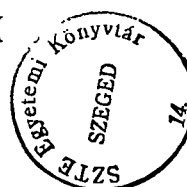


**PEPTIDEK SZEREPE AZ IMMUNOLÓGIAI
FELISMERÉSBEN : SZINTÉZIS,
KONFORMÁCIÓS ÉS IMMUNOLÓGIAI
VIZSGÁLATOK**

EGYETEMI DOKTORI ÉRTEKEZÉS

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BEVEZETÉS

Az immunrendszer a szervezet védelmi rendszere. A külvilágból a szervezetbe jutó, vagy azon belül képződő antigénekkal szemben ellenanyagot termel (humorális immunválasz) és/vagy immunocitát képez (celluláris immunválasz). Az immunválaszban elsődlegesen részt vevő sejtek a limfociták. Legfontosabb képviselőik a T- ill. B-sejtek. A T-sejtek a celluláris immunreakciókban betöltött szerepük mellett az immunválasz szabályozói és a B-sejteket ellenanyag termelésre serkentik. A B-sejtek ellenanyag-termelő plazmasejteké differenciálódnak. Az immunválaszt kiváltó antigének és az ezekre reagáló sejtek az immunrendszer két oldalát képviselik. Megismerésük közelebb vihet bennünket e nagyon sokrétű és bonyolult folyamat szabályozásának lehetőségéhez. Célkitűzésünk ezért információgyűjtés volt részben az antigénekkal, részben a T-sejtekkel kapcsolatban.

Az antigének tanulmányozásához választott modelleken, az influenza és a HIV-1 vírus esetében meg kívántuk vizsgálni egyes vírusfehérjék immunválaszban betöltött szerepét. A vizsgálatok egy része annak felderítését célozta, hogyan befolyásolja az epitópok konformációja az immunválaszban betöltött szerepet. Az influenza esetében a kísérleteket ki kívántuk terjeszteni a H1 szerotípus mellett a H2 és H3 szerotípusokra is.

A limfociták működését számos sejtfelszíni receptor szabályozza, többek között a T-sejt antigén-receptor komplex. Munkánk során a CD3 komplex δ és ζ alegységét kívántuk tanulmányozni, monoklonális ellenanyagok segítségével, részben az antigén felismerés, részben a

jelátvitel szempontjából. Ehhez különböző típusú, antigén tulajdonságú anyagok előállítására volt szükség. Kémiai célkitűzésünknek megfelelően törekvésünk arra irányult, hogy a hagyományosaknál kedvezőbb hatású, egyértelműbb szerkezetű antigéneket szintetizáljunk.

A szervezetben a peptidek, fehérjék egy része poszttranszlációs átalakuláson megy keresztül. A foszforilációnak, defoszforilációnak nagyon fontos szerepe van a regulációban, így az immunválasz jelátviteli folyamataiban is. A vírusok burokfehérjéi gyakran glikoziláltak, egyes peptidhormonok csak szulfátészterként aktívak. A natív fehérjét és ennek módosult formáját az immunrendszer is eltérő módon ismeri fel. Célkitűzéseink között szerepelt szulfát- illetve foszfátészter képzési és glikozilezési módszerek kidolgozása mellett az így átalakított peptidek szintézisére alkalmas polimer előállítása is.

A köpenyfehérjék fragmensei szerepének, a receptor-funkcióknak a tanulmányozása és a poszttranszlációs átalakítások kivitelezése szintetikus vakcinák tervezéséhez vihet közelebb bennünket. Az ismeretek birtokában lehetőség nyílhat új diagnosztikus eljárások kidolgozására is az említett vírusfertőzésekkel kapcsolatban.

ELMÉLETI RÉSZ

I. AZ IMMUNVÁLASZ TANULMÁNYOZÁSA AZ ANTIGÉNEK OLDALÁRÓL

Az első közlemény szintetikus peptidek felhasználásáról vírusok elleni immunizálásban 1982-ben jelent meg a Nature-ben [1]. Ettől kezdve a szintetikus peptidek immunológiai felhasználása rohamos fejlődésnek indult. Az első vírus, amely ellen peptid antigénnel részleges védettséget lehetett elérni, a száj- és körömfájás egyik vírustörzse volt. Azóta már születtek eredmények a hepatitis B, a citomegalovírus, a veszettség, az influenza és legújabban a HIV-1 vírus egyes szintetikus fehérje fragmenseinek felhasználásával.

A vakcinálási stratégiák - fejlődésük ellenére - nem minden vírus ellen alkalmazhatók jó eredménnyel. Ilyen az influenza vírus és az AIDS-et okozó HIV-1. Bár a két különböző törzsbe tartozó vírusban a genetikai mechanizmusok és a vírusfehérjék szerkezete lényegesen eltér egymástól, a burokfehérjék "érési" folyamatában hasonló lépések vesznek részt [2, 3]. Ilyen például az influenza vírus hemagglutinin illetve a HIV-1 burokfehérjéjében a membránfúzióért felelős molekula szakasz hasonló enzimátikus hasítás révén történő aktiválódása [4, 5]. Vizsgálataink ezen enzimátikus hasításnak kitett szakaszok immunfelismerésben betöltött szerepének tisztázására irányultak. A HIV-1 esetében a kísérleti eredmények új diagnosztikus módszer kidolgozását is elősegíthetik.

A, AZ INFLUENZA VÍRUS

Az influenza vírus köpenyfehérjéjének kulcsfontosságú szakasza a hemagglutininnak (HA) nevezett glikoproteid. T-sejtek általi felismerését az utóbbi időben alaposan tanulmányozták [6, 7, 8]. A hemagglutinin összefüggő láncként szintetizálódik, majd egy endopeptidáz hatására két alegységre (HA1 és HA2) hasad [9]. Az alegységek a 329-es pozícióban lévő, a HA1 C-terminálisát képviselő argininen keresztül kapcsolódnak egymáshoz [10]. A hasítás a vírus fertőzőképessége szempontjából nélkülözhetetlen. Az ennek eredményeként létrejövő konformáció változás következtében válik szabaddá a vírus és gazdasejt membránfúziójáért felelős ún. fúziós peptid, a HA2 alegység N-terminális része [11], a membrán fúzió katalizátora [12].

A hemagglutinin immunfelismerésben betöltött szerepének tanulmányozása érdekében számos peptidet állítottunk elő a hasítási hely környezetéből. A szintetizált peptidek a következők voltak:

VTGLRNIPSIQS	H1/HA1 ₃₁₇₋₃₂₈
VTGLRNIPSIQSR	H1/HA1 ₃₁₇₋₃₂₉
GLRNIPSIQS	H1/HA1 ₃₁₉₋₃₂₈
LRNIPSIQS	H1/HA1 ₃₂₀₋₃₂₈
LRNIPSIQSR	H1/HA1 ₃₂₀₋₃₂₉
GLFGAIAGFIEG	H1/HA2 ₁₋₁₂
RGLFGAIAGFIEGR	H1/HA1 ₃₂₉ -HA2 ₁₃
VTGLRNIPSIQSRGLFGAIAGFIEG	H1/HA1 ₃₁₇ -HA2 ₁₂
VTGLRNIPSIQSRGLFGAIAGFIEG	H1/HA1 ₃₁₇ (D-Arg ³²⁹)-HA2 ₁₂

A peptidek szintézise a R. B. Merrifield által 1963-ban publikált szilárd fázisú módszer segítségével történt [13]. Hordozóként általában p-metil-benzhidril-amin polimert használtunk [14, 15], így az előállított peptidek C-terminálisa savamid. Az α -aminocsoportokat t-butil-oxi-karbonil (Boc), az oldallánc funkciókat a következő csoportokkal védtük:

- Asp, Glu : ciklohexil-észter-
- Arg : tozil-
- Ser, Thr : benzil-éter-
- Lys : 2-Cl-benzil-oxi-karbonil-
- Tyr : 2-Br-benzil-oxi-karbonil-
- Cys : p-metil-benzil-
- His : benzil-oxi-karbonil-csoport.

A kapcsolásokat diciklohexil-karbodiimides aktiválással végeztük diklór-metánban, dimetil-formamidban, vagy ezen oldószerek elegyében. Az aminosav beépülést ninhidrin teszttel ellenőriztük [16]. Az Asn, Gln és Arg esetében az aminosavakból in situ 1-hidroxi-benztriazol észtereket képeztünk a mellékreakciók elkerülése érdekében. A semlegesítést 10 % trietil-amin / diklór-metán eleggyel végeztük. A Boc-védőcsoportot 50 % trifluor-ecetsav / diklór-metán eleggyel távolítottuk el. A peptideket a hordozóról folyékony hidrogén-fluoriddal, 0 °C-on, a benne szereplő aminosavaktól függően 30-45 percig hasítottuk. A szolubilizálás ecetsavval történt. Leoldás után az anyagokat liofilizáltuk. Ezt követte a tisztaság-ellenőrzés és tisztítás fordított fázisú HPLC-vel, C_{18} -as oszlopokon, az amid-csoport elnyelési maximumának közelében (220-230 nm-en), az alábbi eluensekkel:

A - 0,1 % trifluor-ecetsav

B - 80 % acetonitril, 0,1 % trifluor-ecetsav.

A termékeket szerkezetét aminosavanalízissel, tömegspektrometriával igazoltuk.

1. táblázat. Az influenza vírus HA kapcsolódási részét reprezentáló szintetikus peptidok tömegspektrometriás vizsgálati eredményei

<i>peptid</i>	<i>számított MS</i>	<i>talált MS</i>
H1/HA1₃₁₇₋₃₂₉	1438,8 [#]	1439,9
H1/HA1₃₁₉₋₃₂₈	1083,25	1083,8
H1/HA1₃₂₀₋₃₂₈	1026,2	1026,9
H1/HA1₃₂₀₋₃₂₉	1182,39	1182,3
H1/HA2₁₋₁₂	1151,3	1151,5
H1/HA₃₂₉-HA2₁₃	1463,7	1463,2
H1/HA1₃₁₇-HA2₁₂	2574,0 [*]	2574,3
H1/HA1₃₁₇(D-Arg)³²⁹-HA2₁₂	2572,97	2573,6

#: monoizotópos MS *: a C-terminális karboxilcsoport, a többi peptidnél savamid

A H1/HA1₃₁₇-HA2₁₂ peptiden, amely a hemagglutinin alegységeinek kapcsolódási részét tartalmazza, két potenciális epitópot jósoltak [17, 18]. Annak ellenőrzése érdekében, hogy a peptid képes-e T-sejt válasz kiváltására, in vitro proliferációs és az interleukin-termelés detektálására irányuló vizsgálatok történtek BALB/c egereken. Ezek bizonyították az

említett peptiden T-sejt epitópok jelenlétét. Proliferációs kísérletekben a H1/HA1₃₁₇₋₃₂₉ fragmens potenciális T-sejt epitópnak bizonyult. További vizsgálatok eredményei alapján ennek C-terminálisán hosszabbított, 25 aminosavból álló formája (H1/HA1₃₁₇-HA2₁₂) is jó epitóp annak ellenére, hogy a hidrofób C-terminális rész az eredetileg helikális konformációt nyújtottá alakítja. Ez utóbbival, illetve D-argininnel módosított változatával és a H1/HA1₃₁₇₋₃₂₉ peptiddel elvégzett dózisfüggőségi vizsgálatok azt mutatták, hogy a rövidebb, valamint a C-terminálison hosszabbított fragmensek T-sejt aktiváló hatása közel azonos. A 25 aminosavból álló epitópok felismerése további enzimes ("tripszin-szerű enzim") hasítás nélkül is végbemegy. Erre abból lehet következtetni, hogy enzim inhibitorok nem képesek gátolni a folyamatot sem a természetes, sem a D-arginint tartalmazó változatnál. Fiziológias körülmények között a 329-es pozícióban lévő arginint egy karboxipeptidáz lehasítja [10, 19], így várható, hogy jelenléte nem szükséges a T-sejtek általi felismeréshez. A H1/HA1₃₁₇₋₃₂₈ fragmens bizonyította a feltevést. A H1/HA1₃₁₉₋₃₂₈, a H1/HA1₃₂₀₋₃₂₈ és a H1/HA1₃₂₀₋₃₂₉ inaktívak, tehát a 13 aminosavas potenciális epitóp N-terminálisán nem rövidíthető a T-sejt aktiváló hatás elvesztése nélkül.

[20]

A vírusfertőzésben létfontosságú konformációváltozás, amely a hemagglutinin alegységekre történő hasadásakor következik be, figyelmünket az epitópként szereplő peptidek térszerkezetének tanulmányozására irányította. A CD spektroszkópiával, trifluoretanolban elvégzett vizsgálatok legmagasabb (35-40%) hélixtartalmat a H1/HA1₃₂₉-HA2₁₃ és H1/HA1₃₁₇-HA2₁₂ peptidek esetében mutattak. A HA1 alegység



√(1) - [21] Ez a levezetés helyes!

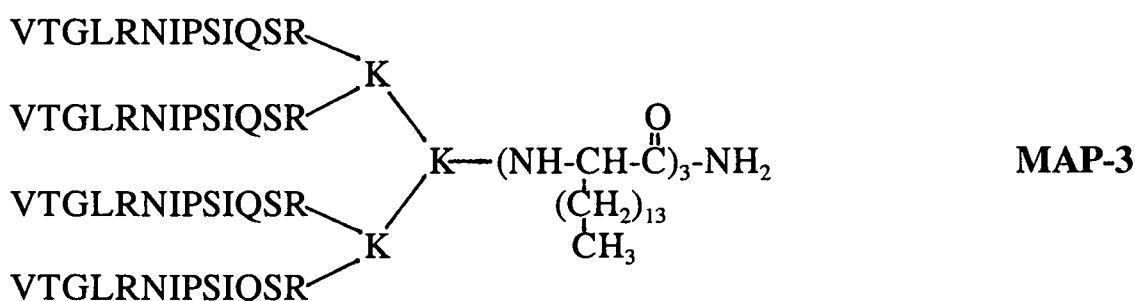
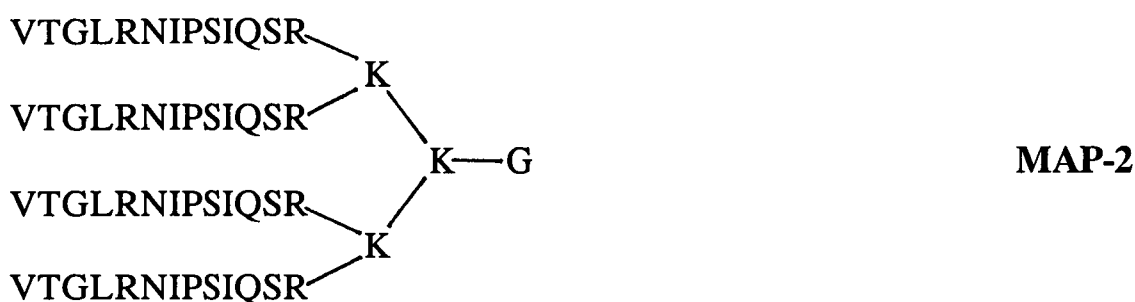
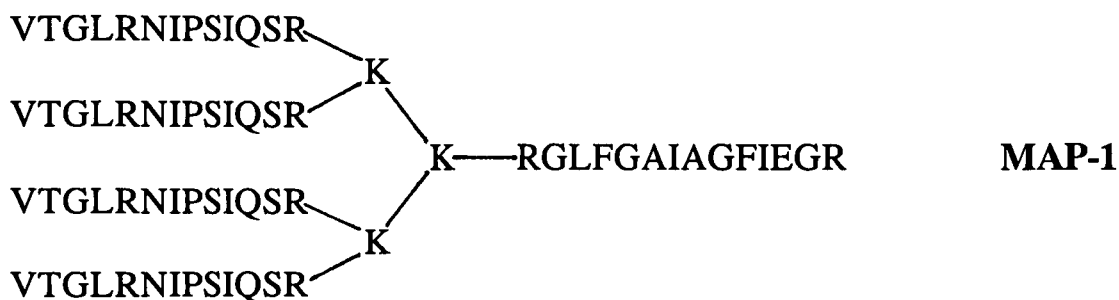
C-terminális részét reprezentáló H1/HA1₃₁₇₋₃₂₉ peptidnél ez az érték mindössze 10%-nak adódott. Ezzel összhangban bizonyos predikciós módszerek és FT-IR vizsgálatok inkább β -turn képződést jeleztek az utóbbi epitópnál.

Tam 1988-ban új típusú, antigén tulajdonságú, elágazó struktúrákat vezetett be az immunológiai kutatásokba [22], amelyeket sikeresen alkalmaztak a száj- és körömfájás [23], illetve a hepatitis vírus [24], valamint a maláriát okozó Plasmodium esetében is [24, 25, 26, 27]. A MAP (multiple antigenic peptide) szerkezetek számos előnnyel rendelkeznek a hagyományos, fehérje hordozókhoz kötött antigénnel szemben:

- az epitópot lényegesen nagyobb százalékban tartalmazzák,
- egyértelműbb, kémiai jobban jellemezhető, reprodukálhatóbb molekulák,
- kiküszöbölik a fehérje hordozó saját immunreakcióit,
- lehetőséget biztosítanak B- és T-sejt epitópokkal együttesen történő immunizálásra,
- segítségükkel egyéb módosítások (pl. a lipofil karakter fokozása) is elérhetők.

A módszer alapelveiből kiindulva az alábbi MAP szerkezeteket szintetizáltuk [28, 29]:

② ~
⑥ ~



A peptideket az I/A fejezetben leírt szilárd fázisú szintézis módszerével állítottuk elő. A hordozóról történt hasítás után a nyers termékeket HPLC-vel tisztítottuk, ezekben az esetekben C₄-es oszlopokon.

A MAP-1 a hemagglutinin alegységei kapcsolódási helyén lokalizált T-sejt epitópokat együttesen tartalmazza. Az ezzel előkezelt BALB/c egerek 22-60 %-ban védetté váltak letális dózisú influenza vírussal szemben. Azonos körülmények között a H1/HA1₃₁₇₋₃₂₉ peptid maximum

2x ②-
⑥-

40%-os védettséget tudott előidézni. (A kontrollcsoportban nem volt túlélő.) [28, 29]

Mivel irodalmi adatok szerint [30, 31] az immunválasz fokozható a peptid lipoproteid-szerű módosításával, ezért szintetizáltuk a MAP-3 szerkezetet.

Az eddig leírtak a H1 influenza szerotípusra vonatkoztak. Hatékony vakcinák tervezéséhez nem elegendő egyetlen típus ismerete, mert a vírus variabilitása miatt ez csak az esetek kis hányadában idézne elő védettséget. Ezért a vizsgálatokat ki kívántuk terjeszteni a H2 és H3 szerotípusokra is. Peptideket szintetizáltuk a hemagglutinin HA1 alegységének C-terminális régiójából.

2. táblázat. A H2 és H3 influenza vírus szerotípusok vizsgálatához szintetizált peptidek

<i>H1 szerotípus</i>	<i>H2 szerotípus</i>	<i>H3 szerotípus</i>
VTGLRNIPSIQSR H1/HA1₃₁₇₋₃₂₉	ATGLRNV PQIESR H2/HA1₃₁₇₋₃₂₉	LATGMRNVPEKQTR H3/HA1₃₁₆₋₃₂₉
GLRNIPSIQS H1/HA1₃₁₉₋₃₂₈	GLRNV PQIES H2/HA1₃₁₉₋₃₂₈	GMRNVPEKQT H3/HA1₃₁₉₋₃₂₈
LRNOPSISQ H1/HA1₃₂₀₋₃₂₈	LRNV PQIES H2/HA1₃₂₀₋₃₂₈	MRNVPEKQT H3/HA1₃₂₀₋₃₂₈
LRNIPSIQSR H1/HA1₃₂₀₋₃₂₉	LRNV PQIESR H2/HA1₃₂₀₋₃₂₉	MRNVPEKQTR H3/HA1₃₂₀₋₃₂₉

A peptideket az I/A fejezetben leírt módszerrel szintetizáltuk, tisztítottuk.

Jellemzésük aminosav analízissel és tömegspektrometriával történt.

3. táblázat. A H2 és H3 influenza vírus szerotípusok fúziós régióját reprezentáló peptidek tömegspektrometriás vizsgálati eredményei

<i>peptid</i>	<i>számított MS</i>	<i>talált MS</i>
H2/HA1₃₁₇₋₃₂₉	1439,63	1440,2
H2/HA1₃₁₉₋₃₂₈	1111,26	1111,9
H2/HA1₃₂₀₋₃₂₈	1054,21	1054,9
H2/HA1₃₂₀₋₃₂₉	1210,4	1211,0
H3/HA1₃₁₆₋₃₂₉	1599,87	1599,7
H3/HA1₃₁₉₋₃₂₈	1158,34	nincs adat
H3/HA1₃₂₀₋₃₂₈	1101,29	1101,6
H3/HA1₃₂₀₋₃₂₉	1257,47	1258,0

Az immunológiai és konformációs vizsgálatok elkezdődtek. A 2. táblázat első sorában lévő három peptid térszerkezetét CD és FT-IR spektroszkópiával összehasonlítva kitűnt, hogy a H1 szerotípushoz tartozó szekvenciánál figyelhető meg a legalacsonyabb α -hélix tartalom, itt inkább β -turn képzés jellemző. Meglepetésünkre a leggyengébb T-sejt epitópnak bizonyult H2 fragmens rendelkezik legmagasabb α -hélix ³-tartalommal, noha korábbi irodalmi adatok ⁽³²⁾ 33, 34, 35] szerint a T-sejtek inkább a helikális szakaszokat ismerik fel. [36]

B, A HIV-1 VÍRUS

A HIV vírus gp160 burokfehérjéjének érése analógiát mutat az influenzáéhoz [2, 3]. A klasszikus fúziós régió a gp160 hasításakor keletkező gp41 9-17 aminosavszekvenciájához köthető [37, 38], amelynek szerepe hasonló az influenza HA fúziós peptidjéhez. Újabb vizsgálatok szerint a gp160 másik hasítási termékén, a gp120 fehérjén is található egy enzimatikusan érintett hely, amelynek szerepe lehet a membránfúzióban [4, 5]. Feltételezhető, hogy az itt történő enzimátikus hasítás az influenza HA-hoz hasonlóan befolyásolja az immunológiai felismerést.

A 120 kDa-os fehérje V3 loopjának vizsgálatához a következő peptideket állítottuk elő:

CTRPNNNTRKSIRIQRGPGRA	FVTIGKIGNMRQAHC	
		gp 120 V3 ₃₀₁₋₃₃₆
NTRKSIRIQRGPGRAF		
		gp 120 V3 ₃₀₇₋₃₂₂
GPGRAFVTIGKIGNMRQAHC		
		gp 120 V3 ₃₁₇₋₃₃₆
AFVTIGKIGNMRQAHC		
		gp 120 V3 ₃₂₁₋₃₃₆

A szintéziseket ebben az esetben is az I/A fejezetben leírtak alapján végeztük. A 36 aminosavból álló peptid előállítása az átlagosnál nehezebbnek bizonyult részben hosszúsága, részben szekvenciája miatt. A hordozóról történt hasítása után a levegő oxigénje segítségével építettük ki a ciszteinek között a diszulfidhidat. A tisztítás RP-HPLC-vel,

C₁₈-as oszlopon történt. A peptidek szerkezetét aminosavanalízissel és tömegspektrometriával igazoltuk. Az immunológiai vizsgálatok folyamatban vannak.

II. A T-SEJT ANTIGÉN-RECEPTOR KOMPLEX VIZSGÁLATA

Az immunválasz során a szervezetbe lépő antigén hatására az immunsejtek aktiválódnak, osztódnak és differenciálódnak. Mindhárom folyamat első lépése a sejtfelszíni receptorok és ligandjaik kapcsolódása. A ligandkötés hatására a receptor konformációja megváltozik és az extracelluláris jelet a sejtbe továbbítja. E két folyamat, tehát az antigén-felismerés és a jelátvitel szabályozza a T-sejt választ.

A, AZ ANTIGÉN KÖTŐDÉSE A RECEPTORHOZ

A T-sejt antigén-receptor komplex minden T-sejt felszínén egy heterooligomer struktúrából épül fel és hat vagy hét különböző alegységből áll [39]. Az alegységek - szerkezetüket tekintve - három különböző csoportba sorolhatók. Az elsőben az immunglobulin géncsaládhoz tartozó α és β lánc található [40]. Az α - β heterodimerhez kapcsolódik a CD3 komplex γ , δ és ϵ lánc, melyek közül az előbbi kettő glikozilált. A harmadik csoportba a ζ családba tartozó dimerek sorolhatók [41]. Az α és β lánc kivételével a T-sejt antigén-receptor komplex többi

alegységének funkciója nem ismert, bár a ζ láncra vonatkozóan kísérleti adatok ismertek az irodalomban [42, 43].

Kísérleteinkben két sejtfelszíni fehérjét, a T-sejt receptor komplex ζ - és δ -láncát vizsgáltuk.

A ζ -lánc aminosav sorrendje:

MKWKALFTAA ILQAQLPITE AQSFG⁷LDPK LCYLLDGILF
szignál peptid extracell. transzmembrán
IYGVILTALF LRVKFSRSAE PPAYQQGQ¹⁹NQ LYNELNLGRR
régio → intracelluláris rész
EEYDVLDKRR GRDPEMGGK²⁹PRRKNPQEGLY NELQKDKMAE
AYSEIGMKGE RRRGKGHDGL YQGLSTATKD TYDALHMQAL
PPR

A δ -lánc szekvenciája:

MEHSTFLSGL VLATLLSQVS PFKIPIEELE DRVFN CNTS
 szignál peptid → extracelluláris rész
 ITWVEGTVGT LLSDITRLDL GKRILDPRGI YRCNGTDIYK

DKESTVQVHY RMSQSCVELD PATVAGIIVT DVIATLLLAL
 transzmembrán régió
 GVFCFAGHET GRLSGAATDQ ALLRNDQVYQ PLRDRDDAQY
 intracelluláris rész
 SHLGGNWARN K

Az első lépés az immunológiailag aktív régiók megkeresése. Ezt különböző predikciós módszerekkel szokás elvégezni [44, 45, 46, 47]. A predikciók elsősorban a peptidlánc hidrofilitását, flexibilitását, illetve

másodlagos szerkezeti elemek (α -hélixek, β -redőzött rétegek, β -turnok) képződését veszik alapul az antigenitási index számításánál. A legjobbnak ígérkező epitópok a magasabb indexű régiókban találhatók. A számítógépes modellezést a WISCONSIN programcsomag felhasználásával végeztük el.

A láncok extracelluláris részeiből immunogén szakaszokat választottunk ki. Az alábbi peptideket szintetizáltuk:

QSFGLLDPKLC	T3 ζ_{22-32}
PIEAQSFGLLDPKLCY	T3 ζ_{17-33}
AQSFGLLDPKLCYLLDG	T3 ζ_{21-37}
TRLDLGKRILDPRGIYR	T3 δ_{35-51}

A ζ -lánc a T-sejt receptorban dimerként fordul elő, amelyben a két láncot diszulfidhíd köti össze. Ezért a levegő oxigénjével történő oxidációval előállítottuk a T3 ζ_{22-32} és a T3 ζ_{17-33} peptidek dimerjeit is. A ζ -láncból szintetizált monomer és dimer peptideket glutárdialdehiddel marha szérum albumin és tireoglobulin hordozókhoz kapcsoltuk pH 6,5-es foszfát pufferben. A konjugátumokat desztillált vízzel szemben 24 órán át tartó dialízissel tisztítottuk, s így használtuk fel. A következő peptid-BSA konjugátumokkal nyulakat immunizáltunk:

BSA - T3 ζ_{22-32}

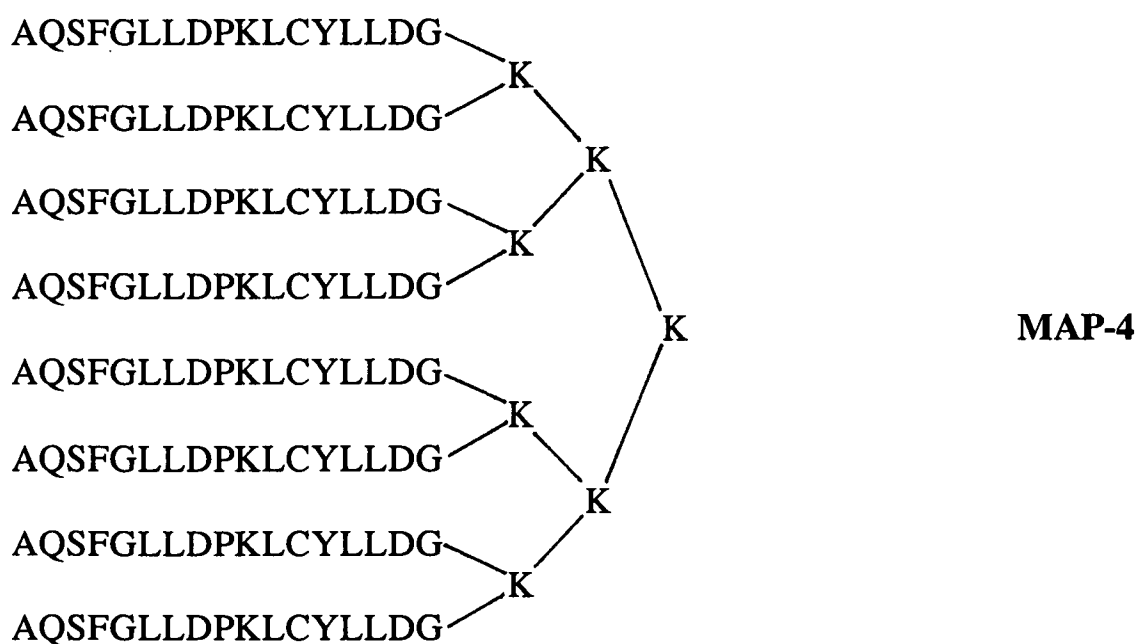
BSA - T3 ζ_{22-32} dimer

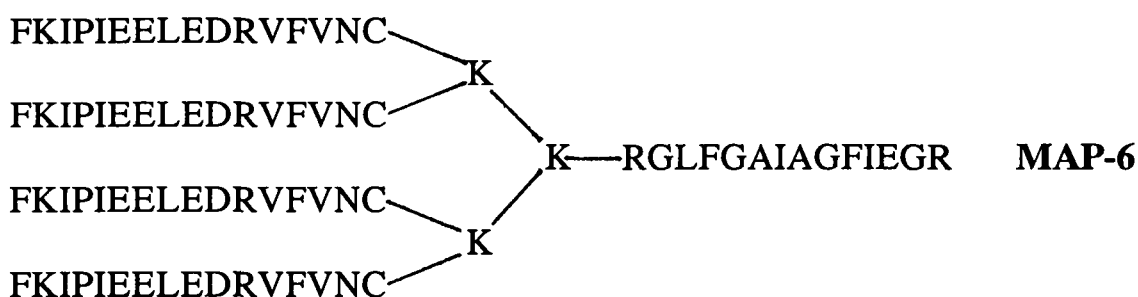
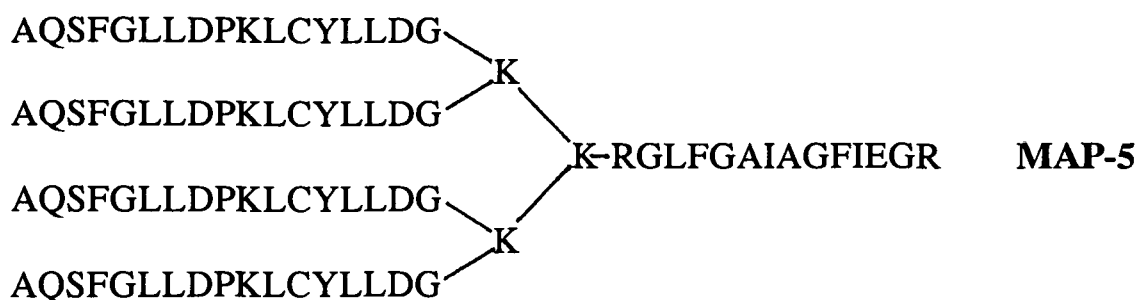
BSA - T3 ζ_{17-33} dimer.

A kapott antiszérumok felismerték az immunizálásra használt peptid tireoglobulinhoz konjugált formáját. Az immunreakció specifikus voltát peptid kompetíciós ELISA kísérletekkel sikerült igazolni. Egyik antiszérum sem ismerte fel viszont a T-sejtekben jelenlévő natív ζ -láncot.

A T3 δ_{35-51} peptidből marha szérumból albumin és tireoglobulin konjugátumokat készítettünk, majd nyulak immunizálására használtuk fel, az előzőekben leírtak szerint. Ebben az esetben is termelődött specifikus immunreakcióban ellenanyag, amely felismerte az oligopeptidet és az immunizálásra használt konjugátumot, de nem ismerte fel a natív δ -láncot.

A fenti kísérletek nem kielégítő sikere miatt megpróbálkoztunk elágazó szerkezetű antigének használatával. A Tam által leírt szerkezetek mintájára szintetizáltunk két MAP struktúrát a ζ -lánc, egyet pedig a δ -lánc vizsgálatához:





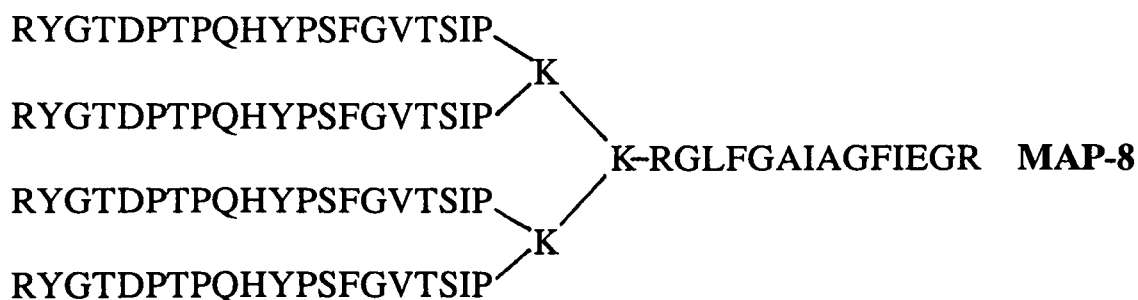
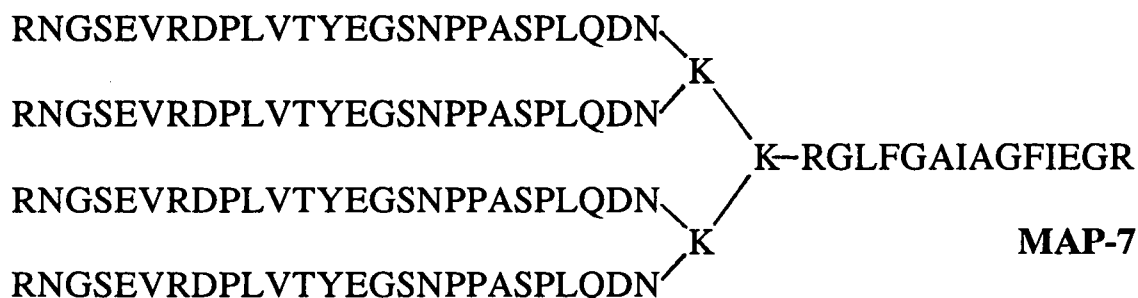
A MAP-4 elágazó szerkezetű antigén, amely egyik T-sejt epitópot oktamerként tartalmazza, az immunizálási kísérletekben toxikusnak bizonyult. A MAP-5 konstrukció szintézisére azért került sor, mert az influenza vírus hemagglutininról nyert eredmények (I/A fejezet) alapján arra következtettünk, hogy a H1/HA₁₃₁₉-HA₂₁₃ peptid (RGLFGAIAGFIEGR) sejtaktiváló szerepet tölt be az immunválasz során. Az így előállított antigénnel szemben termelődött ellenanyag, de az antiszérum ebben az esetben sem ismerte fel a natív ζ -láncot.

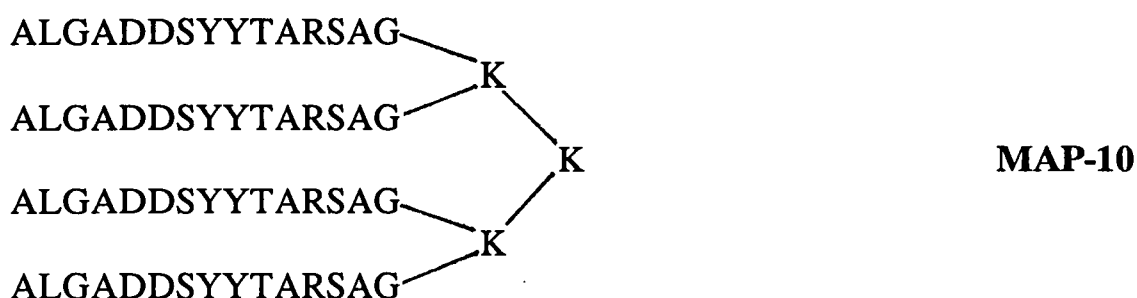
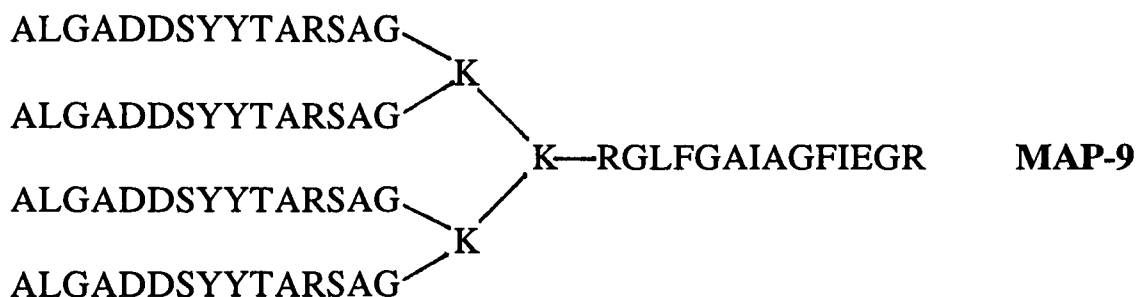
A MAP-6 konstrukció a T-sejt receptor δ -láncának 1-16 szakaszából és az előbb említett H1/HA₁₃₁₉-HA₂₁₃ peptidből épül fel. Immunizálási kísérletekben hasonlóképpen viselkedett, mint a T3 δ_{35-51} szekvenciából készített konjugátumok.

A MAP szerkezetekkel ennél a kísérletsorozatnál nem értünk el jobb eredményt, mint a hagyományos módon előállított antigénnel.

B, A JELÁTVITEL

A T-sejt receptoron keresztül történő sejtaktiválódás során legalább két kináz-útvonal aktiválódik, majd a receptor néhány alegysége foszforilálódik. A kinázokat az antigénnek a receptorhoz való kötődése aktiválja. Ezt követi az alegységek megfelelő aminosavainak foszforilációja. A kináz-utak megismeréséhez azonosítani kell azokat a kinázokat, amelyek az aktivitásért felelősek. Jelenleg két enzimet tesznek felelőssé az említett folyamatokért: a $p56^{lck}$ -t [48] és a $p59^{fyn}$ -t [49]. Kísérleteinket ezekkel, illetve a ZAP70 kinázzal folytattuk. Vizsgálatukhoz az alábbi MAP-szerkezeteket szintetizáltuk:





Az antiLCK és az antiZAP70 szérumok magas affinitással felismerték mind az immunizálásra használt peptid konstrukciókat, mind a natív fehérjét. Az antiFYN szérumok az immunizálásra használt polipeptideket felismerték, de a natív fehérjét felismerő képességüket nem sikerült direkt módon bizonyítani.

A jelátvitelben központi szerepet betöltő, már említett foszforiláció a T-sejt antigén-receptor komplex ζ - és γ -láncát érinti [50]. A folyamatban tirozin és szerin aminosavak hidroxilcsoportjai vesznek részt [51]. A tirozin-foszforiláció a receptor stimulációt követően megy végbe. Eddig nem sikerült bizonyítani, hogy a ζ -lánc intracelluláris részében lévő hét tirozin közül melyek vesznek részt ebben a folyamatban. Ezért vizsgálataink a potenciális foszforilációs helyek feltérképezését célozták. Oligopeptideket szintetizáltunk az intracelluláris régióban lévő tirozin részek környezetéből.

4. táblázat. Oligopeptidek a T-sejt antigén-receptor komplex ζ -lánc intracelluláris régiójában lévő tirozin részek környezetéből

<i>foszforiláció helye</i>	<i>peptid szekvenciája</i>	<i>peptid kódja</i>
Tyr ⁶⁴	PPAYQQG	T3 ζ ₆₁₋₆₇
Tyr ⁷²	NQLYNEL	T3 ζ ₆₉₋₇₅
Tyr ⁸³	REEYDVL	T3 ζ ₈₀₋₈₆
Tyr ¹¹⁰	QEGLYNEL	T3 ζ ₁₀₆₋₁₁₃
Tyr ¹²²	AEAYSEIG	T3 ζ ₁₁₉₋₁₂₆
Tyr ¹⁴¹	DGLYQGL	T3 ζ ₁₃₈₋₁₄₄
Tyr ¹⁵²	KDTYDAL	T3 ζ ₁₄₉₋₁₅₅

Ezen peptidek töltötték be a kináz reakció szubsztrátjainak szerepét. Kináz forrásként HPBALL sejtekből nyert immunoprecipitátumot használva a p56^{lck} képes a Tyr⁶⁴ kivételével az összes tirozin foszforilására. Legjobb szubsztrátnak a Tyr¹²² és Tyr¹⁵² bizonyult. Hasonló figyelhető meg a p59^{fyn} esetében is, bár jóval gyengébb intenzitással. Az előkísérletek azt mutatják, hogy a ZAP70 nem képes foszforilálni egyik peptidet sem. Ha a kináz forrás p56^{lck} immunoprecipitátum, az eredmények kismértékben eltérnek az előzőektől, ebben az esetben a Tyr¹⁴¹ foszforilációja a legkifejezettebb. Megállapítható, hogy különböző kinázok eltérő tirozinokat részesítenek előnyben. Megfigyeléseink jó összhangban vannak az eddig publikált kezdeti eredményekkel.

III. POSZTTRANSLÁCIÓS MÓDOSÍTÁSOK KÉMIAI SZIMULÁCIÓJA

A peptidek és fehérjék egy része az élő szervezetben poszttranszlációs átalakuláson megy keresztül. A szulfatálás, foszforileződés, glikoziláció a biokémiai folyamatok lényeges mozzanatai. Vizsgálatuk az immunválasz tanulmányozása szempontjából is fontos, mivel az immunrendszer eltérő módon ismeri fel a natív és a módosított molekulát.

Noha a rövidebb peptidek szintézise a szilárd fázisú technika elterjedése óta rutin feladatnak számít, az említett utólagos átalakítások ma is kihívást jelentenek a peptidkémikusok számára. Nemcsak maga a szintézis vet fel problémákat. A kapott termékek fokozott érzékenységűek, így stabilitásuk megőrzéséhez kíméletes körülményeket kell biztosítani. Szilárd fázison történő előállításuk új hordozókat igényel, olyanokat, amelyekről a peptid a szokásosnál enyhébb kezeléssel eltávolítható.

A, PEPTID-SZULFÁTÉSZTEREK ELŐÁLLÍTÁSA

Ismeretesek olyan peptidhormonok, amelyek szulfátészter alakjában is megtalálhatók az élő szervezetben. Legtöbbször tirozinon történik ez az utólagos átalakulás. A sokrétű biológiai hatással rendelkező kolecisztokininről kimutatták, hogy nem szulfatált szintetikus fragmensei

a biológiai aktivitásnak csak egy részével rendelkeznek (Johnson, 1970). Kolecisztokinin-szerű peptideket emlősökön kívül béka bőréből is izoláltak (Anastasi és mtsi., 1968). Az így nyert decapeptidet caeruleinnek nevezték el. Az eddigiekkel ellentétben az enkefalinnál a szulfátészter csoport lehasadása aktiválja a molekulát. A 17 aminosavból álló gasztrinnál nem találtak eltérést a szulfatált és nem szulfatált változat között.

Az általunk vizsgált peptideknél, a humán gasztrinnál és a caeruleinnél [52] a tirozin hidroxilcsoportja vesz részt a szulfátészter képzésben. Első lépésben a nem szulfatált peptideket állítottuk elő szilárd fázison, az I/A fejezetben leírtak alapján. A nyers peptideket a hordozóról hasítottuk, majd szolubilizáltuk és liofilizáltuk. A szulfatálásnál két módszert használtunk.

a, Klór-szulfonsav használata

A nyers peptidet piridin/dimetil-formamid elegyben oldjuk. Piridin/DMF/kloroform meghatározott arányú elegybe -10°C -on klór-szulfonsavat csepegtetünk. A két oldatot egyesítjük és szobahőmérsékleten 24 órán át kevertetjük.

b, Piridinium-acetil-szulfát alkalmazása

A csak sötét üvegben, -20°C -on, vízmentes körülmények között tárolható reagens ecetsavanhidridből, kénsavból és piridinből készül -15°C -on. A reagenst a peptid oldatához adjuk és néhány napig szobahőmérsékleten kevertetjük. A reakció lejátszódása után a termékeket a reagensektől elválasztjuk. A szulfátészterek savérzékenységük miatt csak semleges körülmények között tárolhatók. Tisztításukat RP-HPLC-vel végeztük. Az erősen savas körülményeket a kromatografálásnál is elkerültük, 0,5 M,



pH=6,5 ammónium-acetát oldatból készült eluenseket használtunk.

A leírt módszerek alkalmazásával sikerült nagyobb mennyiségben is elfogadható tisztaságú peptid-szulfátésztereket előállítani.

B, GLIKOPEPTIDEK SZINTÉZISE

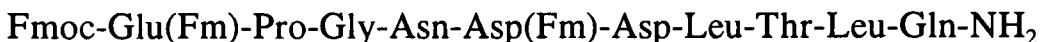
Kísérleteket végeztünk az EPGNDDLTQ peptid-amid glikozilezésére a 6. helyzetű aszparaginsavon keresztül.

N-glikozidok szintézisére két út ismeretes:

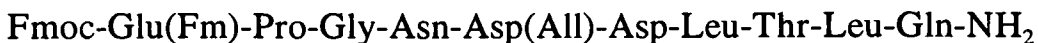
1. a megfelelően védett peptidek glikozilezése [53]
2. glikozilezett aminosavak beépítése [54, 55].

Az 1. módszernél az egyik legnagyobb hibát az jelenti, hogy a glikozilezés körülményei között a módosítandó aszparaginsav β -karboxilcsoportja szukcinimid-származékot képezhet az Asp C-terminálisán lévő amidcsoporttal. Ezt a mellékreakciót eddig csak abban az esetben sikerült elkerülni, ha az aszparaginsavat prolin követte. Célunk ezen 1. szintézisút tanulmányozása volt, különös tekintettel az említett mellékreakcióra.

Az I/A fejezetben leírtak alapján szintetizáltuk a következő peptidet:



A glutaminsav és az 5. aszparaginsav ω -karboxilcsoportjait fluorenil-metil-észterrel védtük. Később, a térgátlás csökkentése érdekében, az Asp⁵ védőcsoportját allil-észterrel cseréltük fel:



Az N-terminális Fmoc-csoportot úgy építettük be, hogy a szintézis után a gyantán lévő peptidet fluorenil-metil-oxi-karbonil-N-hidrox-

szukcinimiddel reagáltattuk. A monoszacharid a 2-dezoxi-2-acetamido-D-glükózamin volt. Számos kapcsolási módszert kipróbáltunk: diciklohexilamin/1-hidroxi-benztriazol, diciklohexilamin/pentafluor-fenol kondenzáció, BOP reagens (benztriazol-1-il-oxi-tris-{dimetil-amino}-foszfónium hexafluoro-foszfát), HBTU reagens (benztriazol-1-il-1,1,3,3-tetrametil-urónium hexafluoro-foszfát). Az első három esetben nem, vagy csak minimális mennyiségben képződött termék. HBTU reagenssel a kapcsolásokat ekvivalens mennyiségű diizopropil-etil-amin jelenlétében, vízmentes dimetil-formamidban végeztük, az irodalomban leírt módszerrel [56]. A reakciókat fordított fázisú HPLC-vel követtük a korábban (I/A fejezet) ismertetett feltételek mellett.

A peptidek reakciókészsége között számottevő különbséget nem tapasztaltunk. A kiindulási anyag mindkét esetben kb. egy óra alatt elfogyott a reakcióelegyből, ezt követően további változást nem észleltünk. Nagyobb része a kívánt terméké alakult, de szukcinimid-származék is képződött. Mennyiségi viszonyukat a kromatogramok integrálásával meghatározva közelítőleg 2:1 arány adódott a glikopeptid javára. Kísérleti eredményeinkből levonható a következtetés, hogy HBTU reagenssel, diizopropil-etil-amin jelenlétében előállíthatók glikopeptidek az 1. módszer alapján.

A II fejezet B pontjában leírt vizsgálatokhoz célul tűztük ki foszfátészter-csoport beépítését. Tirozin tartalmú, hét-nyolc aminosavból álló peptideken végeztük az átalakításokat. Előkísérleteink eddig nem hoztak egyértelmű eredményt. A jövőben tervezzük a megkezdett munka folytatását.

C, EGY PEPTIDSZINTÉZISRE ALKALMAS HORDOZÓ ÚJ ELŐÁLLÍTÁSA

A módosított peptidek szintéziséhez szükséges kíméletesebb körülmények új hordozókat követelnek. Egy ígéretesnek tűnő polimer a Rink által leírt 4-(2',4'-dimetoxifenil-hidroximetil)-fenoximetil-polisztirol [57]. Az igen érzékeny aminosav-hordozó kötés miatt az Fmoc stratégiával előállított peptidek enyhe savas kezeléssel eltávolíthatók a polimerről, így a savérzékeny funkciók gyakorlatilag nem károsodnak. Szintézisére kidolgoztunk egy új módszert [58, 59, 60]. Erre azért volt szükség, mert az eredeti eljárást követve alacsony borítottságú, nem egyértelmű szerkezetű hordozóhoz jutottunk.

Rink először 2,4-dimetoxi-4'-hidroxibenzofenont szintetizált, ezt cézium sóvá alakítva klórmetil-polisztirolhoz kapcsolta. A linkert a polimeren redukálta. A hordozón végzett redukció spektroszkópiai módszerekkel alig követhető, a képződő melléktermékek szinte egyáltalán nem mutathatók ki. Ezen felül a kapott hordozó borítottsága igen alacsonynak, mindössze 0,1 mmol/g-nak adódott.

A felmerült nehézségek miatt új szintézisutat választottunk: előállítottuk a kész linkert és ezt kapcsoltuk polimerhez. Ehhez a 2,4-dimetoxi-4'-hidroxibenzofenon redukciójának körülményeit optimalizáltuk. Megállapítottuk, hogy az irodalomban leírt egy órával szemben 10-15 perc alatt végbemegy a reakció, utána melléktermékek keletkeznek. A cézium sót kálium sóval helyettesítettük, így nemcsak lényegesen olcsóbb előállítási módszerhez, hanem magasabb borítottságú

polimerhez is jutottunk. Hordozónk szubsztitúciós foka 0,4-0,5 mmol/g-nak adódott.

A kidolgozott eljárás segítségével nagy mennyiségben is jó termeléssel, gazdaságosan állíthatunk elő módosított peptidek szintézisére alkalmas polimert.

ÖSSZEFOGLALÁS

Az immunválasz két oldalának, az antigéneknek és az ezekre reagáló sejteknek a tanulmányozása céljából szintetikus peptideket állítottunk elő. Az antigének vizsgálatához az influenza és az ezzel bizonyos hasonlóságot mutató HIV vírust választottuk. Az influenza esetében a vírus fertőzőképessége szempontjából döntő fontosságú membrán glikoproteint, a hemagglutinint tanulmányoztuk. Hasítási helyének környezetéből nyolc célszerűen megválasztott peptidet szintetizáltunk, melyek az enzimatis lebonrás után szabaddá váló alegységek (HA1 és HA2) C- illetve N-terminálisait képviselek. Előállítottunk három elágazó szerkezetű antigént is, melyek az irodalomból ismert MAP (multiple antigenic peptide) szerkezetek eddig nem publikált módon átalakított változatai. A peptidek tisztítását, szerkezetigazolását követően elvégzett immunológiai vizsgálatok bizonyították, hogy a H1/HA1₃₁₇₋₃₂₈ és ennek hosszabbított változatai aktiválják a T-sejteket. A MAP-1 jelű elágazó struktúra túlélési kísérletben 80 %-os védeettséget idézett elő egerekben ötszörös letális dózisú influenza vírussal szemben, ami figyelemre méltó eredmény szintetikus vakcinák készítésének irányában.

Több szerotípus ellen hatásos ellenanyag tervezése céljából a vizsgálatokat kiterjesztettük a H2 és H3 altípusokra is. CD és FT-IR spektroszkópiával a jó epitópnak bizonyult szekvenciáknál rendezett (β -turn, esetenként α -hélix) szerkezeteket sikerült kimutatni.

Az irodalomból ismerve a HIV és az influenza vírusok érési

folyamatai közti analógiát szintetizáltunk négy peptidet a HIV vírus gp120 köpenyfehérjéjéből. Az immunológiai és konformációs vizsgálatok még nem zárultak le.

Az antigénekre reagáló sejtek közül a T-sejtet, közelebbről a felszínén lévő antigén-receptor komplexet tanulmányoztuk. Célunk egyrészt az antigén-felismerés, másrészt az ezt követő jelátvitel vizsgálata volt. A ζ és δ láncokon elvégzett számítógépes predikció eredménye alapján megfelelő epitópnak jósolt régiókból négy rövidebb peptidet és három MAP szerkezetet szintetizáltunk. Az oligopeptideket és esetenként ezek dimerjeit BSA és tireoglobulin hordozókhoz kapcsoltuk, majd az elágazó szerkezetű antigénekkel együtt immunizálásra használtuk. Az előállított antiszérumok felismerték az immunizáló ágenseket, de nem ismerték fel a natív ζ -láncot. Az immunreakció specifikus voltát is igazoltuk.

A jelátvitelt a p56lck, a p59fyn és a ZAP70 kinázok esetében vizsgáltuk. Az enzimekből kiválasztott epitópokból négy MAP szerkezetet szintetizáltunk. Az ellenük termelődött antiszérumon a p59fyn kivételével mind a MAP-ot, mind a natív fehérjét felismerték. A ζ -lánc foszforilációs helyeinek pontos feltérképezése céljából oligopeptideket szintetizáltunk az intracelluláris régió tirozinjainak környezetéből. Különböző forrásokból nyert kinázokat használva megállapítottuk, hogy a Tyr⁶⁴ kivételével mind a hat tirozin szubsztrátja a foszforilezésnek, de az egyes kinázok eltérő módon preferálják egyik illetve másik tirozint.

A peptidek, fehérjék élő szervezetben végbemenő poszttranszlációs átalakulásai közül a szulfatálást, glikozilezést és foszfátálást tanulmányoztuk. Mindhárom esetben azonos stratégiát követtünk: a

módosíthatlan peptidet szintetizáltuk és feldolgozás után ezt vetettük alá az említett utólagos átalakításoknak.

A humán gasztrin és a caerulein szulfatálását két módszerrel, klór-szulfonsav, illetve acetyl-pyridinium-sulfate alkalmazásával valósítottuk meg.

Glikozilezéshez modellként egy aszparaginsavat tartalmazó decapeptidet és a 2-deoxy-2-acetamido-D-glucose-amine-t választottuk. A kapcsolat kivitelezésére legalkalmasabbnak az HBTU reagens bizonyult diisopropyl-ethyl-amine jelenlétében. A termelést csökkentette a mellékreakcióként fellépő succinimide-derivatives képződés.

A poszttranszlációsan módosított peptidek szintéziséhez szükséges enyhe reakciókörülmények biztosítása érdekében foglalkoztunk savérzékeny polimerek előállításával. Egy jó tulajdonságokkal rendelkező hordozó, a Rink által leírt polimer szintézisére új módszert dolgoztunk ki, amely az eredetivel magasabb borítottságú terméket eredményez és az irodalomból ismert eljárásnál gazdaságosabb.

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MELLÉKLET

Conformational and functional properties of peptides covering the intersubunit region of influenza virus hemagglutinin

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The functionally active part of influenza virus hemagglutinin was investigated through the synthesis of a series of peptides representing different parts of the intersubunit region. Secondary structure prediction, circular dichroism and Fourier transform infrared spectroscopic studies were undertaken to investigate the secondary structure of these peptides. The peptide fragments were found to adopt multiple conformations, depending on their concentration in solution, the presence of the non-ionic detergent octyl- β -D-glucoside and the polarity of the solvent. The results of biological studies with these peptide fragments are discussed in relation to their conformation, as inferred from the spectroscopic analysis.

Hemagglutinin, the envelope glycoprotein of the influenza virus, is a trimer of molecular mass 224 640 Da. It is an integral membrane protein characterized by a three-domain structure [1]. A basic requirement for the infectivity of the virus is the post-translational enzymic cleavage of the external domain of hemagglutinin which, by elimination of Arg329 results in two subunits, which we refer to as hemagglutinin 1 (HA1) and hemagglutinin 2 (HA2) (see Scheme 1 for the amino acid sequence of these fragments). This separation exposes the N-terminal of HA2, the so-called fusion peptide [1–3]. It is this peptide which, after a pH-dependent conformational change, enables the fusion of the virus envelope with the endosomal membrane of the host cell. According to earlier circular dichroism and fluorescence spectroscopic studies [4], the 20-amino-acid fragment, but not the 16-amino-acid fragment from the N-terminal fusogenic region of HA2 adopts a helical conformation when bound to vesicles. Using circular dichroism spectroscopy, Wharton et al. [5] found a correlation between fusogenicity and α -helix content of a 23-amino-acid fragment of HA2.

To elucidate whether the conformation of this part of hemagglutinin plays a role in T-cell and B-cell recognition, a series of peptides representing the intersubunit region (IP), the fusion peptide (FP1) and the remnant of the hemagglutinin intersubunit (HA1C-Arg) were synthesized, along with analogues that contain a DArg in the central position [DArg13]IP, lack an arginine at the N-terminal peptide (HA1C), or contain

arginines at both ends of the fusion peptide (FP3; see Scheme 1). As B-cell recognition is highly dependent on determinant conformation [6, 7] and T cell epitopes are characterized by restricted conformational requirements [8], the conformational characterization of these peptides was of particular interest.

Circular dichroism and Fourier-transform (FT-IR) spectroscopic studies were undertaken to explore the secondary structure adopted by these synthetic peptides in solution. The conformation of the peptides was investigated in aqueous solution and in trifluoroethanol, a structure-promoting organic solvent. Changes in peptide conformation are discussed in terms of their impact on antibody and T-cell recognition.

MATERIALS AND METHODS

Peptide synthesis

The hemagglutinin peptide fragments shown in Scheme 1 were all synthesized by solid-phase techniques. Side chain-protecting groups were as follows: Arg (tosyl), Glu (cyclohexyl), Thr and Ser (benzyl). Peptides were synthesized on *p*-methylbenzhydrylamine resin (0.65 mmol/g) and synthesis was carried out manually. Couplings were performed by use of dicyclohexylcarbodiimide, with the exception of Gln, Asn and Arg, which were incorporated as their *N*-hydroxybenzotriazole esters. Amino acid incorporation was monitored by the ninhydrin test. The completed peptide resins were treated with liquid HF/dimethylsulphide/*p*-cresol (88:6:4, by vol.) at 0°C for 1 h. HF was then removed and the free peptides solubilized in 10% aqueous acetic acid. The crude peptides were purified by semipreparative reverse-phase HPLC on a C₁₈ column (16 mm × 250 mm). Peptides were

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Abbreviations. FT-IR, Fourier transform infrared; IP, intersubunit peptide; FP, fusion peptide; HA, hemagglutinin.

IP	1	Val-Thr-Gly-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser-Arg-Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Gly	25
FP1			Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Gly
FP3			Arg-Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Gly-Arg
HA1C		Val-Thr-Gly-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser	
HA1C-Arg		Val-Thr-Gly-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser-Arg	
[pArg13]IP		Val-Thr-Gly-Leu-Arg-Asn-Ile-Pro-Ser-Ile-Gln-Ser-Arg-Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Gly	

Scheme 1. Amino acid sequence and abbreviations of the synthetic peptides from the intersubunit region of influenza virus hemagglutinin used in this report (APR8/34; Murata et al. [12]).

eluted with a linear gradient (0–76%) of acetonitrile containing 0.1% trifluoroacetic acid. Due to this purification procedure, the peptides are present as trifluoroacetate salts. In order to remove the trifluoroacetate counter ion (which has an infrared band around 1670 cm^{-1}), the peptides were additionally purified by chromatography on an Amberlite IR-45 minicolumn. The purified peptides were characterized by amino acid composition and analytical HPLC in two different solvent systems.

Spectroscopy

Circular dichroism measurements were performed on a Jasco J720 dichrograph at room temperature in a 0.2-mm-path-length cell. Double-distilled water and NMR-grade trifluoroethanol (Aldrich) were used as solvents. Octyl- β -D-glucoside was from Sigma. Each measurement was the average of five repeated scans in steps of 0.2 nm. Unless otherwise stated, the peptide concentration was 1 mg/ml. The mean residue ellipticity ($[\theta]_{\text{MR}}$) is calculated using a mean residue molecular mass of 110 Da.

Aqueous peptide solutions for FT-IR spectroscopy [$\approx 100\text{ }\mu\text{l}$; 2% (mass/vol.)] were prepared in D_2O and adjusted to pH 7 with NaOD or DCl as needed. Solutions in trifluoroethanol were prepared at similar concentrations. Solid-state spectra were recorded from KBr discs. For temperature studies, the CaF_2 cell was placed in a temperature-controlled holder. Infrared spectra were recorded on a Digilab FTS-60 FT-IR spectrometer at a resolution of 2 cm^{-1} .

RESULTS

Secondary structure prediction

Application of the Chou-Fasman-Prevelige algorithm [9] to a 48-amino-acid segment of the hemagglutinin protein which contains the IP fragment in positions 18–42, shows that the C-terminal part of IP (represented by peptides FP1 and FP3 from the fusion region) has the ability to adopt both α -helical and β -sheet conformations (Fig. 1). The N-terminal fragment of IP, HA1C-Arg, has no propensity for α -helical conformation; however, it contains an additional β -forming core (Ile-Pro-Ser-Ile). The N-terminal part of IP is also predicted to form repeating β -turns (Val-Leu-Gly-Thr, Ile-Pro-Ser-Ile and Gln-Ser-Arg-Gly or Ser-Arg-Gly-Leu, and possibly Leu-Arg-Asn-Ile). Thus, the critical Arg residue at position 329 of hemagglutinin (indicated by an asterisk in Fig. 1), is situated in a region where a β -turn is likely to occur.

Circular dichroism spectroscopic studies

Circular dichroism spectra, in trifluoroethanol, of the hemagglutinin fragments IP, [pArg13]IP, FP3 and HA1C-Arg are illustrated in Fig. 2. IP gives a helical circular dichroism

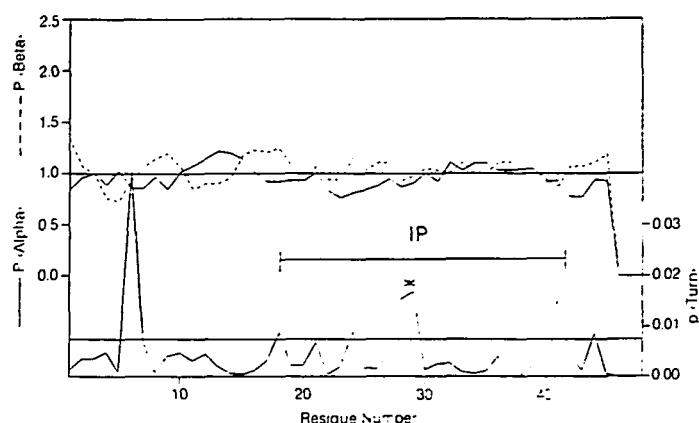


Fig. 1. Secondary structure predictions for a 48-amino-acid segment of hemagglutinin which contains the IP fragment in positions 18–42. The asterisk indicates the position of Arg329 in this segment of hemagglutinin. $P(\alpha)$, $P(\beta)$ and $P(\text{turn})$ represent the probabilities for the respective secondary structural elements; for details see [9].

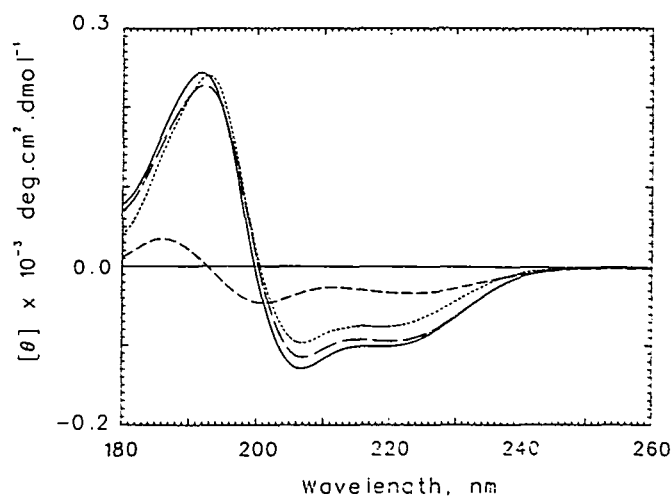


Fig. 2. Circular dichroism spectra, in trifluoroethanol, of the hemagglutinin peptide fragments IP (—), [pArg13]IP (---), FP3 (····) and HA1C-Arg (-·-·-).

spectrum; the α -helical content, calculated by the procedure of Greenfield and Fasman [10], or by that of Yang et al. [11], is approximately 30%, which corresponds to seven or eight residues (two turns of the helix). Fragment FP3 also gives a spectrum with helical features, which, in agreement with the secondary structure predictions, indicates that the helical component of IP is located in its C-terminal half, represented by FP3. A lower absolute intensity and the different relative

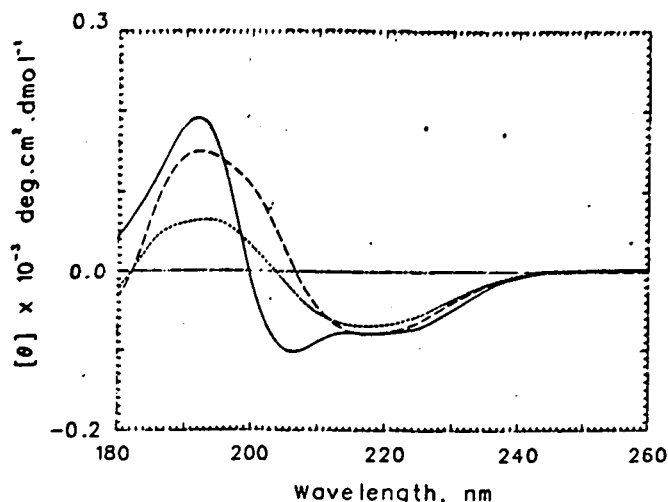


Fig. 3. Circular dichroism spectra of IP in 1:1 trifluoroethanol/D₂O (—), 10/90% trifluoroethanol/D₂O (····) and in D₂O containing 2.5% octyl- β -D-glucoside (—).

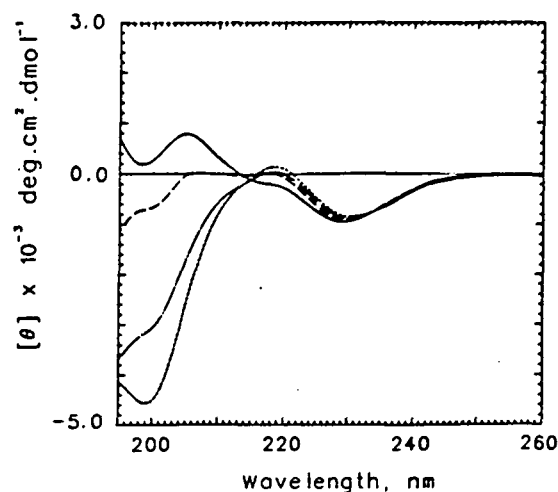


Fig. 4. Circular dichroism spectra of FP3 at different concentrations in water (pH 6): 5.4 mg/ml (—), 2.7 mg/ml (—), 1.35 mg/ml (····) and 0.74 mg/ml (—).

band intensities in the spectrum of FP3 compared to that of IP are compatible with a shorter or looser α -helix in FP3, or with the presence of more than one conformer population of FP3. The fact that IP and [DArg13]IP have very similar spectra suggests that the central arginine in IP, and possibly the adjacent serine and glycine residues, may not play a significant role in determining the chiroptical properties of this peptide. In addition to about 30% α -helix and about 30% unordered conformation, a significant amount of β -sheet ($\approx 35\%$) was calculated for IP, [DArg13]IP and FP3. The peptide fragments HA1C-Arg and HA1C exhibit blue-shifted, low-intensity helical (class C) spectra which can be correlated with certain subtypes of β -turns or their repeats (distorted helices, e.g. 3_{10} helix) [13–15].

Addition of water to trifluoroethanol results in a gradual change in the circular dichroism spectra of IP and FP3; however, the helical character of the peptide segments is preserved until the water content reaches 50%. The spectrum of IP in pure water (pH 5.5) is indicative of an equilibrium between two or more conformers. In an aqueous solution containing 2.5% octyl- β -D-glucoside, a non-ionic surfactant with secondary-structure-promoting properties, IP shows a spectrum with a negative band at 218 nm and a positive band at 183.5 nm (Fig. 3). The single negative band indicates the predominance of a β -sheet conformation, while the shape of the positive band may be related to a small contribution from an aperiodic structure [14]. Thus, octyl- β -D-glucoside has a similar effect to that of low (<25%) trifluoroethanol concentrations in water, whereby β -structures are favoured. The analysis of the IP spectrum shows that the β -sheet contribution to the spectrum is dominant ($\approx 50\%$), though a significant amount ($\approx 25\%$) of α -helix is also present.

While the circular dichroism spectra of IP and FP3 in trifluoroethanol showed no concentration dependence (in the range 0.1–3 mg/ml), a definite spectral change with increasing concentration was observed in water. The spectra at higher concentrations are suggestive of the presence of significant amounts of β -conformers. On dilution in water, the spectral contribution of the β -sheet gradually decreased and, below a 0.5 mg/ml, the spectra revealed the predominance of an aperiodic structure (Fig. 4). These results are consistent with

β -sheet formation stemming from aggregation of IP and FP3 in water at higher concentration. IP and FP3 are switch peptides with expressed α -helicity in helix-promoting environments, and a preponderant β -sheet character at higher concentrations or in the presence of micelles. Below the critical micelle concentration ($\approx 0.7\%$), IP and FP3 showed spectra similar to those measured in dilute aqueous solutions. Since the N-terminal fragment of IP also has some β -forming potential, single chains of this peptide have the ability of forming antiparallel β -pleated sheets with strong H-bonds between adjacent chains.

Infrared spectroscopic studies

The infrared spectrum of IP and those of its C-terminal peptides FP1 and FP3 obtained from lyophilized solids reveal that these peptides are highly aggregated. A strong amide-I band at 1628 cm^{-1} in IP and at 1632 cm^{-1} in FP1 and FP3, along with the presence of a weaker component band at about 1690 cm^{-1} , identify these aggregates as antiparallel β -sheets [16–18]. A broad band at $1650\text{--}1665\text{ cm}^{-1}$ indicates the presence of other populations (aperiodic conformers, α -helical components and turns). The amide-II band shows peaks at 1523 cm^{-1} (typical for β -type structures) and at 1546 cm^{-1} (characteristic of aperiodic and/or α -helical structures), indicative of the existence of at least two populations effectively frozen in a solid KBr matrix.

In aqueous (D₂O) solution, the infrared spectrum of IP also exhibits the strongest amide-I band at 1626 cm^{-1} , with a weaker band at about 1698 cm^{-1} and a broad unstructured band centred at about 1650 cm^{-1} (Fig. 5). Band narrowing by Fourier self-deconvolution [19] leads to a better visualization of the individual bands (Fig. 5). The $1626\text{--}1698\text{ cm}^{-1}$ band pair reflects the prevalence of the β -sheet conformation in D₂O, which was also detected in the solid state. The broad band at 1650 cm^{-1} is compatible with the presence of some unordered and/or α -helical peptide segments. The weak band at 1585 cm^{-1} is characteristic of the arginine side-chain group. The amide-II band at about 1545 cm^{-1} (due to aperiodic and α -helical amide groups) has shifted to about 1450 cm^{-1} as a consequence of NH to ND exchange; however, the presence of a residual amide-II band at 1526 cm^{-1} indicates that even

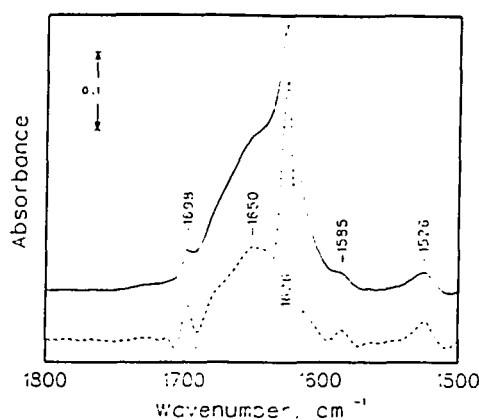


Fig. 5. Infrared spectrum of the hemagglutinin peptide fragment IP in aqueous (D_2O) solution in the region of the amide-I and amide-II bands (—). (---) The same spectrum after reducing the widths of the infrared bands by a factor of 1.5 by use of Fourier self-deconvolution [19].

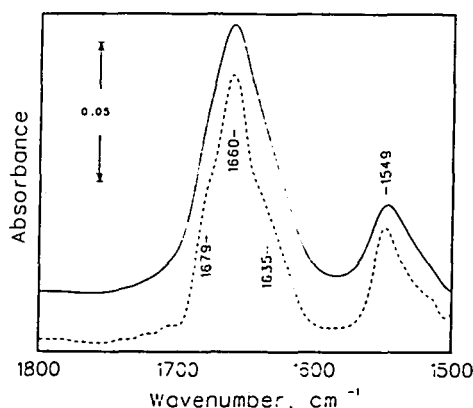


Fig. 6. Infrared spectrum of the hemagglutinin peptide fragment IP in trifluoroethanol solution in the region of the amide-I and amide-II bands (—). (---) The same spectrum after reducing the widths of the infrared bands by a factor of 1.75 by use of Fourier self-deconvolution [19].

after 24 h this exchange is not complete for the β -sheets. However, when a solution of IP was heated from ambient temperature to $80^\circ C$, this band disappeared gradually between $64^\circ C$ and $80^\circ C$, and the 1626-cm^{-1} band shifted to 1622-cm^{-1} , reflecting the complete NH to ND exchange of the β -component.

In trifluoroethanol solution (Fig. 6), the infrared spectrum undergoes a radical change. The major amide-I band is at 1660-cm^{-1} , with shoulder bands at 1635-cm^{-1} and 1679-cm^{-1} . The major amide-II band is at 1549-cm^{-1} band can be attributed to a weakly hydrogen-bonded α -helical conformation, the band at 1679-cm^{-1} is in the region of turns, while the 1635-cm^{-1} band reflects the presence of some residual β -structure (possibly β -turns). The change of solvent from trifluoroethanol to a 1:1 trifluoroethanol/ D_2O mixture has the net effect of increasing the β -sheet component at the expense of the α -helical component. The spectrum of IP in D_2O solution containing 2.5% octyl- β -D-glucoside also shows an increase in the β -sheet components compared to that in D_2O .

FP1 is only slightly soluble in either D_2O or trifluoroethanol; however, FP3 dissolves more readily both in

D_2O and trifluoroethanol and the spectra are similar to those of FP1. The spectrum in D_2O has only a strong band at 1626-cm^{-1} and a weak one at 1688-cm^{-1} . The position of the two bands and their relative intensities are typical of antiparallel β -sheet structures [16–18]. Upon heating an aqueous (D_2O) solution of FP3 to $80^\circ C$, the peak positions did not change. The remarkable stability of this peptide may be attributed to strong hydrogen-bonded β -strands, probably formed between single peptides. The spectrum of FP3 in trifluoroethanol resembles that of IP except that the band at 1635-cm^{-1} is stronger, indicating that FP3 retains a larger proportion of β -sheet conformation.

The infrared spectra in D_2O of the N-terminal peptide fragments HA1C and HA1C-Arg are practically identical and exhibit only a single broad amide-I band centred at approximately 1646-cm^{-1} , typical of unordered peptides proteins in D_2O solution [17, 18].

DISCUSSION

Generally, there is good agreement between the circular dichroism and FT-IR data on the conformation of the hemagglutinin fragments. Minor differences may be related to the nature of the two techniques. While circular dichroism spectra provide information on the relative spatial orientation of the backbone amide groups, the main chromophores of peptides and proteins, FT-IR spectroscopic data obtained from the structure-sensitive C=O stretching vibration (amide-I band) reflect the strength of the H-bonding in the different secondary structures. Both type of spectra indicate a significant α -helical content for IP in trifluoroethanol and show an α -to- β conversion in mixtures containing increasing amounts of water. The position of the amide-I band at 1660-cm^{-1} reflects the weakness of the hydrogen bonds in the helix.

Both circular dichroism and FT-IR spectra provide evidence for β -sheet conformation in IP, FP1 and FP3 in aqueous solution; this is probably due to intermolecular association of the peptides in solution, as evidenced by the low frequency (1626-cm^{-1}) of the amide-I band. The position and relative intensity of the amide-I bands is consistent with their assignment to an antiparallel β -sheet conformation [16–18]. This information cannot be derived from the circular dichroism spectra of the peptides.

The presence of β -turns is supported by an X-ray analysis of the cleaved hemagglutinin, which shows that the N-terminal glycine-rich sequence of chain HA2 (comprised by FP1 and FP3) forms a series of four contiguous reverse β -turns [20].

On the other hand, the infrared spectra of HA1C and HA1C[Arg] differ considerably from those of the other peptide fragments. A single strong band at 1646-cm^{-1} is clearly indicative of a conformation without any secondary structure. Indeed, the secondary structure predictions (Fig. 1) and the class-C circular dichroism spectra of these fragments do not support the occurrence of ordered peptide conformation.

A comparison of X-ray diffraction, circular dichroism and FT-IR spectroscopic data suggests that FP1, FP3 and presumably the N-terminal fusogenic region of HA2, can adopt a variety of conformations (α -helix, β -sheet and repeating β -turns). Structural and environmental factors may, however, bring about the prevalence of a single conformational state. The finding that the sugar-coated octyl- β -D-glucoside micelles prompt the formation of β -sheet structure of IP and FP3, even in dilute aqueous solution suggests that an event preceding

membrane fusion might be chain extension (pleating) of the N-terminal domain on the glycoprotein surface of the endosomal membrane. It is also reasonable to suppose that membrane fusion itself is triggered by a subsequent pH-dependent β -to- α conformational transition of the region, which is initiated by the overall pH-dependent conformational change of hemagglutinin trimers. This assumption is supported by previous findings providing evidence for the correlation of helix formation with fusogenicity [5].

The conformational mobility of peptides comprising the intersubunit region of hemagglutinin may be of importance also in immune recognition as it represents the most conservative amino acid sequence [20, 21]. The functionally essential posttranslational modification of this region occurs late in the secretory pathway [22, 23], and is mediated by host-cell proteases. The event triggers membrane fusion but also influences enzymic degradation and antigenic processing [24, 25].

According to its sequence, the C-terminal half of IP (which comprises FP3) may adopt an α -helix with some amphipathic character (repeating occurrence of glycine residues). FP3 is terminated by a Rothbard motif. Thus, this peptide fulfills both the criteria suggested by Berzofsky and Rothbard for potential T-cell epitopes [26, 27]. Notably, no typical Rothbard motif is contained in the N-terminal part of IP (represented by HA1C-Arg and HA1C).

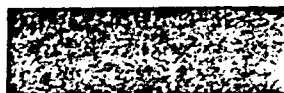
Results of immunological studies on peptides covering the intersubunit area demonstrate that this region of hemagglutinin can be the target of both B-cell and T-cell recognition. A neutralizing monoclonal antibody inhibiting the membrane fusion event could be isolated from the spleen of an influenza virus infected Balb/c mouse. This antibody recognizes the intersubunit peptides IP and [DArg13]IP, but does not react with any of the subunit peptides (Kurucz et al., Rajnavögyi et al., unpublished results). IP in addition carries T-cell epitopes, available also in HA1C and FP3. The T-cell recognition in the context of major histocompatibility class II molecules of d haplotype, however, is influenced by the presence of Arg329 (situated in the center of IP).

According to our comparative spectroscopic approach, both halves of the intersubunit peptide (represented by HA1C, HA1C-Arg and FP1, FP3) are capable of adopting multiple conformations and that different conformations may prevail depending on environmental conditions (nature of solvent, buffer, presence of lipids, pH, etc). It is therefore very likely that the interaction with major histocompatibility structures plays an important role in stabilizing peptide conformations suitable for T-cell recognition. In contrast, antibody recognition requires a more defined conformation adopted by the hemagglutinin molecule and maintained in IP. Based on the circular dichroism and FT-IR evidence discussed earlier, IP and also [DArg13]IP adopt a β -sheet conformation in aqueous solution containing octyl- β -D-glucoside micelles. This finding, in correlation with the high β -turn propensity of the central Arg-containing segment, may suggest a β -sheet structure as a common organization essential for monoclonal antibody recognition.

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Branched Polypeptides as Antigenes for Influenza Virus Hemagglutinin and T-Cell Receptor Subunits

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ABSTRACT

The multiple antigenic peptide (MAP) method was applied to improve the immunogenicity of synthetic peptides representing distinct regions of the influenza virus hemagglutinin (HA). A tetrameric MAP with multiply incorporated overlapping B- and T-cell epitopes was combined with a particular HA sequence representing the slightly modified fusion peptide on the C-terminus of the Lys core (MAP-1). As a result of repeated injections of BALB/c mice with MAP-1 but not with the monomeric HA1C[Arg] peptide, the appearance of MAP-1-specific antibodies crossreactive with the acid-pretreated virus could be observed. In vitro studies revealed the potency of the MAP-1 structure to induce proliferation of HA1C[Arg]-primed T-cells, and in vivo studies demonstrated the protective feature of the immune response

elicited by MAP-1 and to a lesser extent by the monomeric HA1C[Arg]. The increased level of MAP-1-specific antibodies upon viral challenge shows the activation of MAP-1-specific B- and/or T-cells.

The advantage of the previously verified FP3 helper T-cell epitope included in MAP-1 was further utilized to synthesize chimeric structures comprising short fragments of the ζ (MAP-2) or δ (MAP-3) subunits of the T-cell antigen receptor (TCR) complex. The selected peptides of the ζ - and δ -chain regions failed to elicit an antibody response in BALB/c mice as tetra- or octamers, but the inclusion of the modified fusion region resulted in an immunogenic construction. The chimeric MAP-2 and MAP-3 were successfully used to develop polyclonal and monoclonal antibodies recognizing the corresponding multimeric peptides, but they were unable to bind to the cell membrane-expressed form of the subunits.

The MAP constructions that were designed, including appropriately selected B- and helper T-cell epitopes, were proven to be immunogenic; but the crossreactivity of the induced antibodies with the corresponding native proteins was highly dependent on the individual characteristics of the resultant combinations.

INTRODUCTION

The MAP method was successfully used to increase the immunogenicity of various peptide fragments of different proteins (10,17,32,37,40,42). The most promising approaches were aimed at

the enhancement of the protective immune response against distinct pathogens such as the major immunogenic region of the foot-and-mouth disease virus (FMDV) (20), the circumsporozoite antigen of malaria (14,30,38,39), hepatitis (39), *Schistosoma mansoni* (7) or the V3 loop of the human immunodeficiency virus (Levi et al., 22th Eur. Pept. Symp. 1992, A413). This method offers attractive advantages: a) the molar ratio of the peptide epitope to the core molecule is high; b) this kind of structure provides enhanced reproducibility as compared to the previously used peptide-protein carrier conjugates; c) there is no carrier-mediated epitope competition because of the low immunogenicity of the core region.

The effect of copy number was systematically studied with FMDV-derived peptides (20), and tetramers were demonstrated as efficient as octamers. Immunization with MAP in most cases requires adjuvants; however, the widely used but highly toxic Freund's adjuvant can be substituted by other approaches such as Al(OH)₃, the only adjuvant licensed for human application (20), or by the involvement of lipopeptides (13,21,24) or other recent immunopotentiators used for viral subunits (15,36).

Recent improvements to this technology (40), including peptide vaccines containing different incorporated adjuvants (12,13,21,24), opened up wide possibilities for eliciting an efficient memory response involving not only IgG-type antibodies, but also helper and cytotoxic T-lymphocytes (15,36) reactive with both the vaccine and the pathogen. A critical consideration in the development of peptide-based subunit vaccines is the inclusion of one or more potent T-cell epitopes to promote the generation of appropriate T-cell help and long-lasting memory (27,50). The limitation of this alternative lies in the critical orientation of the T- and B-cell epitopes included in the immunogen (27,38). If B-epitopes are linked to T-epitopes in the appropriate position, the immunogenicity can be improved. However, the T-cell-dependent immune response is restricted by the polymorphic major histocompatibility gene complex (MHC). Therefore, a valuable vaccine that is immunogenic in all members of an outbred population requires more than one T-cell epitope. The MAP approach was estab-

lished to also overcome the genetic restriction mediated by MHC (14,31).

The first approach in the present study was aimed at increasing the immunogenicity of a previously identified overlapping T/B-epitope of influenza virus (A/PR/8/34: H1N1) hemagglutinin (HA) (22,33). For these studies, an elongated analog of the HA fusion peptide, characterized by a highly hydrophobic conserved sequence (6,22, 33), was grafted by four tridecapeptides comprising the C-terminal 317-329 amino acids of the HA1 subunit (HA1C[Arg]; see Figure 1, MAP-1). The idea to combine these peptides was based on previously published results demonstrating that the C-terminus of the HA1 subunit encompasses multiple T- and B-cell epitopes (10,35), and that the fusion peptide is also recognized by virus-specific antibodies (6). In our previous studies it was shown that the HA1C[Arg] region and the Arg extended fusion peptide FP3 are efficient in inducing a proliferating T-cell response (33 and É. Rajnavölgyi, unpublished observation). Additionally, the HA1C[Arg] region is immunogenic and can be recognized by anti-peptide or anti-influenza monoclonal antibodies only if it is linked to the fusion peptide (References 22 and 33, and Z. Nagy et al., unpublished observation).

The activation of T-lymphocytes, including the T-helper subpopulation, occurs when they are triggered via the T-cell antigen receptor (TCR) complex. The TCR complex consists of the idiotypic α - and β -chains noncovalently associated with the γ -, δ - and ϵ -polypeptides of the CD3 complex, and additionally with the ζ - and η -chains (23). The α - and β -heterodimers are responsible for the specific recognition and binding of the antigen, while the CD3 complex, as well as the ζ - and η -chains, participates in signal transduction generated by antigen binding. Although a number of studies strongly indicate the participation of the CD3 ϵ - and ζ -chains in signal transduction (23), the roles of the other polypeptides associated with the TCR have not been revealed so far. Monoclonal antibodies are powerful tools used for studying cell-surface receptors or their subunit function. The production of antibodies against cell-surface receptors encounters several difficulties. Isolation or purification of the membrane-bound proteins are laborious, if not impossible—

the antibodies generated against the whole cells, membrane fractions or even the enriched receptor react with a number of uncharacterized epitopes. The close homology of the TCR-related subunits in different species raises further difficulties (47). As the MAP approach was successfully utilized to obtain monoclonal antibodies to the TCR α - and β -chains (32), we applied this method in the development of monoclonal antibodies to the N-terminal extracellular part of the human TCR δ - and ζ -subunits.

In the second part of this study, we synthesized the octameric MAP of the N-terminal extracellular region of the human ζ -chain identical to its murine counterpart. To overcome the inefficiency of this construction to elicit antibodies in BALB/c mice, the tetrameric MAP of these peptides was grafted to the influenza-derived FP3 peptide (Figure 1), introduced as a T-cell epitope and as a hydrophobic region previously shown to support the conformational stability of N-terminal peptide conformation (22). In the MAP-2 and MAP-3 constructions, the orientation of the δ - and ζ -chain peptides was identical to that of the HA1C[Arg] B-cell epitope (Figure 1).

The two approaches applied in this study provided valuable information on the applicability and limitations of the MAP system.

MATERIALS AND METHODS

Chemicals

Dichloromethane (DCM), dimethylformamide (DMF), diisopropylethylamine (DIEA), methanol (MeOH), trifluoroacetic acid (TFA) and HPLC-grade acetonitrile (AcN) were purchased from E. Merck (Darmstadt, Germany) and were used without further purification. Fluka (Buchs, Switzerland) supplied puriss-grade dicyclohexylcarbodiimide (DCC). Protected amino acid derivatives were from Bachem (Torrance, CA). 1-Hydroxy-benzotriazole (HOBt), *p*-cresol and dimethyl sulphide were purchased from Aldrich (Steinheim, Germany). Hydrogen fluoride (HF) was obtained from UCAR (Olen, Belgium).

Peptide Synthesis

The branched peptide antigens were synthesized by a solid-phase technique,

utilizing 'Boc chemistry (29). Side-chain protecting groups were as follows: Arg(Tos), Asp(OcHex), Cys(Meb), Thr(Bzl), Lys(2CIz), Glu(OcHex), Tyr(2BrZ) and Ser(Bzl). The peptide chains were elongated on MBHA resin (0.8 mmol/g) and the syntheses were carried out manually. Couplings were performed with DCC, with the exception of Asn, Gln and Arg, which were incorporated as their HOBt esters. Amino acid incorporation was monitored with the ninhydrin test (25). In the event of incomplete coupling, this step was repeated, but DCM was substituted by DMF. The completed peptide resins were treated with liquid HF/dimethyl sulphide/*p*-cresol (88:6:4, vol/vol), at 0°C for 1 h. HF was removed and the resulting free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized.

Peptide Purification and Characterization

The crude peptides were purified by reverse-phase HPLC (RP-HPLC), using an Astec 300 C4 5- μ column (8 $\times$ 250 mm). The HPLC apparatus was produced by Knauer (Berlin, Germany). The solvent system used was as follows: 0.1% TFA in water, 0.1% TFA + 80% AcN in water; gradient: 0% $\rightarrow$ 90% B in 1.5 h; flow 1.5 ml/min. The appropriate fractions were pooled and lyophilized. Purity was checked by RP-HPLC, isoelectrofocusing and SDS polyacrylamide gel electrophoresis (PAGE). The analytical HPLC investigations were made on a Vydac C4 column (4 $\times$ 250 mm, 214TP54; The Separations Group, Hesperia, CA), at a flow rate of 0.8 ml/min, with detection at 220 nm; the solvent system used was as follows: A: 0.1% TFA in water, B: 0.1% TFA + 80% AcN in water; gradient: 0% $\rightarrow$ 90% B in 20 min. Two-dimensional electrophoresis, with isoelectric focusing vs. SDS PAGE, was carried out by using Servalyt Ag 2-11 (Serva, Heidelberg, Germany) in the first dimension and 15% SDS PAGE as the second dimension.

Mass Spectrometry

The FAB-MS experiments concerning the MAP-1 were performed with a VG ZAB-2SEQ hybrid tandem mass spectrometer, equipped with an LSIMS source (Cs⁺ ion gun used at 30 keV). The tryptic digestion was carried out

"on tip." The matrix-assisted laser desorption ionization measurements were carried out with MALDI III equipment (Kratos, Manchester, UK) in linear mode.

Immunological Methods

a) Induction of anti-MAP-1 antibodies

BALB/c mice (Institute of Oncology, Budapest, Hungary) were immunized with 100 µg MAP-1 (see Figure 1) or with the tridecamer free HA1C[Arg] or FP3 HA peptides emulsified in complete Freund's adjuvant (CFA) (Sigma Chemical, Deisenhofen, Germany) by s.c. injection into the hind footpads and the base of the tail. Eight weeks later, the mice were challenged with the same amount of peptide emulsified in incomplete Freund's adjuvant (IFA) (Sigma). Blood samples were taken 14 days after the last antigen challenge. Both pooled and individual sera were studied.

b) Induction of anti-MAP-2 and anti-MAP-3 antibodies

BALB/c mice were injected with 100 µg antigen into the footpads with CFA. The injections were repeated twice in monthly periods: first subcutaneously with IFA and finally intraperitoneally in phosphate-buffered saline (PBS). The spleen cells of the immune mice were removed and hybridized with SP2/O murine myeloma cells according to Köhler and Milstein (26). For the production of ascitic fluid in mice, 10⁶ hybridoma cells were injected intraperitoneally into BALB/c mice and ascitic fluid was harvested after 2–3 weeks.

After hybrid selection of anti-ζ and anti-δ fusion products, 126 and 89 growing colonies, respectively, were obtained, of which 12 and 4, respectively, produced monoclonal antibodies (MAb), detected by EIA. One of the colonies maintaining the high MAb-producing capacity to ζ or δ was sub-

cloned by the limiting dilution method and further characterized.

Protection experiments

Eight- to ten-week-old male BALB/c mice were preimmunized with s.c. injections of 100 µg MAP-1 or the HA1C[Arg] or FP3 peptides as described above. Five weeks after the second antigen challenge, preimmunized mice were aerosol infected with 5 LD₅₀ dose of the mouse pathogenic A/PR/8/34 influenza virus. Virus dilution corresponding to 50% mortality was determined for each batch of virus in a preliminary survival experiment using different dilutions of the virus for infection. Experimental groups consisted of 5–10 animals. Control mice were treated as described above, but using PBS emulsified in CFA or IFA without the peptides. The number of survivors was given as a percentage of the total number in the groups and was determined daily postinfection.

Antibody detection and quantitation

a) The detection and quantitation of anti-MAP-1 or A/PR/8/34 influenza virus-specific serum antibodies were performed by solid-phase enzyme immunoassay (EIA) using different peptides (10 µg/ml) or purified virus (20 HAU/ml) coated to the solid phase of Maxisorp (NUNC, Roskilde, Denmark) or polyvinyl-chloride (PVC) (Enzy-Plate; Propilen G.M., Pécs, Hungary) plates, respectively. Acidic pretreatment of the virus was performed in a 0.1-M acetate buffer pH = 5.0 for 20 min at RT. Overcoating and antibody dilutions were done with PBS containing 1% bovine serum albumin (BSA) and 2% fetal calf serum (FCS). Excess of reagents was washed out three times with PBS and finally with PBS containing 0.05% Tween 20 (SERVA, Heidelberg, Germany). Bound antibodies were detected with horseradish peroxidase-labeled anti-mouse IgG as a developing second antibody (Sigma Chemical). Ortho-phenylene-diamine (OPD) was used as a substrate (Sigma Chemical). The relative level of polyclonal antibodies was determined by titration of immune sera taken at different points of time after the antigen challenge and characterized by the OD₄₉₂ values corresponding to a defined serum dilution.

b) Monoclonal antibodies against

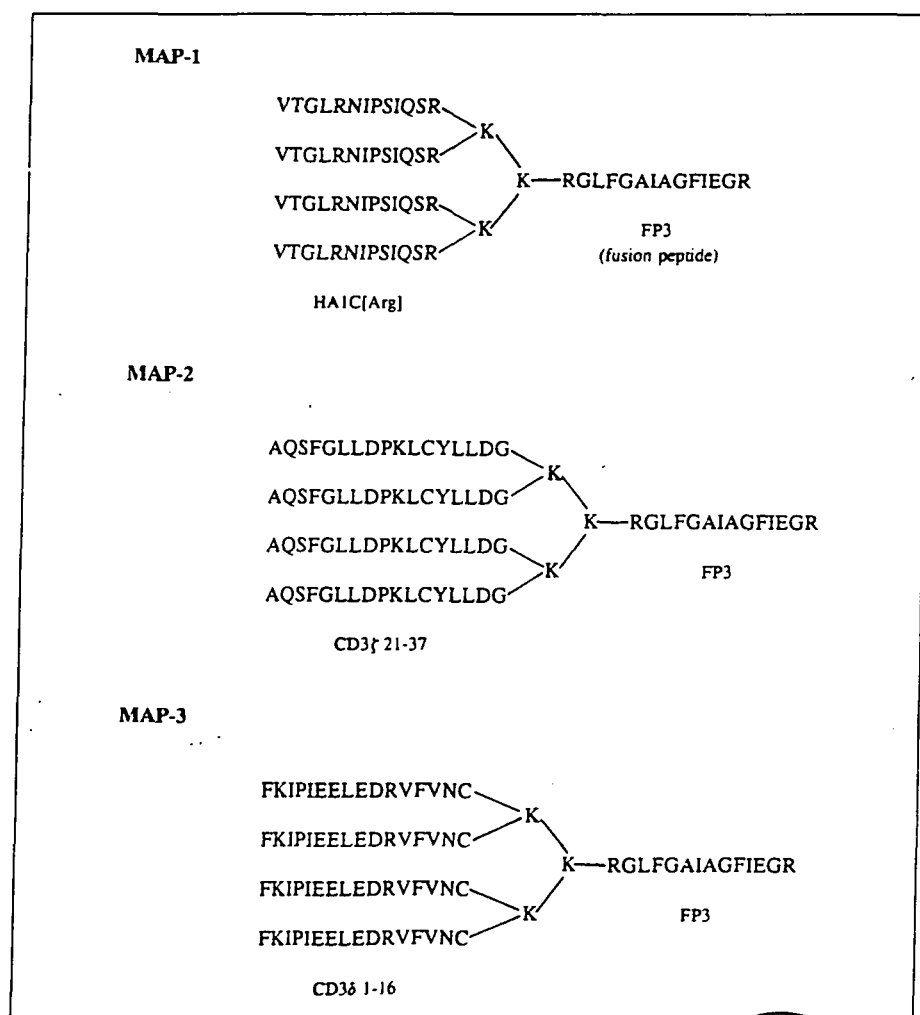


Figure 1. Structure of the synthesized chimeric "multiple antigenic peptides".

MAP-2 and MAP-3 were tested by EIA. Ninety-six-well microplates were coated with 10 µg/ml antigen overnight at 4°C. After washing the wells with PBS containing 0.05% Tween 20, hybridoma supernatants were added for 1 h at 37°C. Finally, horse radish peroxidase conjugated rabbit-anti mouse Ig (DAKOPATTS, Glostrup, Denmark) was used as a second antibody. The reaction was developed by OPD and H₂O₂ as substrates and measured at 495-nm wavelength on a Titertek Multiscan ELISA Reader (EFLAB OY, Helsinki, Finland). Polyclonal antisera and ascitic fluids were tested at different dilutions starting from a dilution of 1:100 to reduce nonspecific binding to the antigen. Hybridoma supernatants were used undiluted, since these reagents contained low concentrations of specific antibody (5–10 µg/ml).

Immunofluorescence and immunoblot

For immunofluorescence analysis, cells were stained with MAb in the optimal dilution (1:100 for ascites and undiluted for hybridoma culture supernatant), and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig (Sigma Chemical) was diluted 1:100 and analyzed on a FACStar IV instrument (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) for fluorescence intensity using a linear amplifier. Immunoblot analyses were carried out as described by Towbin et al. (43)

T-cell proliferation assay

The T-cell proliferation assay was performed by the standard method of Corradin et al. (11), using T-cell-enriched lymph node cells. B-cell depletion was performed by panning on anti-mouse Ig (prepared in our laboratory)-coated Petri dishes. Briefly, 4×10^5 T-cells were incubated together

with 2×10^5 irradiated normal BALB/c spleen cells as antigen-presenting cells (APC) in the presence of different concentrations of the peptides. The reactivity of the T-cells with influenza virus was detected in the presence of APC preinfected with 100 HAU/ml A/PR/8/34 (4 h, 37°C). The APC were washed twice with glucose-containing PBS, irradiated and used as described above. Proliferation was detected by ³H-thymidine (1 µCi, 3.7 kBq/well) incorporation added in the last ten hours of the 4-day culture.

RESULTS

Synthesis and Purification of the Branched Multiple Antigenic Peptides

As specific immune reactions can be mediated by low concentrations of peptides, particular emphasis was put on the purity, homogeneity and compositional studies of the synthetic peptides. The MAPs were synthesized using solid-phase peptide synthesis with Boc methodology. After removing the protecting groups, the solubilized branched peptides were purified by re-

verse-phase HPLC without any difficulties. The resulting purified peptide were characterized by two-dimensional SDS gel electrophoresis (Figure 2) and analytical HPLC (Figure 3). Since amino acid analysis is almost useless in the case of MAPs, we tried to characterize the MAP-1 by mass spectrometry. The Fab MS wasn't able to determine the molecular ion. The smaller fragments became observable only after tryptic digestion (on probe tip), and the peaks identified were as follows: 1307.7 (GLFGAIAGFIEGR, calc 1307.51); 914.6 (NPSIQSR, calc 914.03); 545.6 (VTGLR, calc 544.65); 1440.7 (VTGLRNPSIQSR, calc 1440.66). The Fab MS technique permitted the entire sequence assignment of the branched peptide, with the exception of the branched lysine core. The new matrix-assisted laser desorption ionization mass spectrometry proved to be applicable for determining the molecular ion (Figure 4); however, the accuracy of the method is only 0.5%–0.1%—therefore, alone, it is not applicable for proving the chemical structure. The synthesis and characterization of the control peptides (HAIC[Arg] and FP3) is published elsewhere (22).

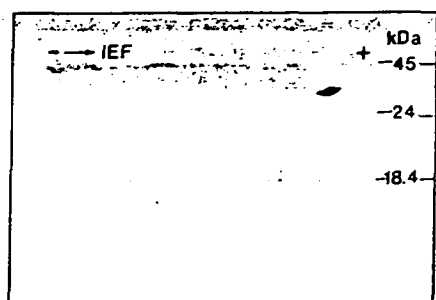


Figure 2. 2D PAGE of MAP-1 structure.

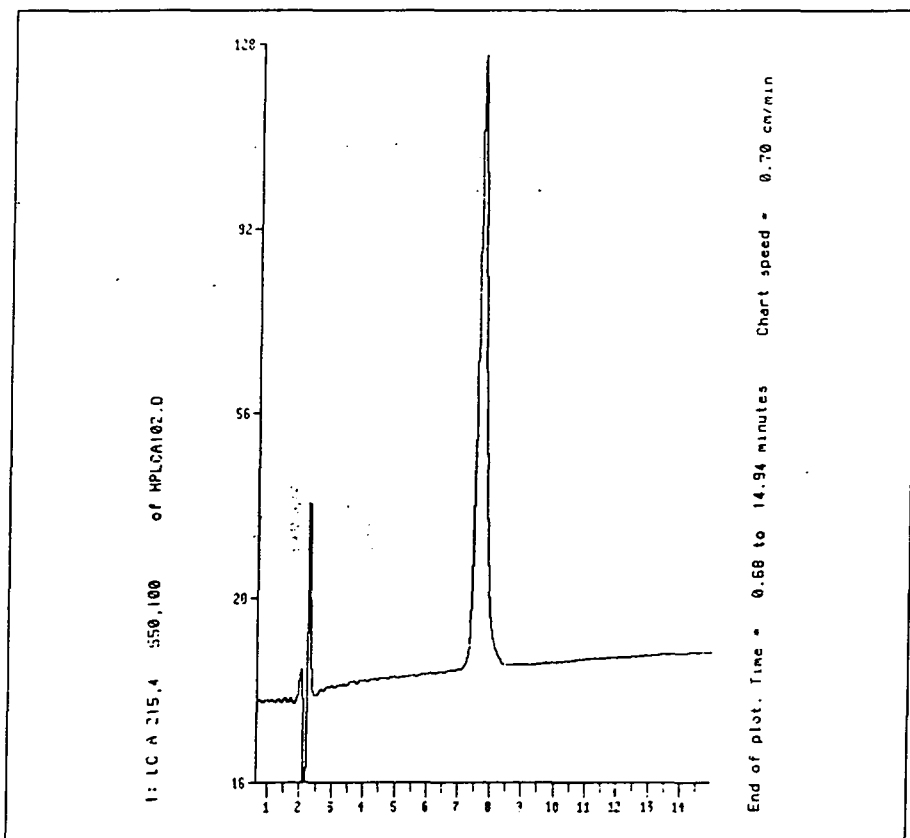


Figure 3. RP-HPLC of purified MAP-1

Table 1. Protection of BALB/c Mice Against a Lethal Dose of A/PR/8/34 Influenza Virus Infection by MAP-1 and HA1C[Arg]

Peptide Used for Preimmunization	Percent of Survivors ^a		
	Control ^b	HA1C[Arg]	MAP-1
Experiment 1 (n = 5)	0	40	60
Experiment 2 (n = 5)	0	20	60
Experiment 3 ^c (n = 10)	0	0	22

^aData registered on day 16 postinfection are documented. Control mice died on day 8–9 in experiments 1 and 2 and on day 7–8 in experiment 3 postinfection.
^bControl mice were treated as other groups using PBS emulsified in CFA instead of the peptides.
^cIn experiment 3, a new batch of the mouse pathogenic A/PR/8/34 influenza virus was used.
n = Mice included in experiment groups.

Characteristics of Immune Response Elicited by MAP-1 Comprising Influenza Virus HA Peptides

As summarized in Figure 5A (dotted columns), repeated injections of MAP resulted in the production of IgG-type antibodies recognizing the MAP-1 structure, but the antibodies were inactive and did not bind to the subunit peptides HA1C[Arg] or the Arg extended fusion peptide FP3 (see Figure 1). MAP-1-specific antibodies showed

weak binding to the infective A/PR/8/34 influenza virus coated to the solid phase, but their binding could be enhanced significantly by acidic pretreatment of the virus (Figure 5A, PR8pH5). As shown in Figure 5B, no HA1C[Arg]-, FP3- or MAP-1-specific antibodies could be detected after HA1C[Arg] immunization. The high level of influenza virus-specific antibodies, detected only after viral challenge (Figure 5B, dark columns), corresponds to antibodies raised against other epitopes of the virus.

T-Cell Recognition of the MAP-1 Structure

Immunization with the HA1C[Arg] peptide induces a proliferative T-cell response that recognizes virus-infected APC and is fully crossreactive with the MAP-1 structure (Figure 6). These results show that the processing and presentation of HA1C[Arg], MAP-1 or the infective virus result in a common epitope recognized by HA1C[Arg]-primed T-cells. In this assay, MAP was even more efficient than the same concentration of HA1C[Arg] used subsequently (Figure 6). In molar terms the monomeric HA1C[Arg] contain four times as many copies as the tetrameric MAP-1; but, in the case of identical $\mu\text{g/ml}$ concentrations, the amount of the presumably processed HA1C[Arg] peptide in the *in vitro* cultures or in preimmunized mice is about 20% less in case of MAP-1, taking into consideration the molar masses (FP3 = 1693 HA1C[Arg] = 1481) of HA1C[Arg] and FP3.

Protection Induced by the MAP-1 Structure

Twenty-two to sixty percent of MAP-1-preimmunized BALB/c mice acquired complete protection against

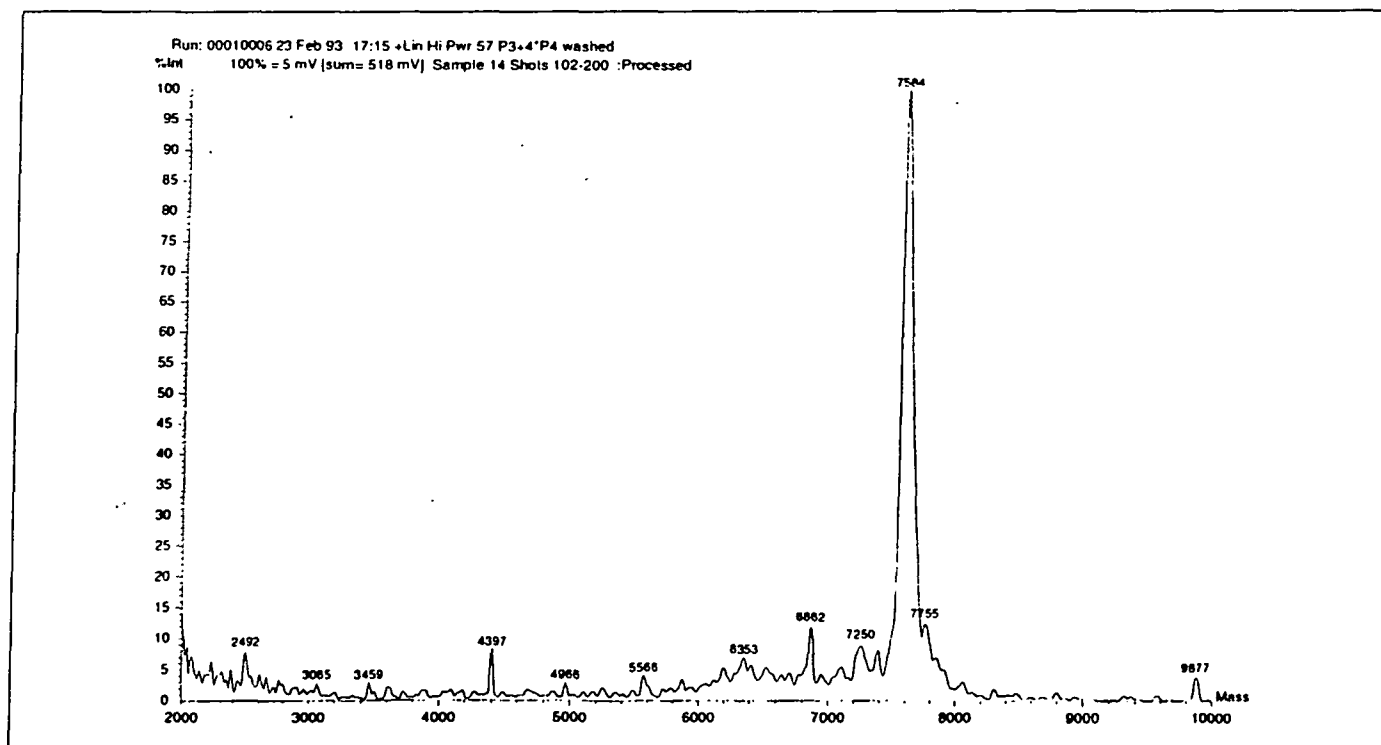


Figure 4. Matrix-assisted laser desorption ionization spectra of MAP-1 structure.

lethal dose of influenza virus as compared to the HA1C[Arg] or control PBS-pretreated mice (Table 1). As summarized in Figure 5B (dark columns), as a result of viral challenge an elevated level of MAP-1-specific IgG-type antibodies could be detected in the serum of the survivors two weeks postinfection. The lack of MAP-1- or HA1C[Arg]-specific antibodies in HA1C[Arg] preimmunized and subsequently infected mice demonstrates that the virus infection itself does not result in the production of MAP-1-specific antibodies (Figure 5B, dark columns). The level of total virus-specific antibodies detected in MAP-1- or HA1C[Arg]-pretreated mice was significantly higher than that in control mice (data not shown).

Characterization of Antibodies Produced Against the ζ - and δ -Chains of Human TCR Complex

Monoclonal antibodies were raised against the peptide antigens derived from the TCR ζ - and δ -chains, respectively (see Figure 1). Table 2, A and B, shows the results obtained by EIA. The conventional antiserum or the monoclonal antibodies were highly reactive with the corresponding ζ - or δ -peptide fragments. Higher than 50% inhibition was achieved when ζ -peptide was used as competitor (Figure 7). This experiment proved the peptide specificity of the antiserum raised against the ζ -peptide. In contrast, indirect immunofluorescence or immunoblotting analysis showed that the anti-peptide antibodies did not recognize the native ζ -chain (data not shown). The δ -peptide antibodies were also tested for reaction with the native δ -chain and gave similar results to the ζ -chain.

DISCUSSION

The recent improvements in peptide chemistry have made feasible the synthesis of large polypeptides or small proteins. However, the purification and structure and purity verification remain problematic. The originally described MAP system (37), consisting of at least 8 epitopic peptides, can easily lead to a molecule with a molecular mass higher than 10–15 kDa. After purification by means of the conventional techniques (RP-HPLC, size-exclusion chromatog-

Table 2. Characterization by EIA of Mouse Antibodies Raised Against TCR ζ - and δ -Peptides

A: OD495 _{sample} –OD495 _{control} ^a		
Dilution of the Antisera	Mouse Anti- ζ Serum ^b	Monoclonal Anti- δ Ascitic Fluid
1:100	2.031	>2
1:300	1.678	>2
1:1000	0.557	0.815
B: OD495 _{sample} –OD495 _{control} ^c		
Monoclonal Anti- ζ Hybridoma Supernatant	Monoclonal Anti- δ Hybridoma Supernatant	
1.185	1.436(1)	
	0.960(2)	

^aOD495 value given by preimmune serum at the proper dilution.

^bMouse anti- ζ serum was obtained from mice as a test blood prior to using the spleen for hybridoma production. Ascites fluid was produced as described in Materials and Methods.

^cOD495 value given by PBS–Tween 20 used as a control. Hybridoma supernatants were harvested from the cells producing monoclonal antibodies.

(1) and (2) show the results obtained with supernatants of two independent hybridoma clones producing anti- ζ and anti- δ Mab.

raphy and ion-exchange), we can establish by HPLC and gel electrophoresis that the resulting purified material is homogeneous, that it has the correct amino acid composition, and that the sequencing resulted in the expected structure; but we cannot be sure

whether the actual purity is 80% or 90%. The separation power of the conventional purification techniques is not sufficient to investigate the actual homogeneity of the products. Only mass spectroscopy seems applicable for acquiring information concerning the

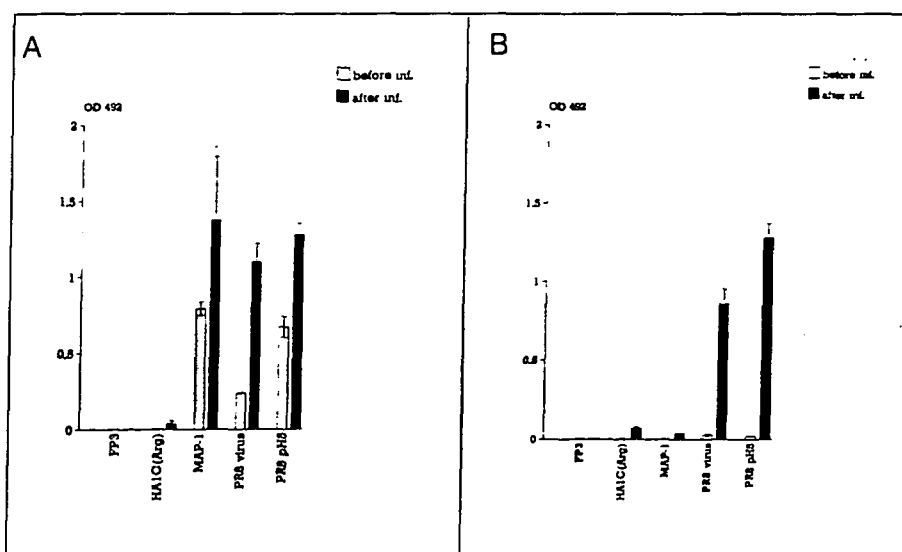


Figure 5. Relative levels of MAP-1- and HA1C[Arg]-induced serum antibodies. BALB/c mice were immunized with MAP-1 (A) or HA1C[Arg] (B), and the relative levels of serum antibodies were measured by EIA, using immune sera taken 14 days after the peptide boost or viral challenge. OD492 values (mean value of duplicates obtained for individual sera $\pm$ SEM) correspond to 10^3 serum dilutions detected with the solid-phase attached peptides HA1C[Arg], FP3, MAP-1 or with infective (PR8) or acid-pretreated (PR8 pH5) A/PR/8/34 influenza viruses. The number of pretreated mice was 3, the number of MAP preimmunized survivors was 6, and the number of HA1C[Arg] preimmunized survivors was 2.

quality and the side-products formed during the synthesis. If the number of epitopic peptides is decreased to 4–5, the efficiency and qualitative parameters of the synthesis can be increased dramatically.

RP-HPLC may be efficient enough to separate the resulting products and side-products, and, if so, verification of the structure and purity by means of mass spectrometry would be much easier. For example, Figure 4 shows the matrix-assisted laser desorption MS of MAP-1. The calculated mass differs slightly from the measured one; since the accuracy of the MALDI spectrophotometer in linear mode is only 0.1%–0.5%, the measured mass can correspond to the expected molecular mass. The small peaks below the molecular ion can correspond to possible side-products. The more accurate Fab-MS proved to be useless for determining the molecular ion, but after enzymic digestion with trypsin, all the possible fragments were detected—so this method verified the structure.

Influenza virus was one of the first subjects of the subunit vaccination approach, and epitopes of different proteins of the virus were applied to elicit

antibodies or T-cells (4). The rationale behind our approach was to combine T- and B-cell epitopes previously identified in the intersubunit region in the HA molecule (22,33). The MAP-1 structure investigated in this study actually mimics the natural sequence of HA present in its uncleaved form in non-infective virions, generated as a result of non-productive infection (16). MAP-1 encompasses two T-cell epitopes (the HA1C[Arg] sequence and the modified fusion peptide (FP3)) in such a way that the overlapping B-cell epitope, localized also in the HA1C[Arg] region, might be able to adopt its appropriate conformation (22). Previous results indicated that the presence of the highly ordered fusion peptide (22,48) is beneficial in stabilizing the overall structure of the less ordered HA1C[Arg] and promotes antibody recognition (33, Z. Nagy et al. unpublished observation). These results, together with the data proving the T-cell-activating capacity of FP3, motivated us to include this peptide in MAP-2 and MAP-3 in order to enhance the immunogenicity of the nonimmunogenic ζ - and δ -chain peptides.

The introduction of an RKKR motif between the two HA epitopes in MAP-1 was designed to provide a sensitive enzyme-susceptibility site, also present

in natural sequences of highly pathogenic avian influenza virus strains (45). This sequence can promote the appropriate processing of MAP-1 by trypsin-like enzymes of antigen-presenting cells favoring elaboration of the T-cell epitope previously localized in the HA1C[Arg] region. Our present results, showing the cross-reactivity of HA1C[Arg], MAP-1 and the infective virus in a T-cell proliferation assay (Figure 6), support this possibility. However, it cannot be ruled out that MAP-1 binds to MHC II molecules as a multivalent ligand, therefore increasing the otherwise low affinity of the linear HA1C[Arg] peptide. Nevertheless, the increased activity of MAP-1 in this assay points to the possible role of enhanced helper T-cell activation in mediating protection of MAP-1-preimmunized mice (Table 1).

Protection against a lethal dose of pathogenic influenza virus was attempted by different approaches including the passive administration of antibodies (22); the induction of neutralizing antibodies, helper or cytotoxic T-cells by viral proteins (1,19,24); or by synthetic peptides (2,3,15,35). Data obtained with CD8+ T-cell-depleted mice revealed that the antibody response has a major role in mediating protection against influenza virus infection (18). Passive administration of IgA-type, but not IgG-type, antibodies was demonstrated to confer complete protection (34). In the case of synthetic peptides, the critical point was to elicit T-cells or antibodies crossreactive with the virus and also neutralizing it (3,5).

The data summarized in Figure 5 demonstrate that the MAP-1 structure is immunogenic and is able to induce IgG-type, MAP-1-specific antibodies crossreactive with the acid-pretreated virus. It is well established that the C-terminus of the HA1 subunit of influenza virus hemagglutinin, comprised in the HA1C[Arg] peptide, is buried in the mature, cleaved form of the HA molecule (49), but it can be exposed for antibody recognition by mild acidic treatment (42). This type of IgG antibody can be detected after repeated MAP-1 administration and at an elevated level after viral challenge in all survivors pretreated with MAP-1. In contrast, the monomeric subunit peptides HA1C[Arg] and FP3 were unable to elicit an antibody response directed to the corresponding peptides, to MAP-

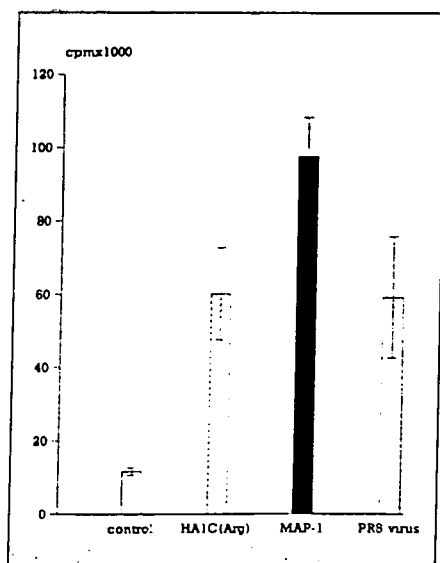


Figure 6. Proliferation of HA1C[Arg]-induced T-cells in presence of HA1C[Arg], MAP-1 or virus preinfected APC. T-cell-enriched lymph node cells of HA1C[Arg]-primed BALB/c mice were stimulated *in vitro* with 20 μ g/ml HA1C[Arg] peptide (rising to right), MAP-1 (dark column) or APC preinfected with 100 HAU/ml A/PR/8/34 influenza virus. Empty columns correspond to control cultures incubated without peptides or the virus. Mean values of cpm $\pm$ SEM of triplicate cultures obtained in a typical experiment are documented.

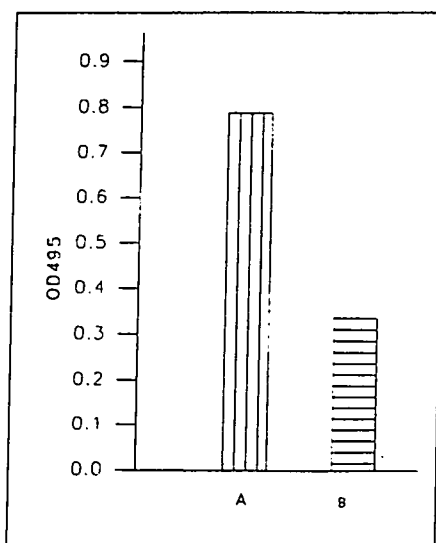


Figure 7. Specificity test of mouse anti- ζ -peptide serum. The plate was coated with 10 μ g/ml of MAP-2 overnight at 4°C. It was then washed and left to react with the mouse anti-MAP-2 serum (1:10000 dilution) in the presence (B) or absence (A) of the 22–32 fragment of the ζ -chain (QSFGLDPKLC, 10 μ g/ml) as a competitor for 1 h at 37°C. The assay was completed as described in Materials and Methods.

1 or to any form of the pretreated mice, compared to the HA1C[Arg]-preimmunized animals. This points to the beneficial participation of the MAP-1-specific antibody response in protection, but raises the possibility of other mechanisms as well. As an alternative explanation, protection induced by MAP-1 can be mediated or supported by efficient help delivered by HA1C[Arg] and/or FP3-specific T-cells cross-reactive with the virus (Figure 6) (33).

The previously detailed and promising results motivated us to include in this study the modified fusion peptide in MAP-2 and MAP-3, comprising oligopeptides of the ζ and δ TCR subunits. MAP-2 and MAP-3 resulted in immunogenic structures, compared to their subunit peptides or even to their tetra- or octamers lacking the FP3 HA peptide. However, polyclonal or monoclonal MAP-2 or MAP-3 antibodies did not recognize the cell-membrane-bound or nitrocellulose-attached forms of the corresponding ζ - or δ -subunits.

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CIRCULAR DICHROISM AND FOURIER-TRANSFORM INFRARED SPECTROSCOPIC
STUDIES ON T-CELL EPITOPIC PEPTIDE FRAGMENTS OF INFLUENZA VIRUS
HEMAGGLUTININ

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Epitopic peptides representing the C-terminal (HA1) region of cleaved hemagglutinin of influenza virus from different serotypes were synthesized. Circular dichroism and Fourier-transform infrared spectroscopic data showed that peptides HS2 and HS3 have a predominantly α -helical conformation in trifluoroethanol. Recently a component band appearing between 1640 and 1635 cm^{-1} in the amide I region of the Fourier-transform infrared spectra of polypeptides has been correlated with strongly H-bonded β -turns (Ref. 8). Using this assignment, HS1 was found to contain less α -helix but have tendency to adopt β -turn(s). Interestingly, fragment HS2 with the highest α -helix content proved to be the poorest T-cell epitope among serotypes HS1-HS3. © 1993 Academic Press, Inc.

There is a growing interest in the understanding of the secondary structure of small (<20 residues) epitopic peptides. It has been reported recently that the peptide-binding site of human class I histocompatibility molecules recognizes 8-11mers (1). Similarly, the antigens recognized by MHC class II-restricted T

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Table 1. Amino acid sequence and abbreviations of the synthetic peptides from the C-terminal region of influenza virus A hemagglutinin from different serotypes

	317.....329
HS1	VTGLRNIPSIQSR
HS2	ATGLRNVPOIESR
HS3	LATGMARNVPEKQTR

cells can be mimicked by small peptides more heterogenous in their size (2). It is well established that MHC-bound peptides are tightly mounted in a groove in extended conformation with a kink in the middle (3,4). Previously, prediction methods suggested amphipathic α -helices for MHC class II restricted T cell epitopes (5).

Small size peptides are expected to adopt multiple conformations in solution. Our earlier spectroscopic studies on synthetic intersubunit fragments of influenza virus hemagglutinin (HA) also demonstrated the presence of conformer mixtures and the dependence of antibody recognition on conformation (6). Circular dichroism (CD) and Fourier-transform infrared (FT-IR) spectroscopies, contrary to NMR, have the capacity of time resolving conformational changes of the peptide chain and therefore both techniques can be applied for the study of conformer mixtures of peptides in solution.

This paper reports CD and FT-IR spectroscopic measurements on peptides (Table 1) comprising the C-terminal of the HA1 subunit of three serotypes of human influenza A viruses. Previous data showed that these peptides were able to elicit MHC II restricted murine T cells cross-reactive with the virus (7).

MATERIALS AND METHODS

The peptides were synthesized by the solid phase technique utilizing Boc chemistry (8). Side chain protecting groups were as follows: Tos(Arg), Bzl(Thr), OchHex(Glu) and Bzl(Ser). The peptide chains were elongated on MBHA resin (0.6-0.8 mmol/g) and the syntheses were carried out manually. Couplings were performed with dicyclohexylcarbodiimide (DCC) (with the exception of Asn, Gln and Arg which were incorporated as their HOBt-esters) using a three-fold excess of Boc-amino acids. Amino acid incorporation was monitored by ninhydrin test (9). In case of incomplete couplings this step was repeated, but the dichloromethane (DCM) was substituted by dimethylformamide (DMF). The completed peptide

resins were treated with liquid HF/dimethyl sulphide/p-cresol/p-thiocresol (86:6:4:2, vol/vol), on 0°C, 1 h. HF was removed and the resulted free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized.

The crude peptides were purified by reverse-phase HPLC (Knauer; Berlin, Germany) using Lichrosorb RP-18 10 μ column (16 x 250 mm). The following solvent systems were used: 0.1% trifluoroacetic acid (TFA) in water (A); 0.1%TFA, 80% acetonitrile (ACN) in water (B); gradient: 0%-50% B in 1.5 hours, flow rate 3 ml/min. The appropriate fractions were pooled and lyophilized. The purity was checked by RP-HPLC (Nucleosil 5 C 18 column, solvent systems as above, gradient: 30%-60% B in 15 min, flow rate 0.8 ml/min, detection at 220 nm, or Eurosil Bioselect 5 C 18 column, flow rate 1.5 ml/min with solvent systems and detection as previously) and proved to be substantially pure (>97%).

The FAB-MS experiments were performed with a VG ZAB-2SEQ type hybrid tandem mass spectrometer, equipped with an LSIMS source (Cs^+ ion gun used at 30 keV). The results of the MS measurements were in all cases in good agreement with the calculated value.

Circular dichroism spectra were measured on a Jobin-Yvon Mark VI dichrograph calibrated with epiandrosterone. Measurements were carried out in 0.02 cm cells at ambient temperature. NMR grade trifluoroethanol (TFE) (Aldrich) and double distilled water were used as solvents. The concentration of the samples ranged between 0.5-1 mg/ml. Mean residue ellipticity, $[\theta]_{\text{MR}}$, is given in $\text{deg.cm}^2/\text{dmol}$.

Infrared measurements (at a resolution of 4 cm^{-1}) were performed at room temperature on a Nicolet 170SX spectrometer. Peptide solution (≈ 1 mg/ml) were prepared in TFE (see above). The IR spectrum of TFE (containing traces of water) was obtained under identical conditions. The deformation vibrational band of water between 1700-1600 cm^{-1} was removed by subtracting the spectrum of TFE on the basis of the combination band of OH stretching and HOH deformation at 5293 cm^{-1} . This procedure allowed an unambiguous comparison of the spectra in the critical 1650-1600 cm^{-1} range. KBr cells of 0.041 cm were used. The spectra were analyzed by a normalized, least-squares curve-fitting program, using products of Gauss and Lorentz curves (Holly et al., unpublished). The selection of component curves was assisted by the Fourier self-deconvolution method of Mantsch et al. (10).

RESULTS AND DISCUSSION

CD studies

In water the CD spectra of peptides HS1-HS3 show a strong negative band between 195-200 nm which is accompanied by a shoulder near 220 nm. In TFE peptides HS1-HS3 adopt helical CD spectra with low intensity bands (Fig. 1). On addition of increasing amounts of water, the spectra (e.g. Fig. 2) are gradually shifted towards the spectrum measured in water. It is the CD spectrum of peptide HS2 which shows the most expressed helical features (Fig. 1). The intensity of the negative and the positive bands of the $\pi\pi^*$ couplet is definitely lower in the CD spectrum of HS1.

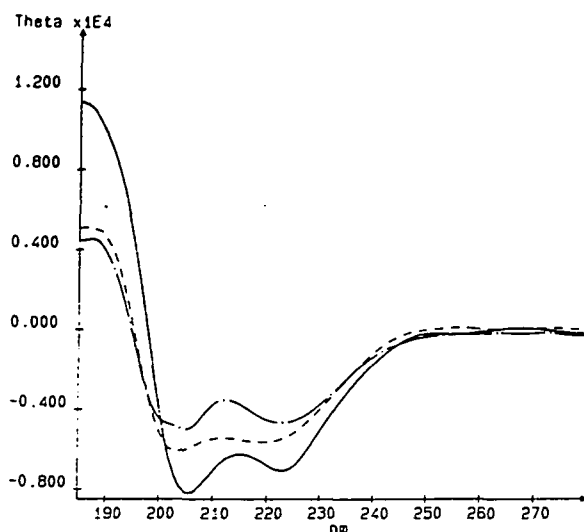


Fig. 1. Circular dichroism spectra in TFE of epitopic peptides HS1-HS3. (---) HS1; (—) HS2; (- - - -) HS3.

FT-IR spectra

Peptides HS1-HS3 are present as TFA salts because of the purification procedure using TFA-containing eluents. The trifluoroacetate anion is known to have an absorption band in the amide I infrared region. In the infrared spectrum of CF_3COONa in KBr pellet the ν_{as} mode of the COO^- group appears at 1694.5 cm^{-1} while the ν_{as} and ν_{s} vibrations of the CF_3 group at 1218 and 1143

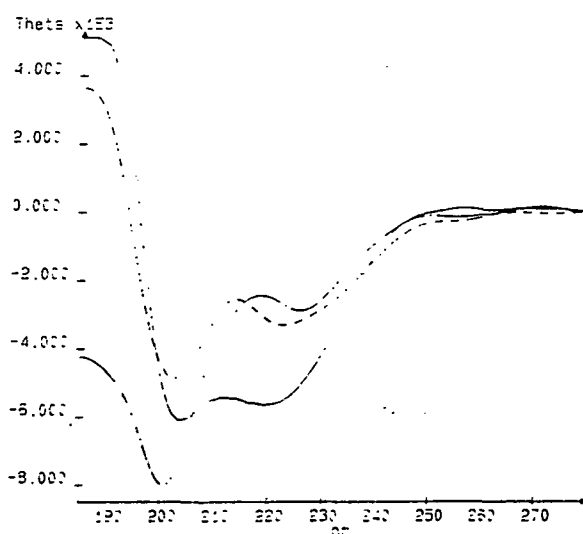


Fig. 2. Circular dichroism spectra of HS3 in TFE/water mixtures. (—) 100%; (---) 75%; (- - - -) 50% TFE.

cm^{-1} , respectively. In the KBr spectra of peptides HS1-HS3 the ν_{S} CF_3 band shows up at $1135 \pm 1 \text{ cm}^{-1}$, accompanied by the ν_{AS} band near 1205 cm^{-1} . In the curve-fitted spectra of the peptides in KBr, a component band was found between 1702 and 1699 cm^{-1} . The ratio of band intensities $I(\sim 1700)/I(\sim 1135)$ in the spectra of peptides and $I(1694.5)/I(1143)$ in the spectrum of CF_3COONa in KBr is practically the same (1.55 ± 0.15). On the basis of this, the component band near 1700 cm^{-1} in KBr and at $1691 \pm 0.5 \text{ cm}^{-1}$ in the TFE spectra of peptides HS1-HS3 was assigned to the COO^- group of the counterion trifluoroacetate.

As reported earlier (6), after subtracting the contribution of trifluoroacetate, the IR spectrum of HS1 in D_2O is dominated by a strong band at 1647.5 cm^{-1} (aperiodic conformation).

The FT-IR spectra in TFE of fragments corresponding to serotypes HS1-HS3 are shown in Fig. 3. All three spectra contain a strong amide I component band between 1678 – 75 cm^{-1} . This band was assigned to the amide I mode of solvent-exposed carbonyls. Peptides HS2 and HS3 show a strong component band, characteristic of weakly H-bonded α -helices, at 1661 and 1659.5 cm^{-1} , respectively (Fig. 3b and c). In the curve-fitted IR spectrum of HS1 (Fig. 3a), a strong component bands appears at 1663 cm^{-1} , above the upper limit of the α -helix (11). (A typical but weak α -helix band is present at 1656 cm^{-1} , Fig. 3a). Weak component bands show up in the spectra near 1645 cm^{-1} (aperiodic conformation) and at 1624 – 1623 cm^{-1} (β -sheet). The β -sheet band is more intensive in the spectra of HS2 and HS3. Component bands between 1639 and 1635 cm^{-1} are also seen in the curve-fitted spectra of the peptides.

Recently an infrared band at 1639 cm^{-1} has been assigned to the repeating 1–4 H-bond of 3_{10} helices (12,13). Similarly, in the FT-IR spectra of the bridged cyclic peptides cyclo[Gly-Pro-Xxx-Gly-NH-(CH_2) $_n$ -CO] (Xxx=Gly, Ser(O^tBu), Ser, Thr(O^tBu); $n=2,4$) a band near 1640 cm^{-1} was assigned to the acceptor C=O of the 1–4 intramolecular H-bond of type I or type II β -turns (14). It was also shown that in TFE the " β -turn band" appears down-shifted in the 1640 – 1635 cm^{-1} amide region. Thus, the low intensity component band between 1641 and 1634 cm^{-1} in the IR spectra of HS1-HS3 (Fig. 3) may be indicative of the presence of H-bonded β -turns.

According to secondary structure prediction, peptide HS1 tends to adopt β -turns: the highest turn propensity is predicted for

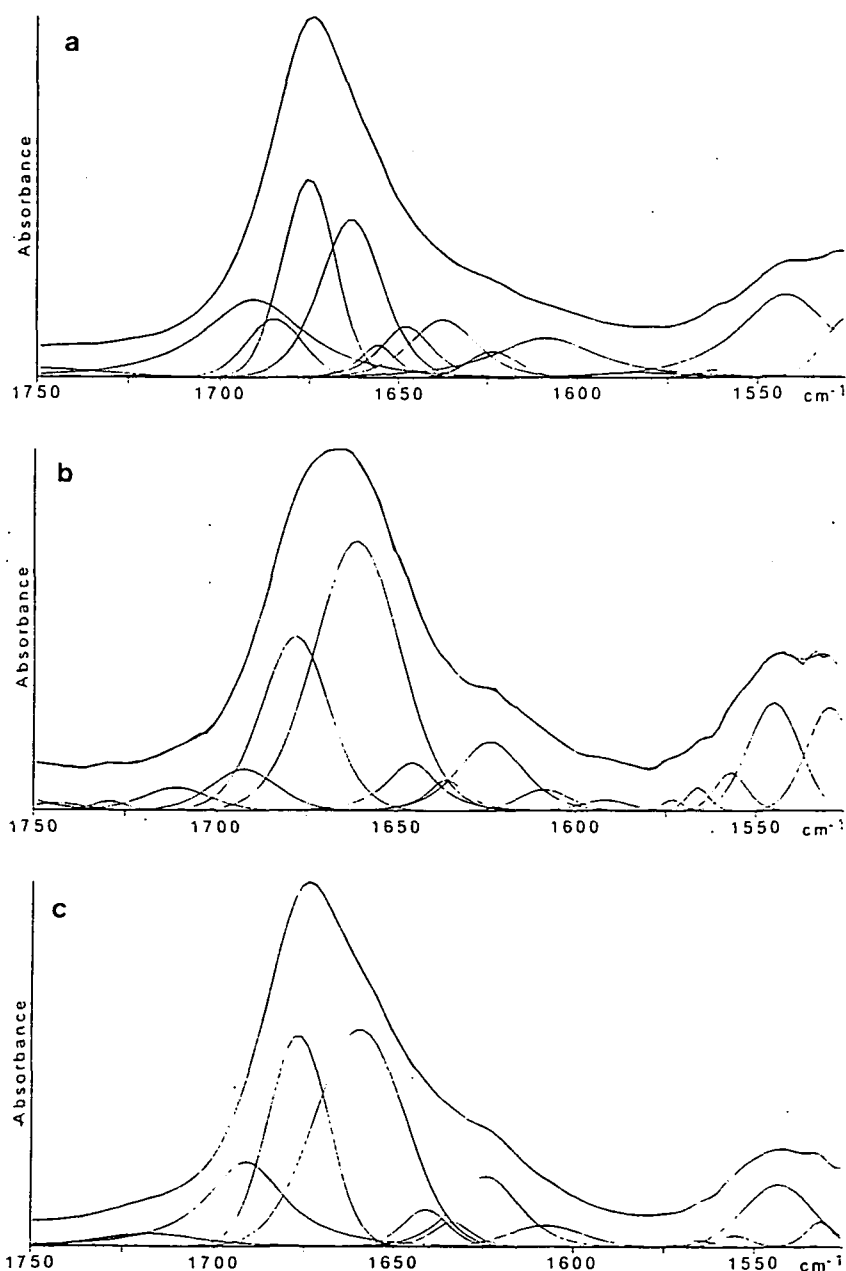


Fig. 3. The amide I and II spectral regions of the FT-IR spectra in TFE of HS1(a), HS2(b) and HS3(c). The measured and curve-fitted (sum) spectra are shown together with the component bands resulting from the analysis (see Materials and Methods).

the Ile-Pro-Ser-Ile tetrapeptide (6). The replacement of Ser for Gln in HS2 and Glu in HS3 destroys the turn-forming Pro-Ser core and increases the helical tendency of these peptides. The structural information inferred from CD and FT-IR studies is in agreement with the prediction. Both methods indicate that the

highest amount of α -helix is present in HS2, but the helicity of HS3 is also significant. The most intensive " β -turn band" at 1637.5 cm^{-1} can be seen in the curve-fitted infrared spectrum of HS1 (Fig. 3a). As reflected by the weak component band at $1624\text{--}23\text{ cm}^{-1}$, peptides HS2 and HS3 contain definite amount of β -sheet conformation in TFE solution. Based on the integrated intensities of the α -helix and β -sheet component bands, it is peptide HS3 in which the β -sheet content is the highest.

We think that, without overinterpreting the curve-fitted FT-IR spectra, two main conclusions can be drawn:

- (1) Peptides HS2 and HS3 in TFE adopt a predominant α -helical conformation which is broken or, more likely loosened at Pro324. Minor conformer(s) or conformational regions with aperiodic and β -sheet conformation are also present. Contrary to this, HS1 occurs as a multicomponent mixture of conformers even in TFE.
- (2) FT-IR spectroscopic data allow the presence of β -turns in all three peptides. Based on secondary structural prediction and the relative intensity of the β -turn band in the infrared spectrum, HS1 contains at least one β -turn which likely encompasses the Pro-Ser dipeptide.

TFE is known to induce α -helical conformation (15). The significant differences in the FT-IR spectra (Fig. 3) clearly indicate that HA fragments HS1-HS3 have different conformational propensities. Interestingly HS2, showing the highest α -helix content on the basis of both CD and FT-IR evidences, was proved to be less potent to induce an *in vivo* T cell response and *in vitro* T cell proliferation compared to peptides HS3 or HS1 corresponding to other serotypes (7). It is reasonable to suppose that in a water rich environment α -helicity does not play a determinant role in the recognition of T cell epitopic peptides from the intersubunit region of influenza virus HA.

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A new synthesis of Rink's polymer, 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxymethylpolystyrene

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For solid-phase peptide synthesis, 2,4-dimethoxy-4'-hydroxybenzhydrol linker was prepared via lithium borohydride reduction of 2,4-dimethoxy-4'-hydroxybenzophenone. The potassium salt of the linker was coupled to chloromethylpolystyrene. This method proved to be better than use of the cesium salt. This new synthesis gave a polymer with appropriate structure and a good degree of substitution. © Munksgaard 1993.

Key words: 2,4-dimethoxy-4'-hydroxybenzhydrol; potassium salt coupling of phenolate; side reactions; substitution level

The solid-phase method introduced by Merrifield is nowadays the most significant process in peptide synthesis. The result of the synthesis depends considerably on the polymer used, especially if the Fmoc strategy is applied. The ideal carrier remains stable during cleavage, and the peptide can be removed by mild acidic treatment after synthesis. Simultaneously, the side-chain protecting groups are also cleaved. Such polymers include the 4-alkoxybenzyl alcohol resin of Wang (1), the 3-xanthroloxymethylpolystyrene of Sieber (2), the 2-methoxy-4-alkoxybenzyl alcohol resin of Mergier (3) and the 4-(2',4'-dimethoxyphenylhydroxymethyl)phenoxymethylpolystyrene of Rink (4).

Rink's polymer is a promising resin. We attempted to synthesize it by following the described method, but two problems arose. Uncontrollable side-reactions occurred, the structure of the polymer could not be determined accurately and the described substitution (4) of the resin could not be reproduced: we obtained only approximately 0.1 mmol/g. We tried reducing the time of the reduction, changing the solvent, and using sodium borohydride or lithium aluminium hydride, but an acceptable level of substitution could not be attained.

Because of these difficulties, we decided on an entirely different strategy: preparing the ready linker and coupling it to the polystyrene. Investigation of the reduction of 2,4-dimethoxy-4'-hydroxybenzophenone showed that this reaction proceeded in 15 min instead of the 1 h described in the literature (4). If we continued the reduction, the amount of by-products gradually increased. (One of them is probably a diphenylmethane derivative.) The structure of 2,4-dimethoxy-4'-hydroxybenzhydrol was confirmed by IR and NMR

spectroscopy. The linker was coupled to the chloromethylated polystyrene via its cesium salt as described previously. This process resulted in an acceptable degree of substitution. Later we used the potassium salt of the phenolic hydroxy group instead of the expensive cesium salt; the product proved better as regards substitution.

The structure of the resin was proved via its IR spectrum and the degree of substitution by coupling of an Fmoc amino acid to the polymer. We achieved a convenient level of substitution (0.35–0.47 mmol/g), suitable for the synthesis of any peptide.

EXPERIMENTAL PROCEDURES

Melting points were taken with a PHMK apparatus and are uncorrected. Microanalyses were carried out with a Heraeus element analyser. IR spectra were recorded with a Nicolet IR 80 spectrometer, and ¹H-NMR spectra with a Bruker Aspect 3000 (400 MHz) spectrometer. Analytical TLC plates were purchased from E. Merck. For TLC, the CHCl₃/acetone (9:1) solvent system was used.

2,4-Dimethoxy-4'-hydroxybenzophenone (1). A solution of 4-hydroxybenzoic acid (69 g, 0.5 mol), resorcinol-dimethyl ether (97 mL, 0.75 mol), zinc chloride (150 g, 1.1 mol) and phosphorus oxychloride (326 mL, 3.5 mol) was stirred at 60–65 °C for 1.5 h. The mixture was then poured onto ice (1.5 kg), and solid KOH was added until the deep purple colour turned yellow. Ethyl acetate (300 mL) was then added to the solution, and the organic phase was separated, dried (Na₂SO₄) and con-

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centrated at reduced pressure. The precipitated product was isolated by suction and washed with diethyl ether; yield: 92 g (71%); m.p. 134–136 °C, RF = 0.53.

Anal. calc. for $C_{15}H_{14}O_4$: C 69.76, H 5.46; found (258.28): C 69.47, H 5.39. IR (KBr): $\nu_{\max}$ 1280, 1605, 3330 cm^{-1} . $^1\text{H-NMR}$ (CDCl_3): δ = 3.70 (s, 3H, 2-OCH₃), 3.85 (s, 3H, 4-OCH₃), 6.52 (m, 1H, 5-H), 6.54 (d, 1H, 3-H), 6.86 (d, 2H, 3',5'-H), 7.33 (d, 1H, 6-H), 7.72 (d, 2H, 2',6'-H).

2,4-Dimethoxy-4'-hydroxybenzhydrol (2) Lithium borohydride (6.34 g, 291 mmol) was added in small amounts to a solution of **1** (50.00 g, 194 mmol) in THF (800 mL). The solution was refluxed for 15 min. After removal of the unreacted lithium borohydride with MeOH (200 mL), the solvent was evaporated at reduced pressure and the residue was partitioned between ethyl acetate (600 mL) and H₂O (300 mL). The organic layer was dried (Na_2SO_4) and concentrated at reduced pressure. After this procedure the product could be obtained as a crystalline substance which was washed with diethyl ether; yield 45 g (89%); m.p. 133–135 °C, RF = 0.32.

Anal. calc. for $C_{15}H_{16}O_4$: C 69.22, H 6.19; found (260.29): C 69.16 H 5.77. IR (KBr): $\nu_{\max}$ 1240, 3240, 3420 cm^{-1} . $^1\text{H-NMR}$ (CDCl_3): δ = 3.79 (s, 6H, 2,4-OCH₃), 4.795 (s, 1H, CHOH), 5.94 (s, 1H, CH), 6.44 (dd, 1H, 5-H), 6.46 (d, 1H, 3-H), 6.77 (d, 2H, 3',5'-H), 7.06 (d, 1H, 6-H), 7.23 (d, 2H, 2',6'-H).

Potassium salt of 2,4-dimethoxy-4'-hydroxybenzhydrol (3) K_2CO_3 (23.9 g, 173 mmol) in ethyl alcohol (200 mL) was added to a suspension of **2** (45.0 g, 173 mmol). The solvent was evaporated off at reduced pressure. The resulting oil was taken up in diethyl ether (250 mL) and the solution was agitated with a glass rod until a crystalline solid formed. The suspension was chilled overnight and filtered to give **3** as a pink solid, which was dried at 45–50 °C and reduced pressure; yield 50.5 g (98%).

IR (KBr): $\nu_{\max}$ 1240, 3240 cm^{-1} .

4-(2',4'-Dimethoxyphenylhydroxymethyl)phenoxyethylpolystyrene (4) **3** (44.8 g, 150 mmol) in DMF (300 mL) was added to a suspension of chloromethylpolystyrene (150.0 g, 150 mmol Cl) in DMF (500 mL). The suspension was stirred at r.t. for 72 h. The resin was then washed in turn with DMF (800 mL), MeOH (800 mL), H₂O (800 mL), MeOH (800 mL), CH_2Cl_2 (800 mL) and MeOH (800 mL), and dried; yield 156 g.

A mixture of Fmoc-Ala-OH (0.31 g, 1.0 mmol), HOBT (0.14 g, 1.0 mmol), DCC (0.21 g, 1.0 mmol) and 4-dimethylaminopyridine (0.024 g, 0.2 mmol) in DMF (5 mL) was added to **4** (0.41 g) in DMF (5 mL). The suspension was shaken at r.t. for 4 h, and the resin was washed in turn with DMF (5 mL), MeOH (5 mL) and DMF (5 mL). The coupling was repeated with half quantities of the reagents. After removal of the Fmoc group with 20% piperidine in DMF (5 mL) for 5 and 15 min, the degree of substitution of the resin was determined by the method of Gisin (5).

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Substituted benzhydryl derivatives as linkers for solid-phase peptide synthesis

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Introduction

A promising resin for the synthesis of peptides with Fmoc chemistry and free carboxyl at the C-terminus is 2,4-dimethoxyphenyl-4'-hydroxymethyl-phenoxyethyl-polystyrene of Rink [1]. Previously we demonstrated the use of 2,4,2'-trimethoxy-4'-succinylamido-benzhydrylamine-polystyrenes for the synthesis of sensitive peptide amides. Based on these results we wanted to design and synthesize a new acid-labile polymer for the synthesis of peptides with free C-terminus. The most commonly used procedure for the preparation of such carriers is the synthesis of the 'keton-resin' and the subsequent reduction. We attempted to synthesize the polymer of Rink by following the above method but we obtained low and unrepeatable substitution. Besides, the structure of the polymer could not be determined accurately.

Results and Discussion

In order to prepare the polymer of Rink we first synthesized the ready linker (2,4-dimethoxy-4'-hydroxy-benzophenone) as described in the literature [1]. Investigation of the reduction showed that it proceeded in 15 min instead of the previously described 1 h. If we continued the reduction, the amount of by-products gradually

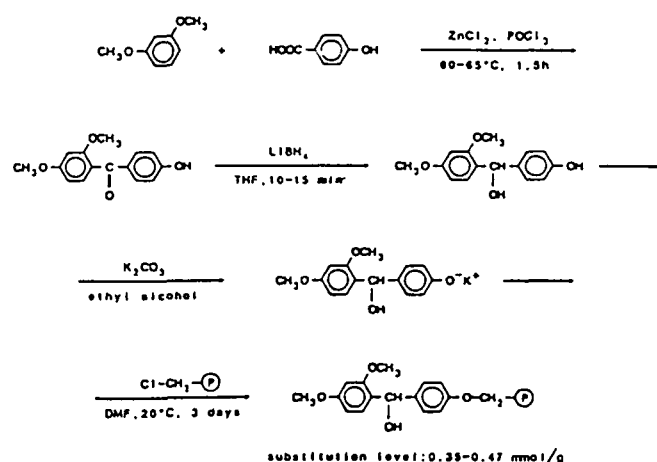


Fig. 1. The new synthesis of 2,4-dimethoxyphenyl-4'-hydroxymethyl-phenoxyethyl-polystyrene of Rink.

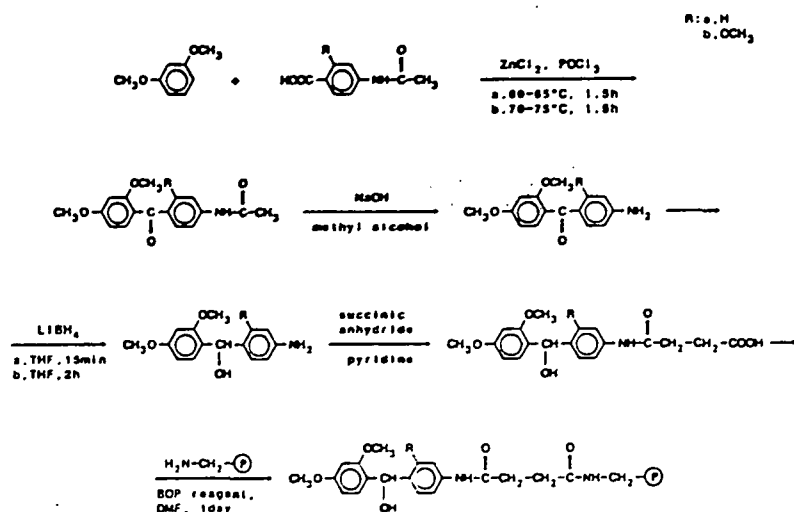


Fig. 2. The synthesis of 2,4-dimethoxy- and 2,4,2'-trimethoxy-4'-succinylamido-benzhydrol-polystyrenes.

increased. One of them is probably a diphenylmethane derivative. For the coupling of the linker to chloromethylpolystyrene, beside the known procedure using cesium salt, we tried to use potassium salt of the phenolate too. This latter process resulted in a cheaper method and better resin as regards substitution than the use of cesium salt (Fig. 1). The structure and purity of the substances were proved via NMR, IR spectra and elemental analyses, the substitution degree by coupling of an Fmoc amino acid. We achieved a convenient level of substitution (0.35–0.47 mmol/g). Based on previous experiments we designed two new, even more acid-labile, carriers for the synthesis of peptides with free C-terminus. These are 2,4-dimethoxy- and 2,4,2'-trimethoxy-4'-succinylamido-benzhydrol-polystyrenes. They were synthesized in analogy of the method worked out in the case of the polymer of Rink. The starting materials (2,4-dimethoxy- and 2,4,2'-trimethoxy-4'-acetamidobenzo-phenone) were prepared from 2-methoxy-4-acetamido-salicylic acid and resorcinol dimethylether. After hydrolysing of the acetyl group the compounds were reduced and the succinyl derivatives of the linkers were coupled then to aminomethylpolystyrene (Fig. 2.). The characterisation of these new resins using model peptide synthesis is in progress.

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Synthesis of branched polypeptides as antigens for influenza virus hemagglutinin and T-cell receptor subunits

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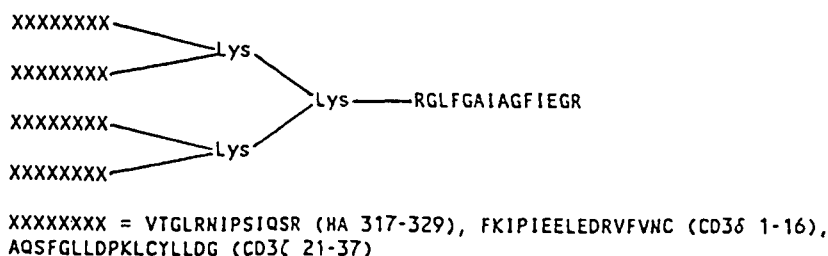
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Introduction

The activation of T lymphocytes occurs via and is regulated by a number of cell-surface receptors, including T-cell antigen receptor (TCR) complex. These receptors have prime importance in the immune response. However, the role of CD3 subunits of TCR and other receptor-associated proteins (CD3 γ , δ , ϵ , ζ) are still poorly understood. Monoclonal antibodies are generally used for studying cell-surface receptor functions. Hemagglutinin, the surface glycoprotein of the influenza virus plays an important role in the infectivity and pathogenicity of the virus. The posttranslational enzymatic cleavage mediated by host cell proteases is necessary for the formation of infective virus. The elucidation of the requirements for the T- and B-cell recognition might lead to important information for subunit vaccine design. In previous experiments we have localized several T- and B-cell epitopes in the intersubunit part of the influenza virus hemagglutinin, including a very good T helper cell activator [1–3]. The multiple antigen peptide (MAP) method [4–7] emerged into a widely used procedure for the production of a strong immune response against various peptide fragments of different proteins. Our aims were the following:

- (1) Raising antibodies against the extracellular part of CD3 δ and CD3 ζ subunits.
- (2) To elucidate the role of hemagglutinin in the immune response.
- (3) To investigate the usefulness of the 'multiple antigen peptide'.



Scheme 1. The structure of the synthesized chimeric 'multiple antigenic peptides'.

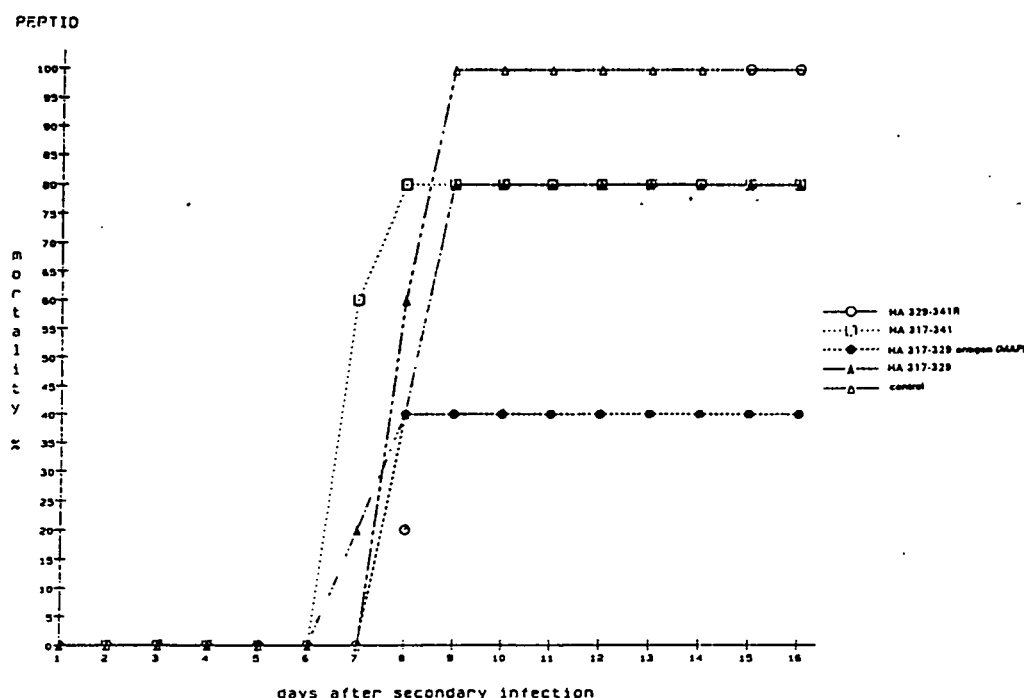


Fig. 1. The effect of the HA antigen construction for the mortality after influenza virus infection.

Peptide Synthesis and Purification

The peptides were synthesized by solid phase technique utilizing *t*-Boc chemistry [8]. Side-chain protecting groups were as follows: Arg(Tos), Asp(OcHex), Cys(Meb), Thr(Bzl), Lys(2ClZ), Glu(OcHex), Tyr(2BrZ) and Ser(Bzl). The peptide chains were elongated on MBHA resin (0.8 mmol/g) and the syntheses were carried out manually. Couplings were performed with DCC, with the exception of Asn, Gln and Arg which were incorporated as their HOBt esters. Amino acid incorporation was monitored by the ninhydrin test [9]. The completed peptide resins were treated with liquid HF/dimethylsulphide/*p*-cresol (88:6:4, vol/vol), on 0°C, 1 h. HF was removed and the resulting free peptides were solubilized in 10% aqueous acetic acid, filtered and lyophilized. The crude peptides were purified by RPHPLC using an Astec 300 C4 5 μ column (8 $\times$ 250 mm). The solvent system used was the following: 0.1% TFA in water, 0.1% TFA, 80% AcN in water, gradient: 0% $\rightarrow$ 90% B in 1.5 h, flow 1.5 ml/min. The appropriate fractions were pooled and lyophilized. The purity was checked by RPHPLC, isoelectrofocussing and SDS gel electrophoresis.

Immunological Results

The immunological tests revealed that all the antisera are highly specific against the used synthetic peptides, however, the antisera in the immunne precipitation test do not recognize the native 16 kDa ζ chain. The antisera against the δ chain were able to recognize the native molecule in indirect immunfluorescence using FACS-

star plus. The hemagglutinin antigen construction resulted in a high antibody response after repeated injection into mice. This new MAP construction was highly potent also in TH cell activation. Additionally, in preliminary experiments it showed a protective effect against influenza infection. The MAP system combined with this TH epitope seems to be a promising approach for subunit vaccine design.

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Comparative Solid Phase Synthesis of Caerulein

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Caerulein was prepared with different liquid and solid phase methods for optimization of the synthesis. Classical liquid phase methods using fragment condensations or solid phase methods using Fmoc- or Boc-chemistry with Met(O) and Trp(For) protection resulted in low yield and/or very impure product. The best method found for the synthesis of caerulein is as follows: solid phase method with Boc-chemistry, Met and Trp without side-chain protection, Thr(Ac) protection, DCC or DCC/HOBt condensation, sulphation of the Thr(Ac) containing peptide with PAS or Pyr/SO₃, deacetylation with basic hydrolysis. Purification can be carried out by MPLC and HPLC.

Caerulein is a decapeptide sulphate ester [Glp-Gln-Asp-Tyr(SO₃H)-Thr-Gly-Trp-Met-Asp-Phe-NH₂], which was isolated from the skin of the frog *Hyla caerulea* [1]. It shows similar biological effects to the peptide hormone cholecystokinin [2-4], and has been used diagnostically for the investigation of different parts of the gastrointestinal tract. The chemical synthesis of caerulein has been a difficult task owing to the following problems:

- a) selective sulphation of Tyr in the presence of Thr;
- b) acid lability of the O-sulphate group;
- c) sensitivity of Met and Trp to sulphating agents (formation of by-products).

Several synthesis strategies with different results have been used for the preparation of caerulein [5,6]. Our aim was to work out an optimal, rapid synthesis for caerulein. In order to optimize the synthesis, we investigated the following strategies:

- A) Classical liquid phase syntheses
 - 1) fragment condensation: 6 + 2 + 2
 - 2) fragment condensation: 1 + 1 + 2 + 2 + 2 + 2
- B) Solid phase syntheses
 - 1) Fmoc-chemistry on SAMBHA resin with direct coupling of Fmoc-Tyr(SO₃Na)-OPFP
 - 2) Boc-chemistry on MBHA resin with protected Trp and Met [Trp(For) and Met(O)]
 - 3) Boc-chemistry on MBHA resin with unprotected Trp and Met and with or without scavenger.

Classical Syntheses

Fragments were built up with Cbz- and Boc-chemistry and using active ester coupling methods (-OPCP or -OPFP). The reactive side-chain functional group of Asp was protected with benzyl ester. In case of A/1, the side-chain of Thr was protected with benzyl ether, Tyr was unprotected, and sulphation was carried out after fragment condensation. In the scheme of A/2, the alcoholic OH of Thr was unprotected and Tyr was coupled as Fmoc-Asp-Tyr(SO₃Na)-N₂H₃ similar to the classical liquid phase synthesis of cholecystokinin octapeptide sulphate ester [7]. Both the azide and DCC/PFPOH method was used for coupling of the fragments. Both syntheses resulted in a small amount of caerulein. Problems observed by using these classical methods are: the hydrolytic cleavage of protecting groups and sulphation of the decapeptide can cause side reactions because of the

presence of unprotected Met and Trp; and both classical methods are very time-consuming, resulting in low yields and very impure product.

Solid Phase Syntheses

Fmoc-chemistry was carried out on SAMBHA (4-succinylamido-2,2',4'-trimethoxybenzhydramino polystyrene) resin using *tert.* butyl ether and ester for side-chain protection of Thr and Asp, respectively. DCC was used for condensation in all cases with the exceptions of Fmoc-Tyr(SO₃Na)-OPFP and Fmoc-Gln-OH, which was coupled with DCC/HOBt. Direct coupling of Fmoc-Tyr(SO₃Na)-OPFP was used instead of sulphation. A mixture of sulphated and unsulphated caerulein (1:1) was obtained after cleavage of the peptide and all the protecting groups with 50% TFA/DCM (containing 2% (CH₃)₂S and 2% ethanedithiol, 20°C, 15 min) with low yield.

Boc-chemistry was performed on MBHA-resin using Thr(Ac), Asp(OcHex), and Tyr(2-Br-Cbz). In the first method, Trp and Met were protected as Trp(For) and Met(O). DCC or DCC/HOBt was used for coupling. The Boc group was removed, after each completed coupling step, by treatment with 50% TFA in DCM. After completion of the synthesis and cleaving the peptide from the resin with HF, Met(O) was reduced with MMA or thioglycolic acid. Sulphation with PAS (pyridiniumacetylsulphate) [8] was followed by basic hydrolysis. The disadvantage of this method was the difficult reduction step with MMA or thioglycolic acid (e.g. partial hydrolysis of the protecting acetyl group of Thr).

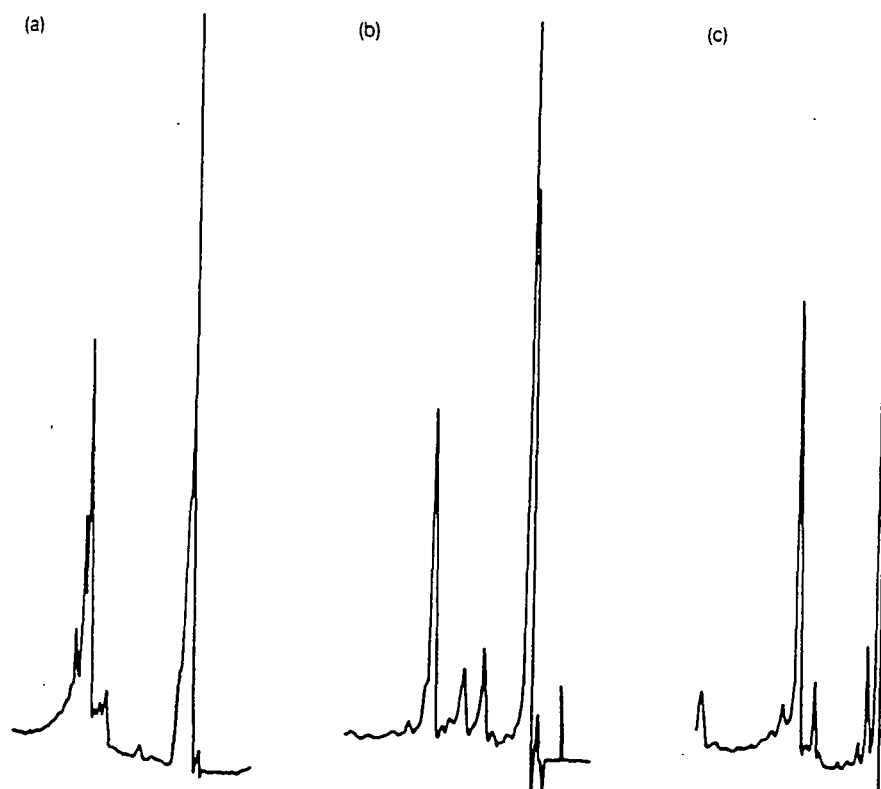


Figure 1. HPLC-chromatograms of crude, Thr(Ac) containing, and unsulphated caerulein from syntheses without (a) or with (b) scavenger and after sulphation (c) of "b". HPLC conditions: column, Lichrosorb RP-18; detection, 220 nm; eluent system, (A) 0.1% aqueous TFA and (B) 0.1% TFA in 80% MeCN; linear gradient, 30% – 50% B in 30 min; flow, 1.5 ml/min.

In the second method of Boc-chemistry, unprotected Trp and Met and DCC or DCC/HOBt coupling was used. The Boc group was removed, after each completed coupling step, by treatment (a) with 50% TFA in DCM, and (b) with 50% TFA in DCM containing 0.5% DTT. After completion of the synthesis, the dried and protected peptide-resin was treated with a mixture consisting of 1.0 ml *p*-cresol, 2 ml $(\text{CH}_3)_2\text{S}$, and 17 ml anhydrous hydrogen fluoride per gram of peptide-resin for 60 min at 0°C to cleave the peptide from the resin and to deprotect the side-chain functional groups with the exception of acetyl of Thr. Sulphation of the Thr(Ac)-containing peptide was carried out with PAS or Pyr/SO_3 . Figure 1 shows the comparative HPLC profile of crude caerulein after HF cleavage using (a) or not using (b) scavenger during the synthesis and sulphation of "5" (c). The peptide was deacetylated via basic hydrolysis. After purification, this strategy resulted in good yield and proved to be the best method for the synthesis of caerulein. Figure 2 represents the HPLC profile of the pure caerulein.

Purification

Crude products were purified first by using a Büchi MPLC system (30 x 300 mm Vydac C18 column, 300 Å, 15-30 µm) using the eluent system containing (A) 0.05 M TEAP (pH = 6.5) and (B) 0.05 M TEAP in 70% MeCN in a linear gradient mode (0% – 70% B in 90 min). The second purification of the peptide was carried out by using a Knauer RP-HPLC system (16 x 250 mm Lichrosorb RP-18 column, 300 Å, 10 µm). The column was eluted with a solvent system consisting of (A) 0.2% aqueous acetic acid and (B) 0.2% acetic acid in 80% aqueous MeCN in a linear gradient mode (0 % – 70% B in 90 min).

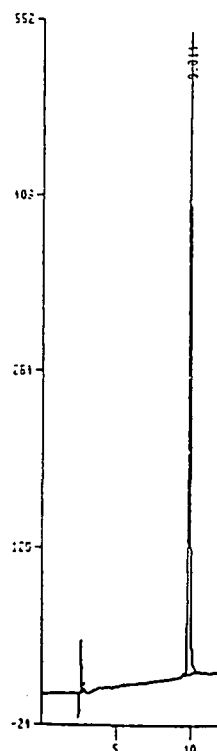


Figure 2. Analytical HPLC profile of purified caerulein. HPLC conditions: column, Nucleosil 5 C18; detection 220 nm; eluent system, (A) 0.1% aqueous TFA and (B) 0.1% TFA in 70% MeCN; linear gradient, 35% – 50% B in 15 min; flow rate 1.2 ml/min.

The chemical and biological properties in different tests of the synthesized and purified caerulein proved to be fully active and identical to the standard product of Farmitalia (TAKUS[®]).

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**T CELL RECOGNITION OF THE POSTTRANSLATIONALLY CLEAVED
INTERSUBUNIT REGION OF INFLUENZA VIRUS HEMAGGLUTININ**

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Running title: T cell recognition of influenza virus

hemagglutinin

*Key words: T cell epitope, antigen processing, influenza
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ABBREVIATIONS

APC	- antigen presenting cell
CD	- circular dichroism
FT-IR	- Fourier-transform infrared spectroscopy
HA0	- immature hemagglutinin
HA	- mature hemagglutinin
Ig	- immunoglobulin
mAb	- monoclonal antibody
PBS	- phosphate buffered saline
G-PBS	- glucose containing phosphate buffered saline

SUMMARY

The influenza virus hemagglutinin is synthesized as a single polypeptide chain, but upon maturation it will posttranslationally be modified by a host cell related trypsin like enzyme. The enzymatic cleavage attacks the so-called intersubunit region of the molecule giving rise to covalently linked HA1 and HA2 subunits. An I-E^d restricted T cell epitope was identified in the highly conservative intact intersubunit region of the influenza virus hemagglutinin. T cell recognition of a 25-mer synthetic peptide comprising the intact intersubunit region does not require further processing and the elimination of the Arg residue coupling the fusion peptide to the C-terminal segment of HA1 does not abolish the T cell activating capacity. Fine specificity pattern of a T cell hybridoma similar to that of the polyclonal T cell response demonstrates that a single T cell receptor is able to recognize peptides of different sizes representing not only the uncleaved but also the cleaved forms of this hemagglutinin region. Based on specificity studies the epitope was localized to the C-terminal 11 amino acids of the HA1 subunit.

The cross reactivity of peptide primed T cells with influenza virus infected antigen presenting cells shows that fragments comprising the identified epitope of the

intersubunit region can be generated as a result of the natural processing of the hemagglutinin molecule. As antigen presenting cells are lacking the enzyme which is responsible for the posttranslational modification of newly synthesized hemagglutinin molecules, the role of immature viral proteins in immune recognition is discussed.

INTRODUCTION

Intracellular processing of viral proteins raises special possibilities for antigen presentation [1-5]. Spike or integral proteins of enveloped viruses are degraded in antigen presenting cells (APC) as exogenous antigens upon entry to the host cell via receptor mediated endocytosis. MHC II molecules play a predominant role in this stage of viral infection presenting viral peptides for virus specific helper T cells [6]. If infective viruses enter the appropriate host cell virus replication occurs generating newly synthesized viral proteins which can predominantly be handled as endogenous proteins in terms of antigen processing. In this process primarily MHC I but, depending on the type of the host cell, also MHC II molecules are involved [7,8]. Influenced by the intracellular destination of viral proteins, MHC I and MHC II molecules have the chance to select from different sets of viral peptides, but some fragments of viral proteins might be able to bind to both types of MHC molecules [9,10].

The hemagglutinin (HA) spike protein of influenza viruses has multiple biological functions and it is one of the main targets of immune recognition [11-13]. T cell epitopes recognized either by helper or cytotoxic T cells in the context of different H-2 [14,15] or HLA [16] haplotypes have been localized in distinct regions of the molecule including

the C-terminal membrane spanning segment of the HA2 subunit [17] or the head region (HA1) accumulating most of the type and subtype specific variations and the corresponding B cell epitopes [1,2,11].

The most conservative sequence of the hemagglutinin molecule from different influenza viruses is the functionally essential fusion peptide representing the N-terminal 1-12 amino acids of the HA2 subunit [18]. In newly synthesized, immature hemagglutinin (HA0) molecules this peptide is covalently linked to the C-terminal of the HA1 subunit via an Arg residue in position 329. This Arg will be deleted in the course of HA0 maturation by subsequent posttranslational enzymatic cleavages [19] giving rise to mature HA built up from disulphide-bridge-linked HA1 and HA2 subunits [20]. This event is the prerequisite of viral infectivity ensuring a pH-dependent conformational change of the HA molecule in endosomes where membrane fusion will be catalysed by the exposed fusion peptide [21]. The amino acid sequence of the C-terminal stretch of the HA1 subunit also shows restricted amino acid variations and comprises an enzyme cleavage motif determining enzyme susceptibility and thus influencing tropism of the virus [22-25]. The enzyme involved in the posttranslational modification was identified as a trypsin like serine protease acting in the post-Golgi compartment of defined cell types such as the epithelial cells of the respiratory tract [20]. Professional antigen presenting

cells such as B cells, macrophages or dendritic cells lack the appropriate enzyme for HA0 cleavage and thus upon infection generate immature, uncleaved molecules giving rise to non infective viruses [6,20].

As we demonstrated (26, Nagy et al. to be published) the 25 amino acid-long synthetic peptide IP corresponding to the uncleaved form of the intersubunit region of the A/PR/8/34 (H1N1) human influenza A virus hemagglutinin (see Table 1) is able to elicit an IgG response in BALB/c mice without the introduction of any carrier. This result implies that T cell epitopes are also present in this region. This assumption was supported by the prediction of an alpha-helical structure and segments resembling to Rothbard motifs in the peptide sequence, analyzed by the combined prediction method of Feller & Cruz [27]. This paper reports a study on the possible recognition of the HA1-HA2 intersubunit region by T cells in relation to the influence of the posttranslational modification. This question was approached by the use of synthetic peptides comprising the C-terminal sequence of the HA1 and the N-terminal sequence of the HA2 subunits. These peptides correspond to the covalently linked intact or cleaved forms of the immature HA0 and the posttranslationally modified HA molecules, respectively. The conformational properties of these overlapping peptides have been characterized by circular dichroism (CD) and Fourier-transform infrared (FT-IR) spectroscopic methods [28,29,

Nagy et al. to be published). They have been used for specificity analysis of peptide or virus primed T cells. Our results revealed that the intersubunit region encompasses a nonpredicted I-E^d restricted T cell epitope located in the C-terminal of the HA1 subunit. This epitope can be recognized in peptides corresponding both to the cleaved and uncleaved forms of the hemagglutinin molecule (Table 1). Peptides of different length covering the core region of this epitope are able to prime and activate T cells which also recognize influenza virus infected APC. These results reveal that the natural hemagglutinin processing pathway can generate peptides comprising this T cell epitope.

MATERIALS AND METHODS

1. Animals

8-10 weeks old female B10 (H-2^b) and CBA (H-2^k) mice were purchased from LATI (Gödöllő, Hungary). Female BALB/c (H-2^d) mice were delivered by the Institute of Oncology (Budapest, Hungary). Animals were housed under conventional conditions.

2. Virus preparation

The A/PR/8/34 influenza virus (H1N1) stock bearing cleaved (grown in chorio-allantoic fluid of hen eggs) or uncleaved (grown in hen egg embryonic fibroblasts) hemagglutinin molecules were prepared as described previously [30]. Heat inactivation of the virus was performed at 56 °C for 30 minutes.

3. Synthetic peptides

The description of peptide synthesis, purification and characterization has been reported elsewhere [28-30]. Characteristics of the peptides used in this study are summarized in Table 1. Prediction of T cell epitopes was performed by the software package TSites [27].

4. In vivo induction of A/PR/8/34 influenza virus primed T cells

BALB/c mice were aerosol infected with 0.03 LD₅₀ dose of the mouse pathogenic A/PR/8/34 influenza virus and 3 months later red blood cell lysed spleen cells were enriched for T cells by passing the single cell suspension through a nylon wool (FENWAL LABORATORIES) column. In an other series of experiments BALB/c mice were infected as described above but three weeks later they were re-challenged with 1 LD₅₀. T cells were isolated 3 month after secondary infection as described above. In a third series of experiments the A/PR/8/34 virus was injected subcutaneously as 100 ug purified virus emulsified in complete Freund's adjuvant and introduced to the base of the tail.

5. In vivo induction of peptide primed T cells

B10, CBA or BALB/c mice were immunized subcutaneously at the base of the tail and to the hindth footpads with 100 ug peptide dissolved in sterile PBS and emulsified in CFA. 14 days later the T cell enriched lymph node cells were used for the proliferation assay. In some experiments peptide administration was repeated on day 21 in combination with incomplete Freund's adjuvant and the T cell enriched lymph node cells were isolated 7-10 days after the secondary peptide challenge. T cell enrichment of the popliteal,

inguinal and paraaortic lymph node cells of subcutaneously immunized mice was performed either by nylon wool (FENWAL LABORATORIES) elimination of adherent cells or by panning on anti-mouse immunoglobulin (Ig) coated Petri dishes. The T cell enriched, eluted cell population (subsequently termed as T cells) was contaminated with 1-3 % B cells detected by cytofluorimetry using FITC-labelled anti-mouse Ig (SIGMA).

6. T cell proliferation assay

The T cell proliferation assay was performed by the method of Corradin et al. [31]. Briefly, 5×10^5 enriched lymph node T cells were cultured in the presence of 2×10^5 irradiated syngeneic spleen cells as APC in the absence or the presence of different concentrations of synthetic peptides. Triplicate microcultures in a final volume of 250 μ l were established in flat bottomed 96 well plates (GREINER) in complete RPMI medium supplemented with 10% fetal calf serum (GIBCO)); 1 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol, antibiotics, nonessential amino acids, and BME-vitamins (all purchased from SIGMA).

Influenza virus infection of normal BALB/c spleen cells was performed by a 4 h preincubation of the cells with different doses of the infective A/PR/8/34 influenza virus. After the preincubation period cells were washed twice with glucose

containing phosphate saline (G-PBS), irradiated or mytomycin-C (SIGMA) treated (25 ug/ml, 37°C, 30 min) and used as APC at 2×10^5 cell/well. Cultures were pulsed with 1 uCi (3.7 kBq) ^3H -thymidine for the final 16 h of the 4-day cultures.

Experiments performed in the presence of the enzyme inhibitor leupeptin (acetyl-Leu-Leu-Arg-SO₃H, BOEHRINGER MANNHEIM) or the tripeptide Boc-D-Phe-Pro-Arg-CHO (RGH-2958), the generous gift of S. Bajusz, Institute of Drug Research, Budapest, Hungary) [32] was performed as described for the T cell proliferation assay except that the inhibitor was present during the whole period of cell culture at 1 mM final concentration.

The inhibitory effect of MHC specific monoclonal antibodies (mAb) I-47 rat IgG2a, anti-H-2D^d [generous gift of G. László (Dept. of Immunol., L. Eötvös University)]; 14.4.4 mouse IgG2a, anti-I-E^{k/d} [33]; M5/114 rat IgG2b, anti-I-A/E^d (ATCC TIB 120) [34]; MKD6 mouse IgG2a, anti-I-A (ATCC HB3) (35) was detected in the presence of 5% hybridoma cell culture supernatants.

7. Isolation of the IP-12-29 T cell hybridoma

The IP-12-9 T cell hybridoma was selected from the spleen of a HA1C[Arg] immunized and virus challenged BALB/c mouse.

HA1C[Arg] immunization was performed as described above and 4 weeks before cell fusion the preimmunized mouse was aerosol infected with 5 LD₅₀ of A/PR/8/34 influenza virus. Spleen cells of the peptide immunized and subsequently virus challenged mouse were reactivated in vitro with the HA1C[Arg] peptide and 5 days later Ficoll separated T cells were fused with the BW-1100.129.237 α^-/β^- [36] thymoma cells according to the method of Rock [37].

8. Detection of IL2 production

Peptide or virus induced IL2 production of activated polyclonal T cells was measured in cell culture supernatants of the proliferation assay harvested after 24 h incubation in the presence of the peptides or virus infected APC. The detection of IL2 in the supernatant of the IP-12-29 T cell hybrid was measured in supernatants taken at 48 h of cell cultures containing 1×10^4 hybridoma cells and 2×10^5 irradiated syngeneic normal spleen cells. IL2 was detected and quantitated by the proliferation of HT2 cells detected by ^3H -thymidine incorporation in secondary cultures using a calibration curve in the presence of different amounts of recombinant human IL2 (kindly provided by Hoffman La Roche).

RESULTS

1. The proliferative response of CBA, B10 and BALB/c mice to the synthetic IP peptide

The proliferative response of in vivo primed lymph node T cells of different inbred mouse strains to the synthetic IP peptide (see Table 1) representing the uncleaved form of the intersubunit region of the influenza virus hemagglutinin molecule is summarized in Table 2. Data demonstrate that IP is able to induce a peptide specific, MHC restricted proliferative response in IP immunized BALB/c but not in B10 or CBA mice. It is also shown that IP induces proliferation and IL2 production in a dose dependent manner in the presence of syngeneic APC. Despite the lack of a predictable I-E^d restricted epitope (see Table 1) inhibition by MHC specific antibodies revealed the I-E^d restriction of the IP specific T cell response (Fig. 1 dark columns).

2. Specificity of the IP induced T cell response of BALB/c mice

The reactivity of IP specific T cells with overlapping synthetic peptides covering the intersubunit region was studied with polyclonal T cells as well as with T cell hybridomas. As it is shown in Table 3 IP primed T cells can be activated by the HA1C[Arg] subfragment peptide but not

with the FP fusion peptide. This observation points to the location of a T cell epitope to the C-terminal segment of the HA1 subunit. The presence of a T cell epitope in the HA1C[Arg] peptide was also verified by the capability of this peptide, but not of the FP fusion peptide to prime T cells (Table 3). HA1C[Arg] primed T cells are cross reactive with the IP peptide (Table 3) and are also recognized in the context of the I-E^d molecule (Fig. 1 plain columns).

To answer the question whether the C-terminal arginine of the HA1C[Arg] peptide has any role in T cell recognition, the capacity of the Arg-truncated HA1C dodecapeptide to induce the activation of HA1C, HA1C[Arg] or IP primed T cells was studied. Although no striking difference in the T cell priming or activating capacity of HA1C[Arg] or HA1C could be observed (Table 3) it should be noted that the Arg extended peptide gave higher response in most cases.

The observations obtained with polyclonal T cells raised the possibility that T cells of differing specificity might be responsible for the recognition of IP and HA1[Arg] and its analogs. The fine specificity pattern of a typical T cell hybridoma (IP12-29) shown in Fig. 2 shows full cross reactivity of IP and HA1C[Arg] supporting the interpretation that HA1C[Arg] can be recognized both in its 13-mer and the fusion peptide elongated 25-mer forms. As in the case of polyclonal T cells (Table 3) Arg truncation at

the C-terminal of the HA1C[Arg] peptide does not abolish recognition by the IP-12-29 T cell hybridoma (Fig. 2B) demonstrating the identical specificity pattern of polyclonal T cells and that of this monoclonal T cell.

On the basis of these results, a T cell epitope localized to the the 317-328 region of the HA1 subunit representing the H1 serotype sequence of influenza virus hemagglutinin could be identified. In good accordance with the prediction data (see Table 1) N-terminally truncated analogs of HA1C up to a deca- (HA1C_{deca}) or nonapeptide (HA1C_{nona}) are not able to activate the IP-12-29 T cell hybridoma (Fig. 2B).

3. Processing requirements of IP recognition

The cross reactivity of IP and HA1C[Arg] primed T cells in both directions does not clarify whether IP is recognized in its full length or will be further processed giving rise to a fragment comprising the HA1C[Arg] peptide. The processing requirements of IP were investigated by studying the activity of the D-Arg substituted analog of IP ([D-Arg]¹³IP) and using different enzyme inhibitors known to interfere with the activity of trypsin like enzymes, which have an inhibitory effect for exogeneous antigen processing and also for certain viral infection (38). As it is shown in Fig. 3 the presence of leupeptin or the Boc-D-Phe-Pro-Arg-CHO trypsin inhibitor previously shown to inhibit trypsin

mediated fusion of influenza virus (unpublished observation) did not abolish IP recognition. This result, in good accordance with the similar dose response curves of HA1C[Arg], IP or its [D-Arg¹³]IP analog to activate the IP-12-29 T hybridoma (Fig. 2A) or polyclonal T cells (data not shown) revealed that the IP peptide can be recognized without further processing at the Arg/Gly cleavage site.

4. Reactivity of HA1C[Arg] or HA1C primed T cells with influenza virus

As it is summarized in Fig. 4 syngeneic normal spleen cells preincubated with the infectious virus activate HA1C[Arg] or HA1C primed T cells in a dose dependent manner. These results show that processing of the HA molecule results in fragments comprising the HA1C[Arg] region which is available for MHC II molecules and can be recognized by peptide primed T cells.

5. Occurrence of IP, HA1C[Arg] or FP specific T cells in influenza virus infected or immunized BALB/c mice

As the HA1C[Arg] epitope was proved to be presented by influenza virus infected APC, peptide reactive T cells were searched in influenza virus infected or immunized BALB/c mice. Synthetic peptides comprising the intersubunit region of influenza virus hemagglutinin were used to activate

influenza virus primed T cells under in vitro conditions. As a positive control APC preinfected with the infective virus or incubated in the presence of heat inactivated A/PR/8/34 influenza virus were used. As shown in Table 4, primary or repeated viral infections do not result in the activation of a substantial proportion of peptide specific T cells. Adjuvant combined immunization with a high dose of infectious A/PR/8/34 virus does not induce the priming of these type of T cells either. These results do not exclude a low frequency of T cells recognizing this region however, efforts for selecting peptide specific T cell lines or clones were unseccessful (data not shown).

DISCUSSION

The influenza virus hemagglutinin can be present in the infected host in two forms; the single chain HA0 precursor and the posttranslationally modified mature molecule. Mature, enzymatically cleaved HA molecules are ready to undergo a pH-dependent conformational change, acquire enhanced sensitivity to further enzymatic cleavage and are characterized by increased hydrophobicity compared to the immature uncleaved form [21]. In this study the question was raised whether the cleaved and/or uncleaved forms of the intersubunit region can be the target of T cell recognition and if so what are the requirements for T cell recognition. Both the AMPHI [39] and the Rothbard & Taylor [40] algorithms revealed the presence of T cell epitopes in the intersubunit region (Table 1). To localize and demonstrate the function of the predicted T cell epitopes in the intersubunit region of the H1 subtype of influenza virus hemagglutinin inbred mice of different MHC haplotypes were injected with the IP peptide and the polyclonal T cell response to the parental and to the overlapping synthetic peptides was measured by in vitro proliferation and IL2 production. Results revealed that the IP peptide induces an intense T cell response in BALB/c, but not in B10 or CBA mice. The proliferative response of BALB/c mice is I-E^d restricted and is accompanied by IL2 production. The

prediction method introduced by Sette et al. [41] did not reveal the presence of the identified epitope (Table 1).

The 24-mer C-terminal segment of the HA1 subunit of the H3 influenza A virus serotype has previously been identified as a region comprising multiple helper T cell epitopes [15,42]. A dominant I-E^d restricted T cell epitope not cross reactive with the virus, was localized to the 305-317 region together with a subdominant epitope in the 319-328 segment [42]. It was of particular interest to investigate whether the additional length at the C-terminal of HA1 representing a hydrophobic natural sequence has any effect on T cell recognition of this region. This C-terminal flanking sequence, comprising the fusion peptide has been demonstrated to undergo a conformational switch from alpha-helical to an extended conformation under appropriate conditions [28]. Another crucial question was whether the C-terminal Arg of the HA1 subunit, which is involved in the predicted alpha helix but not in the Rothbard motif, and presumably being beneficial in I-E^d and peptide interaction [41,43] has any effect on T cell recognition. In our studies the 317-329 HA1 fragment representing the H1 serotype sequence of human A type influenza viruses turned out to be a potent MHC II restricted T cell epitope used either in its 13-mer or C-terminally elongated 25-mer analog to induce or activate proliferating BALB/c T cells. The dose response curves obtained with IP, [D-Arg¹³]IP or HA1C[Arg] to

activate the IP12-29 T cell hybridoma revealed the similar potency of the longer or shorter analogs to activate T cells. In addition, the lack of inhibition by serine and thiol protease inhibitors demonstrated that the longer IP or its [D-Arg¹³]IP analog can be recognized without further processing being mediated by trypsin like enzymes inside or outside of the cell.

The core region of the epitope was localized to the 317/318-328 region as the N-terminally truncated deca- or nonapeptides were inactive (Fig. 2B). Our results show that overlapping peptides of different length comprising this region are able to activate T cells. The fusion peptide elongated 25-mer peptide is as efficient to activate T cells as the shorter HA1 derived peptides (Fig 2A, Table 3). Lack of the C-terminal Arg residue in the HA1C subunit peptide, in good correlation with its elimination under physiological conditions by carboxypeptidase N [19,44] does not abolish T cell recognition (Fig. 2B, Table 3).

These results are consistent with the recent view of the open binding groove of the class II MHC molecule [45]. Data on the size of naturally occurring MHC class II associated and eluted peptides of endogenous or exogenous proteins [46-49] revealed a wide size heterogeneity and provided examples for the enhanced binding efficiency of longer peptides [50]. In functional terms our results demonstrate

that the epitope identified in the intersubunit region can equally well be recognized as peptides of cleaved or uncleaved hemagglutinin molecules if the appropriate fragments are generated by any route of antigen processing. Lysosomal enzymes are not involved in the posttranslational modification of HAO and their activity results in aberrant cleavage products of the immature molecule [51]. These fragments however, can bind to and be protected from further degradation by MHC molecules. The cross reactivity of HA1C[Arg] or HA1C primed T cells with APC, preincubated with the infective virus which subsequently give rise to uncleaved HAO molecules supports this assumption.

The capability of HA1C[Arg] primed T cells to be activated by influenza virus infected APC proves that hemagglutinin fragments comprising the studied peptides will be generated in an available form after natural processing of the molecule. The exact pathway of the route of hemagglutinin processing has however to be elucidated. The infective viral input to the exogenous pathway is much lower compared to the amount of newly synthesized, uncleaved hemagglutinin molecules produced in the course of a single growth cycle (4 h) [14]. This suggests that it is the endogenous pathway which also might be involved in peptide production. This interpretation is consistent with previous results which show, that MHCII molecules can efficiently be loaded also by endogenous influenza virus proteins [7,8,14,52]. Previous

data showing that re-internalization of noninfectious viruses bearing uncleaved HAO molecules, is unefficient [53] also supports this interpretation.

The cross reactivity of peptide primed T cells with virus infected APC argues against the possibility that the low frequency of T cells directing against this region in virus primed mice, is the consequence of inappropriate processing events. It is more likely that the productive growth cycle of the virus results in strong peptide competition in APC mediated by a large excess of dominant T cell epitopes generated from cleaved HA or from other viral proteins entering the endogeneous processing pathway. Consequently, the epitope defined in this study is not an immunodominant T cell determinant in the course of viral infection. However, primary infection of APC producing uncleaved HAO precursors might have an important role in the first recruitment of T and B cells [6]. As it was revealed the covalent attachment of the highly conserved fusion peptide has a beneficial effect on the expression of the B cell epitope located also in the HA1C[Arg] region [26,29,30,54, Nagy et al. to be published]. This is due to the stabile conformation of this region which is maintained also in IP as demonstrated by CD and FT-IR spectroscopy [28]. In an in vivo situation the concomitant presence of a B cell epitope by itself can be beneficial for T cell recognition by promoting uptake of the protein and protection of a particular region against

further degradation. The potency of this region as a subunit vaccine candidate was experimentally demonstrated in survival experiments proving the capability of these peptides and more obviously their modified analogs to elicit a protective immune response [26,29,30, Nagy et al. to be published]. Based on our results it is reasonable to assume that multiple infections generating cleaved HA molecules does not favour antibody recognition of this region and the subsequent internalization of immature HA0 molecules by specific B cell APC suggesting a functional escape mechanism of the virus.

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Fig. 1**MHC restriction of the IP and HA1C[Arg] primed T cell response**

Proliferation of IP () or HA1C[Arg] () primed T cells was studied in the presence of 5 ug/ml IP or HA1C[Arg], respectively in the absence or presence of 5% cell culture supernatants containing mAb reacting with I-A, I-E or H-2D^d molecules. Percentage of inhibition was calculated from cpm values obtained for positive cultures containing the corresponding peptide in the absence of the mAb.

Fig. 2**Reactivity of the IP-12-29 hybridoma with synthetic peptides representing the intersubunit region of influenza virus hemagglutinin**

A - T hybridoma cells were cultured in the presence of irradiated BALB/c spleen cells and different concentrations of the synthetic peptides IP (), [D-Arg¹³]IP () or HA1C[Arg] (). Relative IL2 content of cell culture supernatants was measured by HT2 cell proliferation and given as cpm. Mean values of triplicates of a typical experiment are documented.

B - T hybridoma cells were cultured in the presence of 2.5 ug/ml HA1C[Arg] (), HA1C (), HA1C_{deca} () or HA1C_{nona} () peptides. Negative cultures () did not contain peptide.

Fig. 3

Proliferation and IL2 production of IP primed polyclonal T cells in the presence of trypsin inhibitors

The T cell proliferation assay was performed in the presence of 40 ug/ml IP or 40 ug/ml IP in combination with 1mM leupeptin (Leup) or Boc-D-Phe-Pro-ArgCHO (RGH-2958) (D-FPR). Control cultures did not contain peptide (-) or trypsin inhibitor (+). Mean values of triplicates of a typical experiment are documented.

Fig. 4

Proliferation of HA1C[Arg] or HA1C primed T cells in the presence of A/PR/8/34 influenza virus infected syngeneic APC

Normal BALB/c spleen cells were preincubated with 100, 20 or 4 HAU/ml dose of A/PR/8/34 influenza virus for 4 h at 37 °C, mytomycin treated and cultured with HA1C[Arg] () or HA1C () peptide primed T cells. Control cultures were set up with the same amount of APC not preincubated with the virus.

Table 1

Description, amino acid sequence, purity and epitope prediction of the synthetic peptides

Localization	Code	Sequence	Purity* %
HA ₁₃₁₇₋₃₂₉ +HA ₂₁₋₁₂	IP	VTGLRNIPSIQSRGLFGAIAGFIEG ...AAAA...AAAAAAAAAAAA...RRRRRRRR.....	96
HA ₁₃₁₇₋₃₂₉ +HA ₂₁₋₁₂	[D-Arg ¹³]IP	VTGLRNIPSIQSRGLFGAIAGFIEG	97
HA ₁₃₁₇₋₃₂₉	HA1C[Arg]	VTGLRNIPSIQSR ...AAAAA....	98
HA ₁₃₁₇₋₃₂₈	HA1C	VTGLRNIPSIQS ...AAAAA....	99
HA ₁₃₁₉₋₃₂₈	HAC _{deca}	GLRNIPSIQS ...AAAA...	97
HA ₃₂₀₋₃₂₈	HAC _{nona}	LRNIPSIQS	98
HA ₂₁₋₁₂	FP	GLFGIAGFIEG ..AAAAAAA.. RRRRRRRR...	97

* - determined by HPLC;

A - AMPHI positive segments (Window size 7) [27]

R - Rothbard motifs (Window size 7) [27]

Table 2

The in vitro IP specific proliferative response and IL2 production of lymph node T cells isolated from IP primed BALB/c, B10 or CBA mice

Mouse strain	H-2				T cell proliferation		IL2 production
	K	I-A	I-E	D	IP ug/ml	cpmx10 ⁻³ +SD	cpmx10 ⁻³ +SD
B10					20	2.8 + 0.4	0.2 + 0.1
					4	3.7 + 1.2	0.1 + 0.1
	bb	bb	b-	bb	0.8	3.5 + 1.4	0.8 + 0.2
					-	3.8 + 2.6	0.2 + 0.1
CBA					20	2.0 + 0.7	7.8 + 1.2
					4	1.5 + 0.3	4.9 + 0.7
	kk	kk	kk	kk	0.8	0.7 + 0.1	1.2 + 0.1
					-	0.5 + 0.1	0.3 + 0.1
BALB/c					20	86.3 + 10.7	131.3 +23.2
					4	45.6 + 8.8	128.2 + 5.1
	dd	dd	dd	dd	0.8	13.5 + 9.4	71.5 +14.1
					-	1.9 + 0.6	38.8 + 5.4
					20*	4.9 + 0.5	n.t.

* - tested in the presence of CBA normal spleen cells
as APC

n.t. - not tested

Table 2 (continued)

T cell proliferation was measured by ^3H -thymidine incorporation in the 4-day cultures. IL2 production was measured by the proliferation of HT2 cells in a secondary culture containing 50 μl of culture supernatant harvested 24 h after primary culture in the presence of APC and peptide. Mean values of triplicates + SD of a typical experiment are documented.

Table 3

Cross reactivity of peptide primed lymph node T cells with the intersubunit IP and subunit peptides HA1C[Arg], HA1C and FP

Immunization

In vitro stimulation					
	ug/ml	IP	HA1C[Arg] cpm x 10 ⁻³ + SD	HA1C + SD	FP
IP					
	20	48.9+ 9.9	36.3+ 6.6	33.4+ 4.3	12.2+ 2.3
	4	63.1+ 2.8	33.5+ 3.0	28.5+ 0.5	14.2+ 6.2
	0.8	50.2+ 4.0	19.9+ 5.3	23.0+ 1.7	12.5+ 1.4
	-	7.1+ 0.7			14.1+ 6.1
HA1C[Arg]					
	20	43.3+ 3.4	66.1+17.4	41.9+ 2.7	n.t.
	4	50.1+16.0	41.8+10.5	23.8+12.4	n.t.
	0.8	65.5+ 4.8	33.4+ 6.3	26.7+ 5.5	n.t.
	-	14.6+ 5.2			
HA1C					
	20	22.3+ 6.9	34.8+ 2.3	32.9+ 3.9	n.t.
	4	28.0+ 3.9	22.8+12.7	24.0+ 9.5	n.t.
	0.8	21.1+ 6.3	18.8+ 5.3	21.8+10.4	n.t.
	-	2.8+ 0.3			
FP					
	20	10.7+ 2.9	n.t.	n.t.	5.9+ 0.5
	4	8.8+ 3.4	n.t.	n.t.	5.4+ 0.7
	0.8	7.2+ 0.8	n.t.	n.t.	5.7+ 2.4
	-	5.5+ 1.1			

T cell proliferation was measured as described under Table

2.

Table 4.

Reactivity of influenza virus infected or immunized BALB/c mice with the synthetic peptides covering the intersubunit reigon of influenza virus hemagglutinin

Immunization	In vitro stimulation				
	VIRUS		PEPTIDE		
	A/PR/8/34	A/PR/8/34	IP	HA1C[Arg]	FP
		HIA			
	100 HAU/ml	ug/ml ^{a/b}		20 ug/ml	
A/PR/8/34 infection primary	102.9+14.9	71.9+33.4 ^a	9.4+6.4	4.6+4.2	5.0+3.6
A/PR/8/34 infection repeated	87.9+16.2	86.5+ 4.8 [*]	0	0	0
A/PR/8/34 live s.c. injection	n.t.	73.7+ 9.4 ^{**}	1.8+1.3	n.t.	0

HIA - heat inactivated; n.t.- not tested

* - 4 ug/ml; ** - 20 ug/ml

Primary infection of BALB/c mice was performed with 0.03 LD₅₀. Viral challange was given by 1 LD₅₀. Subcutaneous injection of 100 ug purified live virus was introduced to the base of the tail. Mean cpm values of triplicates + SD of a typical experiment are domucented. Values are corrected with the corresponding cpm values measured in negative control cultures set up in the absence of the virus or peptides.



MHCINH.XLC

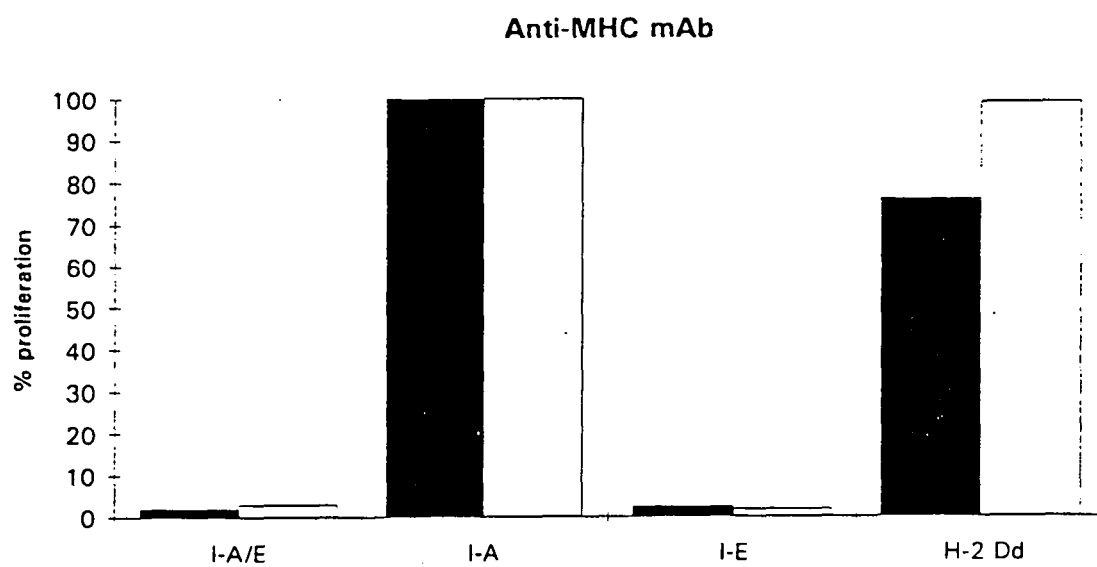


Fig. 2. A

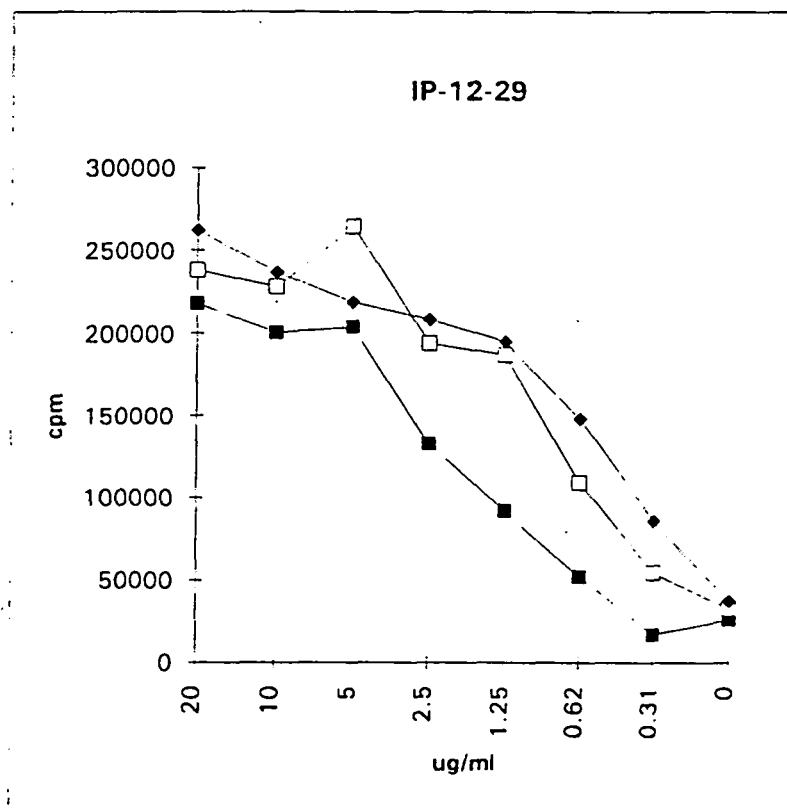


Fig. 2B

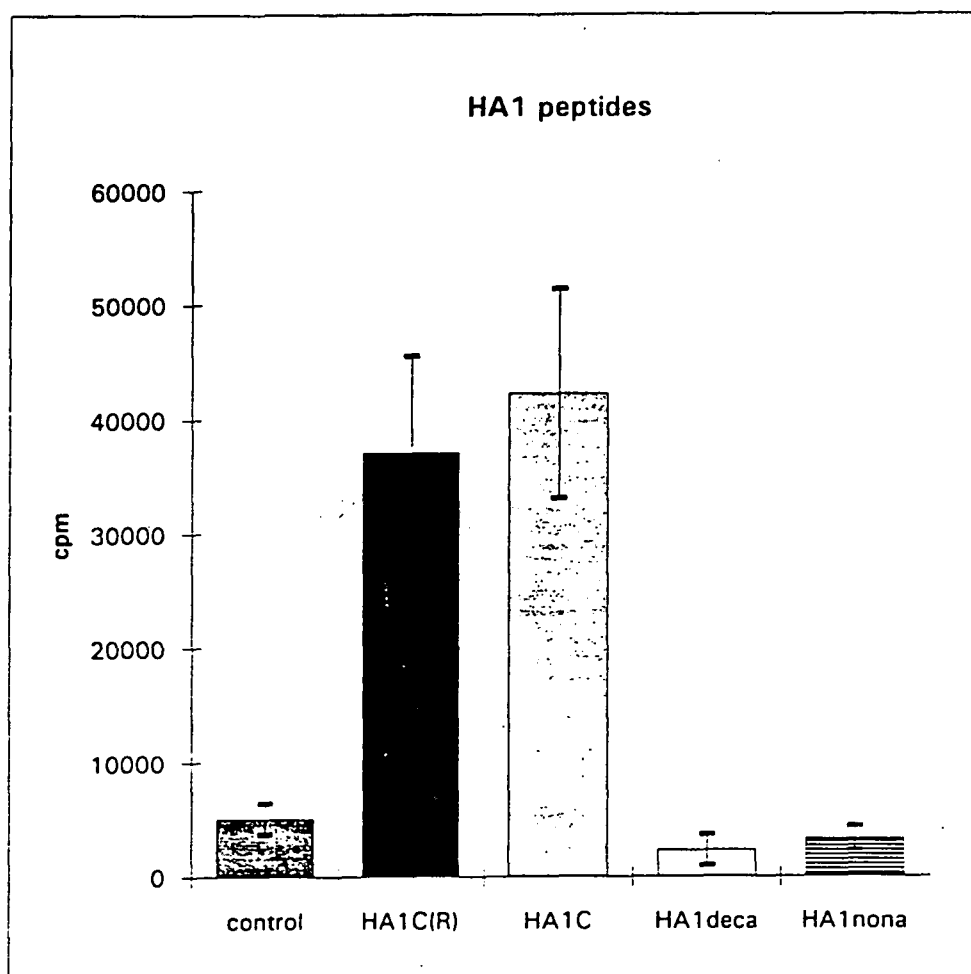


Fig. 3.

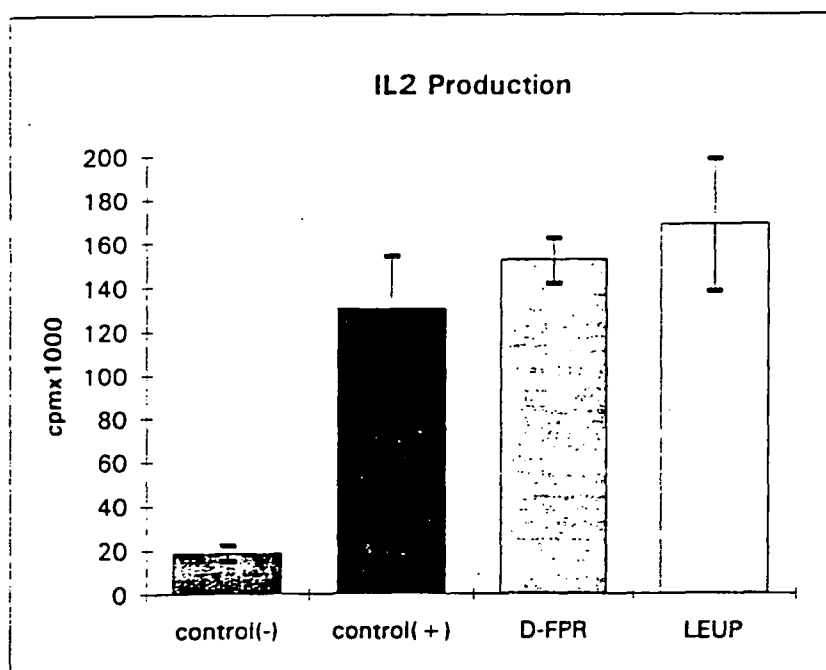
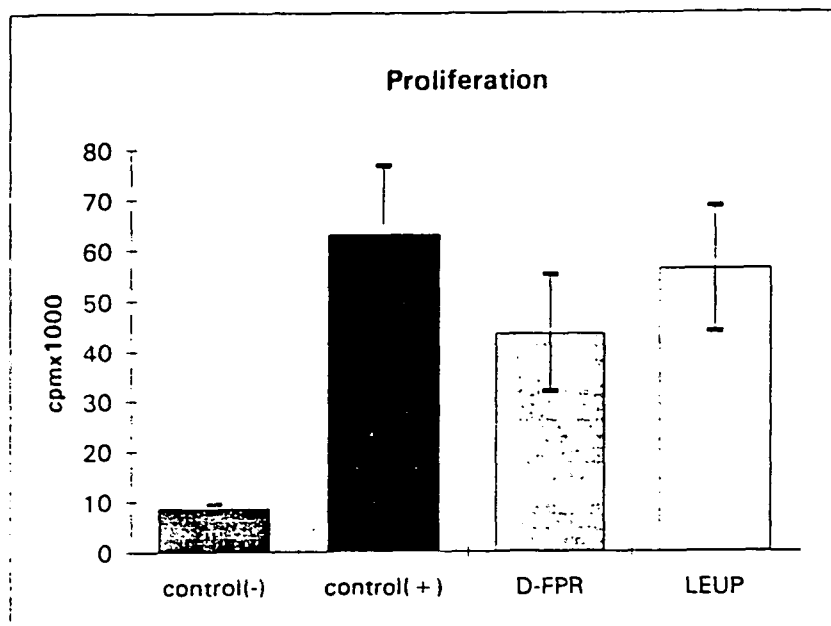


Fig. 4.

