározott epitóp (88-93).

## 6.3.2 Epitóp predikció *in vitro* ellenőrzése

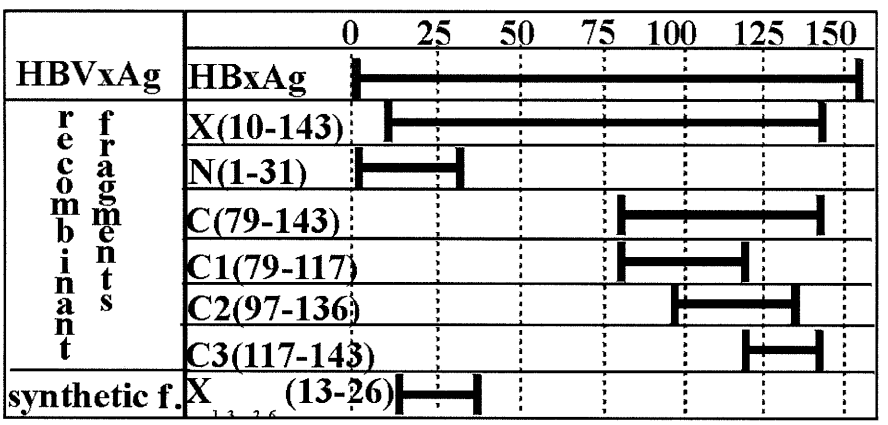
### 6.3.2.1 ELISA eredmények:

**a.) CS:** A legalább az adott csoportban mért szérumok átlagánál két szórással (2SD) nagyobb szérumokat tekintettük pozitívnak. A pozitív anti-CS IgG szérumok aránya 2-4 % minden csoportban. Az IgM szérumok 7-12 %-a volt pozitív, kivétel az autoimmun betegek csoportja, ahol 24 % volt a pozitívok aránya (**II. Táblázat**). Az ELISA pozitív szérumát használtuk további CS vizsgálatainkban.

**II. Táblázat.** Anti-CS pozitív szérumok.

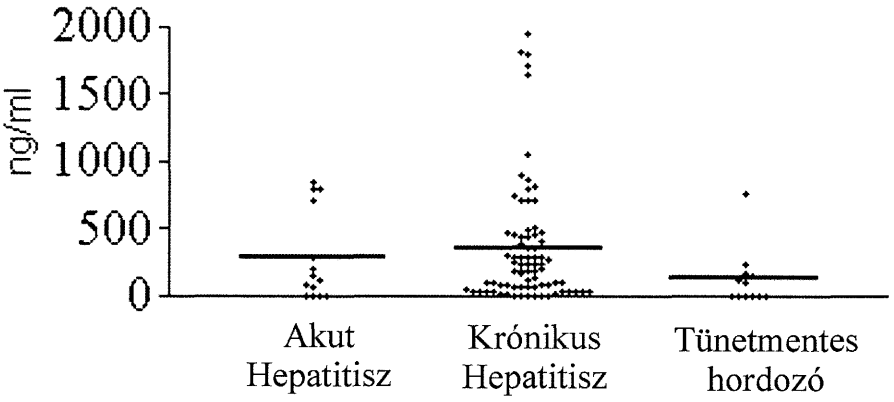
| **Csoport leírása** (Szérumok száma) | **IgG** | **IgM** |
|---|---|---|
| **Magyar véradók** (63) | 3% | 10% |
| **Brit véradók** (51) | 4% | 12% |
| **Finn véradók** (176) | 3% | 9% |
| **Egészséges újszülöttek** (44) | 2% | 7% |
| **Szisztémás autoimmun betegek** (326) | 3% | **24%** |
| **Szívtranszplantált betegek** (52) | **7%** | 12% |

**b.) HBxAg:** A HBxAg szérumszinteket szendvics-ELISA-val vizsgáltuk (Pál, megjelenés alatt). A vizsgálatban használt szintetikus fragmentumok a **21. Ábrán** láthatóak.



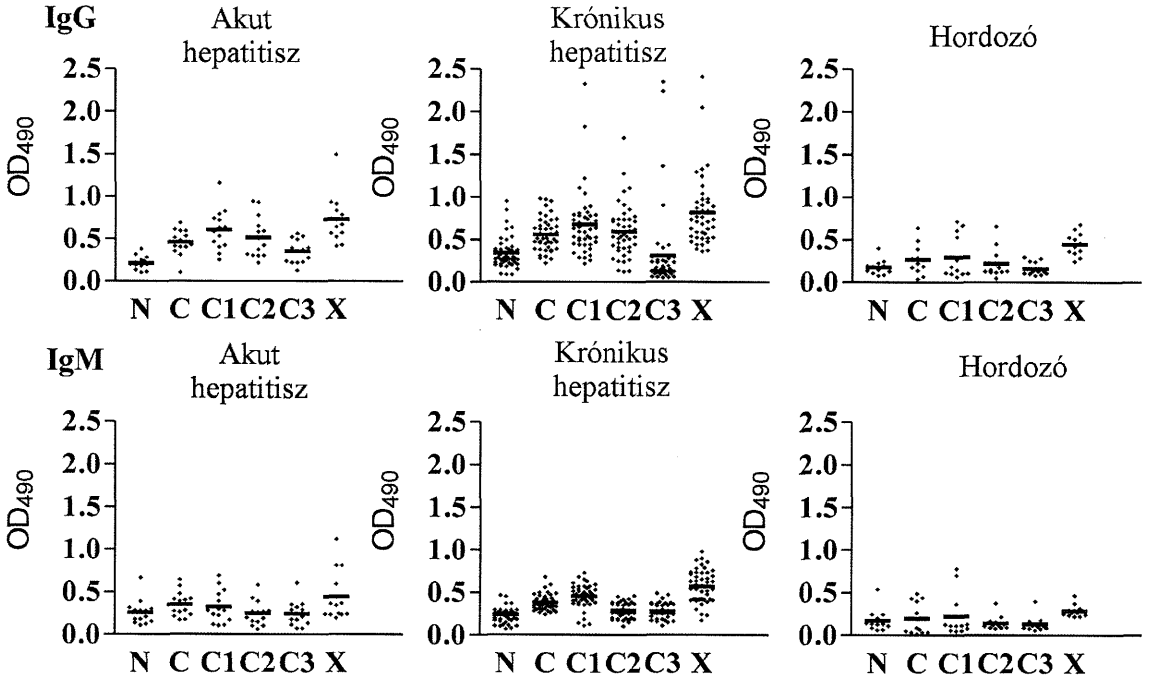

**21. Ábra.** A 154 aminosav hosszúságú HBXAg-t átfedő régiók. Sematikusan jelölve a vizsgált fragmentumok (X: 10-143, N: 1-31, C: 79-143, C1: 79-117, C2: 97-136, C3: 117-143) és a szintetikus ($X_{13-26}$: 13-26) fragmentum egymáshoz való viszonya.

Az abszolút koncentráció 18 – 1800 ng/ml között széles sávban mozgott. A HBxAg koncentráció csoportátlaga magasabb volt az akut és krónikus csoportban, mint a tünetmentes hordozókban. A legnagyobb szórást a krónikus hepatitiszes csoportban találtuk (**22. Ábra**).




**22. Ábra.** HBxAg szendvics-ELISA kvantitatív eredmények.

Az individuális különbségek homogének voltak csoportokon belül. Kivételt a krónikus hepatitiszben szenvedő betegek anti-HBX IgG izotípusában figyeltünk meg. A legerősebb HBX ellenes választ a leghosszabb rekombináns fragmentum (10-143) adta, IgM és IgG izotípusokkal. Az első, rekombináns C fragmentum (C1: 79-117) pozitív volt krónikus hepatitiszes betegek szérumában IgM és IgG izotípusokkal. Ugyanezen a szakaszon az IgG izotípus erős pozitivitást mutatott akut hepatitiszes és egészséges hordozó egyének esetében. A C1 fragmentumon jelentős pozitivitást nem mutatott krónikus hepatitiszben és hordozóknál. A többi vizsgált peptid kismértékű pozitivitást mutatott, minimális individuális variációval. Az egészséges kontrollok konzekvensen nem reagáltak az összes antigén esetében (**23. Ábra**).



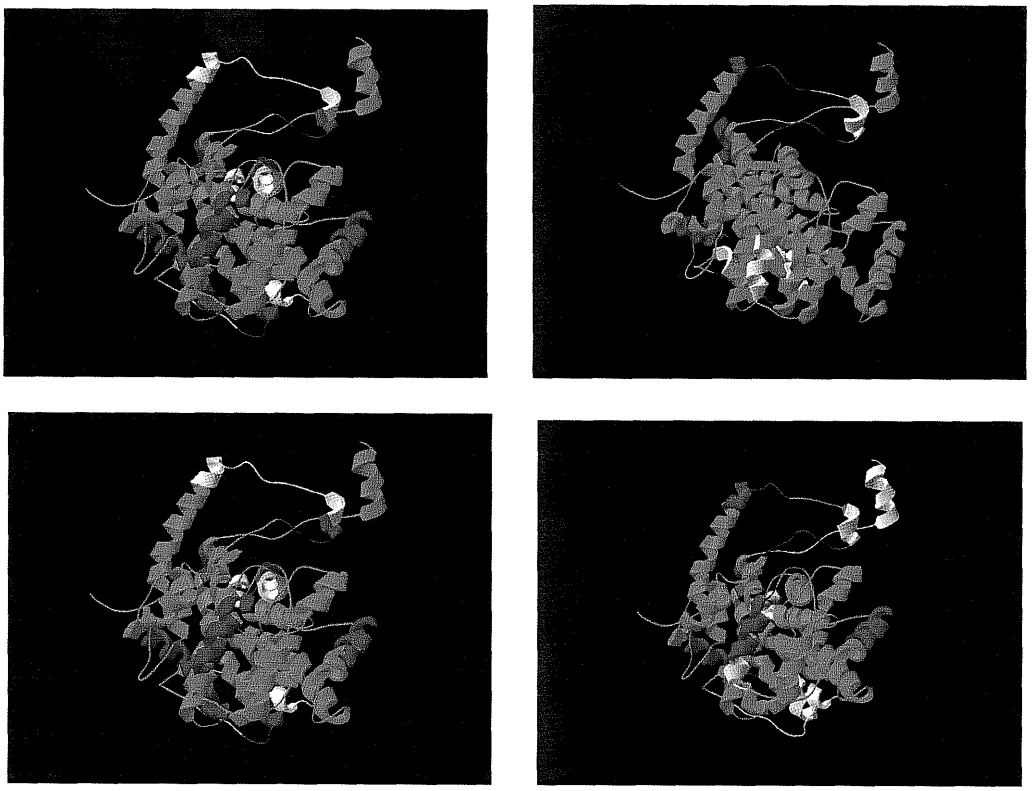

**23. Ábra.** A szintetikus és rekombináns fragmentumok ELISA eredményei (akut, krónikus és hordozók).

### 6.3.2.2 Tűhegy ELISA – immunszerológia epitóp térképezés eredményei:

Az immunszerológiai epitóp térképezés eredményeit táblázatban foglaltuk össze (**III. Táblázat**). Jelentős különbségeket találtuk a különböző szérumcsoportok epitóp felismerési mintázatában.

A vizsgált 40 dekapeptid a humán belső-membrán mitokondriális enzim a CS 49 %-át fedte le. A 40 dekapeptidből 23-at (58%) több mint egy szérumcsoport ismerte fel; 8 peptidet (19%) csak egy valamelyik szérumcsoport ismerte fel. Kilenc eset (23%) egyetlen szérumcsoport sem ismert fel.

Harmincnégy peptid *in silico* jó immunológiai felismeréssel rendelkezett és 27-et (79%) bizonyítottunk ezek közül immunszerológiailag pozitívnak. Tizenegy peptidet (32%) a legtöbb szérum felismert. Tizenhat peptid (47%) legalább 1 csoportban pozitív volt. Hét peptidet (21%) *in silico* prediktáltunk, de nem bizonyultak pozitívnak *in vitro*. Hat peptid nem prediktált régióban helyezkedett el. Ezek a szekvenciák egymáshoz közeli epitópok között helyezkedtek el, ezért történt szintézisük. Három peptidet egyetlen szérum sem ismert fel, hármat legalább 1 csoport felismert (**24. Ábra**; **III. Táblázat**).



**24. Ábra.** Humán CS 3D modellje. Bal oldal IgM, jobb oldal IgG által felismert epitópok. Felső sor egészséges, alsó sor autoimmun beteg széruma által felismert epitópok. (Pirossal jelölve a szérumok legalább 75% által felismert epitópok, sárga legalább a szérumok 50% által felismert epitópok.)

**III. Táblázat.** A különböző betegcsoportba tartozó páciensek szérumai által felismert dekapeptidek. Sárga kiemelés a prediktált epitópokat jelzi. (+ - 0% - 25%, ++ - 25% – 50% and +++ - 50% – 100%)

| Prediktált régió | Aminosav pozíció | Dekapeptid | Egészséges IgG | Egészséges IgM | Autoimmun IgG | Autoimmun IgM | Transzplantált IgG | Transzplantált IgM |
|---|---|---|---|---|---|---|---|---|
| I (51-91) | 51-60 | *FRQQHGKTVV* | + | + | + | + | + | |
| | 56-65 | *GKTVVGQITV* | + | + | | | | |
| | 61-70 | *DQITVDMMYG* | | | | + | | |
| | 66-75 | *GMMYGGMRGM* | + | + | + | | | ++ |
| | 71-80 | *KMRGMKGLVY* | + | | + | | | |
| | 76-85 | *EGLVYETSVL* | | | | | | |
| | 86-91 | *DTSVLDP*DEG | | | | | | |
| II (124-163) | 124-133 | *VTGCIPTEEQ* | | | | ++ | | |
| | 129-138 | *PTEEQVSWLS* | | | | +++ | | |
| | 134-143 | VSWLSKEWAK | + | ++ | ++ | +++ | ++ | +++ |
| | 139-148 | KEWAKRAALP | + | + | ++ | + | +++ | |
| | 144-153 | RAALPSHVVT | + | + | + | + | + | ++ |
| | 149-158 | SHVVTMLDNF | | + | + | | + | + |
| | 154-163 | MLDNFPTNLH | | | | | ++ | + |
| III (176-195) | 176-185 | NSESNFAQAY | | | | | | |
| | 181-190 | FAQAYARGIS | | + | + | ++ | | ++ |
| | 186-195 | ARGISRTKYW | + | +++ | +++ | + | ++ | |
| IV (216-250) | 216-225 | *IYRNLYWEGS* | + | +++ | + | +++ | +++ | ++ |
| | 221-230 | *YWEGSGIGAI* | + | + | + | +++ | +++ | + |
| | 226-235 | *GIGAIDSNLD* | | | | + | ++ | |
| | 231-240 | *DSNLDW*SHNF | | | | + | | |
| | 236-245 | WSHNFTNMLG | + | + | | +++ | + | +++ |
| | 241-250 | TNMLGYTDHQ | | + | | | | + |
| V (261-285) | 261-270 | *IHSDH*EGGNV | | | | | | |
| | 266-275 | EGGNVSAHTS | | | | | | |
| | 271-280 | SAHTSHLVGS | | + | | | + | ++ |
| | 276-285 | HLVGSALSDP | | + | | | | |
| VI (320-339) | 320-329 | GKDVSDEKLR | | | | | | |
| | 325-334 | DEKLRDYIWN | | | | + | | |
| | 330-339 | DYIWNTLNSG | | + | + | +++ | ++ | |
| VII (354-373) | 354-363 | DPRYTCQREF | | + | + | +++ | | + |
| | 359-368 | CQ*REFALKHL* | + | | + | ++ | + | |
| | 364-373 | *ALKHLPNDPM* | | | | + | | |
| VIII (390-414) | 390-399 | EQGKAKNPWP | | | | | | |
| | 395-404 | KNPWPNVDAH | | | | | | + |
| | 400-409 | NVDAHSGVLL | | | | | | |
| | 405-414 | SGVLLQYYTV | | | | + | | |
| IX (447-466) | 447-456 | ERPKSMSTEG | | | + | | | |
| | 452-461 | MSTEGLMKFV | | | | | | |
| | 457-466 | LMKFVDSKSG | + | | + | | | |

Az *in silico* prediktált és *in vitro* analizált CS epitópok azonosság keresésének eredményét a **IV. Táblázat**ban ábrázoltam. A tűhegy ELISA-val bizonyított epitópok analízise azt mutatja, hogy az antigéntulajdonsággal rendelkező peptid szakaszok a CS konzervált régióival mutatnak hasonlóságot.

**IV. Táblázat.** Azonosság keresés eredménye.

| CS szekvenciarészlet | Leírás |
|---|---|
| 51-78 | Konzervált CS |
| 51-60 | Konzervált CS, DNA-damage protein |
| 61-71 | Konzervált CS, Ser-proteáz inhibitor, Enzimek |
| 71-79 | Konzervált CS |
| 85-91 | Konzervált CS |
| 127-132 | Konzervált CS, Bakteriális fehérjék |
| 138-161 | Konzervált CS, DNA-damage függő protein, DNA-repair protein |
| 176-195 | Konzervált CS |
| 187-197 | Konzervált CS, tRNA-szintáz (bakteriális, Ricketsia, Neiseria stb.), P1 fehérje (bakteriális, Pseudomonas) |
| 217-239 | Konzervált CS, Bakteriális fehérje |
| 221-231 | Konzervált CS, Bakteriális fehérjék |
| 236-245 | Konzervált CS, NADH-dehidrogenáz |
| 241-250 | Konzervált CS |
| 261-270 | Konzervált CS |
| 280-285 | Konzervált CS, Konzervált Enzimek |
| 320-329 | Konzervált Enzimek |
| 330-339 | Konzervált CS, Konzervált Enzimek |
| 354-363 | Konzervált CS, Konzervált Enzimek |
| 359-368 | Konzervált CS |
| 364-373 | Konzervált Enzimek |
| 390-404 | Konzervált CS |
| 405-414 | Konzervált CS |
| 448-463 | Konzervált CS |

## 6.4 A biostatisztikai értékelés eredményei

A HBX szérumok koncentrációja és anti-HBX antitest titerek korreláció analízise tisztázta a rejtett összefüggéseket a különböző csoportok és paraméterek között. Az **V. Táblázat** ábrázolja a HBxAg antigén szérum koncentráció és az ellenanyag válasz közötti összefüggést a különböző HBxAg fragmentumok (X, N, C, C1, C2, C3) és a hepatitisz különböző stádiumainak (akut, krónikus, hordozó) viszonylatában.

Akut hepatitiszben erős és homogén korrelációt találtunk a szérum anti-HBxAg IgM antitest és HBxAg szérumkoncentráció között (**V.a Táblázat**). A IgG típusú antitestek magas korrelációt mutattak a C1 fragmentum felismerésében, míg összefüggést az N fragmentummal (N-terminális) nem találtunk. A szérum HBxAg szint és a leghosszabb fragmentum (X: 10-143) közötti szoros összefüggés a matematikai módszer erősségét bizonyítja.

Krónikus hepatitiszben, IgG dominanciát találtunk és a C fragmentum (C: 79-143) felismerésével való fokozott korrelációt. Összefüggés az N-terminális fragmentum felismerésében és HBxAg szérumkoncentráció között nem tudtunk kimutatni. A HBxAg szérumszintje közepes mértékben befolyásolta a C terminális (C: 79-143) és a leghosszabb fragmentum (X: 10-143) elleni IgG választ. Az IgM reaktivitás tekintetében domináns régió az N és C terminális fragmentumok voltak (N: 1-31; C: 79-143), bár statisztikailag szignifikáns összefüggés nem volt kimutatható a hasonló szérumcsoportok között (**V.b Táblázat**).

Tünetmentes HBV hordozók regresszió analízise az akut hepatitiszes betegekéhez hasonló összefüggéseket mutatott. Az immunválaszban az IgM dominancia tipikus volt. IgG válasz esetében erős korrelációt sikerült kimutatni a C1 és N terminális fragmentumokkal (N: 1-31; C1: 79-117). Az IgM dominancia kevésbé volt homogén, szemben az akut hepatitiszben szenvedőkkel. További IgG-vel összefüggő korrelációt találtunk a HBxAg koncentráció és az N terminális fragmentum (N: 1-31) specifikus IgG termelés között (**V.c Táblázat**).

| | | | IgG | | | | | | | IgM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HBxAg | N | C | C1 | C2 | C3 | X | $X^{14-26}$ | N | C | C1 | C2 | C3 | X | $X^{14-26}$ |
| | HBxAg | 1 | - | - | - | - | - | 0,845 | - | - | - | - | - | - | - | - |
| IgG | N | - | 1 | - | 0,976 | - | - | - | - | - | - | - | - | - | - | - |
| | C | - | - | 1 | - | 0,686 | - | - | 0,539 | - | - | - | - | - | - | - |
| | C1 | - | 0,976 | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| | C2 | - | - | 0,686 | - | 1 | - | - | - | - | - | - | - | - | - | - |
| | C3 | - | - | - | - | - | 1 | - | 0,634 | - | - | - | - | - | - | - |
| | X | 0,845 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| | $X^{14-26}$ | - | - | 0,539 | - | - | 0,634 | - | 1 | - | - | - | - | - | - | - |
| IgM | N | - | - | - | - | - | - | - | - | 1 | 0,909 | 0,981 | 0,996 | 0,990 | 0,868 | 0,907 |
| | C | - | - | - | - | - | - | - | - | 0,909 | 1 | 0,949 | 0,922 | 0,951 | 0,970 | 0,956 |
| | C1 | - | - | - | - | - | - | - | - | 0,981 | 0,949 | 1 | 0,986 | 0,995 | 0,915 | 0,945 |
| | C2 | - | - | - | - | - | - | - | - | 0,996 | 0,922 | 0,986 | 1 | 0,995 | 0,883 | 0,915 |
| | C3 | - | - | - | - | - | - | - | - | 0,990 | 0,951 | 0,995 | 0,995 | 1 | 0,917 | 0,940 |
| | X | - | - | - | - | - | - | - | - | 0,868 | 0,970 | 0,915 | 0,883 | 0,917 | 1 | 0,965 |
| | $X^{14-26}$ | - | - | - | - | - | - | - | - | 0,907 | 0,956 | 0,945 | 0,915 | 0,940 | 0,965 | 1 |

**V.a Táblázat.** HBXAg specifikus antitestek regresszió analízise akut hepatitiszes betegek esetében. (R > 0; P=0,05) A jelentős összefüggést mutató értékek (R > 0,8) szürkével kiemelve.

| | | | IgG | | | | | | | IgM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HBxAg | N | C | C1 | C2 | C3 | X | $X^{14-26}$ | N | C | C1 | C2 | C3 | X | $X^{14-26}$ |
| | HBxAg | 1 | - | 0,626 | 0,648 | 0,607 | 0,627 | 0,664 | - | - | - | - | - | - | - | - |
| IgG | N | - | 1 | 0,413 | 0,518 | - | - | 0,540 | - | - | - | - | - | - | - | - |
| | C | 0,626 | 0,413 | 1 | 0,843 | 0,799 | 0,817 | 0,753 | - | - | - | - | - | - | - | - |
| | C1 | 0,648 | 0,518 | 0,843 | 1 | 0,896 | 0,952 | 0,846 | - | - | - | - | - | - | - | - |
| | C2 | 0,607 | - | 0,799 | 0,896 | 1 | 0,930 | 0,781 | - | - | - | - | - | - | - | - |
| | C3 | 0,627 | - | 0,817 | 0,952 | 0,930 | 1 | 0,839 | - | - | - | - | - | - | - | - |
| | X | 0,664 | 0,540 | 0,753 | 0,846 | 0,781 | 0,839 | 1 | - | - | - | - | - | - | - | - |
| | $X^{14-26}$ | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - |
| IgM | N | - | - | - | - | - | - | - | - | 1 | 0,469 | 0,943 | 0,967 | 0,965 | 0,414 | - |
| | C | - | - | - | - | - | - | - | - | 0,469 | 1 | 0,420 | 0,466 | 0,467 | 0,526 | - |
| | C1 | - | - | - | - | - | - | - | - | 0,943 | 0,420 | 1 | 0,938 | 0,955 | - | - |
| | C2 | - | - | - | - | - | - | - | - | 0,967 | 0,466 | 0,938 | 1 | 0,986 | 0,473 | - |
| | C3 | - | - | - | - | - | - | - | - | 0,965 | 0,467 | 0,955 | 0,986 | 1 | 0,467 | - |
| | X | - | - | - | - | - | - | - | - | 0,414 | 0,526 | - | 0,473 | 0,467 | 1 | 0,477 |
| | $X^{14-26}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | 0,477 | 1 |

**V.b Táblázat.** HBXAg specifikus antitestek regresszió analízise krónikus hepatitiszes betegek esetében. (R > 0; P=0,05) A jelentős összefüggést mutató értékek (R > 0,8) szürkével kiemelve.

| | | | IgG | | | | | | | IgM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HBxAg | | N | C | C1 | C2 | C3 | X | $X^{14-26}$ | N | C | C1 | C2 | C3 | X | $X^{14-26}$ |
| | HBxAg | 1 | 0,651 | - | 0,897 | - | - | - | - | - | - | - | - | - | - | - |
| IgG | N | 0,651 | 1 | - | 0,732 | - | - | - | - | - | - | - | - | - | - | - |
| | C | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | C1 | 0,897 | 0,732 | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| | C2 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| | C3 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| | X | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| | $X^{14-26}$ | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - |
| IgM | N | - | - | - | - | - | - | - | - | 1 | - | 0,775 | - | 0,825 | 0,651 | - |
| | C | - | - | - | - | - | - | - | - | - | 1 | - | 0,689 | 0,641 | - | 0,655 |
| | C1 | - | - | - | - | - | - | - | - | 0,775 | - | 1 | 0,657 | 0,802 | - | - |
| | C2 | - | - | - | - | - | - | - | - | - | 0,689 | 0,657 | 1 | 0,933 | 0,781 | 0,766 |
| | C3 | - | - | - | - | - | - | - | - | 0,825 | 0,641 | 0,802 | 0,933 | 1 | 0,696 | 0,615 |
| | X | - | - | - | - | - | - | - | - | 0,651 | - | - | 0,781 | 0,696 | 1 | - |
| | $X^{14-26}$ | - | - | - | - | - | - | - | - | - | 0,655 | - | 0,766 | 0,615 | - | 1 |

**V.c Táblázat.** HBXAg specifikus antitestek regresszió analízise tünetmentes HBV hordozók esetében. ($R > 0$; $P=0,05$) A jelentős összefüggést mutató értékek ($R > 0,8$) szürkével kiemelve.

# 7. Megbeszélés

A molekuláris biológiai és immunológiai laboratóriumi munka szerves részét képezik az *in silico* vizsgálatok (Boguski, 1994a; Baxevanis, 1997). A statisztikai módszerek alkalmazása már a korai időszakban minőségi változást hozott az élő természettudományok eredményeinek kiértékelésében (Attwood, 1999). A számítógépes programok megjelenése tette lehetővé a nagy adathalmazok gyors feldolgozását. További fejlődést hozott a korszerű programozási nyelvek kialakítása és a nagyteljesítményű hardverek – személyi számítógépek - piaci árának gyors csökkenése az 1990-es évek elején. Mivel a felhasználói kör gyorsan bővült és a biológiai kutatásban közvetlenül résztvevők is meg tudták oldani azokat a programozási feladatokat, melyeket korábban csak speciálisan képzett, alkalmazott matematikai és informatikai tudással rendelkező szakemberek voltak képesek. Így, az elmúlt tizenöt év során egy új tudományág, a bioinformatika fejlődött ki. (Attwood, 1999; Alves, 2000a; Alves, 2000b; Womble, 2000; Lazardis, 2002; Greef, 2004)

A fejlődés két irányban folytatódott: egyrészt a nagy adathalmazok kezelése és bonyolult belső összefüggéseinek analizálása, másrészt a meglevő adatokból új, még nem vizsgált, de valószínűsíthető összefüggések feltárása (Lazardis, 2002; Greef, 2004). Az adathalmazok kezelése nemzetközi összefogást is jelentett és kialakultak a világhálón bárhonnan elérhető biológiai adatbázisok, melyek a genetikai és proteomikai kutatásokban napjainkra már nélkülözhetetlenekké váltak (Appel, 1994; Bleasby, 1994; Henikoff, 1996; Etzold, 1996; Bairoch, 1997; Sonnhammer 1998; Brusic, 2000).

Az élő szervezeteket felépítő molekulák 3D szerkezetének vizsgálata már fél évszázados múltra tekint vissza. Alapvető megfigyelések történtek a biomolekulák struktúráinak modellezésével (pl. a DNS vagy a különböző kötőszöveti rostok szerkezete) (Watson, 1953; Appel, 1994; Attwood, 1999; Peitsch, 2002). A bioinformatikai predikció, mint kutatási módszer első alkalmazása már az 1970-es években megkezdődött (Attwood, 1999). Ez a terület is a hardver- és szoftver-fejlesztéseknek köszönhetően az elmúlt évtizedben általánosan használttá vált (Wolf, 1988; Attwood, 1999; Schwede, 2000; Womble, 2000).

Mindezzel az extenzív fejlődési folyamattal párhuzamosan megjelentek a kritikai hangok is és szükségessé vált mind az adatbázis struktúrák, mind a predikciós technikák kritikai elemzése, az élő rendszereken mért adatokkal történő folyamatos összevetése (Boguski, 1994a; Foote, 1998; Peri, 2001; Baxavienis, 1997).

Munkám mind az adatfeldolgozásra, mind az adatbankok segítségével történő predikcióra kiterjedt. Mindkét témakör a biológiai kutatás szolgálata mellett felvetette a bioinformatikai módszerekkel kapott eredmények gyakorlati használhatóságának kérdését.

Az első kutatási témakörben bemutatott, a szekvenálást segítő szoftver-fejlesztés a gélre történő mintafelvitelt és az adatok nyomon követését könnyítette meg és minimalizálta a mintatévesztésből adódó pontatlanságokat, nagyfokban megnövelve a mérési biztonságot. Fejlesztésünk megelőzte az azóta a piacon megjelent hasonló programokat (Nyárády, 1997; Nyárády, 1998). Az ***SWriter program***, melyet 1997-ben fejlesztettem ki, az Uppsalai Egyetemen 1997-től 2002-ig terjedő időszakban volt használatban. A professzionális szoftverfejlesztő cégek termékei hasonló problémákat oldanak meg, különböző algoritmusok alapján, de az általam kifejlesztett program a laboratórium speciális problémáit (az EST és minták elnevezései) figyelembe véve tudta az adatkezelést megoldani. A szoftver GPL

(General Public Licence) engedély birtokában készült, Linux operációs rendszerben. A célalkalmazás – a széleskörű felhasználás lehetővé tétele – nem igényelte az iparjogvédelmet.

A második kutatási témakörben végzett munkám az ***EST szekvenálás*** eredményeinek automatikus ***feldolgozását, annotálását*** és nemzetközi adatbázisokba történő automatikus továbbítását tette lehetővé (Nyárády, 1998; Porcell, 2000). Nagy mintaszámokon végzett szekvenálások esetében ez az automatizált feldolgozás és adattovábbítás meggyorsítja ugyan az eredmények kiértékelését és továbbítását, de bizonyos számú adatvesztéssel is együtt jár. Ez manuális munkával ugyan korrigálható, de az időközben végbement technikai fejlődés, a szekvenálás gyorsabbá és olcsóbbá válása mindenképpen igazolta eredeti elképzelésünket, jelentős munkaidő megtakarítást és költséghatékonyság növekedést eredményezett. Ugyan az operációs rendszerek fejlődése eredeti formájában már nem teszi lehetővé az 1998-ban kifejlesztett szoftverem mai használatát, de adaptálása folyamatban van. Ez a szoftverfejlesztés is GPL alapon történt, Linux operációs rendszerben. Az időközben végbement hardver és szoftverfejlesztések teszik szükségessé az új adaptációt.

A harmadik témakörben több ***immunológiai predikciós*** technika összehasonlítását és kritikai elemzését végeztük el. Az *in silico* epitóp predikció a bioinformatika gyorsan fejlődő ága volt az elmúlt évtizedben (Pongor, 1985; Boguski, 1994a). Mind az elméleti kutatásban, mind a gyakorlati biotechnológiában új perspektívák nyíltak meg az immunológia területén a bioinformatikai módszerek kiterjedt használatával (de Groth, 1999; Guex 1999; Boguski, 2004a; Imani, 2005), ugyanakkor a számítógépes predikció korlátai is hamar felismerésre kerültek (Foote, 1998; van Regenmortel, 1998). Szisztematikus összehasonlító vizsgálatokat azonban csak elvétve végeztek az egyes predikciós technikák tesztelésére. Ezért kezdtünk célzott vizsgálatokat egy jól jellemzett modellben az előzetes *in silico* predikció és az *in vitro* mért immunszerológiai eredmények összehasonlításával.

A biológiai mintákon elvégzett *in vitro* méréseink az esetek jelentős részében egyeztek az *in silico* bioinformatikai eredményekkel, de igen jelentős számban kaptunk attól lényegesen eltérő adatokat is. Az általunk választott citrát szintáz modell alkalmas volt a technikai hibák kizárására azzal, hogy a predikció eredményei alapján kifejlesztett szintetikus peptidkönyvtáron mért adatok, valamint kétféle (M13 filamentózus, ill. lambda) random fágkönyvtárak segítségével végzett mérések eredményeit tudtuk összehasonlítani. A szintetikus peptid könyvtárral 40 szintetikus dekapeptid epitópot vizsgáltunk, melyek előzetes *in silico* predikció alapján kerültek kiválasztásra. Az immunszerológiai mérésekkel 32-50% között találtunk *in vitro* felismerést az egyes csoportokban (egészséges, autoimmun beteg és szívtranszplantálak csoportjai). Az összes szérum a prediktált epitópok 79 %-át ismerte fel, azaz 21%-ban egyetlen szérum sem mutatott pozitivitást. Az *in silico* predikció és az *in vitro* mért eredmények közti eltérések ugyanolyan értékes kutatási eredménynek bizonyultak, mint a bizonyított egyezés az *in silico* és *in vitro* adatok közt és összhangban vannak a korábbi irodalmi adatokkal (Hopp, 1986; Pellequer, 1991). Ugyan az elmúlt években sokat tökéletesedtek a predikciós algoritmusok, az általunk is leírt pontatlanságok felvetették egy „poszt-predikciós" irányzat szükségességét, melyben speciális biobankok mintáin végzett kontroll vizsgálatok felhasználásával a meglevő bioinformatikai technikák speciális irányokba továbbfejleszthetők. A predikciós technikák jelenleg ugyanis egy „általános" immunreaktivitást jósolnak meg, a mérések pedig konkrét egyének, aktuális immunreaktivitását tükrözik. A nagyfokú alkalmazkodóképesség, tehát a változékonyság éppen az immunrendszer egyik alapvető sajátja, ezért elméletileg is evidens, hogy a jelenleg használt adatbázisok finomításra szorulnak, szakosodott adatbankok kialakítására van szükség. Megállapításunk ugyan kézenfekvőnek tűnik, de a témában közölt dolgozatunk az egyik első, valós adatokon történt összehasonlítást bemutató munka volt (Nyárády, 2005) és

összhangban áll korábbi – részben saját - megfigyelésekkel (Pál, 2003; Németh, 2004; Petrohai, 2005; Pál, 2005).

A negyedik témakörben valójában már széles körben és régóta alkalmazott, egyszerű ***biostatisztikai technikákat*** használtuk fel és a korszerű adatfeldolgozás segítségével tettük alkalmassá ***nagyszámú immunszerológiai minta több szempontú kiértékelésére***. A méréstechnikák fejlődése lehetővé teszi napjainkban, hogy nagyszámú mintát párhuzamos, több szempontból vizsgáljunk. Ezeknek a multiparaméteres vizsgálatoknak a kapott eredmények kiértékelése szab határt. Az általunk alkalmazott mátrixstatisztikai eljárás (nagyszámú mérési mintán végzett regresszió analízis) segítségével sikerült jól jellemezhető prognosztikai csoportokat kialakítanunk és bizonyítanunk, hogy a Hepatitisz B vírusfertőzésen átesett és tünetmentes hordozóvá váltak csoportjában kik azok, akik fokozottan veszélyeztetettek az elsődleges májrák kialakulása szempontjából. A technikai szempontból egy adaptációt jelentő munka tehát új összefüggések feltárására vált alkalmassá és az összeállított biostatisztikai panel további, hasonló klinikai és epidemiológiai feldolgozásokban is alkalmazható (Pál, 2005; Pál (elfogadva, megjelenés alatt)).

Mind a négy témakörben munkám a bioinformatika aktuálisan legfontosabb irányaihoz kapcsolódott és a tudományág gyors fejlődését bizonyítja, hogy a tézisek benyújtása előtt nyolc évvel kezdődött munka egyes elemeit ma már túlhaladta a fejlődés. Ugyanakkor mind a négy tématerületen - a nemzetközi publikációinktól függetlenül is - gyakorlati felhasználás bizonyította a kutatási fázis eredményességét.

Az egyes munkák részleteit, a kapott eredményeinknek a szakirodalommal történő összevetését a mellékelt eredeti közleményeink tartalmazzák.

# 8. Köszönetnyilvánítás

Szeretném megköszönni fáradozásukat mindazoknak, akik segítettek és támogattak tudományos munkám megszületésében, összeállításában és végül a cél elérésében.

Ez a munka nem valósulhatott volna meg számtalan barát és kolléga nélkül, akik tanácsaikkal, munkájukkal közvetlenül vagy közvetve segítették téziseim elkészítését.

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# 9. Irodalomjegyzék


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# 10. Saját publikációk

## 10.1 Az értekezés témaköréhez közvetlenül kapcsolódó publikációk:

### 10.1.1 Folyóiratban megjelent közlemények:

1. Czömpöly T, Olasz K, Simon D, **Nyárády Z**, Pálinkás L, Berki T, Németh P: *A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network?* Molecular Immunology (elfogadva, megjelenés alatt, MIMM-D-05-00212)

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4. Czömpöly T, Olasz K, Simon D, Pálinkás L, **Nyárády Z**, Berki T, Czirják L, Németh P: *Az autantitestek epitóptérképezése szintetikus peptid és fágkönyvtárakkal.* Magyar Immunológiai 2005 4: 14.

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### 10.2.1 Folyóiratban megjelent közlemények:

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### 10.2.2 Könyv fejezet:

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1. **Nyárády Z**, Németh Á, Bán Á, Ember I, Olasz L: *Reliveing symptoms of xerostomia with oralpilocarpine (Salagen) during irradiation of the head and neck.* Int J Oral Maxillofac Surg 2005. 34: 149.
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5. Németh Á, Varjas T, Beró A, Olasz L, **Nyárády Z**, Ember Á, Csontos Zs, Arany I, Pázsit E, Czakó Gy, Ember I, Dombi Zs: *Early effects of transplatin on oncogene activation in vivo.* Hungarian Epidemiology 2005 1: S66
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15. **Nyárády Z**, Olasz L, Németh Á: *Reconstruction of massive trough-and-trough facial defects using platysma transpositional flap.* J Craniomaxillofac Surg 2002. 30: Suppl.1. 33.
16. Olasz L, **Nyárády Z**, Németh Á, Rónai A, Gelencsér G: *Regional failure related to alkylating agents in neoadjuvant chemotherapy of oropharyngeal planocellular cancer.* J. Craniomaxillofac Surg 2002. 30: Suppl.1.103.
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# 11. Mellékletek

Az értekezés alapját képező közlemények

# Gene Survey of the Pathogenic Protozoan *Trypanosoma cruzi*

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We have performed a survey of the active genes in the important human pathogen *Trypanosoma cruzi* by analyzing 5013 expressed sequence tags (ESTs) generated from a normalized epimastigote cDNA library. Clustering of all sequences resulted in 771 clusters, comprising 54% of the ESTs. In total, the ESTs corresponded to 3054 transcripts that might represent one-fourth of the total gene repertoire in *T. cruzi*. About 33% of the *T. cruzi* transcripts showed similarity to sequences in the public databases, and a large number of hitherto undiscovered genes predicted to be involved in transcription, cell cycle control, cell division, signal transduction, secretion, and metabolism were identified. More than 140 full-length gene sequences were derived from the ESTs. Comparisons with all open reading frames in yeast and in *Caenorhabditis elegans* showed that only 12% of the *T. cruzi* transcripts were shared among diverse eukaryotic organisms. Comparison with other kinetoplastid sequences identified 237 orthologous genes that are shared between these evolutionarily divergent organisms. The generated data are a useful resource for further studies of the biology of the parasite and for development of new means to combat Chagas' disease.

[The sequence data described in this paper have been submitted to the dbEST database under nos. TENU0001–TENU5214 and the following: AA736292-AA736301, AA738502-AA738535, AA756982-AA756992, AA835598-AA835613, AA866501-AA866550, AA87464-AA874780, AA875669-AA875730, AA875809-AA875824, AA879318-AA897341, AA879376-AA879401, AA882494-AA882518, AA883036-AA883051, AI005678-AI005729, AI007342-AI007441, AI021797-AI021884, AI026370-AI026615, AI037797-AI037846, AI043247-AI043343, AI043427-AI043502, AI046026-AI046290, AI050095-AI050219, AI053146-AI053397, AI057644-AI057957, AI065169-AI065425, AI066117-AI066391, AI069556-AI069908, AI073286-AI073332, AI075466-AI075620, AI077051-AI077281, AI078888-AI079000, AI080790-AI080916, AI083097-AI083245, AI110290-AI110405, AI110412-AI110512, AW324789-AW325325, AW329885-AW330435, and AW621062-AW621094. The sequences are also available at www.genpat.uu.se/tryp/tryp.html.]

Some major health problems in the world are caused by eukaryotic parasites, and genomic studies of these pathogens are of utmost importance for finding new means of treatment. Identification of genes involved in unique metabolic pathways, in pathogenicity, and in mechanisms by which the parasites evade the immune defense is of particular interest. Genome projects for several medically important parasites have therefore been initiated and are in progress.

*Trypanosoma cruzi*, the causative agent of Chagas' disease affecting ~18 million people in Latin America (WHO; www.who.int/ctd/chagas/burdens.htm), is a flagellated protozoan and an evolutionarily ancient organism that belongs to the order Kinetoplastida. Neither vaccines nor safe and efficient drug treatment are presently available against this debilitating disease. The genome project for *T. cruzi* involves both genomic sequencing (Andersson et al. 1998) and identification of functional genes through generation of expressed sequence tags (ESTs) (Brandão et al. 1997; Verdun et al. 1998), a cost-effective technique in gene discovery (Venter 1993; Okubo and Matsubara 1997). The ongoing parasite genome projects of the three kinetoplastids, *Leishmania major* (Blackwell 1997), *Trypanosoma brucei* (Melville 1997), and *T. cruzi* (Zingales et al. 1997), are critical in identifying orthologous genes involved in mechanisms among kinetoplastids. Gene orders are often conserved among Kinetoplastida (Bringaud et al. 1998), and orthologous genes may therefore also be used in physical and transcriptional mapping of any of the parasites, which would accelerate all three genome projects.

In this report we present the analysis of 5013 ESTs generated from a *T. cruzi* epimastigote library. This analysis provides a survey of genes transcribed during the insect stage of the parasite's life cycle. Clustering of all sequences resulted in >3000 different sequences,

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among which a large number of novel genes were identified.

## RESULTS AND DISCUSSION

### Generation and Assembly of ESTs

To accelerate gene discovery in *T. cruzi*, we generated 6059 cDNA sequences from a normalized epimastigote library constructed from the reference clone CL Brener (Urmenyi et al. 1999). Single-pass sequencing was performed from either the 5′- or the 3′-end of the cDNA clones with an average insert size of 650 bp. After assembly and subsequent removal of low-quality sequences, the resulting 5013 ESTs formed a total of 771 clusters (Table 1). Most of the clusters (74%) contained only 2 or 3 sequences and the largest cluster comprised 66 sequences. The consensus sequence generated for each cluster by the assembly program was later used together with all singleton sequences in the subsequent analyses. In total, 3054 transcripts were identified in this study. It is likely that most of these transcripts represent unique genes. However, some fraction of them could be nonoverlapping sequences derived from the same transcript.

The redundancy of the cDNA library, estimated from the fraction of sequences that assembled into clusters was thus ~ 54%, a relatively high value considering the normalization step. The most abundant cDNAs were from the mucin genes, which belong to a large gene family of highly divergent copies in the *T. cruzi* genome (Di Noia et al. 1998). A plausible explanation for a redundancy of genes such as these in the library might be that cross-hybridizing diverged sequences seem to escape the normalization procedure (Bonaldo et al. 1996). Moreover, several clusters showed sequence polymorphisms among different ESTs, suggesting that the sequences were derived from different gene copies and that the actual redundancy is lower. The divergence in the protein-coding regions of the mucin genes caused these cDNAs to resolve into several clusters. Several other multicopy gene families, previously identified in *T. cruzi*, could be found among the largest clusters (Table 2). Only 0.6% of the cDNA clones contained ribosomal RNAs and a few clones encoded known *T. cruzi* genomic repeats (Requena et al. 1996), indicating a low contamination of the cDNA library.

**Table 1.** Summary of the Assembly of All Expressed Sequence Tags (ESTs)[a]

| | |
|---|---|
| Clusters | 771 (2730) |
| Singletons | 2283 |
| Total no. of unique ESTs | 3054 |

[a]The total number of ESTs included in the assembly is 5013, out of which 2620 are 5′-ESTs and 2393 are 3′-ESTs.

**Table 2.** Summary of Most Abundant *Trypanosoma cruzi* ESTs

| No. of ESTs | Identity[a] |
|---|---|
| 107 (2.1%) | Mucin-like proteins |
| 71 (1.4%) | Histone H2A |
| 51 (1.0%) | Histone H3 |
| 31 (0.6%) | Mitochondrial HSP 70 |
| 20 (0.4%) | Unknown |
| 17 (0.3%) | RNA-binding protein |
| 17 (0.3%) | HSP 70 |
| 15 (0.3%) | Ribosomal protein S11A |
| 15 (0.3%) | Ribosomal protein L28 |
| 15 (0.3%) | Histone H1 |

[a]The ten most frequent ESTs are shown. A more detailed result is available at http://www.genpat.tryp/tryp.html.

Other examples of separated clusters, besides the mucin genes, were the two clusters of ESTs encoding succinylCoA ligase, which differ by an in-frame deletion of 40 amino acids (http://www.genpat.uu.se/tryp/tryp.html).

A large number of full-length genes could also be obtained, because both 5′- and 3′-ESTs were generated. In total, 234 ESTs (4.7%) contained the spliced leader sequence or a part thereof. Complete protein-encoding regions of >140 short genes were obtained. Several clusters contained cDNAs, which differed in length of the 3′-UTRs. The 3′-UTRs frequently contained short nucleotide repeats of different lengths and composition as well as other repeat elements (Vazquez et al. 1994). Lists of ESTs showing alternative polyadenylation sites and ESTs containing the spliced leader or repeats are available (see http://www.genpat.uu.se/tryp/tryp.html).

### Biological Survey of Identified Genes in *T. cruzi*

When searching for sequence similarities in public databases, ~20% of the *T. cruzi* transcripts could be assigned a putative identity (Table 3). These identities were classified into different groups according to function (a list is available at http://www.genpat.se/tryp/tryp.html). A representation of the functional groups is shown in Figure 1.

A large fraction of genes with putative identity en-

**Table 3.** Summary of BLAST Searches

| | | No. of unique ESTs |
|---|---|---|
| ID[a] | Putative identity | 610 (20%) |
| | Unknown function | 398 (13%) |
| No ID | | 2046 (67%) |

[a]A putative identity (ID) was assigned to *T. cruzi* ESTs when similarity values exceeded 50% ($P \leq 10^{-5}$ for BLASTN and $P \leq 10^{-4}$ for BLASTX), as described in Methods.

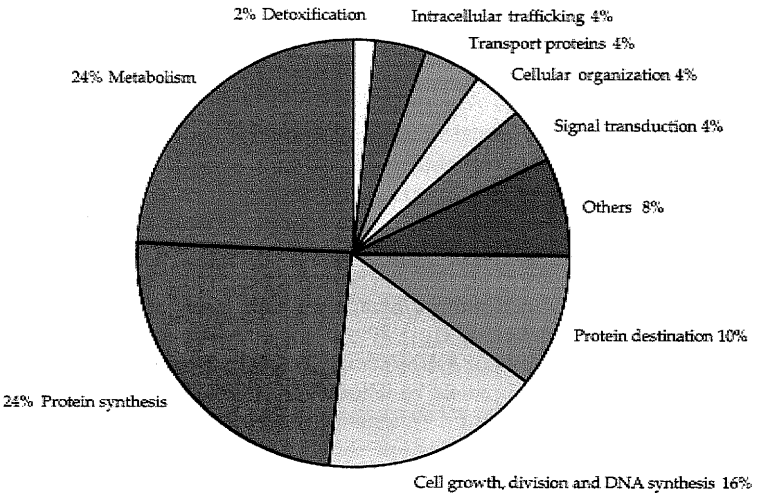


**Figure 1** Functional classification of *Trypanosoma cruzi* ESTs. The transcripts with putative identities to proteins other than highly abundant gene families in *T. cruzi* were divided into functional categories. The largest groups were genes involved in protein synthesis and metabolism. The group designated protein destination included genes for protein modification, folding, and proteolysis and the group named others mainly contained genes with protein kinase, DNA-binding, and Zn-finger motifs (www.genpat.uu.se/tryp/tryp.html).

coded proteins involved in translation (24%), including 61 ribosomal proteins, initiation and elongation factors, and proteins involved in tRNA synthesis.

An interesting group for gene regulatory processes in trypanosomes is the proteins involved in transcription and RNA processing, which amounted to 6% of transcripts and included several RNA polymerase subunits, RNA-binding proteins, and splicing factors.

About 4% of the functionally classified transcripts showed similarities to proteins involved in signal transduction, including multiple rab proteins and MAP kinases. A total of 17 novel genes with similarities to cyclophilins, kinesin-like proteins, and cell division checkpoint proteins were identified among proteins involved in cell cycle regulation and division.

A small group encoded enzymes involved in detoxification. Enzymes unique for trypanosomes participating in the trypanothione biosynthesis, such as glutathionyl spermidine synthetase and trypanothione synthetase, were found. Several thioredoxin-like proteins and different peroxidases were also identified.

Eleven percent of the transcripts were involved in energy metabolism. Multiple ATP synthase subunits, cytochrome C components, and cytochrome 450 were present.

New genes involved in the transport machinery, the secretory pathway, and degradation of proteins were identified and should facilitate studies of these less-defined processes in *T. cruzi*. Five ABC transporters previously not described were identified among the *T. cruzi* transcripts. Among genes involved in cellular organization, several subunits of dynein were identified.

## Comparison of *T. cruzi* ESTs to the Complete Sets of Genes from a Unicellular and a Multicellular Organism

The *T. cruzi* transcripts were compared with the protein sequences of all predicted ORFs from the yeast genome project (Goffeau et al. 1997) and the genomic sequence of *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium 1998), amounting to 6217 and 19,099 ORFs, respectively. In total, 14.7% of the *T. cruzi* genes showed similarity to yeast and *C. elegans* ORFs (Table 4). Of these, 12% were shared by all three organisms and the matches were mainly to proteins with housekeeping functions.

The comparisons of this fraction of ESTs to *C. elegans* and yeast ORFs are not conclusive, but might give an indication as to which *T.cruzi* genes may be shared with other eukaryotes. It is not possible to draw any conclusions from lack of homologs, which would also include genes acquired because of the adaptation to life as an intracellular parasite. The low percentage of similarity between yeast and the protozoan *T. cruzi* might reflect the evolutionary divergence of the trypanosomatids similar to what has been suggested for another protozoon, *Toxoplasma gondii* (Ajioka et al. 1998). The low percentage could also be due to a lower coding poten-

**Table 4.** Comparison of *Trypanosoma cruzi* ESTs to *Saccharomyces cerevisiae, Caenorhabditis elegans,* and Kinetoplastid Organisms

| Organism | No. of unique ESTs |
|---|---|
| *S. cerevisiae* | 448 (14.7%) |
| *C. elegans* | 450 (14.7%) |
| yeast + worm[a] | 365 (12%) |
| yeast-restricted | 83 (2.7%) |
| worm-restricted | 85 (2.8%) |
| Kinetoplastid-restricted[b] | 592 (19.4%) |
| *T. cruzi* | 355 (11.6%) |
| other than *T. cruzi* | 237 (7.8%) |

[a]Number of unique EST sequences with matches to both yeast and worm. Cutoff value used for the comparison = $P \leq 10^{-4}$
[b]Number of unique EST sequences with matches to kinetoplastid-restricted genes. Cutoff value used for the comparison = $P \leq 10^{-4}$ Detailed results are available at http://www.genpat.uu.se/tryp/tryp.html.

tial of 3′-ESTs compared with 5′-ESTs, estimated from singletons with putative identities to be ~ 40% and 60%, respectively.

### Identification of Genes Present in Kinetoplastids

Trypanosomes share a number of unique biological features with other flagellated protozoa of the order Kinetoplastida, such as *trans*-splicing, RNA editing, and the unusual organization of the mitochondrial DNA in the kinetoplast.

To identify genes that are shared among kinetoplastids, we compared all of the *T. cruzi* transcripts with a local database containing public DNA sequences from these organisms. After removal of all sequences with homologs in other organisms, 592 transcripts showed similarity to kinetoplastid sequences (Table 4). The search revealed 237 orthologous genes present in one or more kinetoplastids other than *T. cruzi*, >50% of which were of unknown identity. Among these genes were those encoding surface molecules such as the Gp63-homolog and ESAG from *T. brucei*. The rest of the hits to kinetoplastid sequences matched only *T. cruzi* sequences, a majority being genes or repeats of known identity and also previously known to be specific for *T. cruzi*.

*T. cruzi* can be estimated to have ~12,000 genes from the gene density of about 1 gene per 3.5–4 kb, as revealed by genomic sequencing (Andersson et al. 1998) and the haploid genome size of ~45 Mb in the *T. cruzi* reference clone CL Brener (J. Swindle, unpubl.). Because a considerable part of the *T. cruzi* genome comprises several large gene families as well as other repeat sequences (Requena et al. 1996), this number of genes should be an overestimate. The present study represents the hitherto largest sampling of generated *T. cruzi* ESTs and might correspond to almost one-fourth of the total gene repertoire in *T. cruzi*.

This study also reports the first clustering analysis of a large set of *T. cruzi* ESTs, allowing identification of, for example, alternative polyadenylation sites. Because a larger amount of ESTs than reported previously have been analyzed, a large number of new genes have been identified, giving a better representation of the *T. cruzi* gene content. The larger sampling revealed several new important genes involved in detoxification and a larger set of genes involved in metabolism than those presented in previous works by Brandão et al. (1997) and Verdun et al. (1998) by using the same cDNA library constructed by Urmenyi et al. (1999).

Taken together, the EST analyses performed in *T. cruzi* provide a valuable resource for future studies of parasite biology and for identifying functional genes in a complex genome containing a high number of large gene families.

## METHODS

### Template Preparation and DNA Sequencing

The cDNA library was constructed by using oligo(dT)-primed *T. cruzi* CL Brener epimastigote poly(A)$^+$ RNA (Urmenyi et al. 1999) normalized to reduce the representation of abundant mRNA species (Bonaldo et al. 1996). The cDNA library was transformed into DH5-alpha strain of *Escherichia coli,* and > 23,000 individual colonies were randomly picked and ordered into 384-well microtiter plates. High-quality double-stranded plasmid DNAs were prepared by using the Wizard PLUS SV miniprep DNA purification system (Promega) or the PERFECTprep-96 plasmid DNA purification system (5 Prime-3 Prime Inc.).

Automated fluorescent cycle sequencing reactions were performed by using the ABI Prism-21M13 fluorescent dye-labeled primer kit (Perkin Elmer Cetus) and DYEnamic direct cycle sequencing (Amersham Life Science) with a T7 dye-labeled primer. The samples were analyzed on ABI Prism 377XL DNA sequencers.

### Processing and Annotation of Sequences

The sequences were quality checked by using the software PHRED (Ewing and Green 1998). Vector sequence (GenBank accession no. U13869), including the modified polylinker, the spliced leader sequence (De Lange et al. 1984), and poly(A)-tail sequences, were removed before submission to the dbEST database. Sequences of a quality > 96% accuracy and longer than 100 bp were used for further analysis. The average length of the ESTs was 327 bp. In the similarity searches, low-entropy sequences were masked by using the program DUST (R. Tatusov and D. Lipman, unpubl.). The sequences were searched against the nonredundant Genbank (www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html) database at the National Center for Biotechnology Information by using BLASTN (Altschul et al. 1997) and gapped BLASTX (Altschul et al. 1997) (parameters

$E$ = 1e-5 $E2$ = 1e-5 $B$ = 10 $H$ = 0 $V$ = 10)

and against Swissprot (Bairoch and Apweiler 1997) by using gapped BLASTX (parameters

$E$ = 1e-4 $E2$ = 1e-5 $B$ = 10 $H$ = 0 $V$ = 10).

Results were parsed by using the program btab (Dubnick 1992). The matches in which similarity values exceeded 50% with the BLASTX program and $P \leq 10^{-4}$ were listed as putative identities.

### Assembly of EST Sequences

The *T. cruzi* EST sequences were assembled into overlapping sequences by using the fragment assembly program PHRAP (Green 1996). Initially, raw chromatogram data from 6059 cDNA sequences were used in the assembly. The vector sequences were masked by using cross_match (Green 1996), and the parameters used in the assembly for minmatch and minscore were set to 50 and 39, respectively, whereas the rest of the parameters were default parameters. The parameters for the assembly were tested to optimize the results and the contigs were manually checked for incorrectly assembled sequences.

### Similarity Searches

Similarity searches were performed locally on an IBM SP par-

allel computer at the Parallel Computing Center, Stockholm, Sweden. Local databases included Genbank sequences in Flat File Release 110.0, SwissProt (Bairoch and Apweiler 1997) sequences in release 36, *C. elegans* (http://elegans.swmed.edu), and *Saccharomyces cerevisiae* (http://genome-www.stanford.edu/Saccharomyces) protein sequences. MT-BLAST (M. Tammi, unpubl.) wrapper by using gapped WU-BLASTX (W. Gish 1997, http://blast.wustl.edu) was run on 64 nodes, using default WU-BLAST parameters and matrix. The results were filtered afterward by using a value of $P = 10^{-4}$.

The comparison to the kinetoplastid database was performed locally by using gapped WU-BLAST (W. Gish 1997, http://blast.wustl.edu). The database consisted of 9204 entries from the EMBL database and contained both cDNA and genomic sequences, including the complete sequence of chromosome 1 and parts of chromosome 3 from *L. major Leishmania major*. All *T. cruzi* ESTs already deposited into dbEST were excluded from this comparison.

Clones containing the spliced leader or part thereof were identified among the *T. cruzi* ESTs by BLASTN with search sequences containing the 5′-cloning site, including the tag sequence (GAATTCCAGCTCC) fused to the spliced leader sequence sequentially deleted from the 5′-end down to the four last base pairs.

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# Determination of the fine epitope specificity of an anti-hepatitis B virus X protein monoclonal antibody using microanalytical and molecular biological methods

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**Abstract**

The recombinant form of the 17 kDa, highly hydrophobic and disulfide-bonded hepatitis B virus X protein (HBX) was used for developing a set of monoclonal antibodies (Mab). Our present goal was to determine the fine epitope specificity of our anti-HBX Mab. Based on computer analysis two sequences (amino acids 22–31 and 100–114) were predicted for possessing high immunogenity while the anti-HBX Mab did not recognized them. Limited proteolysis and mass spectroscopic analysis suggested another possible sequence (amino acids 14–26), which also proved to be negative using an immunoserological test. Subsequently, we performed a screen of a phage displayed random peptide library, by which we could localize the epitope to amino acids 88–93. This finding was confirmed using three overlapping fusion peptides spanning amino acids 77–142. Their testing in ELISA assigned the epitope to amino acids 77–95, which supports the result obtained by screening the phage displayed library. Our results suggest the necessity of a complex application of current molecular biological and immunological techniques in fine structure mapping. This approach will be useful to study the prognostic relevance of different antigenic sites on HBX during the development of chronic hepatitis and primary hepatocellular carcinoma.
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*Keywords:* Monoclonal antibody; Hepatitis B virus X protein; Epitope mapping; Mass spectrometry; Phage display

## 1. Introduction

Hepatitis B virus (HBV) is an important etiologic agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. (Szmuness, 1978; Tiollais et al., 1985; Beasley and Hwang, 1994; Feitelson and Duan, 1997). The smallest open reading frame of HBV called X, encodes a 17 kDa protein of 154 amino acid with highly hydrophobic and disulfide-bonded characters (Caselmann, 1996; Gupta et al., 1995). The X protein (HBX) is a multifunctional regulatory protein which modulates transcription, cell responses to genotoxic stress, protein degradation, and signaling pathways, but the precise function of HBX is not well understood (Andrisani and Barnabas, 1999; Murakami, 1999, 2001).

We developed a set of monoclonal antibodies (Mab) against the HBX for research and diagnostic use (Pal et al., 2001). The determination of fine epitope structure recognized by one of our Mab became necessary due to the results of our retrospective study on human liver biopsy specimens. The various epitopes of HBX antigen are attributed with different functional importance (Stemler et al., 1990; Murakami, 1999). Detection of their expression pattern seems to be a suitable prognostic factor in laboratory diagnosis (Feitelson et al., 1990; Vitvitski-Trepo et al., 1990; Kumar et al., 1998). Moreover, for the quantitative measurement of the HBX from human serum, we developed a sandwich type ELISA using a pair of anti-HBX Mabs with no overlapping epitopes. Our goal was to map the fine epitope-specificities of the antibodies that were used in the retrospective immunohistochemical study and in other immunoserological assays.

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## 2. Materials and methods

### 2.1. Materials

Recombinant HBX (amino acids 1–154) was produced as a glutathione *S*-transferase (GST) fusion protein as described (Marczinovits et al., 1997).

Monoclonal antibodies against the recombinant HBX were developed earlier (Pal et al., 2001), the Mab used in this study was the product of clone 3F6-G10. The *E. coli* strain XL1-Blue MRF and the helper phage M13KO7 was purchased from Stratagene (USA). The *E. coli* strain DH5-alfa was from Invitorgen (USA). Nitrocellulose membrane was purchased from Pharmacia Biotech (Sweden). Alkaline phosphatase (AP) conjugated anti-mouse IgG antibody and horseradish-peroxidase (HRPO) conjugated anti-mouse IgG antibody were purchased from Dako (Denmark). Luria-Bertani (LB) bacterial media and all the other chemicals were purchased from Sigma (Germany).

### 2.2. Antigenity prediction and synthetic peptides

The Chou–Fasman and the Garnier–Osguthorpe–Robson secondary structure predictions (Chou and Fasman, 1974; Garnier et al., 1978; Gibrat et al., 1987; Prevelige and Fasman, 1990) of HBX were performed with the Protein Structure program of the Wisconsin-GCG package (Genetics Computer Group Inc., University Research Park, Madison, WI, USA) peptide sequences chosen on the basis of Jameson–Wolf antigenity index were synthesized using fmoc chemistry and purified with HPLC at the Department of Chemistry, Faculty of Medicine, University of Szeged.

### 2.3. Limited proteolysis and mass spectrometry

Recombinant X protein was reduced by dithiothreitol, alkylated with iodoacetamide and digested by trypsin in solution (Kele et al., 1998). The peptide fragments were separated on a C18 reversed-phase HPLC column (Nucleosil 5C18, 300 Å) using 40 min linear gradient from 20 to 60% solvent (0.1% trifluoroacetic acid in 80% aqueous acetonitrile) at the flow rate of 1 ml/min solvent. Column effluent was monitored at 215 nm and was collected in 1 ml fractions. Binding characteristic of the separated peptides to monoclonal antibody against HBX were tested by ELISA. The amino acid sequence of the fractions showing the highest positive reaction in ELISA was determined by nano-ES/MS/MS using a TSQ 7000 triple quad mass spectrometer (Wilm and Mann, 1996).

### 2.4. Screening of the phage displayed random peptide library

The filamentous phage library displaying nine amino acid random peptides as a fusion to the N-terminal of the M13 major coat protein VIII was constructed previously (Felici et al., 1991), and was a generous gift from Dr. Alessandra Luzzago (Instituto di Ricerche di Biologia Molecolare, Italy). The affinity selection of phages with our anti-HBX Mab was performed using the biopanning technique (Parmley and Smith, 1988). In brief, a plastic petri dish was coated with the Mab overnight (40 and 4 μg/ml in PBS during the first and the second round of panning, respectively), after washing with PBS/0.05% Tween-20 and blocking with a solution containing PBS/3% BSA, $10^{10}$ ampicillin transducing unit (ATU) blocked phage was added and incubated for 2 h at 37 °C. The plate was washed with PBS/Tween-20 (0.05% in the first or 0.5% in the second round of panning) and the bound phage were eluted with 1 mg/ml BSA/0.1 M glycine, pH 2.2. Following neutralization with 2 M Tris–base, 10 ml XL1-Blue ($O.D._{600}$: 0.5) was infected and plated on LB agar plates containing 50 μg/ml ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with $10^{11}$ plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37 °C phage were precipitated with 16.7% PEG8000/3.3 M NaCl twice and resuspended in TBS. Following the second round of panning 10 ml XL1-Blue ($O.D._{600}$: 0.5) was infected with $10^4$ ATU phage, superinfected with $10^{11}$ PFU M13KO7 helper phage and plated on LB agar plates at a low density to allow immunoscreening to be performed. After 5 h of incubation nitrocellulose membranes were applied and left on the plates overnight. The membranes were blocked with TBS/0.05% Tween-20/5% non-fat dry milk and incubated with the anti-HBX Mab (1 μg/ml in blocking solution) for 2 h. Positive clones were visualized with an AP conjugated anti-mouse IgG secondary antibody.

### 2.5. Phage ELISA

For each clone three wells of an ELISA plate (NUNC, USA) were coated with $10^{10}$ phage particles overnight at 4 °C. After blocking with TBS/2% non-fat dry milk the wells were incubated with the anti-HBX Mab (2 μg/ml in TBS/1% non-fat dry milk). Reactions were developed with a HRPO conjugated anti-mouse IgG secondary antibody.

### 2.6. Sequencing

The DNA of the selected phage clones was isolated by phenol extraction and sequenced with BigDye 1.0 chemistry on an ABI Prism 310 instrument (Applied Biosystems, USA).

### 2.7. Construction of recombinant overlapping HBX fragments and production of bacterial fusion proteins

For the production of recombinant overlapping HBX fragments we used the glutathione S-transferase Gene

Table 1
PCR primers used for the generation of DNA encoding for fragments of HBX

| Sequence of primers (5′–3′) | Peptide fragments, AA |
|---|---|
| GAGGATCCATGGAGACCACCGTGAAAGC<br>CTGAATTCCCCAAACAGTCTTTGAAGTATGCC | 77–116 |
| GAGGATCCAGGACTCTTGGACTCTCTG<br>CTGAATTCCCGCCTCCTAATACAAAGACCT | 96–135 |
| GAGGATCCTTTAAAGACTGGGAGGAGTTG<br>CTGAATTCCCGGCAGAGGTGAAAAAGTTGC | 116–142 |

Recognition sites of the restriction endonucleases are underlined (*Eco*RI: GAGGATCC; *Bam*HI: CTGAATTC). AA: corresponding amino acids of the HBX.

Fusion System (Amersham Pharmacia Biotech, Sweden). Three overlapping parts of the HBX gene (subtype ayw, Bichko et al., 1985) were amplified by PCR using primers with *Bam*HI and *Eco*RI restriction sites (Table 1). The fragments were cloned into the expression vector pGEX-6P-1. Each construct was verified by sequencing. Fusion proteins were expressed in *E. coli* DH5-alfa, and the recombinant proteins were purified using the GST–glutathione affinity system with modifications according to Marczinovits et al., 1997.

### 2.8. ELISA with overlapping fragments

Microtiter plates (Nunc, USA) were coated with 0.1 μg antigen in 0.1 M bicarbonate buffer, pH 9.6, at 4 °C overnight. PBS-gelatin was used to block the free binding sites. The anti-HBX Mab was used at final concentration of 1 μg/ml for 1 h at 37 °C. Reactions were developed by a HRPO labeled anti-mouse IgG secondary antibody.

## 3. Results

Computer analysis of the HBX secondary structure with the Chou–Fasman and the Garnier–Osguthorpe–Robson structure predictions revealed that there were a few sequences exhibiting turn structures along the N-terminal half of the molecule and at the C-terminal as well. The sequence between amino acids 75–130 contains alpha-helices. Beta-sheets are predicted all along the polypeptide chain. There are no sites presumed to be glycosylated. The protein contains 84 hydrophobic amino acids amounting to 54% of the whole molecule. On the basis of the Jameson–Wolf antigenity index, two sequences (amino acids 22–31 and 100–114) were chosen and synthesized for further testing (Fig. 1). The prediction was controlled immunoserologically with simple binding ELISA technique using synthetic peptides (sequences 22–31 and 100–114) as antigens. Unfortunately, none of the Mabs recognized these epitopes (data not shown).

Using our anti-HBX Mab we screened a random peptide library displayed on filamentous phage. Based on the immunoscreening and ELISA tests we chose 10 phage clones for DNA sequencing. A comparison of the deduced amino acid sequences of these clones revealed a consensus sequence of LPxxLH (Table 2). This sequence can be found in the primary structure of HBX (amino acids 88–93). This is in agreement with our earlier Western-blot result performed with recombinant HBX antigen under reducing conditions (Pal et al., 2001).

Limited proteolysis and mass spectroscopic analysis were performed to compare the results of computer prediction and phage display techniques. Limited proteolysis of the recombinant HBX was performed with trypsin digestion, and the peptides separated by reversed-phase HPLC were tested in ELISA. We found strong reactions with anti-HBX Mab in

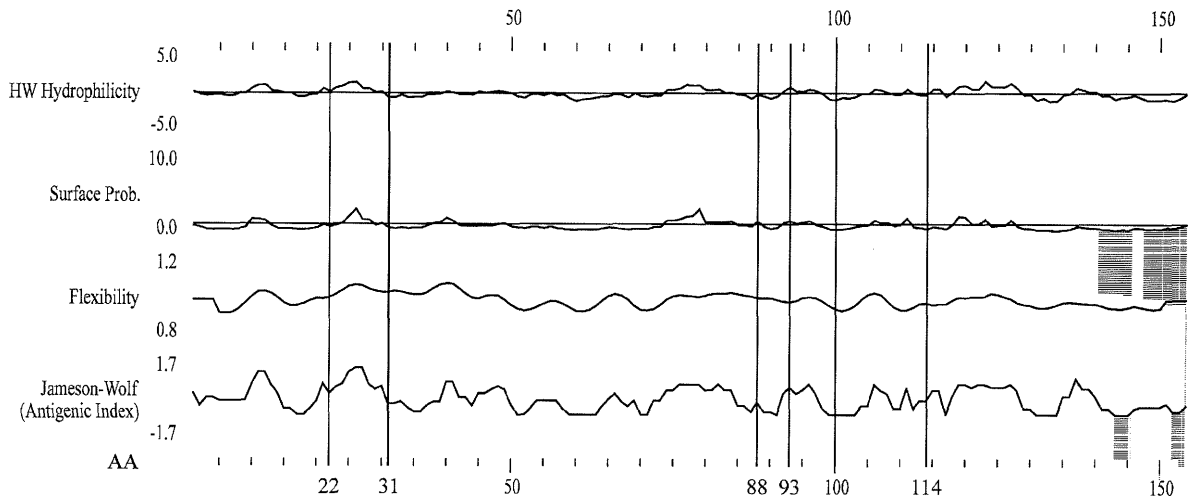


Fig. 1. Computer aided structure analysis of HBX. Peptides chosen on the basis of Jameson–Wolf antigenic index were tested negative for binding to our anti-HBX Mab (amino acids 22–31 and 100–114). The epitope localized by screening a phage displayed random peptide library is between amino acids 88 and 93.

Table 2
Deduced amino acid sequences of clones selected with anti-HBX Mab from a library of nine amino acid random peptides displayed on filamentous phage

| |
|---|
| PFLPKVLHK[a] |
| RMLPASLHL |
| RRLPEELHR |
| ARLPPSLHL |
| RNLPRALSP |
| RRLPSAFHP |
| LRSLHLVRG |
| SRPPSLFRP |
| RNVFLIRTE |
| GRVFFLVRP |
| DRNIYLVRQ |

[a] Amino acids 86–94 of HBX.

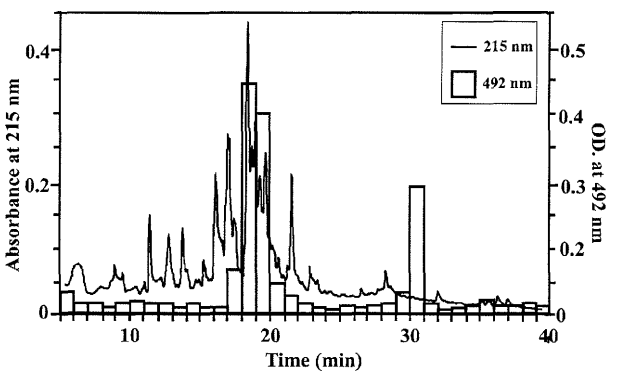


Fig. 2. RP-HPLC separation of peptide fragments after digestion with trypsin. UV-absorbance of the eluted fractions is indicated on the left vertical axis. Optical density following an ELISA with our anti-HBX MAb is indicated on the right vertical axis.

three fractions (Fig. 2). Fractions #18 and #19 showed high immunoreactivity and contained several peptides (with the molecular mass of 1471.7, 2524.0 and 2551.3 of the three main peaks, respectively). Only the first peptide could be assigned to an HBX tryptic fragment: amino acids 14–26 (DVLCLRPVGAESR). The nano-MS/MS spectrum of the double charged ion of this peptide confirmed the sequence. However, this result did not fit in the phage display prediction, and there was another fraction (#30) with positive result in ELISA. We could not identify any peptide in fraction #30 despite the positive ELISA result (Fig. 2).

In order to unequivocally confirm the result obtained by random peptide library we constructed three overlapping GST fusion peptides spanning amino acids 77–142 of the HBX (Table 1). The anti-HBX Mab recognized only the segment representing amino acids 77–116. No reaction was found when peptides containing sequence 96–135 and 116–142 were tested. According to the alignment of the segments only the sequence 77–95 can possibly contain the epitope bound by the anti-HBX Mab (Table 3). This calculation further supports the results of the random peptide library screen, which identifies the epitope sequence as LPxxLH (amino acids 88–93 of the HBX).

## 4. Discussion

The exact identification of epitope regions of antigens recognized by natural and genetically engineered (monoclonal) antibodies appears to be of crucial importance both in biotechnological research and routine diagnostics (Rapley, 1995; Davis et al., 1999; Milstein, 1999). Several techniques have been used for mapping the precise epitope structures. Various procedures based on overlapping synthetic peptide sequences are frequently used in the case of known primary antigen structures. These methods are sufficient in the majority of proteins and peptides; however, the correct mapping of non-linear epitopes is difficult (Helfrich et al., 1994). Mass spectrometric analysis represents a cost-effective, time saving, and well reproducible alternative (Zhang et al., 2002). The efficiency of these techniques can be enhanced by computer modeling and calculation of probable immunogenic regions on the antigen molecules.

Antigens with unusual physico-chemical features impair the efficiency of the application of classical immunoserological and spectrometric mapping techniques, and limit the value of computer aided structure analysis, as we found during the mapping of the epitope specificity of our anti-HBX monoclonal antibody.

Computer analysis of the molecular model suggested different possible immunogenic regions on HBX, however, the identified epitope is estimated to posses a relatively low predicted immunoreactivity (Fig. 1). This contradiction could be explained partially by the application of usual physico-chemical parameters on this highly hydrophobic protein.

Repeated reviewing of our data could indicate a possible explanation for the controversial results of our mass spectrometric analysis. The epitope sequence obtained by the random peptide library screen (LPKVLH 88–93) contains a

Table 3
Overlapping recombinant HBX fragments

| Fragment | AA | ELISA |
|---|---|---|
| METTVKAQPFLPKVLHKRTLGLSVMSTTDLEAYFKDCLF | 77–116 | + |
| TLGLSVMSTTDLEAYFKDCLFKDWEELGEEIRLKVFVLGG | 96–135 | – |
| FKDWEELGEEIRLKVFVLGGCRHKLVC | 116–142 | – |

AA: corresponding amino acids of the HBX protein; ELISA: reactivity with the anti-HBX Mab in ELISA.

trypsin cleavage site. If we consider fragments of the partially digested HBX, sequence 79–95 has one such site, sequence 79–96 has two and sequence 79–112 has three missed cleavage sites, respectively. The hydrophobicity of these fragments calculated by the method of Guo et al. (1987), Browne et al. (1982), Rekker (1977) is higher than that of peptide 14–26. Higher hydrophobicity results in a longer retention time in reverse-phase chromatography. Although the digestion of proteins by trypsin is usually complete in the applied process, partial cleavage may always occur. There was no UV absorbance around 30 min, and even MS measurement could not detect any peptide in this fraction (Fig. 2). The fraction #30 may contain any of these peptides with missed digestion site. As our Mab proved to be strongly reactive against the in vivo expressed HBX in pathologic hepatocytes (chronic active hepatitis, chronic persistent hepatitis, cirrhosis, primary hepatocellular carcinoma) in paraffin embedded tissue sections, it could be sufficiently sensitive to detect the minute amount of partially digested peptide in fraction #30.

Application of molecular biological techniques offered new possibilities in antigen mapping. The combination of phage displayed peptide libraries with immunoserological determination of the expressed antigen fragments and antibodies results a new potential. The fine epitope specificity of anti-HBX Mab verified by phage display technique was confirmed by immunoserological control on synthetic peptide antigen and on overlapping recombinant antigen constructs.

In summary, all mapping techniques require regular control measurements using independent assays in a complex application of current molecular biological and immunological techniques. Our method seems to be useful for the analysis of epitope specificity of natural serum antibodies reacting with HBX molecule in the near future. These investigations would help to clarify the prognostic relevance of different antigenic sites on HBX protein.

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The nine amino acid random peptide library displayed on filamentous phage kindly provided by Dr. Alessandra Luzzago (Instituto di Ricerche di Biologia Molecolare, Italy) is greatly appreciated. This work was supported by grants National Health Foundation (ETT) 05-393, National Plan for Research and Development (NKFP) 01/48/2001 and 01/26/2001 of Hungary.

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Research paper

# Sandwich type ELISA and a fluorescent cytometric microbead assay for quantitative determination of hepatitis B virus X antigen level in human sera

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**Abstract**

The hepatitis B virus X protein (HBxAg) is responsible for severe complications of HBV infections including primary hepatocellular carcinoma. A sandwich type ELISA and a flow cytometric microbead assay for quantitative determination of serum levels of Hbx-Ag are introduced. We have previously developed monoclonal antibody families against well-conserved epitopes on HbxAg, characterized by different immunohistochemical and immunoserological techniques. Special selection of the antibody pairs provided highly sensitive and highly specific tools for quantitative immunoassay development. The resulting assays were tested on human sera (208 samples) collected from patients suffering from different clinical forms of HBV infection. The sensitivity range of the sandwich type ELISA was between 4 and 2000 ng/ml as measured on both the recombinant antigen and the sera of chronic hepatitis patients. A further flow cytometric microbead assay was established and tested in parallel with the ELISA. The quantitative results of these two immunoserological techniques were in strong

*Abbreviations:* DMEM, Dulbecco's modified Eagles's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal cow serum; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; HBcAg, hepatitis B virus core antigen; HBsAg, hepatitis B VIRUS surface antigen; HBV, hepatitis B virus; HBxAg, hepatitis B virus X antigen; HCC, hepatocellular carcinoma; OD, optical densities; oPD, *ortho*-phenylenediamine; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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correlation and they were found to be highly specific and sensitive on clinical samples. The HBxAg ELISA technique is applicable for routine clinical laboratory measurements, and our HBxAg microbead technique is recommended for complex multiparametric measurements combined with other markers.
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*Keywords:* Chronic hepatitis; Recombinant viral antigen; Immunoserology; Monoclonal antibody; ELISA; Flow cytometric microbead assay

## 1. Introduction

Persistent hepatitis B virus (HBV) infection is most frequent in cases of chronic hepatitis, liver cirrhosis and primary hepatocellular carcinoma (HCC). The incidence of this infection is approximately 4–5% of the entire human population. Every year, 500,000 lethal consequences of HBV-related chronic hepatitis or HCC are registered (Blumberg, 1997; Schafer and Sorrell, 1999; Malik and Lee, 2000; Murakami, 2001; Pal et al., 2001). Perinatal transmission results in lifelong symptomless but infectious carrier status with great epidemiological importance. The probability of perinatal transmissions has been found to be about 55% in the case of first-born babies, and about 40% in the case of later pregnancies of symptomless carrier mothers (Ordog et al., 2003).

Hepatitis B virus belongs to a family of DNA viruses called Hepadnaviridae possessing partially double-stranded circular DNA of about 3200 base pairs (Ganem and Varmus, 1987). Hepatitis B virus has one of the shortest viral genomes but its virus cycle is also unique. Three forms of envelope protein HBxAg, the "core" protein, the polymerase protein and the HBxAg are coded for by the genome. The X gene is the smallest coding unit of the HBV genome, which encodes a 17 kDa polypeptide (pX) of 154 amino acids (Kodama et al., 1985; Bichko et al., 1985; Hann et al., 2004). This product of the HBx gene is usually called HBx antigen (HBxAg) in the literature.

HBxAg is a multifunctional protein that serves as an activator of the mitogenic signaling cascade in the cytoplasm and as a specific element of the transcription in the nucleus. HBxAg modulates several host functions via direct and indirect protein–protein interactions, e.g. modulation of transcription, alteration of the protein-kinase signaling cascade with involvement of numerous host genes in cell proliferation (Andrisani and Barnabas, 1999; Murakami, 1999, 2001). Direct correlation was found between the intensity of viral replication and the amount of intracellular HBxAg in liver samples taken from chronic HBV carrier patients (Levrero et al., 1990, 1991). Circulating anti-HBx antibodies were identified in the sera of HBV-infected patients with significant peak periods during the viral replication stage (Hann et al., 2004).

Clinical practice may require continuous monitoring of patients suffering from acute and chronic HBV infections with special attention to monitoring primary HCC progression. However, due to certain technical difficulties, standard, large-scale routine laboratory assay methods are currently not available for quantitative determination of serum HBxAg content. In this paper, we present the development of a sensitive and highly selective sandwich type ELISA and we applied a recently introduced flow cytometric microbead assay (Carson and Vignali, 1999; Cook et al., 2001) for quantitative measurements of HBxAg in human serum samples.

## 2. Materials and methods

### 2.1. Antigens

Recombinant HBxAg and synthetic peptide fragment were used for the assay development.

#### 2.1.1. Construction of HBx recombinant plasmids

The recombinant construct of HBxAg (10–143 amino acids) was described previously (Marczinovits et al., 1997). Briefly, the truncated X gene was obtained by cutting the pHB320 plasmid with *BamHI–FspI* restriction enzymes and cloned through the pHSG 395 vector into the *BamHI–SmaI* restriction sites of the fusion-expression vector pGEX-3X (Amersham Pharmacia Biotech), giving rise to the pGEX-3XXBF recombinant plasmid. The X protein

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is truncated by 9 and 11 amino acids at the N- and C-termini, respectively. According to the cloning strategy, the truncated X protein is flanked by five amino acids at the N-terminus and by four amino acids at the C-terminus, derived from the pHSG 395 and pGEX-3X plasmids, respectively.

*2.1.2. Synthetic peptide antigen*

Peptides of 13–26 amino acid sequences were synthesized using fmoc chemistry and purified with HPLC at the Department of Medical Chemistry, University of Szeged, Hungary.

*2.2. Development and characterizations of monoclonal antibodies*

*2.2.1. Development of the capture antibody*

The affinity purified recombinant HBxAg-glutathione *S*-transferase (GST) construct was the first antigen and the hybridoma clones were characterized by various methods. A clone (3F6/G10) was chosen as the capture antibody. Selectivity was tested in human liver biopsy samples from a retrospective immunohistochemical study (Pal et al., 2001).

*2.2.2. Epitope determination of the anti-HBxAg 3F6/G10 by random peptide library*

Fine epitope specificity of the capture antibody was determined by phage display library as published recently (Pal et al., 2003). Briefly, the phage library displayed random peptides of nine amino acids fused to the N-terminus of the M13 major coat protein VIII (Felici et al., 1991). Phage affinity selection was performed by anti-HBX (3F6/G10) biopanning technique (Parmley and Smith, 1988) and DNA was isolated from selected phage clones and sequenced. The results were confirmed by overlapping recombinant HBxAg probes.

*2.2.3. Development of the detecting monoclonal antibody*

Detecting antibody was developed against synthetic peptide fragments (13–26) of HBx. The 13–26 peptide antigen was conjugated to carrier molecules. Balb/c mice (Charles River Laboratories, Raleigh, USA) were sequentially immunized. Hybridomas were produced (Kohler and Milstein, 1975; Najbauer et al., 1986) and cultured in DMEM (Sigma Chemical Co., USA) containing 10% FCS (Gibco, USA). The purified monoclonal antibodies were labeled by biotin (Sigma, USA). The best clone (4F1/A9) was selected for equally highest affinity to the synthetic antigen, to the recombinant HBxAg, and to human serum samples.

*2.3. Serum samples*

All sera were obtained from the laboratory services of the First Department of Medicine, Medical Center of the University of Pécs and the Division of Virology, Bela Johan National Center for Epidemiology, Budapest, as residual samples after completion of all laboratory testing. The results obtained with sera of patients suffering from HBV positive acute (14) and chronic (80) hepatitis and those taken from healthy HBV carriers (12) were compared with healthy, HBV negative, serum samples (22) and sera from symptomless HBsAg-carrier pregnant women (80). The sera were pretested for HBV by specific ELISA for HBsAg and HBcAg (Dialab, Hungary).

Low pH pretreatment was used in serum samples before the immunoassays to decompose the immunocomplexes and the non-specific aggregates present in sera stored in the frozen state at −20 °C. GST-HBxAg standard and serum samples were diluted in glycine (pH 2.0, 10 min, 37 °C) and then restored to pH 8.0 in Tris-base to a final dilution of 1/10.

*2.4. Construction of sandwich type ELISA*

Assay conditions were optimized by checkerboard titration of recombinant HBxAg-GST standard. Microtitre plates (Nunc, France) were coated with 100 μl of mouse monoclonal anti-HBx (3F6/G10) antibody at a concentration of 10 μg/ml in 0.05 M bicarbonate buffer (pH 9.6) at 4 °C overnight, followed by 60 min incubation at 37 °C. After repeated washing, the plate with 0.15 M phosphate-buffered saline (PBS) non-specific binding sites was blocked by adding 1% gelatin-PBS (300 μl) at 37 °C for 1 h. Then, the plate was washed four times with PBS-Tween 20 (PBST). Following the last wash, GST-HBxAg standard and serum samples were diluted in glycine buffer (pH 2.0, 10 min, 37 °C) and then restored to pH 8.0 by adding Tris-base to a final dilution of 1/10. The plate was incubated at 37 °C

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for 4 h. After washing, 100 μl of biotin-conjugated mouse monoclonal anti-HBx (4F1/A9) antibody (2 μg/ml) were added to each well and incubated at 37 °C for 1 h and washed again. Then, 100 μl of streptavidin–horseradish peroxidase (Sigma, USA) diluted to 1/3.000 in PBST were added to each well and incubated at 37 °C for 1 h. Finally, the plate was washed, the color reaction was developed by *ortho*-phenylenediamine (oPD) (Sigma, USA), and was stopped by 0.46 M sulfuric acid (100 μl/well). Optical densities (ODs) were read at 490 nm in a plate reader (Thermo Labsystems IEMS Reader MF, Finland). The levels of HBxAg in the serum samples were interpolated from a GST-HBx standard calibration curve using an automatic calculation program (Labsystems Ascent Software 2.4 for IEMS Reader MF).

### 2.5. Cytometric bead array immunoassay

Microparticles were prepared and coupled to monoclonal antibody as previously described (Carson and Vignali, 1999). Briefly, polystyrene beads, 7.5 nm diameter (BD Biosciences USA), were coupled via a covalent linkage based on thiol-maleimide chemistry with monoclonal anti-HBx (3F6/G10) capture antibody according to the labeling kit and the manufacturer's protocol. The labeled microbeads were stored for sequential investigations at +4 °C in storage buffer. Before the measurement, 1000 beads per sample were reconstituted in washing buffer by ultrasound (37 kHz, 100 W, 2 min) at room temperature. Each serum sample was pretreated as described in the ELISA protocol. The samples were incubated with 1000–1000 beads for 4 h at 37 °C. Then, the samples were washed and incubated with biotin conjugated anti-HBx (4F1/A9) detecting antibody (2 μg/ml) for 1 h. After three washing steps, the samples were incubated with streptavidin–FITC (DAKO, Denmark) in 1/100 dilution for 1 h at 37 °C. The samples (after three washing steps) were acquired within 1 h using a FACSCalibur flow cytometer (BD Biosciences USA) and list mode files were further analyzed with FCAP Array software (SoftFlow, Hungary). Comparing the mean intensity values of the FITC reporter fluorescence of the samples to a standard curve, the software generates quantitative data on the HBxAg concentration in patients' sera.

### 2.6. Immunoblot

The sera were boiled for 10 min in SDS sample buffer and were separated by 15% SDS-PAGE using Mini Protean 3 devices (Bio-Rad, Hercules, USA). The gel was transferred to a Hybond ECL membrane (Amersham Biosciences), blocked with 5% non-fat dry milk in PBS for 1 h and incubated with anti-HBX monoclonal antibodies (1/500, 3F6/G10, 4F1/A9) in PBS containing 1% non-fat dry milk. After the membrane was washed three times with PBS, horseradish peroxidase-coupled anti-mouse IgGs (1/5000 Amersham Biosciences) were added and the signal was detected with the ECL Plus Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions, using Kodak X-ray film.

### 2.7. Statistical analysis

Statistical analysis was performed by Student's *t* test. The data were analyzed with SigmaPlot software 2000 (SPSS Inc., USA).

### 2.8. In silico homology analysis of HBxAg epitopes

In silico analyses was performed to check for known homologies of 13–26 and 89–94 subsequences of HBxAg (gi:4704317). Subsequences were searched against the non-redundant protein database (database size as of September 2004=688,443,072) using the BLASTP2.2.9 program (Altschul et al., 1997) (expect=20000; Matrix PAM30; gap cost existence=9; extension=1; word size=2). Results were limited by the Entrez option: hepatitis B virus [ORGANISM] AND X protein [PRODUCT]. (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

## 3. Results

The fine epitope specificity of monoclonal anti-HBxAg (3F6/G10) capture antibody was localized on 89–94 amino acid sequences as published (Pal et al., 2003). The detecting monoclonal anti-HBxAg (4F1/A9) antibody was developed against a synthetic 13–26 amino acid sequence of HBx. These epitopes are located in two different positions on the molecular surface and make possible the development of sand-

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4F1/A9
MAARLCCQLDPARDVLCLRPVGAESRGR

PFSGSLGTLSSPSPSAVSTDHGAHLSLRGL

PVCAFSSAGPCALRFTSARRMETTVKAQP
3F6/G10
FLPKVLHKRTLGLSVMSTTDLEAYFKDCL

FKDWEELGEEIRLKVFVLGGCRHKLVCA

PAPCNFFTSA

Fig. 1. The anti-HBxAg antibody clones recognize two epitopes (underlined) located in two distinct locations of the antigen molecule. The capture antibody (clone 4F1/A9) specific to the 13–26 aa sequence, the detecting antibody (clone 3F6/G10) specific to the 89–94 aa sequence.

wich type immunoassays with the application of this antibody pair (Fig. 1).

The immunoglobulin isotype of anti-HBxAg 3F6/G10 is IgG2a and the capture antibody (anti-HBxAg 4F1/A9) is IgG1 (data not shown).

The specificity of the monoclonal antibody pair was tested with both recombinant HBxAg and serum samples from HBV-infected patients using the immunoblot technique (Pal et al., 2001). Sera from 12 chronic hepatitis patients were analyzed; highly specific labeling was found in all samples at the 17 kDa band. Fig. 2 shows the characteristic Western blot results.

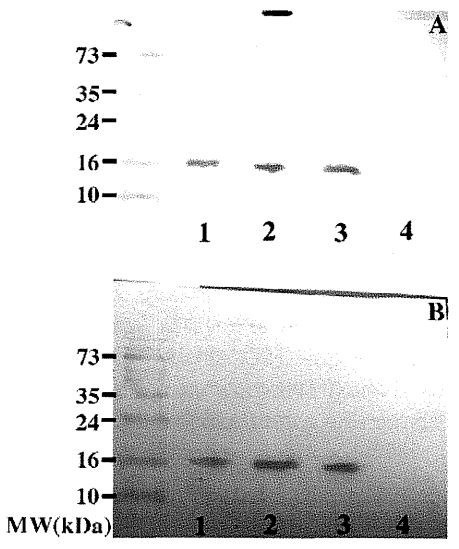


Fig. 2. The specificity of the monoclonal antibody pair was tested in HBV-infected patients and healthy, HBV negative serum samples using the immunoblot technique. Mw, protein molecular weight standard. (A) Result of the capture antibody (clone 4F1/A9); (B) result of the detecting antibody (clone 3F6/G10). 1, 2, 3, serum samples of HBV-infected patients; 4, serum sample of a healthy individual.

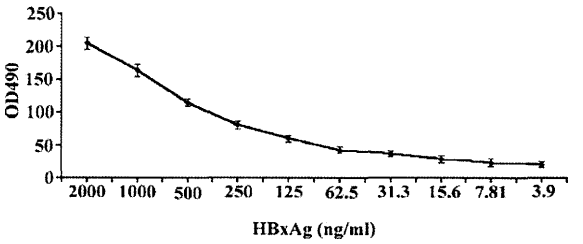


Fig. 3. Calibration curve of a quantitative sandwich ELISA for recombinant HBxAg (three serial dilutions from 2000 to 4 ng/ml). Standard deviation shown as bars. $p < 0.05$.

Homology searches with subsequence 13–26 of HBxAg resulted in 442 positive blast hits. In 50 cases there was a difference of one amino acid in the query sequence, and in two cases there was a difference of two amino acids. In subsequence 89–94, we received 436 blast hits. In 42 cases there was a difference of only one amino acid, and in 45 cases there was a difference of two amino acids in the query sequence. All hits were of different HBxAg variants (data not shown). This can be interpreted that these subsequences of HBxAg are highly conserved and specific.

A sandwich type ELISA was developed first and optimized using recombinant HBxAg as standard. The sensitivity range was found to be between 4 and 2000 ng/ml using checkerboard titration of HBxAg. The lowest optimal sensitivity was found to be 4 ng/ml and the highest detected level was 2000 ng/ml. The original titration curve was an exponential curve typical of measured optical densities (OD490) (Fig. 3).

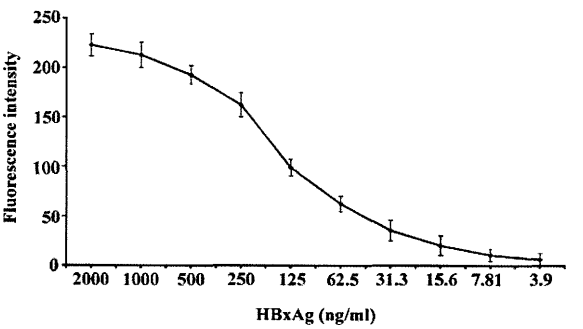


Fig. 4. Calibration curve of a quantitative sandwich type microbead assay for recombinant HBxAg (200 serial dilutions from 2000 to 4 ng/ml). Standard deviation shown as bars. $p < 0.05$.

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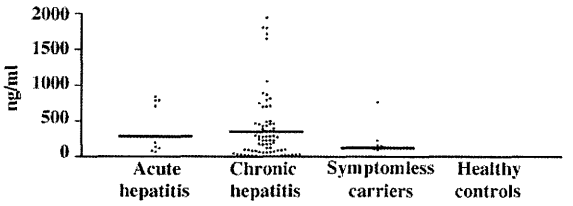


Fig. 5. Quantitative detection of HBxAg in sera of hepatitis B patients by sandwich ELISA type. The figure was generated using SigmaPlot software 2000. HBV positive acute ($n=14$) and chronic ($n=80$) hepatitis and those taken from symptomless carriers ($n=12$) were compared with healthy, HBV negative, serum samples ($n=22$) (The "0 ng/ml" level is calculated automatically by the Labsystems Ascent Software 2.4 from the measured OD490 values.).

We developed a sandwich type flow cytometric microbead assay technique based on the results of the HBxAg ELISA measurement using the same monoclonal anti-HBxAg pair and the same recombinant HBxAg standard. During the test optimization, we found similar dynamic range as in ELISA: 4–2000 ng/ml. The original titration curve showed sigmoid character (Fig. 4).

The selectivity of HBxAg ELISA was tested on 128 human serum samples: the HBV negative samples (22 healthy control sera) were consistently negative in the assay (the OD490 was in all cases under 2SD of the OD value of the blank and the control sera). The presence of the HBxAg on 106 HBV infected patients was detected by ELISA in 64% of acute patients, in 76% of chronic hepatitis patients and in 50% of the healthy carriers. The cut off range was >4 ng/ml according to the results of the healthy controls. (The previous literature data are suggest a similar range (Kumar et al., 1998).) The concentrations were between 18.2 and 1803.86 ng/ml. The observation range of the quantitative data is shown in Fig. 5.

The microbead assay was tested with the sera of chronic hepatitis patients (32), sera of symptomless HBsAg-carrier pregnant women (80) and healthy individuals (22). Fig. 6 demonstrates characteristic positive and negative samples.

The results were compared to the results of the ELISA measurements performed in parallel. Comparative analysis of the two assays showed the same sensitivity and selectivity; the correlation coefficient

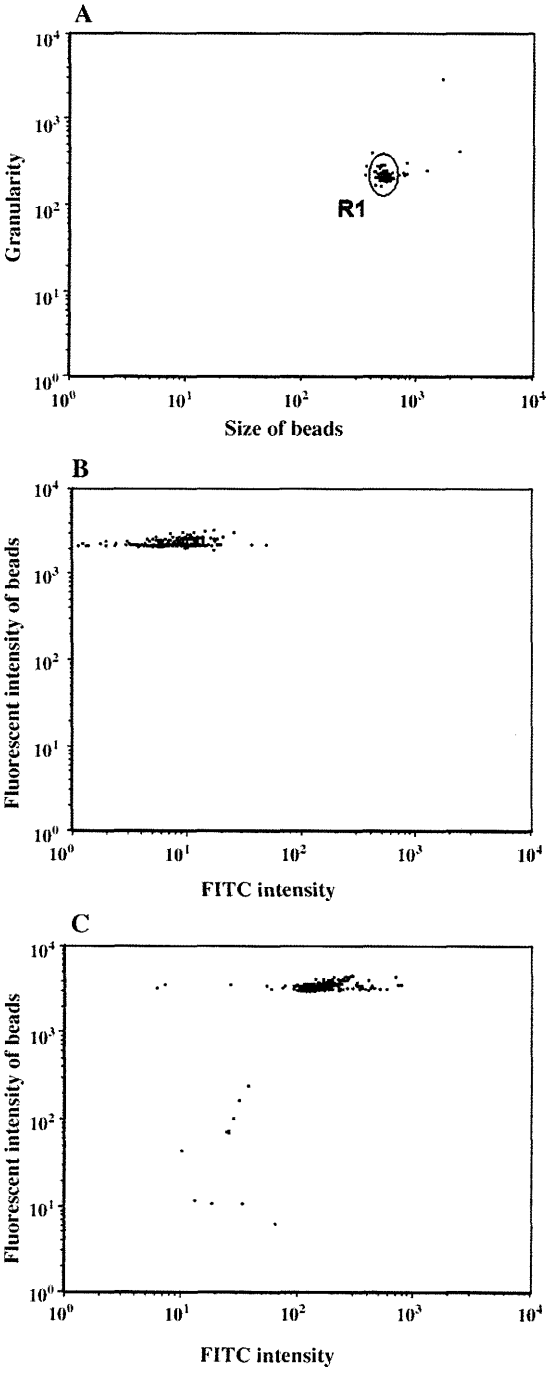


Fig. 6. These charts show typical results from HBx microbead assay of human serum samples. In the granularity vs. size of beads dot plot (A), the microbead population was gated (R1) and only beads falling into this gate were further analyzed. The two other dot plots show the results of HBxAg negative (B) and positive (C) serum. FL3 (fluorescence intensity of beads) was the clustering parameter for bead detection and FL2 (FITC intensity) was the reporter parameter for HBxAg content of the sera.

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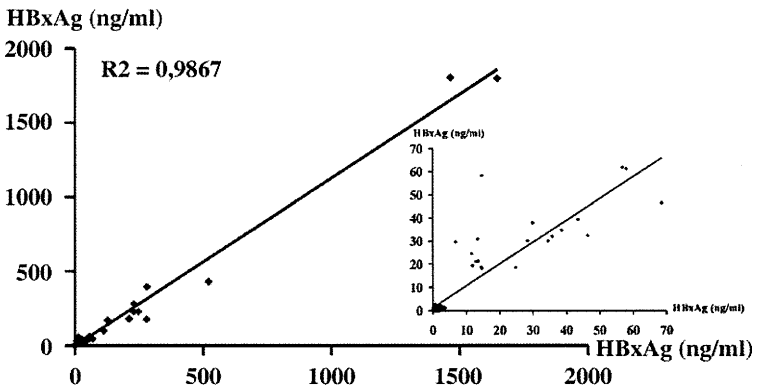


Fig. 7. The figure shows the results of regression analysis on measured serum HBxAg concentrations between sandwich type ELISA ($y$ axes ) and flow cytometric microbead assay ($x$ axes). The measurements were performed on 112 hepatitis B-infected patients. The regression was 98.68% ($R^2$, regression coefficient). The right-hand side of figure demonstrates the data in the lower scale.

of the data (measured on the same serum samples) was 98.67%. The microbead assay resulted in similar sensitivity as the ELISA (Fig. 7).

## 4. Discussion

Quantitative immunoserological determination of the HBxAg level in the sera of HBV-infected patients is important in clinical pathological research and daily laboratory routine because of the pathological consequences of chronic B virus infection in the development of primary hepatocellular carcinoma. Studies are required for comparative analysis of the serum HBxAg level in different groups of patients to clarify the pathological events and mechanisms of disease progression during the natural history of persistent HBV infections.

Nucleic acid probes exist for HBxAg detection but routine laboratory services need conventional immunoassays for large-scale measurements. The available immunological techniques for measuring HBxAg in sera have been limited by various methodological and technical difficulties. The immunoblot technique and anti-HBx antibodies were used for the detection of serum HBxAg (Hwang et al., 2003). Monoclonal antibodies were developed against different epitopes of HBxAg and used in immunohistology and immunoserology (Su et al., 1998; Pal et al., 2001, 2003; Wang et al., 2004). Kumar et al. (1998) published a competition ELISA used for HBxAg measurements in different groups of HBV-infected patients. However, these investigations faced different methodological problems and could not be standardized for routine diagnostic applications.

The development of a highly selective and sensitive antibody family mainly depends on the nature of the antigen used for immunization and standardization of the assays (Regenmortel, 2000). The generation of specific polyclonal or monoclonal antibody against HBxAg protein is technically difficult because of its special physico-chemical feature: more than the half of the molecule (54%) is composed of hydrophobic amino acids. The classical biochemical methods for isolation of HBxAg are inefficient because of the absence of a specific substrate for culturing the virus, and the risks are high in connection with handling of pathologic liver samples. Synthetic peptide fragments were used in some cases for antibody development (Stemler et al., 1990). However, both the selectivity and sensitivity of these antibodies are altered compared to antibodies produced against natural antigen, because the synthetic peptides exhibit different conformations from the natural antigens. The generally used bioinformatic predictions on the HBxAg are less useful (according to our results) due to the special physico-chemical character of the molecule (Pal et al., 2003).

The most useful antibodies were developed by immunization with recombinant HBxAg protein (Marczinovits et al., 1997; Henkler et al., 2001). We have developed an anti-HBxAg monoclonal anti-

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body family against a truncated recombinant antigen (Pal et al., 2001). The antibodies were tested with both the recombinant and natural antigens, the latter being present in human tissue samples (Pal et al., 2003). The starting point of our work was the previously published study of Stemler et al. (1990) who identified a major immunodominant epitope residing in amino acid sequence 89–102 on HBxAg. It has been confirmed that this is a characteristic epitope of human pathogenic HBV. By using epitope mapping principles, we selected a clone (anti-HBxAg 3F6/G10) specific to the 89–94 sequence which is located on the surface of the natural antigen molecules (Pal et al., 2003). The anti-HBxAg 3F6/G10 was selected as the capture antibody for the assay because of its high selectivity. The selectivity of this clone was proved on human liver tissue samples from hepatitis patients using immunohistochemistry (Pal et al., 2001).

Further sequences on the N terminal region (amino acids 1–20 and 13–26) were described on HBxAg with significant pathogenic importance in the development of primary hepatocellular carcinoma in chronic hepatitis patients (Misra et al., 2004). We used the synthetic 13–26 peptide sequence as antigen to develop a monoclonal antibody family against HBxAg. Hybridoma clones were tested against both the synthetic and the recombinant antigens. Based on the satisfactory sensitivity and specificity of the clone, anti-HBxAg 4F1/A9 was chosen as the detection antibody during the next step of the immunoassay development.

The selectivity of both chosen clones was tested in different samples with various immunoserological and immunomorphological techniques. We found strong specificity for HBxAg without any non-specific cross-reactions.

With application of the recombinant antigen, we optimized a sandwich type ELISA to a broad sensitivity range. The previous literature data forecast the minimum sensitivity to be around 5 ng/ml (Kumar et al., 1998). Different serum samples from healthy controls, acute and chronic hepatitis patients and persistent HBV carriers were measured by the HBxAg ELISA. The assay conditions are suitable for routine immunoserological laboratory techniques. The measured data are in harmony with the results of the surface (HBsAg) and core (HBcAg) antigen measurements as described in other studies (Song et al., 2004).

Previous publications have reported correlation between the well-established serological markers (i.e., HBsAg and HBV DNA) of ongoing viral replication and either X antigen expression or the serum level of anti-HBxAg circulating antibodies (Levrero et al., 1990, 1991; Vitvitski-Trepo et al., 1990; Hann et al., 2004). The clinical significance of the relationship between different HBV antigens and the production of circulating antibodies against them is poorly understood. Multiparametric comparative studies are required to analyze the pathogenic role of different HBV antigens and to identify new prognostic markers (Kumar et al., 1998).

Sandwich type ELISA is generally used for routine laboratory measurements on a large-scale for a limited number of antigens, however, comparative examinations in multiparametric studies performed on the same samples are difficult with ELISA. For our HBxAg ELISA development, we constructed a microanalytical immunoserological assay using the same monoclonal antibody pairs as the quantitative measurements in a flow cytometric microbead assay.

Polystyrene microbeads as carriers are used in various immunoassays (Carson and Vignali, 1999; Cook et al., 2001). Flow cytometry measurements are optimal for the analysis of large-scale samples in different parallel assays on the same sample. The comparative measurements by our anti-HBxAg monoclonal antibody pair made on the same set of human serum samples showed strong correlation with the results of the ELISA and the flow cytometric microbead assay. The results of 208 clinically verified serum samples confirm the theoretical and practical advantages: our assays are suitable for investigating the pathomechanism of HBV infection, and finding new prognostic markers for hepatitis patients (Pal et al., submitted for publication).

## Acknowledgments

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# Detection of citrate synthase-reacting autoantibodies after heart transplantation: an epitope mapping study

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**Abstract** Autoimmune mechanisms play an important role in the pathogenesis of allograft vasculopathy following heart transplantation, but the autoantigens involved have been only sparsely studied. Citrate synthase (CS) enzyme is a conserved molecule, and, as an important mitochondrial autoantigen, it is protected by the "immunological homunculus". Tissue destruction and alteration of the immune regulatory mechanisms can induce pathological immune response against CS in other autoimmune diseases. In our present study we aimed to detect CS-specific autoantibodies in heart transplant patients, therefore, prospective, randomised clinical tests were conducted on 33 heart transplant patients and compared with 130 healthy blood donors. The level and isotype of CS antibodies were detected by simple binding indirect enzyme-linked immunosorbent assay (ELISA). The epitope specificities of the autoantibodies were measured on synthetic overlapping peptide sequences of CS enzyme by an indirect multi-pin ELISA method. Mainly IgM isotype CS autoantibodies were found in healthy controls, while IgG was found at higher levels and frequency (four-times higher) in heart transplant patients. Autoantibodies of IgG isotype recognise different epitopes than do autoantibodies of IgM isotype, even within the same group and individual. New epitope-specific IgG and IgM isotype autoantibodies appeared in heart transplant patients when compared with the controls. Our findings suggest a possible role of CS-specific autoantibodies in the pathomechanism of allograft vasculopathy.

**Keywords** Citrate synthase · Autoantibodies · Heart transplantation · Allograft vasculopathy · Anti-mitochondrial autoantibodies

## Introduction

According to recent data, circulating immunoglobulins play an important role in the pathogenesis of both acute rejection and accelerated coronary artery disease following human heart transplantation. The role of antibodies in heart graft dysfunction remains to be defined, although few would dispute their importance. The vast majority of cardiac transplant centres monitors and detects acute cellular rejection by the use of surveillance endomyocardial biopsies and treats T cell-mediated rejection with appropriate anti-T cell cytolytic therapy. Only a few centres perform post-transplant monitoring of antibody formation, although the need for more focused attention on the role of antibodies has become apparent in recent years due to two clinical problems.

The first is the syndrome that has been called vascular rejection, whereby the heart shows poor ventricular

function, with sparse or no cellular infiltration in repeated biopsies [1, 2]. The second problem is transplant-associated cardiac allograft vasculopathy [3, 4]. More than half of our patients have suffered from cardiomyopathy before transplantation. The exact cause of this illness is unknown; however, an autoimmune origin is suspected, which underlines the possibility of an altered immune response in these patients [5, 6]. As the presence of autoantibodies is an important pathological factor in dilated cardiomyopathy, their role in post-transplantation allograft vasculopathy was also suspected. Minor antigens, released from damaged parenchymal cells within the graft, are thought to be responsible for the chronicity of the antibody response after transplantation [7, 8].

According to the "cognitive theory of the immune system" postulated by Cohen [9], discrimination between "physiological" and "pathological" self-reacting antibodies is not only theoretically relevant but also has major clinical consequences [10]. Different genetically well-conserved target antigens were analysed, both in human and mouse models, during the past decade, and they proved to be significant participants of the normal "immunological homunculus" protected by low-affinity physiological autoantibodies with IgM isotype [11]. Mitochondrial inner-membrane enzymes are among the oldest proteins with conserved structures and possess basic biological functions. Citrate synthase (CS) is a well-conserved molecule with a very similar amino acid (aa) sequence and the same function in both prokaryotic and eukaryotic cells [12, 13]. Low-affinity IgM isotype autoantibodies are present in the human circulation against these structures to protect them from a targeting immune response [14]. Severe bacterial infection or tissue destruction can cause the formation of these low affinity molecules, but a parallel malfunction of the immune regulation may result in the appearance of high-affinity IgG or IgA isotype autoantibodies against these proteins [15, 16], mediating further tissue destruction via an autoimmune response.

So far, several tests have been conducted to detect autoantibodies in heart transplant patients, such as myosin [17, 18], phospholipids [19] and endothelial cells [20]. However, we did not find any records of test on autoantibodies against mitochondrial inner-membrane enzymes in cardiac transplant patients [21], although these have been previously described in the pathomechanism of other diseases with autoimmune backgrounds, such as primary biliary cirrhosis and anti-glomerular basement membrane disease [22, 23, 24]. In this study our aim was to detect the presence and isotype of citrate synthase-specific autoantibodies, one of these anti-mitochondrial autoantibodies, in heart transplant patients. We also wanted to compare the epitope specificity of these antibodies, using an overlapping peptide library of the CS enzyme.

## Materials and methods

### Patients

Altogether, 52 heart transplantations were completed in our clinical department between 1992 and 2002. Of these 52 patients, 33 were alive during our clinical study. Prospective, randomised clinical tests were conducted on these patients. Within the first year after the heart transplantation 16 patients in the studied group required hospital therapy. These cases were considered only as postoperative complications: rejection was observed in five patients (International Society for Heart and Lung Transplantation, ISHLT, I-B-II-A) and infection occurred in 11 patients. There were no complications in 17 patients. The basic therapy after the heart transplantation was cyclosporin, mycophenolate mofetil and steroids. The supporting therapy was decided according to the needs of the given patient and included anti-diabetic, antihypertensive and various other additional therapies.

It is the usual practice in our department to investigate the transplant recipients for vascular complications on a monthly basis. We used this opportunity to measure autoantibody levels. Serum samples were collected freshly at appropriate intervals and stored at −70°C before examination. The control group comprised of 51 English, 17 Finnish and 62 Hungarian blood donors with matched age and gender ratios.

### Immunoserological detection of anti-CS autoantibodies

We used an indirect enzyme-linked immunosorbent assay (ELISA) to detect anti-citrate synthase autoantibodies from human sera [25]. Citrate synthase from porcine heart (Sigma) was incubated for 12 h at 4°C followed by 60 min incubation at 37°C on 96-well polystyrol plates (NUNC, France). Following the saturation of non-specific binding sites with 0.5% gelatine (Sigma Chemical Company, USA) three washing steps with phosphate-buffered saline solution (PBS) containing 0.1% Tween-20 (Sigma) were performed. The serum samples and controls were incubated in 1:100 dilutions for 60 min; finally, the plate was incubated with anti-human IgG HRPO or anti-human IgM HRPO secondary antibodies (Dako, Denmark) for 60 min. The colour reaction was carried out with ortho-phenylenediamine (Sigma), and was measured on a Dynatech MR7000 (USA) microphotometer at 492 nm [optical density (OD)], and the results were compared with the control samples.

### Epitope mapping of anti-CS autoantibodies using overlapping decapeptides

Epitope prediction on human citrate synthase was made by Chou–Fasman epitope prediction and Swiss model epitope prediction [26, 27, 28]. Synthetic peptide fragments of CS were synthesised on a multi-pin system using Fmoc/tBu methodology. Decapeptides overlapping of five amino acid residues were synthesised on $\beta$-alanylglycine functionalised polyethylene pins on a block (Mimotopes, Australia) with Fmoc/tBu chemistry according to Geysen's method [29]. We used $^{t}$Bu (Thr, Ser, Tyr), O$^{t}$Bu (Asp, Glu), Trt (Cys, His, Gln, Asn), Pbf (Arg) and Boc (Lys, Trp) as sidechain-protecting groups [30].

Peptide epitopes of citrate synthase were bound on multi-pin systems. Two hundred microlitres per well of all reagents and serum samples were dispensed in a 96-well polystyrol plate (NUNC). The incubation, washing and developing steps of the multi-pin system were preformed in the 96-well plate. All steps were performed with agitation. First we incubated the multi-pin system for 60 min at room temperature in PBS containing 0.5% gelatine (Sigma) for saturation of non-specific binding sites. Later, three washing steps were performed with PBS containing 0.1% Tween-20 (Sigma). The serum samples and controls were incubated in 1:100 dilutions in PBS containing 0.5% gelatine, 0.1% Tween-20 for 60 min.

After incubation three 10-min washing steps were performed with PBS containing 0.1% Tween-20. Then, the multi-pin system was incubated with affinity isolated anti-human IgG HRPO or anti-human IgM HRPO secondary antibodies (Dako) for 60 min at room temperature. After three 10-min washing steps the colour reaction was carried out with ortho-phenylenediamine (Sigma) and measured on an iEMS MF microphotometer (ThermoLabsystem, Finland) at 492 nm (OD). The results were analysed with Ascent Software v.2.4.2 (ThermoLabsystem).

The multi-pin system was regenerated after each measurement according to the manufacturer's instructions. The regeneration was tested by conjugate test.

### Statistical analysis

Since antibody titres measured in different groups did not match a Gaussian distribution, a non-parametric test was used. Results of the ELISA measurements were comparatively analysed by the Mann–Whitney U test. $P < 0.05$ was considered statistically significant.

## Results

### Serological screening studies

We compared the isotype and the titre of anti-CS autoantibodies using repeated indirect ELISA measurements. Citrate synthase enzyme-specific autoantibodies were found in both healthy control groups (blood donors) and in heart transplant patients, with significant individual differences in their titre and isotype. The average titre of IgM autoantibodies was much higher than that of the IgG and IgA isotype autoantibodies in all groups investigated (Fig. 1). We defined autoantibodies as "high titre" when the OD of a given sample was higher than (or equivalent to) the average OD plus two standard deviations (OD + 2SD) of the samples

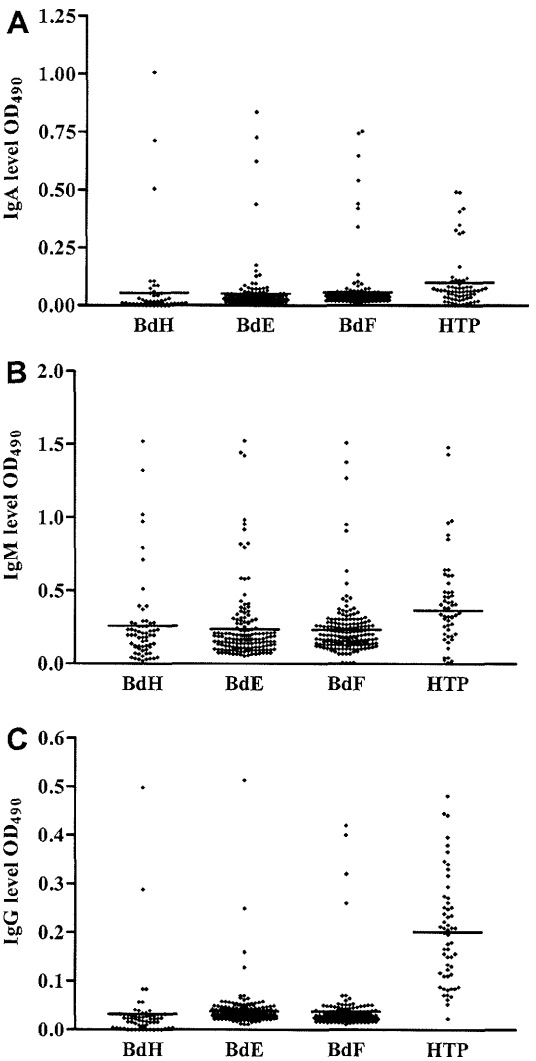


**Fig. 1A–C** The isotype of CS-specific autoantibodies was measured by indirect ELISA. The concentration of IgA (**A**) isotype anti-CS autoantibodies was higher in heart transplant patients than in healthy individuals. The average concentration of IgM isotype (**B**) anti-CS autoantibodies was higher in each group investigated than the concentration of IgA and IgG isotype autoantibodies (aABs). Significant elevation of anti-CS aAb concentration with IgG isotype (**C**) was detected in heart transplant recipients

**Table 1** Frequency of high-titre autoantibodies against CS (*BD* healthy blood donors, *HTP* heart transplant patients, *a-CS* anti-citrate synthase)

| Samples | a-CS IgG | a-CS IgM | a-CS IgA |
|---|---|---|---|
| BD from Hungary | 3% | 10% | 4% |
| BD from England | 4% | 12% | 5% |
| BD from Finland | 3% | 9% | 5% |
| HTP | 17% | 12% | 3% |

from healthy individuals. The frequency of high-titre autoantibodies was mainly of IgM isotype in all healthy blood donor groups and in heart transplant patients as well. However, remarkable differences could be observed in the prevalence of anti-CS IgG isotype autoantibodies between the controls and the heart transplant patients. Compared with the average 3% frequency of high-titre IgG autoantibodies in healthy donors, 17% of the transplanted patients showed high-titre IgG anti-CS autoantibodies (Table 1). Therefore, the prevalence of high-titre IgG anti-CS autoantibodies in heart transplant patients was four times higher than in other study groups.

The specific and individually characteristic IgM level of healthy blood donors and heart transplant patients was stable in each individual during repeated measurements (Fig. 2A). Autoantibody levels with IgG isotype varied in time both in the case of heart transplant patients (Fig. 2B) and in healthy blood donors (data not shown).

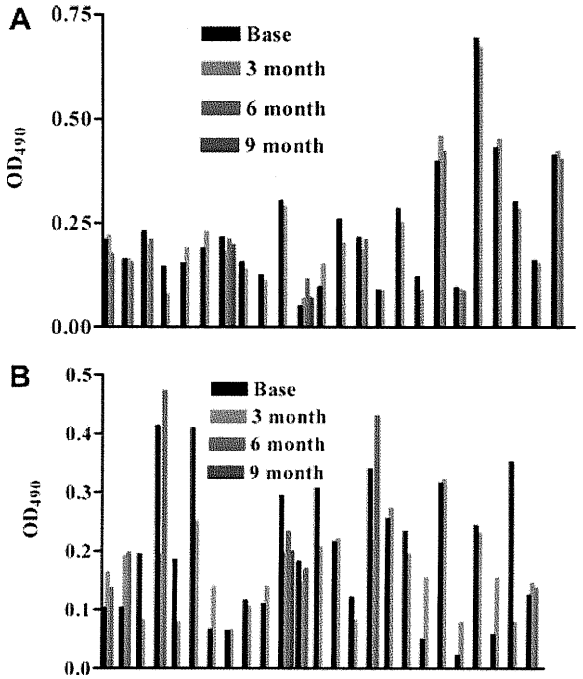


**Fig. 2A,B** Persistence of anti-CS autoantibodies with IgM isotype (**A**) and time-dependent alteration of anti-CS autoantibodies with IgG isotype (**B**) from heart transplant patients was detected by repeated indirect ELISA measurements

### Epitope specificities of anti-CS autoantibodies

Computer analysis showed characteristic immunodominant epitopes of the citrate synthase enzyme (Fig. 4A). We used synthetic overlapping (5–5 aa) decapeptides to compare the epitope specificities of IgG and IgM isotype autoantibodies of healthy blood donors (5–5) and heart transplant patients (5–5). Epitope reactivity of individuals belonging to identical study groups showed minimal differences. Autoantibodies of IgG isotype recognised different epitopes than did autoantibodies of IgM isotype, even within the same group and individuals. There are common conserved antigenic regions found on the citrate synthase enzyme recognised by the autoantibodies (Fig. 3). However, there are variations in the epitope reactivity pattern of sera of healthy individuals (Fig. 4B, D) and the heart transplant patients (Fig. 4C, E) against these conserved regions of CS. Besides the "physiological" autoantibody pattern of healthy individuals, new IgG and IgM isotype autoantibodies with new epitope specificities appeared in the heart transplant patients (Fig. 4B, C, D, E).

## Discussion

Cellular and humoral immune reactions participate in the development of allograft vasculopathy following heart transplantation [7, 18, 20]. Most of the cardiac transplant centres monitor and detect acute cellular rejection by the use of endomyocardial biopsies. Although the role of autoantibodies in the development of chronic rejection is ever more accepted, only a few centres perform post-transplant monitoring of antibody formation. Since the most frequent indication of heart transplantation is idiopathic cardiomyopathy—which is a well-recognised mitochondrial disease associated with defects of mitochondrial function [31, 32]—it was obvious to detect anti-mitochondrial autoantibodies in heart transplant patients. Antibodies against distinct mitochondrial antigens are not necessarily pathogenic but represent markers of immune-mediated injury.

Appearance of anti-cardiac antibodies against myosin and other unknown antigens in myocarditis supports the involvement of autoimmunity in one-third of patients. However, reduction in the autoantibodies during disease progression and development of dilated cardiomyopathy was also observed [33]. Unfortunately, we do not have information about the anti-CS antibody levels of our patients before transplantation, but continuous

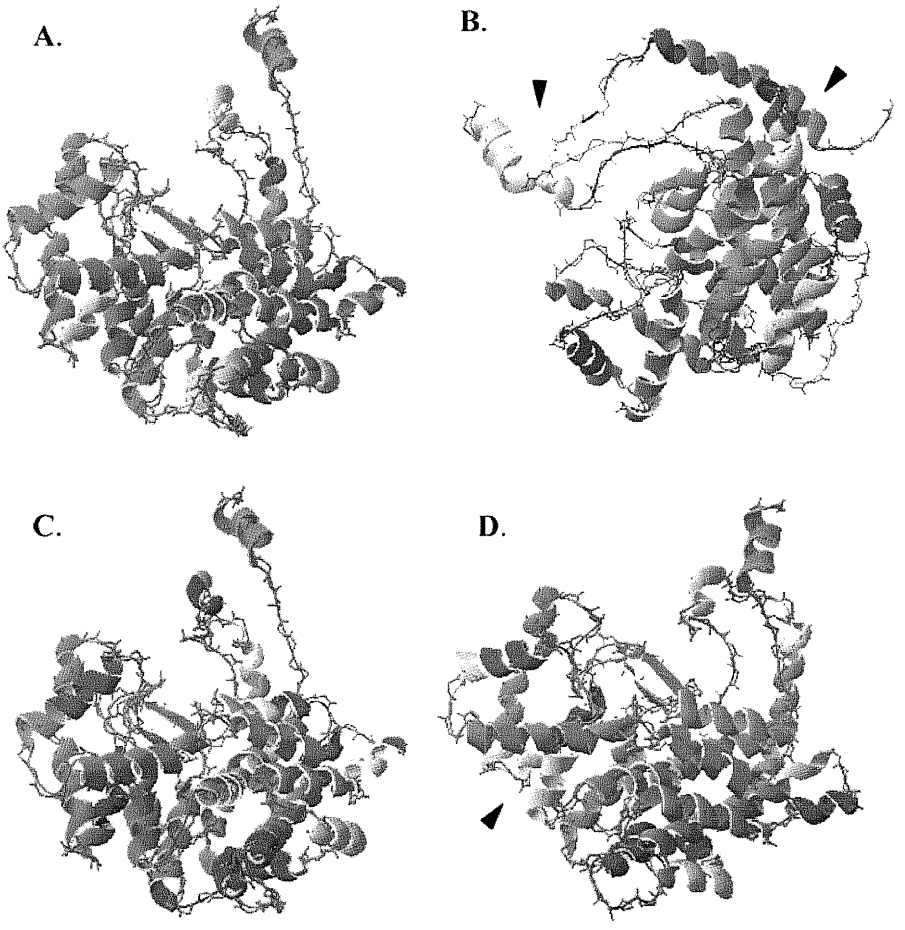


**Fig. 3A–D** 3D model of CS enzyme was rotated to a position that illustrated the highest number of epitopes detected by anti-CS autoantibodies. Dominant epitopes detected by autoantibodies with IgG isotype from healthy blood donors (**A**) were compared with the epitopes of autoantibodies in sera of heart transplant patients (**B**). Less difference between the IgM isotype anti-CS autoantibody epitopes of healthy individuals (**C**) and heart transplant patients (**D**) was observed. *Arrowheads* point to the differences

detection of post-transplantation levels of anti-CS antibodies may help us to follow-up the immune-mediated myocardial injury.

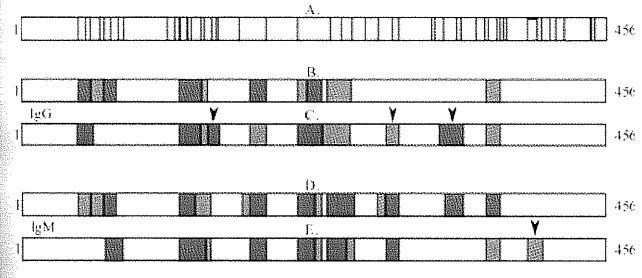


**Fig. 4** Citrate synthase epitopes recognised by autoantibodies of healthy individuals and heart transplant patients. *Line A* represents immuno-dominant epitopes (*yellow*) of CS enzyme predicted by computer analysis. IgG (*line B*) and IgM (*line C*) isotype autoantibodies of healthy controls showed different (*arrowheads*) epitope specificities (*red and orange*) on overlapping peptide sequences of the CS enzyme. Besides this characteristic epitope pattern, new epitope-specific IgG (*line D*) and IgM (*line E*) isotype autoantibodies (*arrowheads*) appeared in sera of heart transplant patient. The main epitopes recognised by autoantibodies are coloured *red*, and the secondary, alternative, epitopes are coloured *orange*

Citrate synthase enzyme is one of the mitochondrial antigens that is detectable in the serum of patients with mitochondrial injury [34]. As the key enzyme of energy production in the citrate cycle, citrate synthase is an evolutionally well-conserved protein, with distinct and important biological functions. It is known that low-affinity autoantibodies with IgM isotype specific for well-conserved structures are present in the blood to serve as protection against a destructive immune response [10, 31].

Tissue damage, infection [35] and a parallel malfunction of the immune regulation or the apoptotic process [36] may result in the appearance of conserved self-molecules and cause the formation of high-affinity IgG-type or IgA-type autoantibodies, mediating further severe tissue destruction [9, 14]. The level of IgM-type and IgG-type autoantibodies with the same antigen specificity may predict the presence of an underlying autoimmune response. The finding that the frequency and the level of high-affinity IgG isotype anti-citrate synthase autoantibodies were higher in heart transplant patients than in any of the control groups shows that an imbalance in the

level and pattern of citrate synthase-specific antibody isotypes can be found in heart transplant patients. This imbalance may result in a shift towards an unfavourable, destructive, autoimmune response that—as the literature [10, 16, 35] suggests—can take place in the formation of allograft vasculopathy.

The presence of anti-citrate synthase autoantibodies with IgG isotype in heart transplant patients poses a question: is the fine epitope (molecular surface peptide sequence) specificity the same as in the case of the "physiological autoantibodies" (with IgM isotype) or are there new molecular surface antigen targets that the "pathological" IgG antibodies react with? The results of our analysis to determine the peptide fragment specificities, of antibodies show remarkable changes in the epitope specificity of IgG autoantibodies for the citrate synthase molecular surface compared to that of IgM autoantibodies and to that of IgG found in healthy individuals. Our data with anti-CS-specific autoantibodies suggest a new field of active tolerance controlled by the immunological homunculus [37, 38] and provides new insight into the pathogenesis of allograft vasculopathy, thus giving us hope of finding new monitoring techniques.

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# Validation of in silico prediction by in vitro immunoserological results of fine epitope mapping on citrate synthase specific autoantibodies

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**Abstract**

In silico antibody–antigen binding predictions are generally employed in research to rationalize epitope development. These techniques are widely spread despite their technical limitations. To validate the results of these bioinformatic calculations evidence based comparative in vitro studies are necessary.

We have used a well-conserved mitochondrial inner membrane antigen–citrate synthase to develop a model for comparative analysis of the predicted and the immunoserologically verified epitopes of circulating autoantibodies. Epitopes were predicted using accepted tools: the *GCG Wisconsin* package and *TEPITOPE 2000*. An overlapping multipin ELISA assay – covering 49% of the citrate synthase molecule – was developed to map autoantibody epitopes of individuals (healthy, systemic autoimmune, and heart transplanted) in different immunopathological conditions.

From the 40 synthesized decapeptides 34 were predicted in silico and 27 were validated in vitro. Thirty-two percent of epitopes were recognized by majority of sera 47% by at least one sera. False positive predictions were 21%. There was major difference in the recognized epitope pattern under different immunopathological conditions.

Our results suggest that special databases are needed for training and weighing prediction methods by clinically well-characterized samples, due to the differences in the immune response under different health status. The development of these special algorithms needs a new approach. A high number of samples under these special immunological conditions are to be mapped and then used for the "fine tuning" of different prediction algorithms.
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*Keywords:* Autoimmunity; In silico epitope prediction; Autoantibody; Citrate synthase; Multipin ELISA; Immunoserology; Heart transplantation

## 1. Introduction

The diagnosis of autoimmune diseases shows an increasing tendency in highly developed countries and causes growing socio-economic problems. We have no specific therapy or prevention strategy against them, and there is no common theory for their pathomechanism. Detailed analysis of the targeted epitopes by autoreactive T-cells and autoantibodies has an outstanding importance in immunopathological research and development as well as in the design of novel diagnostic tools, therapeutic drugs or vaccines (Laver et al., 1990;

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Sturiniolo et al., 1999; van Regenmortel, 2000; Németh et al., 2004).

Efficient fine molecular mapping techniques are required for the better understanding of the relation between the biomolecules' structure and functions (Pellequer et al., 1991). State-of-the art microanalytical techniques are able to analyze distinct submolecular characters and combine them with biological functions; however, the extended applications of conventional laboratory methods are time and resource consuming. The development of computation power and algorithms to analyze biological data increased the efficiency of laboratory research and diagnostics during the last decade, and by the use of in silico research tools, the development of drugs or vaccines became more efficient and cost effective (van Regenmortel and de Marcillac, 1988; Peri et al., 2001).

Various algorithms are available for calculation of immunogenity index and predicting epitopes on antigens for further immunological studies (Hopp, 1986; van Regenmortel, 2000). Most of these techniques involve calculations based on scales of values for the twenty amino acids reflecting their likelihood of occurrence at the surface of proteins or as part of secondary structures, such as helices or beta-bends. The most successful of these algorithms use scales related to the water solubility of the individual amino acids. In particular, the highest success rates are obtained by using hydrophilicity scales that emphasize the charged and polar amino acids, but are not overly selective for either positive or negative charges. Such a method can correctly pinpoint major antigenic sites on the proteins of most well-characterized infectious organisms (Hopp, 1986).

Drug design – including vaccine development – needs efficient assistance in selecting the possible candidate molecules for novel and improved drugs in all fields of treatment and in the prevention of infectious diseases caused by resistant microorganisms, the hazard of bio-terrorism, or bio-therapies for cancer, organ transplantation as well as autoimmune diseases (Cohen and Young, 1991; Schawarz and Cohen, 2000; Németh et al., 2004). By predicting epitope one would be able to develop specific monoclonal antibodies with short peptide immunization raising antibodies cheaper and quicker (van Regenmortel, 1986; van Regenmortel, 1989; Schwede et al., 2000).

The genome-based vaccine research opened new potential issues for the selection of the targeted antigens using in silico predictions for analysis of the genomes and using set of recombinant techniques to express the potential antigen fragments. (Serruto et al., 2004; Masignani et al., 2004) Laboratory diagnostics, drug design and vaccine developments are intensively growing areas of current medical biotechnology where computing techniques play an essential role (Guex et al., 1999; Andersen et al., 2000; Masignani et al., 2004). However, the evidence based verification of these bioinformatically predicted epitopes targeted by autoantibodies has been the focus of recent studies only (Peri et al., 2001).

Our major goal was to compare and evaluate the data of in silico predictions with the results of in vitro immunoserological measurements in a well-characterized model: fine epitope specificity of circulating autoantibodies was determined and analyzed on the mitochondrial citrate synthase (CS) enzyme to assess the value of predicted and immunoserologically verified epitopes under normal and different pathological conditions.

## 2. Materials and methods

### 2.1. B-cell epitope prediction

The human mitochondrial CS sequence (EC 2.3.3.1; GenBank accession no. PIDO75390; Goldenthal et al., 1998) used for prediction analysis was obtained from the protein sequence database (Benson et al., 1999; http://www.ncbi.nlm.nih.gov/Entrez/index.html).

To localize the potentially antigenic sites, we have predicted β-turns of CS protein segments using the Chou-Fasman secondary structure prediction method (Chou and Fasman, 1978; Prevelige and Fasman, 1990) and hydrophobicity predictions by Eisenberg et al. (1990). In both cases, windows of seven amino acids were used in MS Excel applying the original matrices of the authors. Segments with high probability of β-turn secondary structure ($P_{\beta\text{-turn}} > 1$) and low probability of hydrophobicity ($P_{\text{hydrophobicity}} < 0$) were selected. We also used the *PepPlot, PeptideStructure* and *PlotStructure* programs of the *GCG Wisconsin Package* (http://www.gcg.com) for surface probability ($P_{\text{surface}} > 1$), Jameson-Wolf antigenity index ($P_{\text{JW antigenity}} > 1$) (Jameson and Wolf, 1988) and hydrophilic regions ($P_{\text{HW hidrophilicity}} > 1$) (Hopp and Woods, 1981) to fine tune our prediction results.

Swiss-Model Automated Protein Modelling Server (http://www.embl-heidelberg.de/predictprotein) was used to obtain a 3D model of human CS, based on homology *E. coli* CS (EC 4.1.3.7, Brookhaven Protein Data Bank No. 1K3P; Nguyen et al., 2001) (Peitsch, 1996; Guex et al., 1999; Berman et al., 2000; Schwede et al., 2000; Peitsch, 2002).

### 2.2. T-cell epitope prediction

T-cell epitope prediction was done by a virtual matrixes method formed by assigning and combining pocket-specific quantitative binding values derived from one HLA allele to other alleles via HLA sequence comparison, using the *TEPITOPE 2000* (Juergern Hammer, Vaccinome 1999) program (Hammer et al., 1994; Sturiniolo et al., 1999). Scanning was performed with all the 25 HLA-II alleles available in the program (DRB1*0101; DRB1*0102; DRB1*0301; DRB*0401; DRB1*0402; DRB1*0404; DRB1*0410; DRB1*0421; DRB1*0701; DRB1*0801; DRB1*0802; DRB1*0804; DRB1*0806; DRB1*1101; DRB1*1104; DRB1*1106; DRB1*1107; DRB1*1305; DRB1*1307; DRB1*1311;

DRB1*1321; DRB1*1501; DRB1*1502; DRB1*0101). HLA binding pockets were weighed evenly. A 1% threshold value was used to select the high specificity probable epitopes.

### 2.3. Serum samples

Serum samples, from healthy individuals collected during a 5-year period (63 healthy Hungarian, 51 healthy British and 176 healthy Finnish adult blood-donors, and 44 serum samples from healthy infants), samples of patients with systemic autoimmune diseases (326 clinically well-documented cases) and sera of 52 heart transplant patients were analyzed. Serum samples were stored at −70 °C before examination. The age and sex ratios of patient groups were matched.

### 2.4. Immunoserological detection of anti-CS autoantibodies by ELISA

An indirect ELISA was used to detect anti-CS autoantibodies from human sera: CS from porcine heart (Sigma) as antigen was incubated for 12 h at +4 °C followed by 60 min at 37 °C on 96-well polystyrene plates (NUNC, France). Following the saturation of non-specific binding sites with 0.5% gelatine (Sigma, USA) in PBS (pH 7.3), three washing steps with PBS containing 0.1% Tween 20 (Sigma, USA) were performed. The triplicated samples were incubated at 1:100 dilutions (diluted in washing buffer) for 60 min. Finally, the plate was incubated with anti-human IgG-HRPO or anti-human IgM HRPO secondary antibodies (Dako, Denmark) for 60 min. The color reaction was developed with *o*-phenylenediamine (Sigma, USA), and measured on Dynatech MR7000 (USA) microphotometer at $\lambda = 492$ nm (OD, optical density). The results were compared to controls containing no CS but all the other components, no primary sera, and no secondary anti-human IgG-HRPO or anti-human IgM HRPO.

### 2.5. Multipin peptide synthesis

Forty decapeptides overlapping five amino acid residues were synthesized, corresponding to the nine regions predicted, on β-alanyl-glycine functionalized polyethylene pins (Mimotopes, Australia) with Fmoc/tBu chemistry according to Geysen's method (Geysen et al., 1984). We used $^t$Bu (Thr, Ser, Tyr), O$^t$Bu (Asp, Glu), Trt (Cys, His, Gln, Asn), Pbf (Arg) and Boc (Lys, Trp) as side chain protecting groups. Briefly, the Fmoc α-amino protecting group was removed with 20% piperidine/DMF (v/v). Coupling was performed with DIC/HOBt methodology and monitored with bromophenol blue added to the coupling mixture (Krchnák et al., 1988). Peptides were acetylated at the *N*-terminus, then the side chain protecting groups were removed with TFA – EDT – anisole 38:1:1 (v/v/v), but the unprotected peptides remained covalently attached to the pins. Two control peptides were also prepared: PLAQGGGGGG and GLAQGGGGGG.

### 2.6. Immunoserological epitope mapping of anti-CS autoantibodies using overlapping decapeptides in multipin system

Ninety-six-well polystyrene ELISA plates (NUNC, France) and pin-bound peptides were the hardware of epitope mapping. All steps were preformed with agitation. First, we kept the pins in PBS containing 0.5% gelatine for 60 min at room temperature for saturation of non-specific binding sites. Then the pins were washed three times with PBS containing 0.1% Tween 20. The serum samples and controls were added to the plates in the dilution of 1:100 (diluted in PBS containing 0.5% gelatin and 0.1% Tween-20). After 60 min of incubation, the pins were washed again for 3 × 10 min with PBS containing 0.1% Tween-20. Finally, pins were incubated with anti-human IgG-HRPO or anti-human IgM HRPO secondary antibodies for 60 min at room temperature. After washing (3 × 10 min) the color reaction was developed with *o*-phenylenediamine and measured on iEMS MF micro photometer (ThermoLabsystem, Finnland) at 492 nm (OD). The data were analyzed with Ascent Software V.2.4.2 (ThermoLabsystem, Finnland).

### 2.7. Homology searches

In addition to the antibody binding experiments, we have compared the sequence of epitopes of human CS with that of proteins present in the non-redundant protein database by BLASTP2 program. (Expect = 20,000; Matrix PAM30; Gap cost Existence: 9 Extension: 1; Word size = 2, database size as of September 2004 = 688,443,072.) Immunologically relevant hits showed homology with proven or hypothetical CS, but only negligible similarity was found between CS and non-CS proteins (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

## 3. Results

The complete epitope mapping of the well-conserved mitochondrial inner membrane CS enzyme using autoantibodies obtained under normal and pathological conditions was made, and the results of in silico antigenity prediction with the in vitro immunoserological results were compared.

### 3.1. Results of B-cell epitope prediction

Assuming that antibody epitopes are usually found at or near to hydrophilic β-turn parts of proteins, fifteen segments with high probability of β-turn secondary structure ($P_{\beta\text{-turn}} > 1$) and low probability of hydrophobicity ($P_{\text{hydrophobicity}} < 0$) were considered for selection. These segments cover the following sequences: 51–78, 85–91, 103–119, 127–132, 138–161, 176–195, 217–239, 244–250, 261–269, 281–285, 302–309, 320–331, 354–364, 390–398, 409–414, 448–463 (Fig. 1).

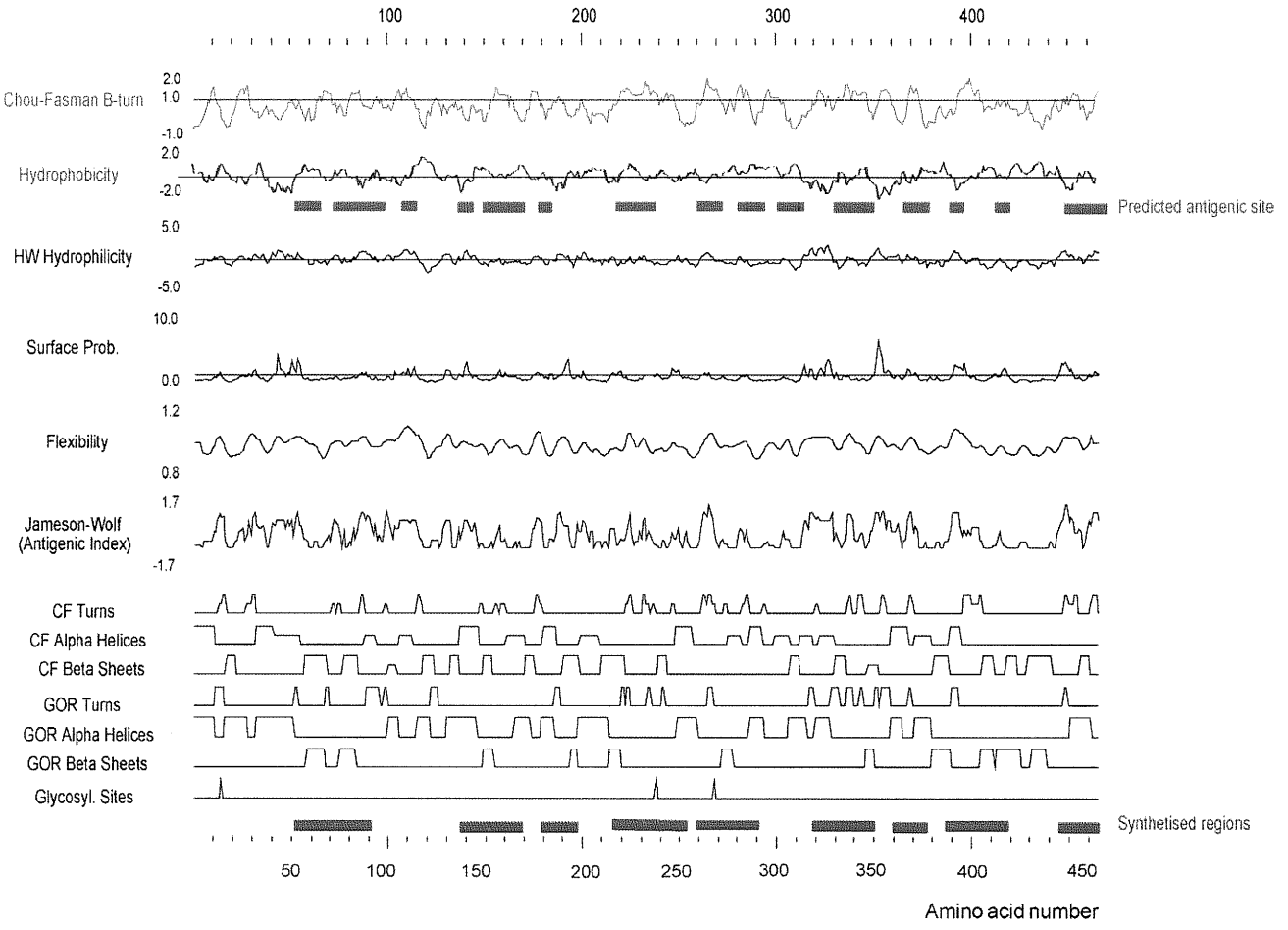


Fig. 1. The analysis of CS by the most commonly used prediction methods for immunogenity are plotted in the figure. Chou-Fasman β-turn prediction (purple; $P_{\beta\text{-turn}} > 1$), Hydrophobicity (green; $P_{\text{hydrophobicity}} < 0$); predicted antigenic sites (blue) and synthesized overlapping sequence used in vitro (red).

Considering the results of 3D homology modeling, two of the fifteen segments were rejected. Regions 103–119 and 302–309 had only very limited or no accessibility for antibody binding, as they are located not on the surface of the molecule (Fig. 2). Based on scores derived from the prediction and modeling results, the following nine segments were selected for further studies: 51–90 (I), 124–163 (II), 176–195 (III), 216–250 (IV), 261–285 (V), 320–339 (VI), 354–373 (VII), 390–414 (VIII), and 447–466 (IX). For immunoserological validation of predicted epitopes, a multipin system with set of overlapping decapeptides covering regions I–IX were prepared by solid phase chemistry (Table 1).

### 3.2. Results of T-cell epitope prediction

Amino acid sequences 18–29, 35–88, 92–115, 119–131, 139–165, 195–237, 254–266, 306–319, 361–393, and 406–446 were predicted by binding to at least one of virtual HLA allele. Nine segments, showing binding potential to more than 10 virtual HLA alleles were regarded as highly potential for T-cell epitopes (18–29, 57–83, 91–103, 119–131, 201–215, 254–266, 306–319, 373–393 and 415–427). 3D modeling showed that two of these regions are located on α-helices positioned in the inner part of the protein (Fig. 2). It is interesting to note, that four of the nine segments partly overlap with the predicted B-cell epitope regions: 57–60, 57–79, 255–266, 306–319, and 415–427. One of these overlapping sequences (306–319) is located also in the intramolecular space. Three T-cell predicted highly potential sequences on the surface of the molecule were just adjacent to predicted B-cell epitopes (T|B: 91–103|103–119, 119–131|103–119, 415–427|409–414).

### 3.3. Results of ELISA

Sera having an OD value at least two standard deviation (2 S.D.) higher than the average of measured OD of sera in the group were selected as positive. The positive anti-CS IgG cases were 2–4% of all subgroups, except among heart transplant patients, where 7% of all cases were positive. IgM sera were positive in 7–12% of all cases, except the autoimmune patients, where 24% of all cases were positive

Table 1
In silico selected regions and synthetic overlapping decapeptides used for studies in the multipin system

```
I.
(51) FRQQHGKTVVGQITVDMMYGGMRGMKGLVYETSVLDPDEG(90)
     FRQQHGKTVV            KMRGMKGLVY
          GKTVVGQITV            EGLVYETSVL
               GQITVDMMYG            ETSVLDPDEG
                    DMMYGGMRGM

II.
(124) VTGCIPTEEQVSWLSKEWAKRAALPSHVVTMLDNFPTNLH(163)
     VTGCIPTEEQ            RAALPSHVVT
          PTEEQVSWLS            SHVVTMLDNF
               VSWLSKEWAK            MLDNFPTNLH
                    KEWAKRAALP

III.
(176) NSESNFAQAYARGISRTKYW(195)
     NSESNFAQAY
          FAQAYARGIS
               ARGISRTKYW

IV.
(216) IYRNLYWEGSGIGAIDSNLDWSHNFTNMLGYTDHQ(250)
     IYRNLYWEGS            WSHNFTNMLG
          YWEGSGIGAI            TNMLGYTDHQ
               GIGAIDSNLD
                    DSNLDWSHNF

V.
(261) IHSDHEGGNVSAHTSHLVGSALSDP(285)
     IHSDHEGGNV
          EGGNVSAHTS
               SAHTSHLVGS
                    HLVGSALSDP

VI.
(320) GKDVSDEKLRDYIWNTLNSG(339)
     GKDVSDEKLR
          DEKLRDYIWN
               DYIWNTLNSG

VII.
(354) DPRYTCQREFALKHLPNDPM(373)
     DPRYTCQREF
          CQREFALKHL
               ALKHLPNDPM

VIII.
(390) EQGKAKNPWPNVDAHSGVLLQYYGM(414)
     EQGKAKNPWP
          KNPWPNVDAH
               NVDAHSGVLL
                    SGVLLQYYGM

IX.
(447) ERPKSMSTEGLMKFVDSKSG(466)
     ERPKSMSTEG
          MSTEGLMKFV
               LMKFVDSKSG
```

Light-shaded background in the synthesized region shows predicted epitopes. Dark-shaded background in decapeptide sequence shows amino acids differing from the selected regions, due to the limitations of the multpin system. *Italics* represents the possible HLA-binding sites.

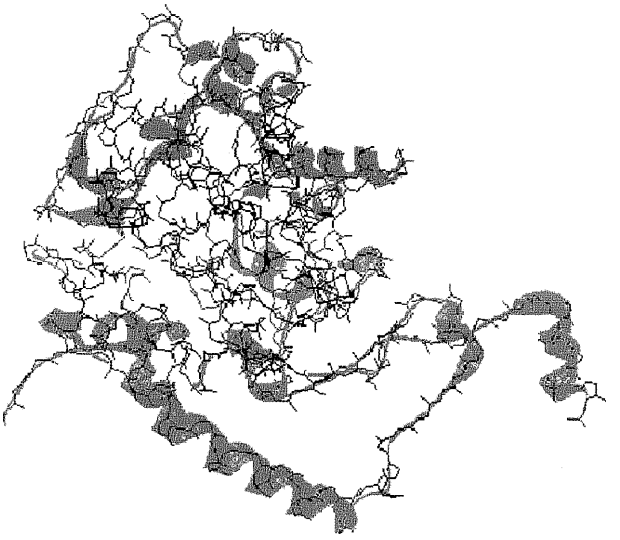

Fig. 2. Location of predicted linear B-cell epitopes on 3D homology model of mammalian CS. Based on Prevelige-Fasman prediction and hydrophobicity calculations, sixteen regions of different length, 4–23 amino acid residues were selected (backbone in red). Potentially antigenic sites according to the tertiary structure model are represented (green ribbons).

(Table 2). Serum samples found positive during the simple binding ELISA test were used for the further epitope mapping.

### 3.4. *Immunoserological epitope mapping in multipin system*

Results of epitope mapping are summarized in Table 3. Major differences were found in the recognition pattern of different types of sera. Differences in epitope pattern of healthy and heart-transplanted patients have been published recently (Petrohai et al., 2005). The results of detailed immunopathological analysis on the differences between the results of the autoimmune and healthy samples are under publication (Czömpöly et al., under publication).

The examined 40 decapeptides (synthetic epitope) covered 49% of the whole CS molecule. From 40 decapeptides 23 (58%) were recognized by more than one group of sera; 8 (19%) were recognized only by one group of sera, while 9 (23%) did not had any recognition.

From the 40 synthetic decapeptides 34 were predicted in silico for good immunological recognition, and 27 (79%) of

Table 2
Distribution of anti-citrate synthase positive sera

| Description of group (no. of serum samples) | IgG (%) | IgM (%) |
|---|---|---|
| Hungarian blood-donors (63) | 3 | 10 |
| British blood-donors (51) | 4 | 12 |
| Finnish blood-donors (176) | 3 | 9 |
| Healthy infants (44) | 2 | 7 |
| Systemic autoimmune patients (326) | 3 | *24* |
| Heart transplanted patients (52) | 7 | 12 |

Table 3
Epitopes recognized by IgG and IgM autoantibodies of different groups of patient sera

| Predicted region | AA position | Decapeptide | IgG healthy | IgM healthy | IgG autoimmune | IgM autoimmune | IgG transplanted | IgM transplanted |
|---|---|---|---|---|---|---|---|---|
| I (51-91) | 51-60 | FRQQHGKTVV | + | + | + | + | + | |
| | 56-65 | GKTVVGQITV | + | + | | | | |
| | 61-70 | DQITVDMMYG | | | | + | | |
| | 66-75 | GMMYGGMRGM | + | + | + | | | ++ |
| | 71-80 | KMRGMKGLVY | + | | + | | | |
| | 76-85 | EGLVYETSVL | | | | | | |
| | 86-91 | DTSVLDFDEG | | | | | | |
| II (124-163) | 124-133 | VTGCIPTEEQ | | | | ++ | | |
| | 129-138 | PTEEQVSWLS | | | | +++ | | |
| | 134-143 | VSWLSKEWAK | + | ++ | ++ | +++ | ++ | +++ |
| | 139-148 | KEWAKRAALP | + | + | ++ | + | +++ | |
| | 144-153 | RAALPSHVVT | + | + | + | + | + | ++ |
| | 149-158 | SHVVTMLDNF | | + | + | | + | + |
| | 154-163 | MLDNFPTNLH | | | | | ++ | + |
| III (176-195) | 176-185 | NSESNFAQAY | | | | | | |
| | 181-190 | FAQAYARGIS | | + | + | ++ | | ++ |
| | 186-195 | ARGISRTKYW | + | +++ | +++ | + | ++ | |
| IV (216-250) | 216-225 | IYRNLYWEGS | + | +++ | + | +++ | +++ | ++ |
| | 221-230 | YWEGSGIGAI | + | + | + | +++ | +++ | + |
| | 226-235 | GIGAIDSNLD | | | | + | ++ | |
| | 231-240 | DSNLDWSHNF | | | | + | | |
| | 236-245 | WSHNFTNMLG | + | + | | +++ | + | +++ |
| | 241-250 | TNMLGYTDHQ | | + | | | | + |
| V (261-285) | 261-270 | IHSDHEGGNV | | | | | | |
| | 266-275 | EGGNVSAHTS | | | | | | |
| | 271-280 | SAHTSHLVGS | | + | | | + | ++ |
| | 276-285 | HLVGSALSDP | | + | | | | |
| VI (320-339) | 320-329 | GKDVSDEKLR | | | | | | |
| | 325-334 | DEKLRDYIWN | | | | + | | |
| | 330-339 | DYIWNTLNSG | | + | + | +++ | ++ | |
| VII (354-373) | 354-363 | DPRYTCQREF | | + | + | +++ | | + |
| | 359-368 | CQREFALKHL | + | | + | ++ | + | |
| | 364-373 | ALKHLPNDPM | | | | + | | |
| VIII (390-414) | 390-399 | EQGKAKNPWP | | | | | | |
| | 395-404 | KNPWPNVDAH | | | | | | + |
| | 400-409 | NVDAHSGVLL | | | | | | |
| | 405-414 | SGVLLQYYTV | | | | + | | |
| IX (447-466) | 447-456 | ERPKSMSTEG | | | + | | | |
| | 452-461 | MSTEGLMKFV | | | | | | |
| | 457-466 | LMKFVDSKSG | + | | + | | | |

Shaded background represents predicted epitopes. (AA: amino acid; +: 0%–25%, ++: 25%–50% and +++: 50%–100% of sera bind to the given decapeptide.)

them were confirmed by immunoserological measurements. Eleven decapeptides (32%) were recognized by the majority of sera, 16 (47%) by at least one. Seven (21%) decapeptides were predicted in silico but not validated in vitro. There were six peptides, which were not predicted but synthesized, three were not and three were recognized by autoantibodies. One unpredicted synthetic peptide (330–339 amino acid sequence) had strong reactivity with the different groups of sera (Table 3.).

The results of the homology searches of the in silico predicted and in vitro analyzed epitopes are presented in Table 4. The comparison of binding data obtained from ELISA experiments and scores from this analysis clearly show that all peptides recognized by autoantibodies possess high score hits to the conserved parts of CS first, and some well-conserved – mostly bacterial – hits were found as well.

## 4. Discussion

To make a difference between "physiological" and "pathological" self-reacting antibodies is not only a theoretically relevant question, but it has major clinical consequences in diagnosis, treatment, monitoring, and prevention of autoimmune diseases. Factors regulating and determining the tolerance of the most important self-structures are studied intensely. The postulation of "immunological homunculus theory" opened new aspects in autoimmunity research during the last decade (Cohen and Young, 1991; Cohen, 1992; Schawarz and Cohen, 2000). Several molecular biological and immune physiological studies were initiated based on this theory, however, the role of physiological autoimmunity in the active tolerance and in the occurrence of different autoimmune diseases remain mostly unclear. Only complex studies

Table 4
Homology search results. All subsequences recognized by autoantibodies possess high score hits to the conserved parts of CS first, and some well-conserved – mostly bacterial – hits were found as well

| CS subsequences | Description |
|---|---|
| 51–78 | Conserved CS |
| 51–60 | Conserved CS, DNA-damage protein |
| 61–71 | Conserved CS, Ser-protease inhibitor, Enzymes |
| 71–79 | Conserved CS |
| 85–91 | Conserved CS |
| 127–132 | Conserved CS, bunch of bacterial proteins |
| 138–161 | Conserved CS, DNA-damage dependent protein, DNA-repair protein |
| 176–195 | Conserved CS |
| 187–197 | Conserved CS, tRNA-synthase (bact, Ricketsia, Neiseria, etc.), P1 protein (bacterial, Pseudomonas) |
| 217–239 | Conserved CS, bacterial proteins |
| 221–231 | Conserved CS, several bacterial proteins |
| 236–245 | Conserved CS, NADH-dehidrogenase |
| 241–250 | Conserved CS |
| 261–270 | Conserved CS |
| 280–285 | Conserved CS, conserved enzymes |
| 320–329 | Conserved enzymes |
| 330–339 | Conserved CS, conserved enzymes |
| 354–363 | Conserved CS, conserved enzymes |
| 359–368 | Conserved CS |
| 364–373 | Conserved enzymes |
| 390–404 | Conserved CS |
| 405–414 | Conserved CS |
| 448–463 | Conserved CS |

using molecular and cellular biological experiments and clinical epidemiological surveys are able to clarify the alteration in the most important regulatory pathways of autoimmunity.

Analyzing the fine molecular structures of the targeted epitopes is important both for the prognostic values and for the further therapeutic consequences in different autoimmune diseases. In our experiments, we investigated the immunoreactivity of a genetically conserved antigen: CS is one of the earliest proteins with basic biological functions in the citric acid cycle and subsequent ATP generation. Autoantibody production against this antigen was analyzed in healthy individuals, autoimmune, and heart transplant patients during our previous studies. Remarkable evidences were found for differences in epitope pattern and the participation of anti-CS autoantibodies in the formation of physiological and pathological autoimmune reactions (Németh et al., 2004; Petrohai et al., 2005; Czömpöly et al., under publication). However, the results of the in silico epitope predictions performed earlier were not fitting to the measured data in some cases. This finding initiated a systematic comparative analysis between in silico predictions and in vitro immunoserological measurements. The CS study was an appropriate model for the validation of bioinformatic predictions.

The verification of bioinformatically predicted epitopes could be achieved by the use of synthetic peptides and appropriate antibodies. An overlapping peptide library was created and applied according to our prediction data.

Theoretically, the entire accessible surface of a molecule may be regarded as a large number of overlapping epitopes of a size of roughly 700 $Å^2$ (Benjamin et al., 1984). Not all of these potential epitopes are relevant immunologically. The immunological recognition and the antigen specific responses depend on the recipient immune system and on the antigen's actual conformation (Attasi, 1984). Most of the epitopes are assembled or conformational, only some of them are continuous ones (van Regenmortel and de Marcillac, 1988; van Regenmortel, 1989). The most precise way of identifying an epitope is either X-ray crystallography or NMR of the given peptide and antibody bound to it. Both approaches have their technical limitations as they require specific criteria for deciding which residues of the antigen are in contact with the paratope and are functionally part of the epitope (van Regenmortel, 1989).

The limitations in computational power, our knowledge of epitope structure of proteins and the paratope–epitope binding as well as the lack of reliable tertiary structure data of proteins focused the attention on epitope prediction from primary structure of proteins (van Regenmortel, 2000). These algorithms have the limitation of predicting potential continuous linear epitopes only, which certainly will not represent all the recognized immunologically active epitopes of an individual in its given state.

Short synthetic peptide sequences are suitable tools for exploring the molecular subregions (epitopes) of larger proteins recognized by antibodies (Lerner, 1982; Lerner, 1984).

The multipin overlapping peptide synthesis combined with ELISA is an ideal way of searching for continuous linear epitopes. This 96-position array format provides a simple and cost effective entry point into multiple parallel organic syntheses as our CS experiment. The chosen 10 amino acid long peptides are suitable for simultaneous synthesis of a large number of compounds without the requirement for complex filtration systems. These decapeptides are able to mimic the linear parts of the original protein (Maeji et al., 1995).

For the majority of proteins known today, the only available structural information is their primary amino acid sequence, usually deduced from their nucleotide sequence of the corresponding gene. Many attempts were made to utilize this information to predict the position of continuous epitopes. Parameters such as hydrophilicity, mobility and accessibility of short segments of polypeptide chains were been correlated with the location of continuous epitopes in a few well-characterized proteins. This led to the development of several prediction scales to locate continuous epitopes (van Regenmortel, 1986; Hopp, 1986). For our study, we had chosen some well-proven, widely accepted and available methods, collected in the *GCG Wisconsin Package*.

The Chou-Fasman secondary structure prediction method is an accepted approach, which proved to be 75% efficient in prediction of continuous epitopes (Hopp, 1986; Prevelige and Fasman, 1990). The Hopp and Woods hydrophilicity approach is one of the most efficient methods by predicting up to 100% of epitopes among certain conditions (Hopp

and Woods, 1981; Hopp, 1986; Pellequer et al., 1991). The antigenity index, proposed by Jameson-Wolf, combines profiles of hydrophilicity, accessibility, flexibility and secondary structure. This method is able to predict continuous epitopes with 90% probability (Jameson and Wolf, 1988; Thornton et al., 1986).

Advances in understanding antigen presentation at the molecular level accelerated the development of computer models capable of predicting T-cell epitopes (Hammer et al., 1994; Honeyman et al., 1998; Andersen et al., 2000). Interaction of peptide fragments with HLA molecules was characterized thoroughly. HLA peptide binding is proved to be the major bottleneck in the selection of epitopes, as indicated by the finding that most peptide sequences lack the capacity to interact with HLA molecules. The T-cell epitope prediction approach with virtual HLA II matrices incorporated into *TEPITOPE*, like quantitative matrices, provides a detailed model in which the contributions to binding of each amino acid (ligand) with each pocket/position (HLA binding cleft). *TEPITOPE* proved to be capable of recognizing a wide variety of HLA II epitopes (Hammer et al., 1994; Sturiniolo et al., 1999; de Groth and Rothman, 1999).

The in silico analysis coincided frequently, but not always, with the immunoserologically determined epitopes. Synthesized regions covered 49% of CS, nearly the whole immunologically accessible region of the molecule. Individual sera groups recognized 32–50% of all predicted epitopes with remarkable differences between the healthy, autoimmune or heart transplanted groups. Antibodies of various groups of sera recognized 79% of predicted epitopes. This is comparable to the literature, where 60–100% of all epitopes are identified by in silico methods (Hopp, 1986; Pellequer et al., 1991). Predictions under different immunological conditions (individual sera groups) differ from the ideal data in the literature. This is because, methods currently employed in attempts to identify antigenic determinants and other features of proteins are currently trained under "healthy" immunological conditions, and tuned for recognizing the immunologically possible epitopes. From the 34 predicted epitopes, 27 were validated immunoserologically. There were seven false positive predictions (these were predicted but not validated), i.e. 21% of all predicted epitopes. In the detailed analysis of individual sera groups this incidence increases drastically to 50–68%. The variability between the analyzed groups can be explained by the differences in the immune response under different health status. The immunological conditions of the host immune system can determine the recognition pattern of the selected epitopes.

Our results suggest that special databases are needed for training and weighing prediction methods by clinically well-characterized samples. The development of these special algorithms needs a new approach. A high number of samples under these special immunological conditions are to be mapped and then used for the "fine tuning" of different prediction algorithms. This can introduce a new era of post-prediction in bioinformatics.

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# The C-terminal sequence (aa79-117) of HBxAg plays key role in the immune response shown by fine epitope mapping and bio-statistical analysis

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## Abstract

The pathogenetic significance of Hepatitis B virus x-protein (HBxAg) in chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma has been studied recently, but the comparative analysis of both the actual serum HBxAg levels and antibody production against various HBx epitopes are less studied. We investigated the relationship between anti-HBxAg antibody production (IgG and IgM) and HBxAg level in sera of patients with acute (14) or chronic hepatitis (80) and symptomless carriers (12) using overlapping recombinant and synthetic antigens and a sandwich type ELISA for HBxAg. IgM dominance was found against the whole C terminal region of the HBx molecule, however, a strong IgG response localized to a characteristic C-terminal region (C1: 79-117) in parallel with a low antibody response against the N-terminal part of the HBxAg in acute hepatitis. Correlations were found between the HBxAg concentrations and the anti-HBx-specific antibody (mostly IgG) levels in chronic hepatitis. The pathophysiological role of C1 sequence is described. Our results suggest a "casual" immune response against this epitope in acute hepatitis. The significant difference between the IgG levels of chronic hepatitis patients and symptomless chronic carriers might indicate that HBxAg-specific ADCC possesses pathogenetic role in the development of chronic hepatitis.

***Key Words:*** Hepatitis B virus, HBxAg, immunoserology, epitope mapping, antibody response

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## Introduction

Hepatitis B virus (HBV) infection causes severe and permanent public health problems world wide, the incidence is approximately 4-5 % within the population with strong geographical differences. Chronic hepatitis, liver cirrhosis and primary hepatocellular carcinoma cause more than half a million deaths every year according to different morbidity and mortality surveys[2,10,15,20]. The perinatal transmission is close to 50% and may result in lifelong symptomless carrier state with high epidemiological risk[17].

The HBV has one of the shortest viral genomes and its virus cycle unique as well. The X gene is the smallest coding unit of the HBV genome which encodes for a 17 KD polypeptide (pX) of 154 – mostly hydrophobic - amino acids, and this product is known as the HBx antigen (HBxAg)[3,5,8,23]. Different epitopes of the HBx antigen have various biological functions[14,22]. Several experimental data reflect the basic importance of the HBxAg in the development of chronic hepatitis, liver cirrhosis and primary hepatocellular carcinoma. The pathogenetic role of different epitopes in disease formation and in the antiviral immune response is intensively studied[5,6,14,15,20,21,22]. However, no comparative analysis has been performed of the serum HBxAg level and the specific antibody production against different epitopes of the antigen by technical difficulties.

Our study is based on a previously developed sandwich type ELISA for quantitative determination of serum HBxAg level, and we constructed an overlapping epitope library for epitope mapping of the reacting antibodies[17]. The hidden correlations between the isotype of specific antibody response against the various epitopes of HBxAg and the serum concentration of the antigen were analyzed by bio-statistical methods. We investigated the sera of patients with acute and chronic hepatitis and symptomless carriers. Significant differences were found in both the targeted epitopes and the isotype of the reacting antibodies among the groups of patients. A well-characterized C terminal part of the HBxAg was found to be the specific target of the IgG type antibody response.

## Materials and Methods

*Antigens*

Recombinant HBxAg overlapping fragments and synthetic peptide fragments were used for the development of the assay.

*1. Construction of HBx recombinant plasmids*

1.1. Recombinant X antigen (10-143. amino acid):

The development of the recombinant construct of HBxAg was described previously[11]. Briefly, the truncated X gene was obtained by cutting of the pHB320 plasmid with *BamHI*-*Fsp*I restriction enzymes and cloned via the pHSG 395 vector into *BamHI*-*Sma*I restriction sites of the fusion-expression vector pGEX-3X (Amersham Pharmacia Biotech), creating the pGEX-3XXBF recombinant plasmid. The X protein is truncated by nine and eleven amino acids at the N- and C-termini, respectively. According to the cloning strategy, the truncated X protein is flanked by five amino acids at the N-terminus and by four amino acids at the C-terminal, derived from the pHSG 395 and pGEX-3X plasmids, respectively.

1.2. N terminal fragment (10-90 amino acid) of HBxAg:

The original plasmid[1] (pHB320) containing the HBV DNA subtype *ayw* was used to subclone the entire HBx coding region between the *Nco*I site (position 1370 on the HBV genome) and *Bgl*II site (position 1986) into the pGEX-3X vector (Amersham Pharmacia Biotech) producing the pGEX-3XX recombinant plasmid. Clones containing only the N-terminal portion of the HBx coding region were obtained by cutting the pGEX-3XX recombinant plasmid with *Ava*I (position 1461 on HBV genome) and *EcoR*I (linker sequence

of the vector), blunt-ending with Klenow fragment of DNA polymerase I followed by ligation.

1.3. C terminal overlapping fragments:

The pGEX-3XX plasmid was used to produce the recombinant overlapping HBx fragment. Three overlapping parts of the X gene were amplified by PCR using primers with *BamHI* and *EcoRI* restriction sites we published previously[18]. The fragments were cloned into the pGEX-6P-1 expression vector (Amersham Pharmacia Biotech). Each construct was verified by sequencing.

The recombinant plasmids were transformed into *E. coli* strain DH5α, induced with isopropyl-thio-B-D-galactopyranoside (IPTG) and the recombinant fusion proteins were purified on glutathione-S transferase resin according to Marczinovits[11].

The **Figure 1.** summarizes the location of the analyzed HBxAg fragments on the recombinant and synthetic protein.

*2. Synthetic peptide antigens*

Peptides of 13-26 amino acids were synthesized using fmoc chemistry and purified by HPLC using routine techniques at the Department of Chemistry, Faculty of Medicine, University of Szeged, Hungary.

***Serum samples***

All sera were obtained from the laboratory services of the First Department of Medicine, Medical Center of the University of Pécs and the Division of Virology, Bela Johan National Center for Epidemiology, Budapest, as residual samples after completion of all laboratory testing. Results obtained with the sera of patients suffering from 14 HBV positive acute and 80 chronic hepatitis and those taken from 12 healthy HBV carriers were compared with 22 healthy - HBV negative - serum samples. The sera were pre-tested for HBV positivity by HBsAg and HBcAg specific ELISA (Dialab, Hungary).

Low pH pretreatment was used in serum samples before the immunoassays to decompose the immunocomplexes and the non-specific aggregates present in the frozen sera stored at –20°C. GST-HBxAg standard and serum samples were diluted in glycine pH 2.0 (10 min 37 °C) than restored to pH 8.0 in Tris-base to final dilution of 1:10.

*Construction of Sandwich type ELISA*

Assay conditions were optimized by checkerboard titration of recombinant HBxAg-GST standard. A microtitre plate (Nunc, France) was coated with 100µl of mouse monoclonal anti-HBx (3F6/G10) antibody at a concentration of 10 µg/ml in 0.05 M bicarbonate buffer, pH 9.6. at 4°C overnight, followed by 60 min incubation at 37 C°. Following repeated washing with 0.15 M phosphate-buffered saline (PBS) non-specific binding sites were blocked by adding of 1% gelatin-PBS (300 µl) at 37°C for one hour. Then, the plate was washed four times with PBS-Tween 20 (PBST). Following the last wash, GST-HBxAg standard and serum samples were diluted in glycine buffer at pH 2.0 (10 min 37 °C) and then restored to pH 8.0 by adding Tris-base to a final dilution of 1:10. The plate was incubated at 37°C for four hours. After washing, 100µl of biotin conjugated mouse monoclonal anti-HBx (4F1/A9) antibody (2µg/ml) was added to each well and incubated at 37°C for 1 hour and washed again. Then, 100µl of streptavidin-horseradish peroxidase (Sigma, USA) diluted to 1:3.000 in PBST was added to each well and incubated at 37°C for 1 hour. Finally, the plate was washed and the color reaction was developed by adding the substrate buffer solution containing ortho-phenylenediamine (oPD) (Sigma, USA). The color reaction was stopped by 0.46 M sulfuric

acid (100µl/well). Optical densities (ODs) were read at 490 nm in an ELISA plate reader (Thermo Labsystems IEMS Reader MF, Finnland). The levels of HBxAg in serum samples were interpolated from the GST-HBx standard calibration curve using an automatic calculation program of the microphotometer (Labsystems Ascent Software 2.4 for IEMS Reader MF)[19].

***ELISA on HBx antigen fragments***

Microtitre plates (Nunc, USA) were coated with an even amount of antigens relative to the peptide component of each construct (recombinant protein or synthetic peptide fragment) in 3$\mu$g/ml concentration dissolved in 0.1M bicarbonate buffer, pH 9.6 overnight at 4°C. Followed by incubation for 1 hour at 37°C. We washed the plates three times with PBS containing 0.05% Tween 20 (Sigma, USA) and PBS-gelatin (0.5%) (Sigma, USA) treatment was used for 30 min to eliminate the unbound antigen and to saturate the free bindings sites of the assay plates. After a washing step serum samples (1:100 diluted in PBS-Tween 20) were added for 1 hour at 37°C. Following the next wash with PBS-Tween 20, HRPO labeled rabbit anti-human IgG and IgM (DAKO, Denmark) was added to the wells in 1:2000 dilution (Diluted in PBS-Tween 20) and incubated for 1 hour at 37°C .The plates were repeatedly washed with PBS-Tween 20. Enzyme acivity was visualised using o-phenylenediamine (Fluka, Germany) and 0.02% $H_2O_2$ added in 0.1M citric acid buffer (pH 5.0). The reaction was blocked by sulfuric acid (4M) and the results were counted by a microphotometer (Labsystems Ascent Software 2.4 for IEMS Reader MF) at 490 nm wavelength. The measurements were performed in parallel under standard conditions, the samples were applied in triplicate. The quantitative measurements of serum HBxAg concentrations yielded constant values (the inter-assay variability scatters were less than 5%), the HBx specific antibody measurements showed standard tendencies during the repeated measurements.

**Statistical analysis**

The descriptive statistics of the examined variables are seen on Table 1. Statistical analysis of the collected data was done using on an IBM PC, running SPSS for Windows, release 9.0 (SPSS Inc., 233 S Wacker Drive, Chicago, 60606, Ill., USA). A linear regression analysis of the ELISA results was done for all three groups all variables. Absolute regression values under 0.4 were rejected, as well as when the P value exceeded 0.05. Results were plotted on Figures 2. cases of different disease groups ("x" axes) were plotted against the serum anti-HBxAg in ng/ml levels ("y" axes) by "Prizma 3" software.

## Results

We used the recombinant 10-143 HBxAg and six recombinant antigen fragments and a synthetic N-terminal peptide fragment (13-26) for mapping the antibodies present in the blood sera of HBV infected patients (**Figure 1.**). Serum samples of patients with 14 acute or 46 chronic hepatitis, 12 symptomless carriers, and 22 healthy donors were analyzed for anti-HBxAg antibodies of IgG and IgM isotypes.

The HBxAg serum levels were measured by sandwich type ELISA developed previously[19]. The absolute concentrations were scattered in a wide range between 18 – 1800 ng/ml. The averages of the serum HBxAg concentrations were higher in both acute and chronic hepatitis samples, than in symptomless carriers, however, the individual variations were also the highest in chronic hepatitis patients. (**Figure 2.**)

**Figure 3.** demonstrates the distribution of serum titers of circulating anti-HBx antibodies of IgG and IgM isotypes. The individual differences were relatively homogenous except for chronic hepatitis patients with anti-HBx IgG isotype. The strongest anti-HBx reactivity was found on the longest fragment of the recombinant antigens (10-143) with both IgG and IgM isotypes. The first recombinant C terminal fragment (C1: 79-117) was characteristically positive in chronic hepatitis patients with both IgG and IgM isotypes. The IgG antibodies were strongly positive on the same sequence in acute hepatitis and in symptomless carriers. The anti-HBx IgM positivity was not significantly stronger on C1 sequence in any of the group examined. The other investigated sequences were slightly positive with minimal individual variations. Healthy controls proved to be consequently non-reactive on all antigens included into the study.

Correlation between the HBx serum concentration and anti-HBx antibody titers was analyzed by regression analysis enables to clarify the hidden correlations among different parameters and groups. **Table 1.** shows the results of regression analysis between HBxAg serum concentrations and the antibody response against different HBx fragments (both IgG and IgM) measured in the same serum samples. Strong and homogenous correlations were found in ***acute hepatitis*** (**Table 1.a.**) for the all measured HBxAg fragments in IgM response. The IgG antibodies showed correlation between the high recognition of C1 fragments and consequent ignorance of the N terminal end. The strong correlation between the serum HBxAg level and the antibody production against the longest fragment (X: 10-143) shows good probability of the mathematical analysis.

In ***chronic hepatitis*** B patients (**Table 1.b.**) we found IgG dominance and correlations among C terminal fragments but no correlation with N terminal fragments. The serum level of HBxAg moderately influenced the correlations in the IgG response against the C terminal fragments and the longest fragment (X: 10-143). The regression analysis of IgM reactivity with N terminal and C terminal fragments were dominant and no correlation was found with the antigen concentrations in the same sera.

The regression analysis of the results of ***symptomless HBV carriers*** (**Table 1.c.**) showed close similarities to acute hepatitis B: the IgM dominance was typical and there was a strong correlation in the IgG response between the C1 and N terminal fragments. However, the IgM dominance was less homogenous in comparison to the patients suffering from acute hepatitis, and further IgG related correlations were detected between the HBxAg concentration and the N-terminal fragment-specific IgG production probably indicating, that HBxAg immuncompexes are composed of IgG and antigen.

## Discussion

The need for a relevant prognostic serum marker and a well-characterized molecular target for biotherapies (e.g. antigen specific vaccination) in HBV infection have increased recently. Laboratory detection of HBs and HBe or HBc antigens are appropriate by general diagnostic value, however, the immunopathological studies reflected for the basic role of the HBx antigen in the pathogenesis of chronic hepatitis B and primary hepatocellular carcinoma.

HBxAg appears in serum during peak periods of active viral replication as evident from immunserological analysis of chronic hepatitis B[4,19]. The antigenity of HBxAg protein has been mapped earlier by 15mer overlapping peptides using the sera of hepatitis B positive patients. The immunodominant epitopes were located close to the C-terminal end (85-110 sequences) of HBxAg[22].

Several research groups have identified numerous T-cell epitopes using synthetic polypeptides. Most of them were located to the carboxyterminal of the HBxAg protein[6,7].

Murakami[13] suggested the differentiation of the functional domain structures of HBxAg. A regulatory domain and a transacting domain have been proposed. The physiological role of a highly conserved nine amino acid sequence (132-140) on the C terminal part was described as the regulator of HBV replication[21]. The HBx 58–140 residue was described as the regulator of the AP-1 binding activity in the nucleus, and a regulator of the MAPK pathway activation in the cytoplasm. According to literature data the 58-140 sequence is involved in transactivation function[9].

The N-terminal (1–20) region of HBxAg is the most conserved region among mammalian hepadnavirus genomes; their role has been described as a negative regulator of transactivation[12].

The pathophysiological roles of different epitopes have been mapped by the use of overlapping synthetic peptide libraries and recombinant antigen fragments in this study, however, the complex analysis of the antibody response against these epitopes was not performed. Our ELISA assay previously developed for large scale quantitative measurement of the circulating HBxAg made the study of fine correlations possible between the serum concentration of X antigen and the antibody response against it in different stages of HBV infection.

In accordance with the classical IgM dominance in acute hepatitis B we found a significant IgG response against the well-characterised C-terminal epitope (C1: 79-117). The key role of this sequence is proved by the increased antibody production of both IgG and IgM isotypes in chronic hepatitis B. Regression analysis showed strong correlation between the serum concentration of HBxAg and the IgG antibody production against C1 in chronic hepatitis B and symptomless carriers. Our observation is in harmony with the data of Kumar *et al*, who described the significance of the C terminal subregion of the HBxAg also in the efficient T cell response. The fine mapping of the epitopes by recombinant antigen fragments localized the relevant sequence to the region between 79-117 amino acids. This is a pathophysiologically active part of the molecule that is responsible for transactivation, but no remarkable antibody response could be detected against the negative regulator N-terminal part of the HBxAg. The strong correlation between the intense IgG production against C1 and the low antibody responses against the N-terminal sequences suggest a "casual" humoral immune response in acute hepatitis B. Presenting more C-terminal epitopes (C1, C2, C3, and the whole C) as the target of the HBxAg-specific humoral immune response might indicate that these are participating in the immuncomplex formation or probably participate in the pathogenesis of the chronic hepatitis B through an ADCC reaction. This hypothesis is supported by the significant difference between the IgG values of symptomless HBV carriers without any detectable inflammation and those of chronic hepatitis patients. One might speculate that the IgG epitopes of symptomless chronic carriers participate in immunocomplex formation, but are unable to initiate ADCC related inflammation.

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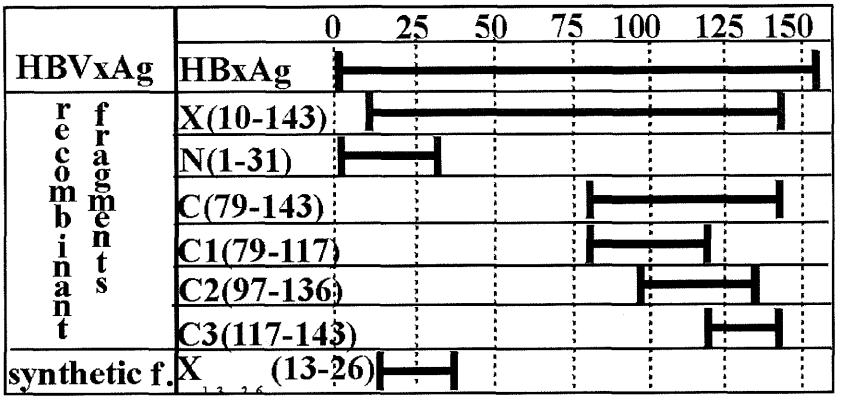


**Figure 1.:** The 154aa.-long HBxAg sequence (first band) has been seven regions. Schematic alignment of the six recombinants (X: 10-143, N: 1-31, C: 79-143, C1: 79-117, C2: 97-136, C3: 117-143) and the synthetic ($X_{13-26}$: 13-26) fragment. Fragments are shown by dark bands.

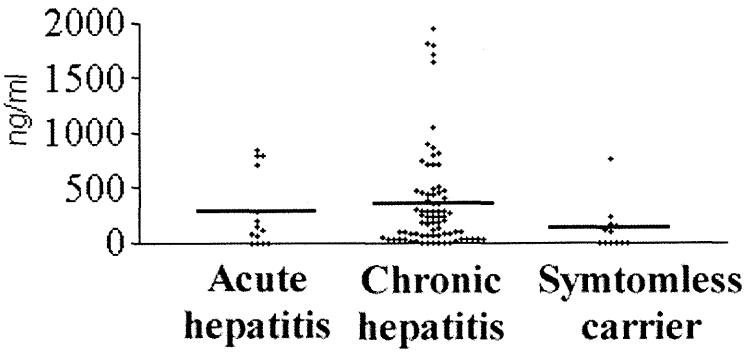


**Figure 2.:** Quantitative detection of HBxAg in Hepatitis B patients' sera by sandwich ELISA.

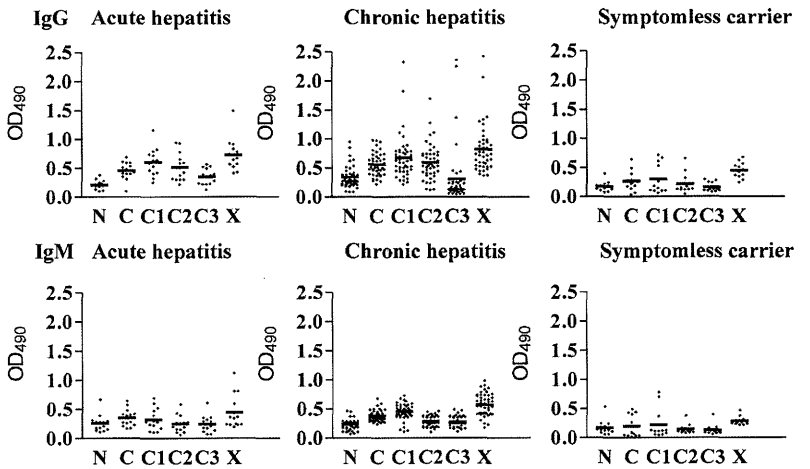


**Figure 3.:** ELISA results for recombinant and synthetic fragments in serum samples taken from patients suffering from confirmed acute B hepatitis, chronic B hepatitis and symptomless carriers of HBV.

| | | | IgG | | | | | | | IgM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HBxAg | N | C | C1 | C2 | C3 | X | $X^{14-26}$ | N | C | C1 | C2 | C3 | X | $X^{14-26}$ |
| | HBxAg | 1 | - | - | - | - | - | 0.845 | - | - | - | - | - | - | - | - |
| IgG | N | - | 1 | - | 0.976 | - | - | - | - | - | - | - | - | - | - | - |
| | C | - | - | 1 | - | 0,686 | - | - | 0,539 | - | - | - | - | - | - | - |
| | C1 | - | 0.976 | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| | C2 | - | - | 0,686 | - | 1 | - | - | - | - | - | - | - | - | - | - |
| | C3 | - | - | - | - | - | 1 | - | 0,634 | - | - | - | - | - | - | - |
| | X | 0.845 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| | $X^{14-26}$ | - | - | 0,539 | - | - | 0,634 | - | 1 | - | - | - | - | - | - | - |
| IgM | N | - | - | - | - | - | - | - | - | 1 | 0.909 | 0.981 | 0.996 | 0.990 | 0.868 | 0.907 |
| | C | - | - | - | - | - | - | - | - | 0.909 | 1 | 0.949 | 0.922 | 0.951 | 0.970 | 0.956 |
| | C1 | - | - | - | - | - | - | - | - | 0.981 | 0.949 | 1 | 0.986 | 0.995 | 0.915 | 0.945 |
| | C2 | - | - | - | - | - | - | - | - | 0.996 | 0.922 | 0.986 | 1 | 0.995 | 0.883 | 0.915 |
| | C3 | - | - | - | - | - | - | - | - | 0.990 | 0.951 | 0.995 | 0.995 | 1 | 0.917 | 0.940 |
| | X | - | - | - | - | - | - | - | - | 0.868 | 0.970 | 0.915 | 0.883 | 0.917 | 1 | 0.965 |
| | $X^{14-26}$ | - | - | - | - | - | - | - | - | 0.907 | 0.956 | 0.945 | 0.915 | 0.940 | 0.965 | 1 |

**Table 1. a:** Regression analysis of the HBxAg-specific antibody results of taken from acute hepatitis patients (ELISA). Only regression values above 0.0 are presented. P=0.05. High regression values (above 0.800) are with grey background.

| | | | IgG | | | | | | | IgM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HBxAg | N | C | C1 | C2 | C3 | X | $X^{14-26}$ | N | C | C1 | C2 | C3 | X | $X^{14-26}$ |
| | HBxAg | 1 | - | 0,626 | 0,648 | 0,607 | 0,627 | 0,664 | - | - | - | - | - | - | - | - |
| IgG | N | - | 1 | 0,413 | 0,518 | - | - | 0,540 | - | - | - | - | - | - | - | - |
| | C | 0,626 | 0,413 | 1 | 0.843 | 0,799 | 0.817 | 0,753 | - | - | - | - | - | - | - | - |
| | C1 | 0,648 | 0,518 | 0.843 | 1 | 0.896 | 0.952 | 0.846 | - | - | - | - | - | - | - | - |
| | C2 | 0,607 | - | 0,799 | 0.896 | 1 | 0.930 | 0,781 | - | - | - | - | - | - | - | - |
| | C3 | 0,627 | - | 0.817 | 0.952 | 0.930 | 1 | 0.839 | - | - | - | - | - | - | - | - |
| | X | 0,664 | 0,540 | 0,753 | 0.846 | 0,781 | 0.839 | 1 | - | - | - | - | - | - | - | - |
| | $X^{14-26}$ | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - |
| IgM | N | - | - | - | - | - | - | - | - | 1 | 0,469 | 0.943 | 0.967 | 0.965 | 0,414 | - |
| | C | - | - | - | - | - | - | - | - | 0,469 | 1 | 0,420 | 0,466 | 0,467 | 0,526 | - |
| | C1 | - | - | - | - | - | - | - | - | 0.943 | 0,420 | 1 | 0.938 | 0,955 | - | - |
| | C2 | - | - | - | - | - | - | - | - | 0.967 | 0,466 | 0.938 | 1 | 0.986 | 0,473 | - |
| | C3 | - | - | - | - | - | - | - | - | 0.965 | 0,467 | 0.955 | 0.986 | 1 | 0,467 | - |
| | X | - | - | - | - | - | - | - | - | 0,414 | 0,526 | - | 0,473 | 0,467 | 1 | 0,477 |
| | $X^{14-26}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | 0,477 | 1 |

**Table 1. b:** Regression analysis of HBxAg-specific antibody results (ELISA) in the sera taken from chronic hepatitis B patients. All other values are OD averages. Only regression values above0 .0 are presented. P=0.05. High regression values (above0 .800) are with grey background.

| | | IgG | | | | | | | IgM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HBxAg | N | C | C1 | C2 | C3 | X | $X^{14-26}$ | N | C | C1 | C2 | C3 | X | $X^{14-26}$ |
| HBxAg | 1 | 0,651 | - | 0,897 | - | - | - | - | - | - | - | - | - | - | - |
| IgG | N | 0,651 | 1 | - | 0,732 | - | - | - | - | - | - | - | - | - | - | - |
| | C | - | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | C1 | 0,897 | 0,732 | - | 1 | - | - | - | - | - | - | - | - | - | - | - |
| | C2 | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| | C3 | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - |
| | X | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | - |
| | $X^{14-26}$ | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - |
| IgM | N | - | - | - | - | - | - | - | - | 1 | - | 0,775 | - | 0,825 | 0,651 | - |
| | C | - | - | - | - | - | - | - | - | - | 1 | - | 0,689 | 0,641 | - | 0,655 |
| | C1 | - | - | - | - | - | - | - | - | 0,775 | - | 1 | 0,657 | 0,802 | - | - |
| | C2 | - | - | - | - | - | - | - | - | - | 0,689 | 0,657 | 1 | 0,933 | 0,781 | 0,766 |
| | C3 | - | - | - | - | - | - | - | - | 0,825 | 0,641 | 0,802 | 0,933 | 1 | 0,696 | 0,615 |
| | X | - | - | - | - | - | - | - | - | 0,651 | - | - | 0,781 | 0,696 | 1 | - |
| | $X^{14-26}$ | - | - | - | - | - | - | - | - | - | 0,655 | - | 0,766 | 0,615 | - | 1 |

**Table 1. c:** Regression analysis of the HBxAg-specific antibody results (ELISA) in the sera taken from symptomless hepatitis B virus carrier sera. Only regression values above 0.0 are presented. P=0.05. High regression values (above 0.800) are with grey background.

# A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network?

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## Abstract

Natural antibody (nAb) producing B-1 B cells are considered an intermediate stage of evolution between innate and adaptive immunity. nAbs are immunoglobulins that are produced without antigen priming. nAbs can recognize foreign targets and may serve in the first line of immune defense during an infection. Natural autoantibodies (nAAbs) present in the serum of both healthy humans and patients suffering from systemic autoimmune diseases recognize a set of evolutionarily conserved self-structures. Because of their endosymbiotic evolutionary origin, proteins compartmentalized into mitochondria represent an interesting transition from prokaryotic foreign (non-self) to essential (self) molecules. We investigated the possible overlap in recognized epitopes of innate and self-reactive nAbs and surveyed changes in physiological autoreactivity under pathological autoimmune conditions. Epitope mapping analysis of a mitochondrial inner membrane enzyme, citrate synthase (CS) (EC 2.3.3.1) by synthetic overlapping peptides and phage display libraries using sera from healthy individuals and from patients having systemic autoimmune disease revealed CS recognizing nAAbs with IgM isotype. We analyzed cross reactive epitopes on human CS, bacterial CS, and various standard autoantigens. The anti-CS nAAbs by participating in the nAb network, could function in innate defense mechanisms and at the same time recognize a target antigen (nucleosome) in a systemic autoimmune disease. Thus, at the level of recognized epitopes there is a possible new link between the innate like component and the adaptive-autoimmune arm of the humoral immune system.

**Keywords:** natural antibody; mitochondrial inner membrane enzyme; epitope mapping; phage display; multi-pin ELISA; systemic lupus erythematosus; autoimmunity

## Introduction

Several lines of evidence indicate that natural antibody producing B-1 B cells represent an intermediate stage of evolution between innate and adaptive immunity. In fact they play an important role during the early phases of immune responses (Martin et al., 2001, Wardemann et al., 2002, Baumgarth et al., 1999). While they need to be under control and regulation because of their autoreactivity, these innate B cells themselves play an important role in controlling autoimmunity (Boes et al., 2000, Cocca et al., 2001).

Natural antibodies (nAbs) are immunoglobulins that are produced without immunization with antigen (Couthino et al., 1995). These molecules can recognize foreign targets and may serve in the first line of immune defense during an infection (Ochsenbein et al., 1999). In contrast, natural autoantibodies (nAAbs) present in the serum of both healthy humans and patients suffering from systemic autoimmune diseases recognize a set of self-structures that have been conserved during evolution (Cohen and Young, 1991). Most nAAbs belong to the IgM or IgG isotype (Mouthon et al., 1995, Avrameas, 1991) and show polyreactivity with a broad range of affinities for the recognized epitopes (Lacroix-Desmazes et al., 1998). Several functions have been suggested for nAAbs: they may participate in the selection of immune repertoires, play a role in the acceleration of primary immune responses (Ehrenstein et al., 1998), aid the clearance of apoptotic cells (Peng et al., 2005), possess anti-inflammatory effects (Miletic et al., 1996) and contribute to the maintenance of immune homeostasis (Lacroix-Desmazes et al., 1996).

Because of their endosymbiotic evolutionary origin, proteins compartmentalized into mitochondria represent an interesting transition from prokaryotic foreign to essential self molecules. To date there are a limited number of epitope mapping analyses performed on human antigens recognized by nAAbs. In particular little is known about the possible overlap in recognized epitopes of innate and self-reactive nAbs. Moreover, the possible changes in physiological autoreactivity under pathological autoimmune conditions remain unclear.

To address these problems we have chosen a mitochondrial inner membrane enzyme, citrate synthase (CS) (EC 2.3.3.1) for epitope mapping using sera of healthy individuals and patients having systemic autoimmune disease (systemic lupus erythematosus, rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyosits, systemic sclerosis, Raynaud syndrome and Sjörgen syndrome). We investigated the presence of CS recognizing nAAbs both in healthy and autoimmune patients. The molecular recognition pattern on CS was analyzed and compared under physiological and pathological (autoimmune) conditions. We analyzed cross reactive epitopes on human CS, bacterial CS, and various standard autoantigens. Our data indicate that the anti-CS nAAbs have an individually characteristic fine epitope pattern which is independent from the actual condition of the immune system by participating in the nAb network.

## Materials and Methods

### Patients and control sera

Serum samples from healthy individuals: 63 Hungarian blood donors from the Blood Transfusion Service of Baranya county, Pécs (age: 36.8 ± 17.1 years; 32 women, 31 men); a standardized panel from 51 British blood donors and 176 Finnish blood donors (by the courtesy of professor G. Füst and Z. Prohaszka, 3rd Department of Internal Medicine at the Semmelweis University, Budapest); 44 serum samples from healthy infants from the Pediatrics Clinic, University of Pécs (age: 11.5 ± 4.5 months; 26 females, 18 males) and samples of patients with systemic autoimmune diseases: 326 clinically well-documented cases

of systemic lupus erythematosus (SLE), rheumatoid arthritis, undifferentiated connective tissue disease, polymyositis/dermatomyosits, systemic sclerosis, Raynaud syndrome and Sjörgen syndrome from the Immunology and Rheumatology Clinic, University of Pécs (age: 44,2 ± 14,1 years; 284 women, 42 men) were used in this work with the permit of the Ethical Committee of the Medical Center of the University of Pécs.

**Detection of mitochondrial enzyme specific autoantibodies by ELISA**

96-well polystyrene plates (NUNC) were coated with CS, malate dehydrogenase (MDH; EC 1.1.1.37) and pyruvate dehydrogenase (PDH; EC 1.2.4.1) from porcine heart (Sigma) in 0.1M bicarbonate buffer, pH 9.6. Following the saturation of non-specific binding sites with 0.5% gelatin (Sigma) in PBS (pH 7.3), serum samples were incubated in triplicates at 1:100 dilutions in washing buffer (PBS, 0.05% Tween 20) for 60 min. Finally, the plate was incubated with HRPO conjugated anti-human-IgA, or -IgG or –IgM specific secondary antibody (Dako) for 60 min. The reaction was developed with *o*-phenylenediamine (Sigma), and measured on an iEMS MF microphotometer (ThermoLabsystem) at 492 nm. Cut off values of each groups examined were calculated from the average of measured OD492 data. Sera having higher OD value than average + 2SD were considered positive. All measurements were standardized with a monoclonal anti-citrate synthase antibody (Clone 4H3-E5) we produced previously (Nemeth P et al, 1991).

**Affinity purification of sera on CS**

CS from porcine heart was coupled to cyanogen-bromide activated sepharose 4B (Sigma) according to the manufacturer's instructions. Fifteen ml sera of 30 healthy blood donors and 14 patients with autoimmune disease were passed three times through the CS-sepharose resin. After washing antibodies were eluted in glycine-HCL pH 2.5, fractions were neutralized with 1 M TRIS and were tested for CS reactivity with indirect ELISA using HRPO conjugated anti-human-IgA, or -IgG or –IgM specific secondary antibody (Dako).

**Cross reactivity testing of CS affinity purified sera**

Cross reactivity with additional mitochondrial inner membrane enzymes was tested with indirect ELISA using MDH and PDH from porcine heart (Sigma) as antigens.

Reactivity with *E.coli* CS was tested with pin-bound overlapping decapeptides as described previously. (Petrohai et al., 2004; Nyarady et al., 2005)

Recognition of autoantigens implicated in various autoimmune diseases were tested using indirect ELISA kits developed for the quantitative measurement of double stranded DNA, nucleosome, Cenp-B, MPO, PR3, alpha-fodrin, gastric parietal cell, intrinsic factor, Asca, gliadin, tissue transglutaminase, cardiolipin, β2-glycoprotein-1, phosphatidyl serine, prothrombin (Orgentech) and Sm, RNP, SSA, SSB, Scl-70, Jo-1, CCP, thyreoglobulin, glomerular basal membrane (Hycor) specific autoantibodies.

**Construction of a CS antigen fragment library**

Total RNA was isolated using TriReagent (Sigma) from $3x10^6$ mononuclear cells obtained by Ficoll Paque (AmershamPharmacia) gradient centrifugation from peripheral blood of a healthy blood donor. 5 μg total RNA was reverse transcribed with Superscript II RT (Invitrogen) according to the manufacturer's instructions. cDNA encoding for the full length human mitochondrial citrate synthase was amplified with the following primers: 5'-ATGGCTTTACTTACTGCGGC-3' and 5'-TTACCCTGACTTAGAGTCCAC-3'. The PCR reaction contained 300mM of each dNTP, 1.5 mM $MgSO_4$, 1 μM of each primer, 5 μl cDNA and 5 units of ProofStart DNA polymerase (Qiagen) in a 100 μl final volume, cycling was done with the following profile: 95 C 5min, 35 cycles of 95 C 1min, 51 C 30s, 72 C 2min,

final extension at 72 C for 10 min. The PCR product was separated on a 1.5% agarose gel and purified using the Quiaquick Gel Extraction Kit (Qiagen). Following A-addition it was cloned into a T/A vector using the InsT/Aclone PCR Product Cloning Kit (Fermentas). The identity of insert was verified by sequencing on an ABI3100 Avant genetic analyzer.

Library construction was done using the lambdaD-bio phage display vector (a kind gift from Dr. Alessandra Luzzago; Instituto di Ricerche di Biologia Molecolare, Italy) as described (Ansuini et al., 2002). In brief, inserts were produced by tagged random primed elongation and amplification using SpeI and NotI tagged random primers (Santi et al., 2000) and CS cDNA as template excised with BamHI and EcoRI (Promega) digestion from the plasmid mentioned above. Following purification with the Quiaquick PCR purification kit (Qiagen) and size selection on Wizard columns (Promega) inserts were digested with SpeI and NotI (Promega). Twenty ligations were set up containing 1 $\mu$g of SpeI/NotI digested lambdaD-bio DNA, 25 ng of SpeI/NotI digested insert, 30U of T4 DNA ligase (Fermentas) in a final volume of 5$\mu$l and incubated 48 hours at 4C . The ligation mixture was phenol-chloroform extracted, ethanol precipitated and packaged with the Ready To Go Lambda Packaging Kit (AmershamPharmacia). Phage were amplified by infecting log phase E.coli BB4 cells and plating them on LB agar plates. After plaque formation phage were eluted by an overnight incubation in SM buffer (100mM NaCl, 8.1 mM $MgSO_4$, 50mM Tris-HCl pH 7.5), concentrated with polyethylene glycol precipitation and resuspended in SM buffer supplemented with Complete EDTA Free Proteasae Inhibitor Cocktail (Roche).

**Affinity selection of CS antigen fragment library**

Affinity selection of CS antigen fragment library with CS affinity purified sera was performed essentially as described (Santini et al., 1998). Briefly, microtiter plates were coated with affinity purified anti-CS sera or anti-CS monoclonal antibody (mAb) 4H3E5 (developed in our lab) at 10 $\mu$g/ml in coating buffer. After blocking $10^{10}$ phage were incubated for 2 h at room temperature. Wells were washed five times and bound phages were recovered by in well infection of E.coli BB4 cells. The infected bacteria were plated on LB agar plates and phage were eluted then concentrated as described above. The affinity selection was repeated one more time and individual clones were picked up for DNA sequencing.

**Random peptide library screening**

The filamentous phage library displaying cyclic nine amino acid random peptides as a fusion to the N-terminal of the M13 major coat proteinVIII was constructed previously (Felici et al., 1991). Affinity selection of phages with CS affinity purified sera from SLE patients was performed using the biopanning technique (Parmley and Smith, 1988). In brief, microtiter plates were coated with affinity purified anti-CS sera (40 $\mu$g/ml during the first, and 4 $\mu$g/ml during the second and third rounds of panning). After washing with PBS/0.05%Tween-20 and blocking with a solution containing PBS/3%BSA, $10^{10}$ ampicillin transducing unit (ATU) phage (blocked with PBS/1%BSA) was added and incubated for 2h at room temperature. The plate was washed with PBS/Tween-20 (0.05% in the first or 0.5% in the second an third round of panning) and the bound phage were eluted with 1mg/ml BSA /0.1 M glycine pH 2.2. Following neutralization with 2M TRIS, 10 ml XL1-Blue ($O.D._{600}$:0.5) was infected and plated on LB agar plates containing 50 $\mu$g/ml ampicillin. The next day the colonies were scrapped off the plates, were resuspended in 10 ml LB and were superinfected with $10^{11}$ plaque forming unit (PFU) M13KO7 helper phage. After an overnight incubation at 37°C, phage were precipitated with 16.7% PEG8000/3.3 M NaCl twice and resuspended in TBS. Enrichment was monitored by indirect ELISA with phage pools after each selection step. Following the third round of panning, randomly chosen clones were picked up and tested for

reactivity with the selecting sera by indirect ELISA. Based on the ELISA results forty clones were selected for DNA sequencing.

## Results

### Anti-mitochondrial enzyme specific antibodies in healthy individuals and systemic autoimmune patients

Using simple binding ELISA we demonstrated the presence of antibodies recognizing CS, MDH, and PDC both in the sera of healthy individuals and systemic autoimmune patients. Isotype specific ELISA showed that enzyme specific antibodies with IgM isotype are more frequently present in all investigated groups than that of IgG or IgA isotypes (**Table 1.**). No differences were found among the subgroups of healthy individuals; however, the incidence of anti-CS and anti-MDH autoantibodies with IgM isotype was significantly higher in autoimmune patients compared to the healthy controls.

We continued our investigations with CS specific IgM autoantibodies because this group showed the most characteristic pattern of distribution. We followed the titer of anti-CS IgM antibodies in 53 healthy individuals selected from British and Hungarian blood donors with repeated sample collection minimum 3 times during a 5 year period. We have found that the CS reactivity of individual sera remained permanently constant over this time period (**Figure 1**).

### Affinity purification and cross reactivity testing of CS reactive sera

To exclude the masking effects of nonspecific bindings we purified anti-CS antibodies from 44 human sera (30 healthy and 14 autoimmune patients: 9 with SLE, 3 with systemic sclerosis and 2 with rheumatoid arthritis) by affinity chromatography for further experiments. Affinity purification was successful only in those cases (2 healthy and 2 SLE patients) when the actual serum had extraordinary high (OD492 >1.5) anti-CS reactivity. The eluted anti-CS antibodies were exclusively with IgM isotypes (data not shown).

Cross reactivity of affinity purified anti-CS antibodies with other mitochondrial inner membrane enzymes (MDH and PDC) was tested by indirect ELISA. The affinity purified anti-CS antibodies have not recognized these enzymes (data not shown).

Subsequently, we applied a synthetic peptide fragment library in multi-pin ELISA to test whether antibodies affinity purified on CS from porcine heart (identity with human CS: 96%) would be able to recognize epitopes on bacterial CS from *E.coli* (identity with human CS: 25%). Only three cross reacting sequences were found: amino acids 124-133: FRRDSHPMAV (identity with human CS: 40%, similarity: 60%); amino acids 174-183: MCYKYSIGQP (identity with human CS: 30%, similarity: 40%) and amino acids 351-360: YFIEKKLYPN (identity with human CS: 40%, similarity: 60%).

In order to examine the cross reactivity of affinity purified anti-CS sera on autoantigens having a role in various autoimmune diseases, we performed several indirect ELISAs with commercially available autoantibody kits. The affinity purified anti-CS sera from two SLE patients recognized nucleosome antigen (**Figure 2A**). To exclude the possibility of CS contamination in the nucleosome antigen preparation we used our anti-citrate synthase mAb (Nemeth P et al, 1991) in the same ELISA system. For further verification of our results we carried out competition ELISA experiments using CS from porcine heart as competitor (**Figure 2B**).

In order to obtain further support for these findings we screened 46 additional SLE patients for CS and nucleosome reactivity and performed CS affinity purification from the 11 double positive (high CS and nucleosome reactivity) patients' sera. All of the above mentioned 11 CS affinity purified sera recognized the nucleosome antigen, which reactivity

could be inhibited in competition with CS (mean nucleosome reactivity OD (450): 0.38 ± 0.15 without competition; 0.21 ± 0.09 in competition with 10 μg CS and 0.08 ± 0.03 in competition with 50 μg CS). In addition we performed fluorescent immunocytochemistry on Hep-2 cells using a commercially available kit from Diasorin. All of the CS affinity purified SLE patients's sera resulted a low-intermediate staining intensity in the nucleus - and the nucleoli of the nuclei were consequently negative - characteristic for the recognition of nucleosomes. We found an intermediate-high staining intensity in the cytoplasm, characteristic for the recognition of CS (data not shown). Our results show that there is indeed a cross reactivity of anti-CS antibodies from SLE patients with nucleosome antigen.

**CS antigen fragment library construction**

For analysis of fine epitope structure of anti-CS autoantibodies we constructed a CS antigen fragment library displayed on bacteriophage lambda. The library contains approximately $10^7$ insert bearing independent clones. First we tested the library by performing an affinity selection with an anti-CS mAb. After the second round of affinity selection 30 clones were chosen for DNA sequencing. Among them 4 distinct sequences were found repeatedly (**Table 2**). These sequences could be aligned with amino acids 7-65. of human CS and the minimal epitope of our anti-CS mAb could be restricted to amino acids 31-59.

**Affinity selection of CS antigen fragment library with CS purified sera**

After the effectiveness of our phage displayed CS antigen fragment library for epitope mapping was demonstrated, we moved forward to the epitope mapping of affinity purified anti-CS sera. Following two rounds of affinity selection 20 clones selected with each serum were picked up for DNA sequencing. In contrast to the selection with our anti-CS mAb these clones carry short peptide sequences which could be also aligned to human CS (**Figure 3**). These short sequences are scattered through the human CS sequence and it seems that practically the same regions of the molecule are recognized by the two groups of sera. However, it is important to note that the fine epitope pattern is different in the two groups examined.

**Random peptide library screening with CS purified sera**

To get additional data about the basis of CS-nucleosome cross reactivity we screened a nine amino acid random peptide library with CS affinity purified sera from two patients with SLE. The 40 sequenced clones carry similar peptides which show partial homology with human CS (**Table 3**) We performed competition ELISA with the phage clone most resembling CS (YAAPSHQSH, phage#5) both for CS and nucleosome antigen.(**Figure 4**). According to our results, phage#5 inhibited the CS reactivity of CS affinity purified sera from SLE patients but not from healthy individuals. When tested for blocking nucleosome reactivity, phage#5 proved to be an efficient inhibitor of the nucleosome reactivity measured with CS affinity purified sera from SLE patients. These results indicate that the CS-nucleosome cross reactivity at least in part is caused by antibodies recognizing the CS epitope (amino acids 145-150.) mimicked by phage#5.

## Discussion

In this study we demonstrated the presence of natural antibodies recognizing CS both in healthy individuals and in patients with systemic autoimmune disease. Our finding that the majority of these antibodies have IgM isotype, their presence in infants and the long term stability of serum titers in adults indicates that these specificities belong to the nAAb repertoire established early in postnatal life (Mouthon et al., 1995, Stahl et al., 2000).

Since CS has not been implicated as target antigen in any autoimmune disease we investigated, it seems to be an appropriate model protein to examine potential changes in physiological autoreactivity under conditions of systemic autoimmune disease. In our previous study (Petrohai et al, 2004) we performed comparative epitope mapping on human CS with sera from healthy individuals and heart transplanted patients using overlapping synthetic peptides. We detected similar recognition patterns in healthy group as in the present study, but in heart transplanted patients both epitope pattern and the isotype of the anti-CS autoantibodies were different: strong dominance of IgGs was found with altered fine epitope specificities in strong relationship with the clinical stage (graft rejection).

Epitope mapping with overlapping synthetic peptides is a widely used technique (Maeji et al., 1995, Uray et al., 2003), but it's constraints include the *in silico* B-cell epitope prediction used for selection of antigenic regions (Nyarady et al., 2005), the partial coverage of primary sequence by synthetic peptides and the possible loss of all unpredicted or conformational epitopes. Since these effects could have influenced our results, we performed the epitope mapping using a basically different technique: bacteriophage surface display of peptides is an extensively used technique for a variety of applications (Scott and Smith, 1990, McCafferty et al., 1990, Marks et al., 1991, Farilla et.al., 2002). The most commonly used systems are based on fusion to a filamentous phage coat protein. However, the life cycle of these phages limits the size of the displayed peptide, therefore we have chosen phage lambda for construction of a CS antigen fragment library. The library contains fragments of CS with random starting point and length; consequently it overcomes the theoretical and technical limitations associated with the overlapping synthetic peptide approach. According to our results obtained with phage displayed antigen fragments, while there is no favored region of the CS molecule recognized exclusively either by healthy individuals or patients with SLE, the fine epitope pattern is different in the two groups examined.

Since previous studies suggested that natural antibodies play an important role in the innate like component of the humoral immune response (Martin et al., 2001, Wardemann et al., 2002, Baumgarth et al., 1999), we investigated the possible overlap in nAAb recognized epitopes on mammalian and bacterial CS. Due to the prokaryotic origin of mitochondria, CS represents an attractive target molecule to examine the self-reactive nAAbs' capability to recognize epitopes on the foreign counterpart of the same molecule. To achieve this, we used sera affinity purified on mammalian CS for epitope mapping on CS from *E.coli*, using the overlapping synthetic peptide method. The three recognized sequence shows a limited homology with human CS, though identical amino acids with a possible anchor function are present at corresponding positions. These amino acids contain either polar or charged side chains, which is in agreement with previous reports about the preferential amino acid composition of nAAb epitopes (Novick et al., 1992, Pashov et al., 2002). The three peptides, according to the three dimensional model, are located on the surface of the molecule. Moreover, two of the peptides (124-133 and 174-183), though separated by 50 amino acids in the primary sequence, are in close proximity on the structural model, indicating that they represent the same antigenic region.

We tested whether CS affinity purified nAAbs cross react with other self antigens implicated in various autoimmune diseases. The surprising cross reactivity of SLE patients' CS affinity purified sera with nucleosome antigen could not have been unequivocally explained by the results of epitope mapping performed either by the synthetic overlapping peptide or phage displayed antigen fragment method. Therefore we screened a random peptide library displayed on filamentous phage. In this system random peptides are presented at high copy number, making the identification of low affinity interactions easier. We isolated a phage clone (phage#5) which carries a peptide corresponding to amino acids 145-150. of human CS, and while it has no effect on sera from healthy individuals, it is capable of

inhibiting both the CS and nucleosome reactivity of CS affinity purified SLE patients' sera. Reviewing our data obtained by phage displayed CS antigen fragment library revealed that a lambda phage clone, carrying practically the same part of the CS molecule as the filamentous phage clone #5, was isolated with the SLE patients' sera. Therefore, on the basis of evidence substantiated by the random peptide and CS antigen fragment library methods, we conclude that this CS epitope (145-150.) is preferentially recognized by the SLE patients' sera. The cross reactive epitope identified on human CS (145-150.) is located on the surface of the molecule. It is interesting to note that it is part of the region which contains two of the E.coli CS cross reactive determinants (124-133 and 174-183). We hypothesize that this (124-183.) part of the molecule is the major target for both the self-reactive (pathological) and innate like nAAbs. We did not find homology in the primary structures between the isolated huCS 145-150 peptide fragment and the nucleosome proteins; however, the isolated sequence motif (AALPSH) is hydrophobic and it is able to induce similar immunoreactivity as the also strongly hydrophobic nucleosome sequences. Our data call the attention for the general changes in self-recognition network under pathological conditions resulting changes on cross-reacting epitope patterns.

In conclusion, we demonstrated the presence of CS recognizing nAAbs. We showed that, the fine epitope pattern on CS is different under physiological and pathological (SLE) conditions. We identified cross reactive epitopes on human and bacterial CS. In addition we demonstrated cross reactivity of CS affinity purified sera with nucleosome antigen. These data indicate that in theory, nAAbs „specific" for a given self antigen could fulfill the function of participating in innate defense mechanisms and at the same time recognize a target antigen in a systemic autoimmune disease. Thus, at the level of recognized epitopes there is a possible new link between the innate like part and the adaptive-autoimmune arm of the humoral immune system.

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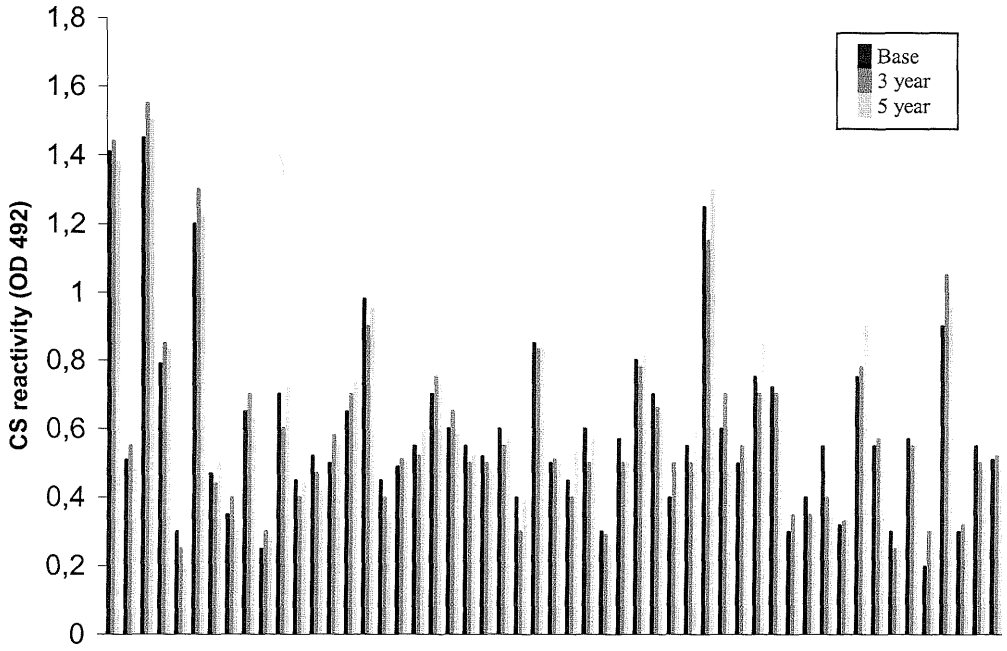
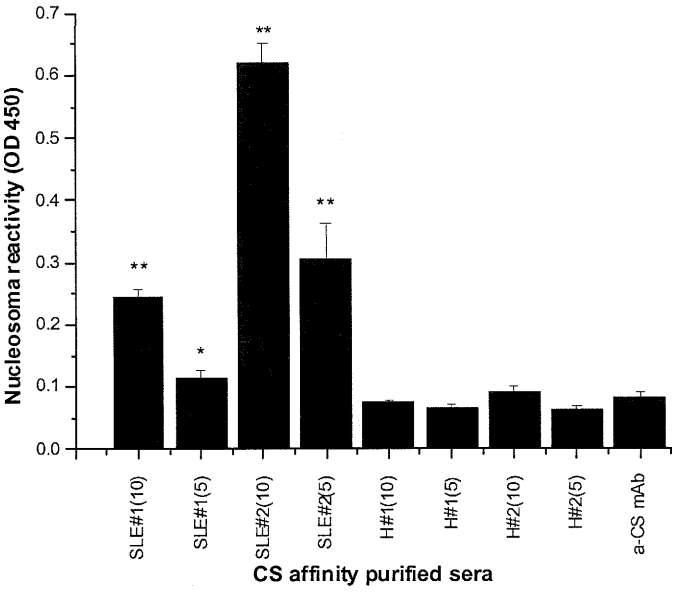



**Figure 1.** CS reactivity in healthy individuals over a five year period.
CS reactivity of sera from 53 healthy individuals was followed up during a five year period with IgM isotyp
specific indirect ELISA.

**A**




**B**

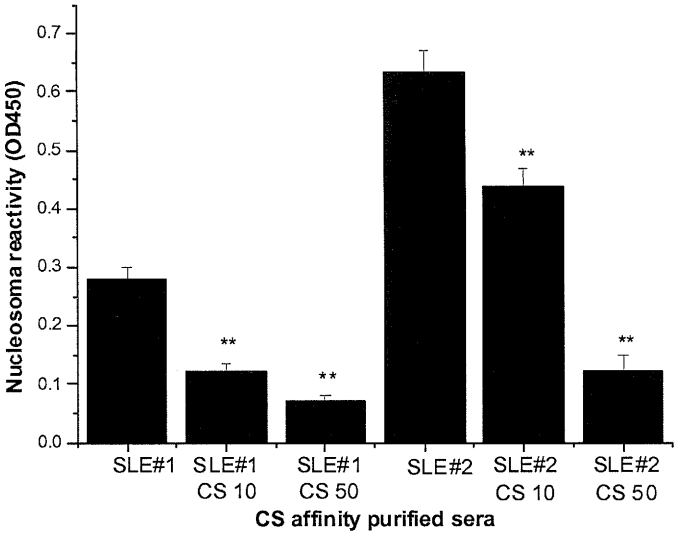



**Figure 2.** Anti-CS antibodies from SLE patients recognize nucleosoma antigen.
A: Affinity purified anti-CS sera from two SLE patients (SLE) and two healthy (H) individuals were tested for nucleosome reactivity. Numbers in parentheses indicate the protein concentration in $\mu$g/ml. CS contamination of the nuclesome antigen was controlled with an anti-CS mAb 4H3E5 (a-CS mAb).
B: Competitive ELISA with CS: Affinity purified anti-CS sera from two SLE patients (10 $\mu$g/ml) were preincubated with the indicated amount of CS ($\mu$g/100$\mu$l) for 1h then reactivity for nucleosome antigen was tested. All measurements were performed in triplicates and were repeated in three independent experiments. * $p < 0.05$, ** $p < 0.001$

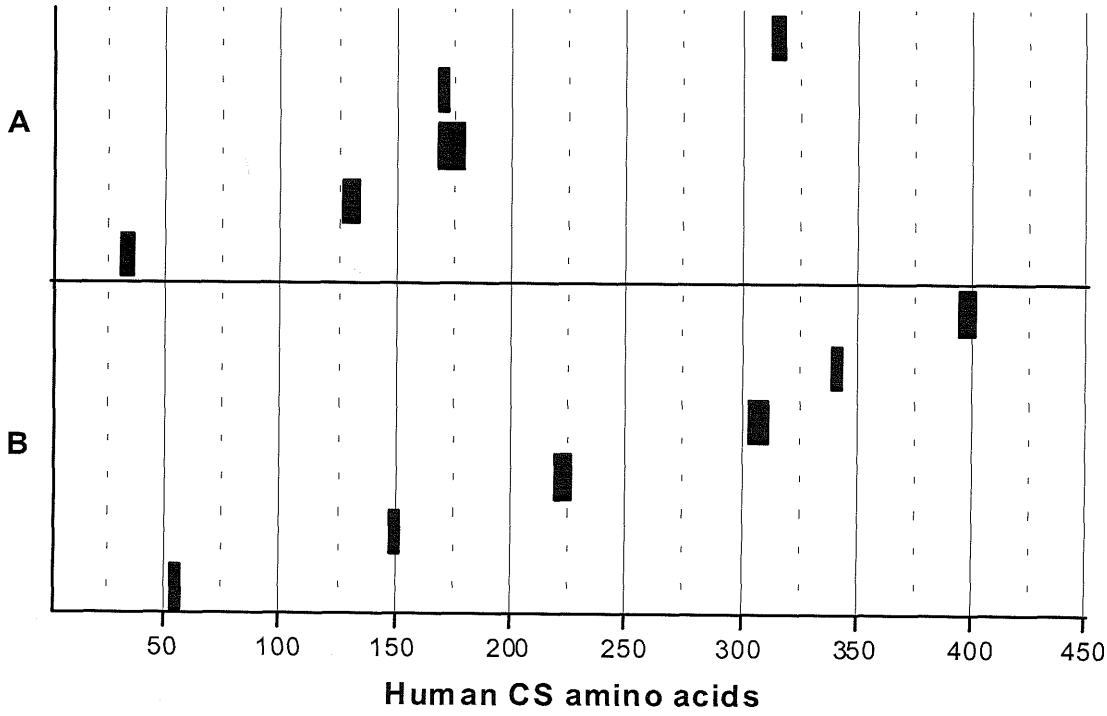
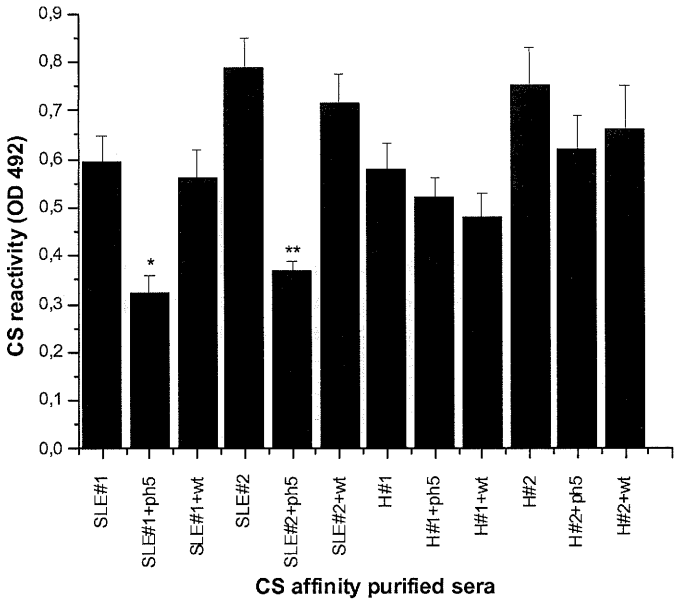



**Figure 3**. Alignment of anti-CS sera selected phage clones with human CS.
Deduced amino acid sequences of phage clones selected with affinity purified anti-CS sera prepared from healthy individuals (A) and patients with SLE (B) are plotted along the human CS sequence.

**A**




**B**

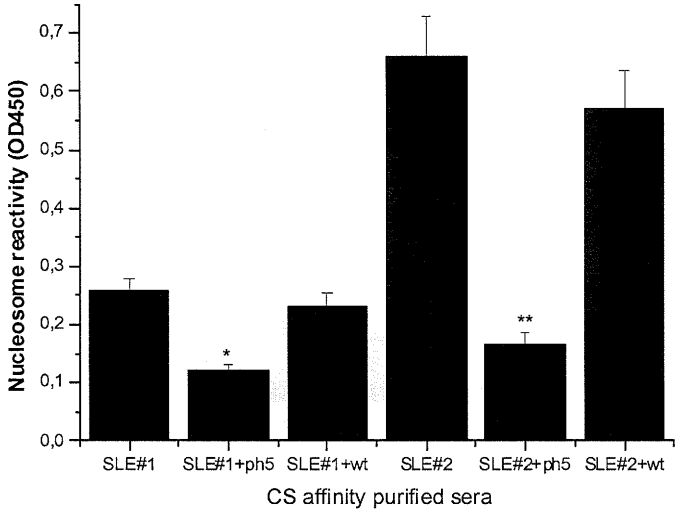



**Figure 4.** Competition for CS and nucleosome antigen with a phage clone selected from a random peptide library. We performed competition ELISA with phage#5 (YAAPSHQSH), selected with CS affinity purified SLE patients' sera from a random peptide library. A: CS affinity purified sera from patients with SLE or healthy individuals (SLE#1, #2 and H#1, #2 at 1 $\mu$g/ml) were preincubated with $10^{10}$ phage#5 (ph5) or wild type (wt) particles for 1h at 4°C, then reactivity for CS was measured by indirect ELISA. B: CS affinity purified sera from patients with SLE (SLE#1, and #2 at 10 $\mu$g/ml) were preincubated with $10^{10}$ phage#5 (ph5) or wild type (wt) particles for 1h at 4°C, then reactivity for nucleosome antigen was measured by indirect ELISA. * $p < 0.05$, ** $p < 0.005$.

| | CS IgA | CS IgG | CS IgM | MDH IgA | MDH IgG | MDH IgM | PDC IgA | PDC IgG | PDC IgM |
|---|---|---|---|---|---|---|---|---|---|
| **Hungarian blood-donors (63)** | 4% | 3% | 10% | 3% | 2% | 8% | 3% | 3% | 10% |
| **British blood-donors (51)** | 5% | 4% | 12% | 3% | 3% | 9% | 3% | 4% | 10% |
| **Finnish blood-donors (176)** | 5% | 3% | 9% | 2% | 2% | 8% | 3% | 5% | 10% |
| **Healthy infants (44)** | 2% | 2% | 7% | 4% | 4% | 9% | 2% | 4% | 9% |
| **Systemic autoimmune patients (326)** | 5% | 3% | 24% | 4% | 4% | 16% | 4% | 4% | 6% |

**Table 1.** Prevalence of mitochondrial inner membrane enzyme specific antibodies in human sera
Sera were tested for citrate synthase (CS), malate dehydrogenase (MDH) and pyruvate dehydrogenase (PDH) reactivity with isotype specific indirect ELISA. Values in parentheses indicate the number of individual serum samples tested.

| Number of independent isolations | Deduced amino acid sequence |
|---|---|
| 5 | AARLLGTKNASCLVLAARHASASS**TNLKDILADLIPKEQARIKTFRQQHGKTV**VGQITV |
| 8 | **TNLKDILADLIPKEQARIKTFRQQHGKTV** |
| 9 | ASCLVLAARHASASS**TNLKDILADLIPKEQARIKTFRQQHGKTV**VG |
| 6 | ASCLVLAARHASASS**TNLKDILADLIPKEQARIKTFRQQHGKTV** |
| huCS | AARLLGTKNASCLVLAARHASASS**TNLKDILADLIPKEQARIKTFRQQHGKTV**VGQITV |

**Table 2.** Alignment of anti-CS mAb selected phage clones with human CS.
The number of independent isolations refers to the clones sequenced and found identical for the four distinct inserts. huCS indicates amino acids ***7-65***. of human CS.

| Number of Independent Isolations | Deduced amino acid sequence |
|---|---|
| 2 | APASPYQCG |
| 1 | APASPYQSG |
| 5 | EAASPYQSH |
| 5 | YAAPSSQSH |
| 9 | YAAPSRQSH |
| 18 | YAAPSHQSH |
| huCS 145-150 | AALPSH |

**Table 3.** Alignment of CS affinity purified sera selected random peptides with human CS.
The number of independent isolations refers to the clones sequenced and found identical for the given peptide. huCS indicates amino acids 145-150. of human CS.