Appendix 1

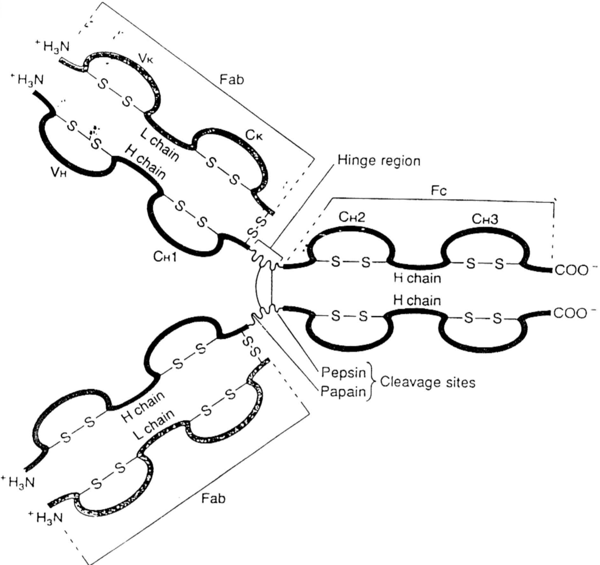
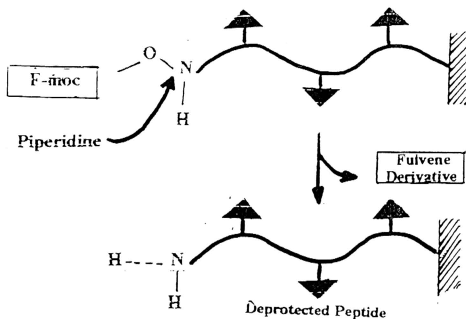
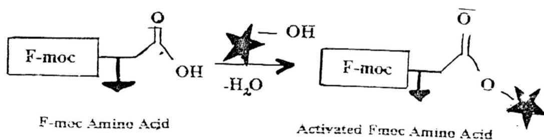


Figure showing simplified model for an IgG human antibody molecule showing the basic 4-chain structures and domains (V_H , C_H , etc). V indicates the variable region. C indicates the constant region. Sites of enzyme cleavage by pepsin and papain are shown. (Reproduced, with permission, from Sites DP & Terr AI [editors]: Basic & Clinical Immunology, 7th ed. Appleton & Lange, 1991.

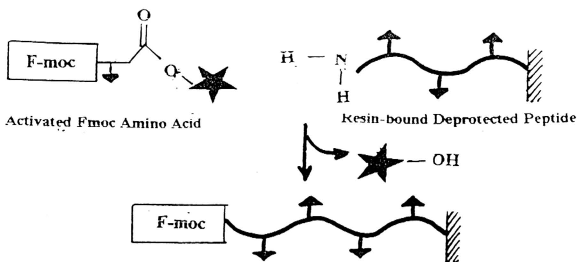
Appendix 2



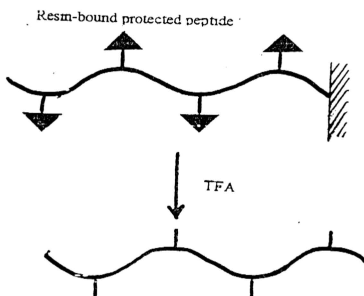
Deprotection of resin-bound peptides



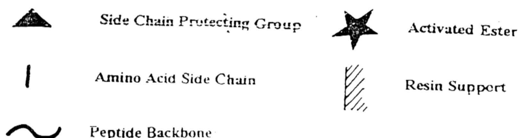
Activation of F-moc protected amino acids



Coupling of an activated amino acid to a resin-bound peptide

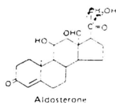


Cleavage

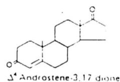


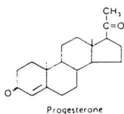
Symbol key for figures 1.9, 1.10, 1.11, 1.12

Appendix 3

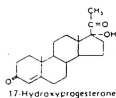


Aldosterone

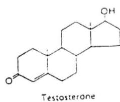

Δ^4 Androstene-3,17 dione



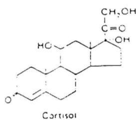
Progesterone



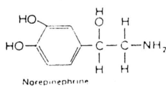
17-Hydroxyprogesterone



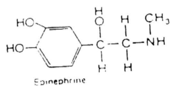
Testosterone



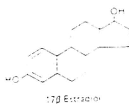
Cortisol



Norepinephrine



Epinephrine



17β Estradiol

Structures of hormones

Appendix 4

Solutions and buffers used during standard ELISA

*** Phosphate buffer saline (PBS) working solution (10 X) 1 liter**

NaCl..	80 gm
KCl...	2 gm
$Na_2 HPO_4$....................................	11.5 gm
$KH_2 PO_4$.....................................	2 gm
Distilled water upto..........................	1000 ml
	pH 7.2

PBS was kept at room temperature

*** PBS (1 X) washing solution (1 Liter)**

PBS 10 X......................................	100 ml
Distilled water................................	900 ml

PBS washing solution was used in washing ELISA microtiter plates. It was kept at 4 °C.

*** Diluent buffer (100 ml)**

Phosphate buffer saline (PBS) 1X................	100 ml
1% Bovine Serum..................................	1 gm

This buffer was used in blocking the ELISA microtiter wells. Buffer was kept at 4 °C.

* **Citrate phosphate buffer (200 ml)**

Citric acid.. 0.805 gm

Na_2HPO_4.. 1.6465 gm

Distilled water upto.................................... 200 ml

pH 5.6

This buffer was used in substrate reaction and kept at 4 °C.

* **Ortho - phenyl - diamine (OPD) (10 mg/ml)**

OPD powder... 500 mg

Citrate phosphate buffer............................ 50 ml

* **4M H_2SO_4 stopping reagent (1 Liter)**

Sulphuric acid....................................... 213.2 ml

Distilled water....................................... 786.8 ml

This stopping reagent was used to stop the calorimetric reaction of the ELISA. This was kept at 4 °C.

Appendix 5

Amino Acids	Three letters Abbreviation	One letter Abbreviation
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

Appendix 6

```
GENERAL NET SYNTHESIS SCHEDULE NUMBER : 20                        Page 1
  Description: Apolipoprotein E synthesized as 13 mers


   13-mer peptides based on the sequence APO-E2~1.ASC
           Peptide spacing increment is 7


   Segment 1:  43 peptides starting at residue 1
       First peptide: [MKVLWAALLVTFL]
       Last  peptide: [AGLVEKVQAAVGT]

     Protein sequence: APO-E2~1.ASC (317 residues)


  1:  MKVLWAALLV   TFLAGCQAKV   EQAVETEPEP   ELRQQTEWQS   GQRWELALGR
 51:  FWDYLRWVQT   LSEQVQEELL   SSQVTQELRA   LMDETMKELK   AYKSELEEQL
101:  TPVAEETRAR   LSKELQAAQA   RLGADMEDVC   GRLVQYRGEV   QAMLGQSTEE
151:  LLVRLASHLR   KLRKRLLRDA   DDLQKRLAVY   QAGAIEGAER   GLSAIRERLG
201:  PLVEQGRVRA   ATVGSLAGQP   LQERAQAWGE   RLRARMEEMG   SRTRDRLDEV
251:  KEQVAEVRAK   LEEQAQQIRL   QAEAFQARLK   SWFEPLVEDM   QRQWAGLVEK
301:  VQAAVGTSAA   PVPSDNH

           Amino Acid set to be used - AASET1


aaset 1:Free acid L-Fmoc amino acids - DIC/HOBt chemistry


           Positive Control Peptide PLAQGGGG

           Negative Control Peptide GLAQGGGG


           Number of copies of each peptide  2


        Schedule based on a 150 microlitre fill/well

              (Well concentration is 30 mM)


          Details of pins used in the synthesis

          Pin Batch No. .......................
          Acid level      .............. microMole
          HMD level       .............. mMole
          Beta-Alanine    .............. mMole


                    Single block required


          Input Data checked and found to be correct


              Synthesis authorized to proceed
```

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 20

Amino terminus is printed on the left

```
 1 A  1(1,2)PLAQGGGG     <
 2 A  2(1,2)GLAQGGGG     <
 3 A  3(1,2)MKVLWAALLVTFL<
 4 A  4(1,2)LLVTFLAGCQAKV<
 5 A  5(1,2)GCQAKVEQAVETE<
 6 A  6(1,2)QAVETEPEPELRQ<
 7 A  7(1,2)EPELRQQTEWQSG<
 8 A  8(1,2)TEWQSGQRWELAL<
 9 A  9(1,2)RWELALGRFWDYL<
10 A10(1,2)RFWDYLRWVQTLS<
11 A11(1,2)WVQTLSEQVQEEL<
12 A12(1,2)QVQEELLSSQVTQ<
13 A  1(3,4)SSQVTQELRALMD<
14 A  2(3,4)LRALMDETMKELK<
15 A  3(3,4)TMKELKAYKSELE<
16 A  4(3,4)YKSELEEQLTPVA<
17 A  5(3,4)QLTPVAEETRARL<
18 A  6(3,4)ETRARLSKELQAA<
19 A  7(3,4)KELQAAQARLGAD<
20 A  8(3,4)ARLGADMEDVCGR<
21 A  9(3,4)EDVCGRLVQYRGE<
22 A10(3,4)VQYRGEVQAMLGQ<
23 A11(3,4)QAMLGQSTEELLV<
24 A12(3,4)TEELLVRLASHLR<
25 A  1(5,6)LASHLRKLRKRLL<
26 A  2(5,6)LRKRLLRDADDLQ<
27 A  3(5,6)DADDLQKRLAVYQ<
28 A  4(5,6)RLAVYQAGAIEGA<
29 A  5(5,6)GAIEGAERGLSAI<
30 A  6(5,6)RGLSAIRERLGPL<
31 A  7(5,6)ERLGPLVEQGRVR<
32 A  8(5,6)EQGRVRAATVGSL<
33 A  9(5,6)ATVGSLAGQPLQE<
34 A10(5,6)GQPLQERAQAWGE<
35 A11(5,6)AQAWGERLRARME<
36 A12(5,6)LRARMEEMGSRTR<
37 A  1(7,8)MGSRTRDRLDEVK<
38 A  2(7,8)RLDEVKEQVAEVR<
39 A  3(7,8)QVAEVRAKLEEQA<
40 A  4(7,8)KLEEQAQQIRLQA<
41 A  5(7,8)QIRLQAEAFQARL<
42 A  6(7,8)AFQARLKSWFEPL<
43 A  7(7,8)SWFEPLVEDMQRQ<
44 A  8(7,8)EDMQRQWAGLVEK<
45 A  9(7,8)AGLVEKVQAAVGT<
```

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 20

Bulk solutions for activator and/or additives (90 wells)

Chemistry-Group 1 data for synthesis coupling 1

Activator : DIC requires 66.3 mg in 3.50 ml of DMF

Additive 1: HOBt requires 92.9 mg in 13.5 ml of DMF

WEIGHTS FOR INDIVIDUAL AMINO ACID SOLUTIONS

AA	#	Amino acid description	Batch	Weight (mg) Target	Weight (mg) Actual	DMF (ml)	DIC (ml)	HOBt (ml)
A	10	Fmoc-L-Ala-OH.H2O	.....	18.0	......	0.00	0.36	1.46
D	4	Fmoc-L-Asp(OtBu)-OH	.....	10.9	....	0.00	0.18	0.70
E	12	Fmoc-L-Glu(OtBu)-OH.H2O	.....	28.5	......	0.00	0.43	1.71
G	2	Fmoc-Gly-OH	.....	5.0	......	0.00	0.11	0.45
I	2	Fmoc-L-Ile-OH	.....	6.0	......	0.00	0.11	0.45
K	6	Fmoc-L-Lys(Boc)-OH	.....	16.8	......	0.00	0.24	0.96
L	20	Fmoc-L-Leu-OH	.....	36.0	......	0.00	0.68	2.72
Q	12	Fmoc-L-Gln(trt)-OH	.....	39.2	......	0.00	0.43	1.71
R	10	Fmoc-L-Arg(PMC)-OH.3IPE	.....	38.1	......	0.00	0.36	1.46
S	2	Fmoc-L-Ser(tBu)-OH	.....	6.5	......	0.00	0.11	0.45
T	2	Fmoc-L-Thr(tBu)-OH	.....	6.7	......	0.00	0.11	0.45
V	4	Fmoc-L-Val-OH	.....	9.0	......	0.00	0.18	0.70
						0.00	3.31	13.24

Amino acids weighed by : Shabana Wafaq. Date: 16·5·99

Solutions prepared by : Shabana Wafaq. Date: 18·5·99

Solutions dispensed by : Shabana Wafaq. Date: 18·5·99

Comments:-

PIN POSITIONS for Synthesis coupling 1

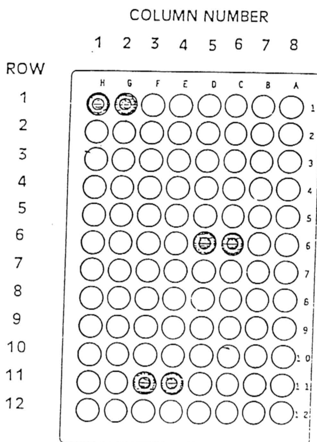
NEW PIN POSITIONS

A 3(1,2) TO A 9(7,8)

Well positions for amino acid dispensing

```
A  A 4(3,4)  A 6(3,4)  A 4(5,6)  A 3(7,8) TO A 4(7,8)

D  A 1(3,4)  A 7(3,4)

E  A 5(1,2)  A 3(3,4)  A 9(3,4)  A 9(5,6) TO A11(5,6)

G  A 7(1,2)

I  A 5(5,6)

K  A 2(3,4)  A 1(7,8)  A 8(7,8)

L  A 3(1,2)  A 8(1,2) TO A 9(1,2) A11(1,2)  A 5(3,4)  A 1(5,6)  A 6(5,6)
   A 8(5,6)  A 5(7,8) TO A 6(7,8)

Q  A 6(1,2)  A12(1,2)  A10(3,4)  A 2(5,6) TO A 3(5,6) A 7(7,8)

R  A 8(3,4)  A12(3,4)  A 7(5,6)  A12(5,6)  A 2(7,8)

S  A10(1,2)

T  A 9(7,8)

V  A 4(1,2)  A11(3,4)
```

Appendix 7



Numbering system of tray in Mimotope as used by the software supplied with the synthesis kit. Wells are identified by a letter that represents the block, followed by the column number(s) in parenthesis. Assuming that this is block A, filled wells (dark) are identified as A1(1,2), A6(5,6) and A11(3,4); from top to bottom

Appendix 8

Recommended order for activating and dispensing amino acids:

A D E F G I L M P S T V Y W Q N K C H R

Appendix 9

Solutions and buffers used during peptide synthesis

*** Phosphate buffer saline (PBS) stock solution (4 Liters)**

$Na_2HPO_4 . 2H_2O$	53.7 gm
$NaH_2PO_4 . 2H_2O$	15.6 gm
NaCl	340 gm
Hot deionized water	4000 ml
	pH 7.2

All the salts were dissolved in hot deionized water to give a final volume of 4L. It was kept at room temperature.

*** PBS working solution (1 Liter)**

PBS stock solution	100 ml
Deionized water	900 ml

*** PBS washing solution (1 Liter)**

PBS working solution	100 ml
Deionized water	900 ml

* **Pre-coat buffer (500 ml)**

This buffer was used to pre-coat pins and also used as antibody diluent

2% w/v bovine serum albumin..................	10 gm
0.1% v/v Tween 20.................................	0.5 ml
0.1% w/v sodium azide............................	0.5 gm
0.01M PBS working solution.....................	500 ml

This solution could be kept in the refrigerator for 24 hrs.

* **Conjugate buffer (500 ml)**

This was the diluent for the rabbit anti-species conjugate in the ELISA.

1% v/v rabbit serum....................................	5 ml
0.1% v/v Tween 20....................................	0.5 ml
0.1% w/v sodium casienate............................	0.5 gm
0.01 M PBS working solution	500 ml

This buffer kept at - 20 °C.

* **Substrate buffer (500 ml)**

This buffer was used as the solvent for the chromogenic substrate in ELISA.

$Na_2 HPO_4 . 2H_2 O$....................................	7.1 gm
Citric acid monohydrate..............................	8.4 gm
	pH 4.0

Kept at 4 °C.

* **Sonication bath solution (2000 ml)**

0.1 M PBS working solution……………………..	1800 ml
1% w/v sodium dodecyl sulphate (SDS)…………..	18 gm
? 0.1% v/v Mercaptoethanol……………………….	1.8 ml
	pH 7.2

Appendix 10

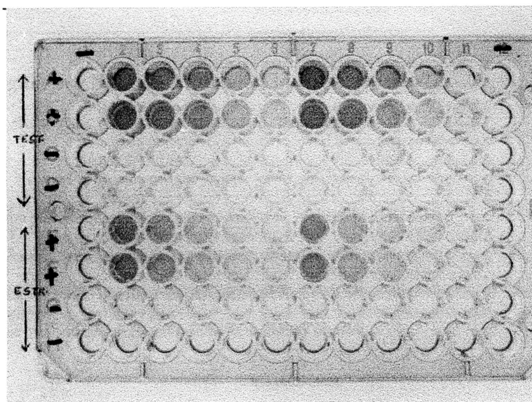


Figure showing IgG antibody binding to testoterone (TEST) and β-estradiol (ESTR) detected by ELISA. Two sera (columns 2 - 6 and 7 - 11, respectively) were used for the dilution studies. Each serum was incubated at 1 : 50, and double diluted to 1 :800.

Appendix 11

Hypertension
Volume 34, No. 5, 1999
On-line e 10
http://hyper.ahajournals.org

Regulatory Natural Antibodies in Hypertension?

Hwee-Ming Cheng, Shabana Aafaqi, Sam Choon-Kook

We read with interest the recent report by Wu et al[1] on the reduction of both IgG and IgM autoantibodies to oxidised LDL (OxLDL) in individuals with borderline hypertension compared with normotensive controls. We would like to extend the implication of their data with results from our own studies of natural antibodies in healthy persons.

We recently detected in all normal human serum (NHS), natural antibodies to oxysterols (including cholesterol epoxide, 7keto-, 19hydroxy- and 7hydroxy- cholesterol), which are main components of LDL that has undergone oxidative modification[2]. The consistent presence of anti-oxysterol (and by extension, anti-OxLDL) was postulated to be important in regulating the concentration and bioactivity of OxLDL (which comes from either dietary source or from in vivo oxidation[3]) that are thought to be involved in endothelial dysfunction and other events in the atherogenic process. The lowered serum anti-OxLDL in Wu et al's study might therefore be related to this suggestion.

Additional role of natural antibodies in regulating blood pressure is also indicated by the finding of natural antibodies that recognise renin, angiotensin II and cathecolamines [4-6.] Diet appear to influence these natural humoral immunoreactivity. We have now also observed autoantibodies to aldosterone in normal subjects. In an ELISA system, all eleven NHS tested contained specific IgG and IgA and 8 of the 11 sera also had IgM anti-aldosterone antibodies (Aafaqi et al., unpublished).

Natural antibodies are thought to function as immunotransporters besides being involved in physiologic clearance of aged molecules and cell membrane fragments[7]. The natural antibody binding of hormones involved in vascular and blood volume control of arterial pressure may serve to modulate and maintain an appropriate hormonal level amidst physiological fluctuations. Dietary determinants that affect blood pressure may thus act through antioxidative/prooxidative factors, vascular modulators as well as the level of natural antibody activity[8,9].

It would be of interest if Wu and coworkers could also analyse their hypertensive serum samples for autoantibodies to some of the above hormones.

References

1. Wu R, De Faire U, Lemne C, Witztum JL, Frostegard J. Autoantibodies to OxLDL are decreased in individuals with borderline hypertension. Hypertension 1999; 33: 53-59.
2. Cheng HM, Sundram K. Cholesterol oxides and natural autoantibodies. Artherosclerosis 1998; 141: 347-348.
3. Osada K, Inoue T, Nakamura S, Sugano M. Dietary soybean protein moderates the deleterious disturbance of lipid metabolism caused by exogenous oxidised cholesterol in rats.
   Biochim Biophys Acta 1999; 1427: 337-350.
4. Castedo M, Pelletier L, Pasquier R, Guettier C, Huygen K, Michel JB, Druet P. Anti-renin T cells trigger normal B cells to produce anti-renin antibodies and normalize blood pressure in spontaneous hypertensive rats. Int Immunol 1993; 12: 1569-1576.
5. Pogozheva AV, Rozanova IA, Miagkova MA, Sorokovoi KV, Panchenko ON, Trubacheva ZN. Effect of antiatherosclerotic diet enriched with polyunsaturated omega-3 fatty acids on the level of natural antibodies to catecholamines in patients with cardiovascular diseases. Vopr Pitan 1998; 4: 32-35 (Russian).

6. Rozanova IA, Pogozheva AV, Miagkova MA, Savitskaia IA. Effect of anti-atherosclerotic diet with polyunsaturated omega-3 fatty acids on natural antibodies to bradykinin and angiotensin II in patients with cardiovascular diseases. Vopr Pitan 1998; 2: 32-34. (Russian)
7. Cheng HM. Natural cryptic antibodies (review). Autoimmunity 1998; 27: 99-108.
8. Sata M, Walsh K. Oxidised LDL activates Fas-mediated endothelial cell apoptosis. J Clin Invest 1998; 102: 1682-1689.
9. Cheng HM, Sundram K. Oxidised LDL, diet and natural antibodies. Amer J Clin Nutr 1999; 70: 104-105.