3.0 Results

The hormones tested in this study for natural antibody binding were:

a) Adreno-cortical hormones (hydrocortisone, aldosterone, androstenedione)

b) Adreno-medullary hormones (adrenaline, noradrenaline)

c) Other steroid hormones (progesterone, 17-hydroxyprogesterone, testosterone, β-estradiol)

All three antibody isotypes, IgA, IgG & IgM of natural antibody binding were investigated. For each hormone tested, the same serum was used for IgA, IgG and IgM analysis in ELISA.

3.1 IgA Natural Antibody to Hormones

3.1a Adreno-Cortical Hormones

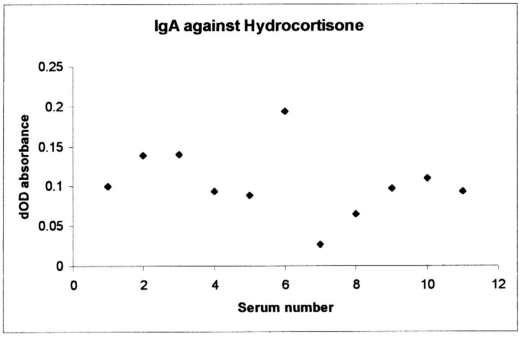
Eleven normal human sera (NHS) were used to detect the IgA binding to different cortical hormones. Table 3.1a shows the ELISA results and Figure 3.1, the same ELISA data graphically. All the 11 NHS contained IgA antibodies reactive with aldosterone. In contrast to IgA anti-aldosterone, there was no serum that had positive IgA antibody binding to hydrocortisone and androstenedione.

Dilution curve of natural IgA binding to aldosterone was done. Two positive sera, for natural IgA was used in normal ELISA. The delta absorbance (dOD) was assigned positive when, dOD > 0.2. IgA still bound to aldosterone at 1 : 200 dilution for both sera. The dilution curves for 2 IgA positive sera to aldosterone are shown in Figure 3.2.

Table 3.1a: NATURAL IgA AGAINST ADRENO-CORTICAL HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
HC	0.100	0.139	0.141	0.094	0.089	0.195	0.028	0.065	0.098	0.11	0.094
ALDO	0.349	0.916	0.249	0.693	0.605	0.504	0.482	0.773	0.322	0.447	0.291

(Each hormone was tested with 11 different NHS). Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbance between hormone positive and hormone negative microtitre wells. There was no IgA binding to hydrocortisone and androstenedione (not shown). dOD > 0.2 was considered as positive (red).
HC = Hydrocortisone, ALDO = Aldosterone, ANDR = Androstenedione



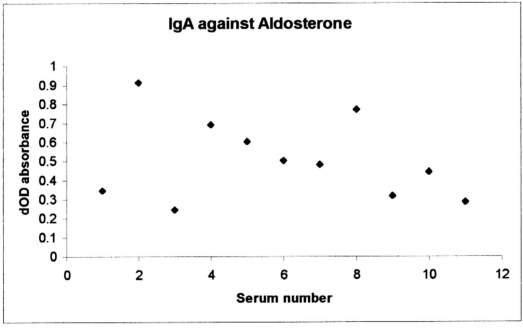


Figure 3.1: Natural IgA against Adreno-Cortical Hormones
A dOD > 0.2 was considered as positive (See Materials and Methods)
As was the case for IgA anti-hydrocortisone, there was no IgA bound to Androstenedione in all 11 normal human serum (not shown)

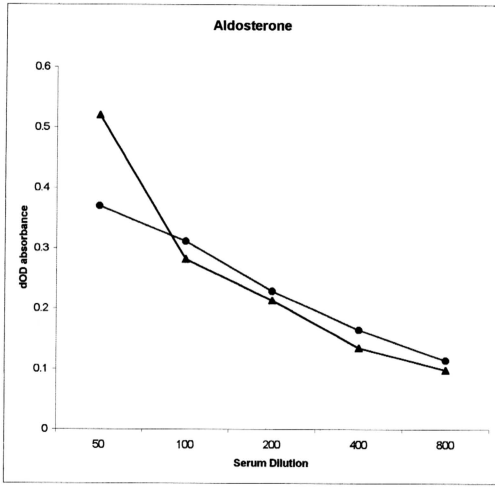


Figure 3.2: Dilution Curve for Natural IgA against Aldosterone
A dOD > 0.2 was considered as positive

3.1b Adreno-Medullary Hormones

IgA antibodies against the two catecholamines, adrenaline and noradrenaline were measured in eleven different NHS. Table 3.1b shows the ELISA results and Figure 3.3, the same ELISA data graphically. Seven of the 11 sera had IgA reactive with adrenaline while ten out of 11 sera contained IgA antibodies to noradrenaline.

3.1c Other Steroid Hormones

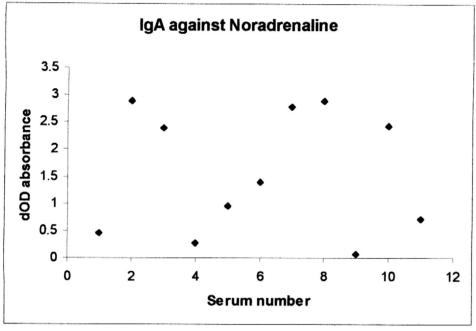
IgA against some of the related steroid hormones were also studied. Table 3.1c shows the ELISA results and Figure 3.4, the same ELISA data graphically. Different NHS was used for each hormone. Three of 5 NHS had IgA to progesterone while 11/11 had IgA for hydroxyprogesterone. Two out of 5 NHS had IgA to testosterone but none of the 5 NHS bound to β-estradiol.

Dilution curve of natural IgA binding to some of these steroid hormones was done. Two positive sera, with natural IgA were used in normal ELISA. IgA was still reactive with progesterone at 1 : 200 dilution for serum 1 and 1 : 400 dilution for serum 2. IgA was still reactive with testosterone at 1 : 200 dilution for serum 1 and 1 : 400 dilution for serum 2. The dilution curves for two IgA positive sera to progesterone and testosterone are shown in Figure 3.5.

Table 3.1b: NATURAL IgA AGAINST ADRENO-MEDULLARY HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
ADR	0.196	0.616	0.161	0.015	0.253	0.327	0.259	0.431	0.182	0.274	0.282
NADR	0.459	2.894	2.388	0.269	0.955	1.394	2.776	2.893	0.066	2.439	0.724

(Each hormone was tested with 11 different NHS). Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbance between hormone positive and hormone negative microtiter wells. A dOD > 0.2 was considered as positive (red).
ADR = Adrenaline, NADR = Noradrenaline



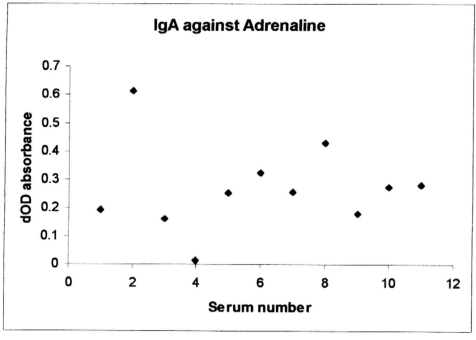
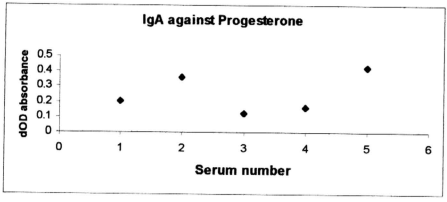


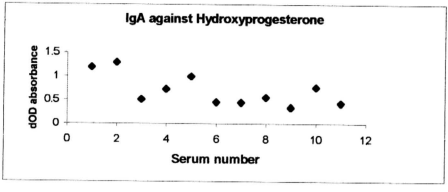
Figure 3.3: Natural IgA against Adreno-Medullary Hormones
A dOD > 0.2 was considered as positive

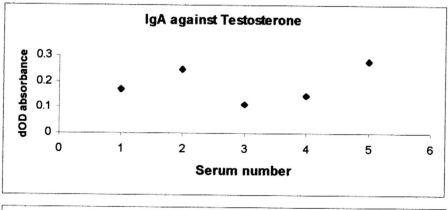
Table 3.1c: NATURAL IgA AGAINST OTHER STEROID HORMONES

Hormones	Serum										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
HYPR	1.194	1.299	0.518	0.735	0.996	0.460	0.454	0.561	0.349	0.779	0.437
PROG	0.202	0.360	0.125	0.166	0.429						
β-ES	0.049	0.124	0.046	0.057	0.148						
TEST	0.170	0.249	0.113	0.149	0.282						

Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbance between hormone positive and negative microtiter wells. A dOD > 0.2 was considered as positive (red)
HYPR = Hydroxyprogesterone, PROG = Progesterone, TEST = Testosterone, β- ES = Beta-Estradiol







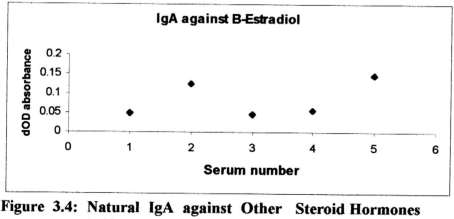
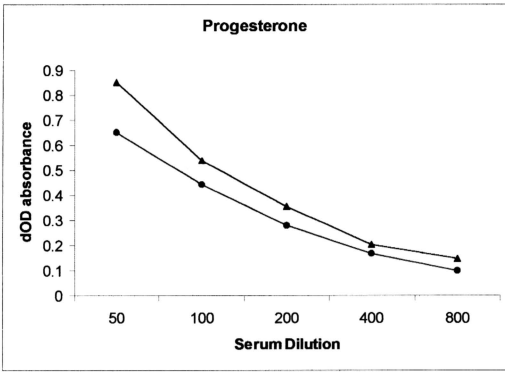


Figure 3.4: Natural IgA against Other Steroid Hormones
A dOD > 0.2 was considered as positive



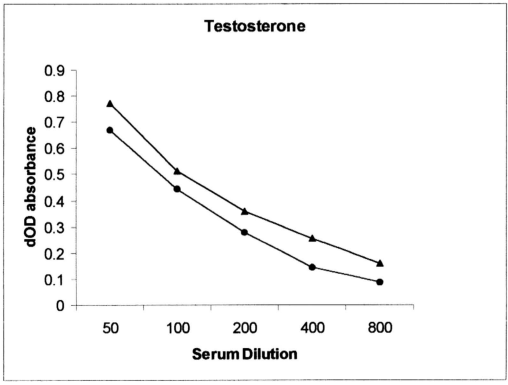


Figure 3.5: Dilution curve for Natural IgA against Other Steroid Hormones
A dOD > 0.2 was considered as positive

3.2 IgM Natural Antibody to Hormones

3.2a Adreno-Cortical Hormones

As for IgA, eleven different NHS were used to detect the IgM binding to different cortical hormones. Table 3.2a shows the ELISA results and Figure 3.6, the same ELISA data graphically. Eight out of 11 NHS had IgM to aldosterone while six out of 11 contained IgM antibodies to hydrocortisone. As was the case for IgA, there was no serum that had positive IgM binding to androstenedione.

Dilution curve of natural IgM binding to adreno-cortical hormones was done. Two positive sera, for natural IgM were used in normal ELISA. IgM was still reactive with hydrocortisone at 1 : 200 diiution for serum 1 and 1 : 100 dilution for serum 2, while IgM was reactive with aldosterone at 1 : 100 dilution for serum 1 and weakly bound to serum 2. The dilution curves for two IgM positive sera to hydrocortisone and aldosterone are shown in Figure 3.7.

3.2b Adreno-Medullary Hormones

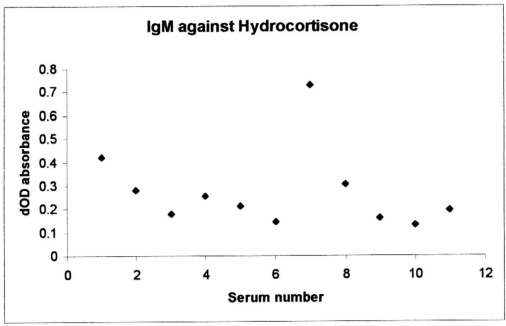
IgM antibodies against the two catecholamines, adrenaline and noradrenaline were measured in 11 different NHS. Table 3.2b shows the ELISA results and Figure 3.8. the same ELISA data graphically. Two out of 11 sera had IgM reactive with noradrenaline while all NHS showed negative IgM binding to adrenaline.

Dilution curve of natural IgM binding to noradrenaline was done. IgM was still reactive with noradrenaline at 1 : 50 dilution for serum 1 and 1 : 200 dilution for serum 2. The dilution curves for two IgM positive sera to noradrenaline are shown in Figure 3.9.

Table 3.2a: NATURAL IgM AGAINST ADRENO-CORTICAL HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
HC	0.423	0.284	0.178	0.257	0.216	0.145	0.73	0.306	0.161	0.133	0.197
ALDO	0.129	1.504	0.612	0.699	1.648	0.686	0.263	0.175	0.689	1.093	0.121

(Each hormone was tested with different 11 NHS) Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbances between hormone positive and hormone negative microtitre wells. There was no IgM binding to androstenedione. A dOD > 0.2 was considered as positive (red).
HC = Hydrocortisone, ALDO = Aldosterone, ANDR = Androstenedione



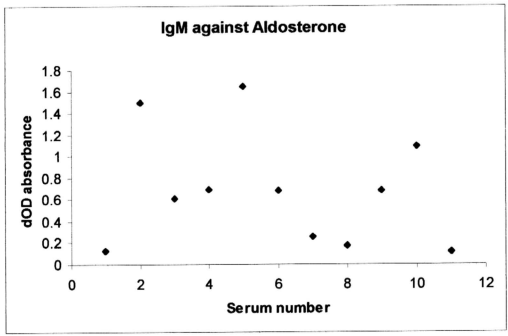
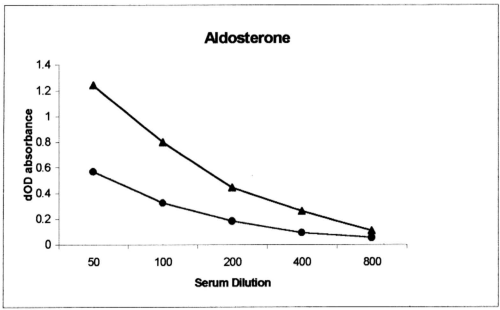


Figure 3.6: Natural IgM against Adreno-Cortical Hormones
A dOD > 0.2 was considered as positive
There was no IgM bound to androstenedione in all 11 NHS (not shown)



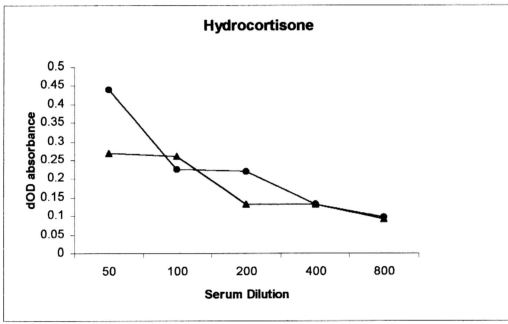
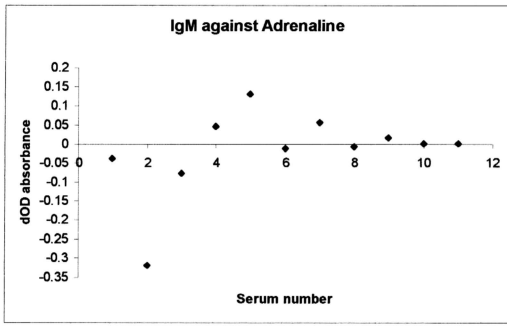


Figure 3.7: Dilution Curve for Natural IgM against Adreno-Cortical Hormones
A dOD > 0.2 was considered as positive
There was no IgM bound to adrenostenedione in all NHS (not shown)

Table 3.2b: NATURAL IgM AGAINST ADRENO-MEDULLARY HORMONES

Hormones	Serum										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
ADR	←				< 0.05						→
NADR	0.204	0.020	0.044	0.178	0.278	0.068	0.092	0.068	0.079	0.058	0.046

(Each hormone was tested with different 11 NHS) Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbances between hormone positive and negative microtiter wells. A dOD > 0.2 was considered as positive (red).
ADR = Adrenaline, NADR = Noradrenaline



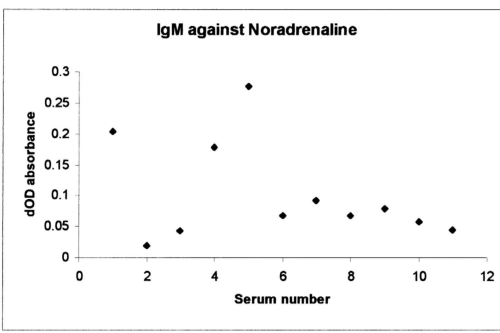


Figure 3.8: Natural IgM against Adreno-Medullary Hormones
A dOD > 0.2 was considered as positive
IgM anti-adrenaline was negative in all normal human serum. Negative dOD is an unexplained ELISA phenomenon which is occasionally observed.

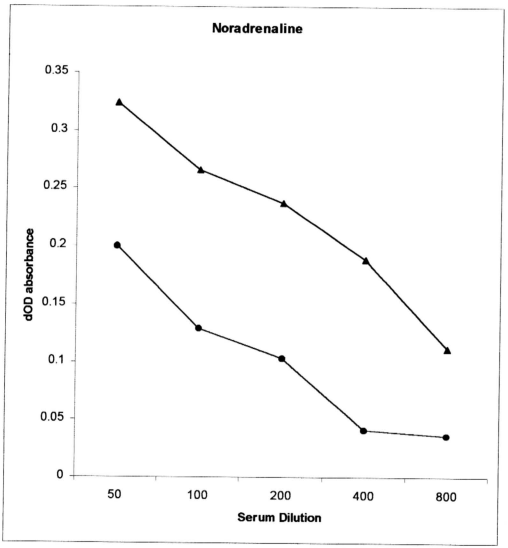


Figure 3.9: Dilution Curve for Natural IgM against Noradrenaline
A dOD > 0.2 was considered as positive
There was no IgM bound to adrenaline in all NHS

3.2c Other Steroid Hormones

IgM against some other steroid hormones were also tested. Different NHS was used for each hormone. IgM only bound to progesterone, testosterone, and β-estradiol. Table 3.2c shows the ELISA results and Figure 3.10, the same ELISA data graphically. All 5 NHS showed IgM reactive with testosterone, three out of 5 sera showed IgM reactive with β-estradiol while seven out of 11sera showed IgM binding with progesterone. There was no serum that had positive IgM antibody binding to hydroxyprogesterone.

Dilution curve of natural IgM binding to other steroid hormones was done. Two positive sera, for natural IgM was used in normal ELISA. IgM was still reactive with progesterone at 1 : 200 dilution for serum 1 and 1 : 400 dilution for serum 2. Testosterone was also reactive at 1 : 400 dilution for serum 1 and 1 : 800 dilution for serum 2. β-Estradiol was highly reactive still at 1 : 200 dilution for both sera. The dilution curves for two IgM positive sera to progesterone, testosterone and β-estradiol are shown in Figure 3.11.

Table 3.2c: NATURAL IgM AGAINST OTHER STEROID HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
PROG	0.154	0.180	0.226	0.236	0.246	0.213	0.243	0.620	0.151	0.089	0.329
HYPR	◄					< 0.05					►
TEST	0.338	2.957	0.751	1.074	2.564						
β- ES	0.047	0.939	0.193	0.376	0.853						

Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbances between hormone positive and negative microtiter wells. A dOD > 0.2 was considered as positive (red)
PROG = Progesterone, HYPR = Hydroxyprogesterone, TEST = Testosterone, β - ES = Beta-Estradiol

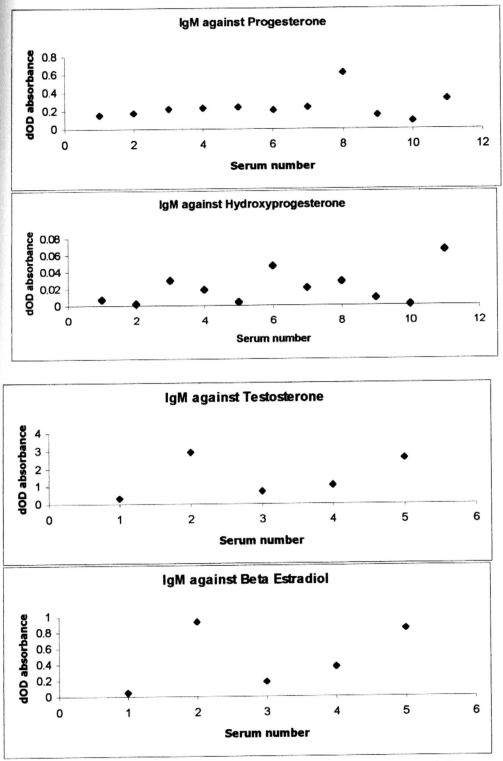
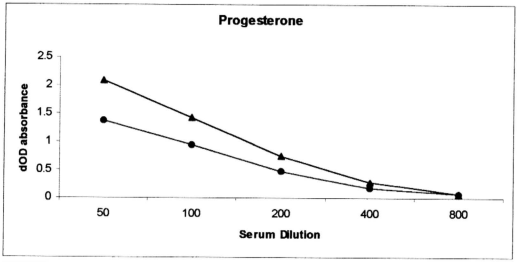
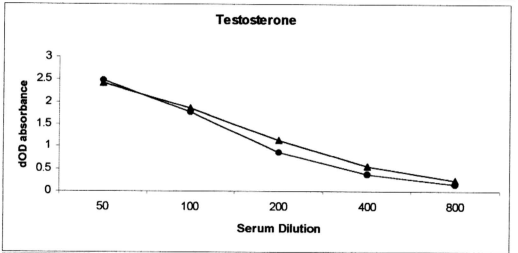


Figure 3.10: Natural IgM against Other Steroid Hormones
A dOD > 0.2 was considered as positive





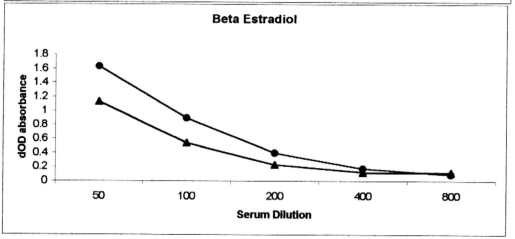


Figure 3.11: Dilution curve for Natural IgM against Other Steroid Hormones
A dOD > 0.2 was considered as positive

3.3 IgG Natural Antibody to Hormones

3.3a Adreno-Cortical Hormones

As for natural IgA and IgM, eleven different NHS were used to detect the IgG binding to different cortical hormones. Table 3.3a shows the ELISA results and Figure 3.12, the same ELISA data graphically. Ten out of 11 NHS had IgG to hydrocortisone while all 11 NHS showed IgG reactivity with aldosterone. As was the case for IgA and IgM, there was no serum that had positive IgG antibody binding to androstenedione.

Dilution curve of natural IgG binding to adreno-cortical hormones was done. Two positive sera, for natural IgG was used in normal ELISA. The delta absorbance (dOD) was assigned positive when, dOD > 0.2. Natural IgG still bound to hydrocortisone as well as to aldosterone at 1 : 800 dilution for both sera. The dilution curves for two positive IgG sera to hydrocortisone and aldosterone are shown in Figure 3.13.

3.3b Adreno-Medullary Hormones

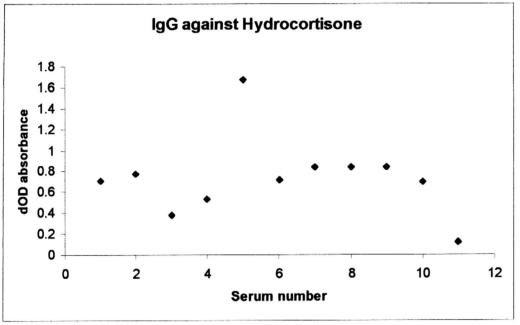
IgG antibodies against the two catecholamines, adrenaline and noradrenaline were measured in eleven different NHS. Table 3.3b shows the ELISA results and Figure 3.14, the same ELISA data graphically. One of the 11 sera had IgG reactive with adrenaline while 9/11 sera contained IgG antibodies to noradrenaline.

Dilution curve of natural IgG binding to noradrenaline was done. IgG was still reactive with noradrenaline at 1 : 800 dilution for both sera. The dilution curves for two IgG positive sera to noradrenaline is shown in Figure 3.15.

Table 3.3a: NATURAL IgG AGAINST ADRENO-CORTICAL HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
HC	0.707	0.779	0.380	0.538	1.674	0.722	0.842	0.842	0.844	0.710	0.126
ALDO	0.868	1.663	0.607	1.148	0.938	0.83	0.633	1.025	1.053	0.665	0.664

(Each hormone was tested with different 11 NHS) Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbance between hormone positive and hormone negative microtiter wells. A dOD > 0.2 was considered as positive (red) There was no IgG anti-androstenedione in all the 11 NHS.
HC = Hydrocortisone, ALDO = Aldosterone



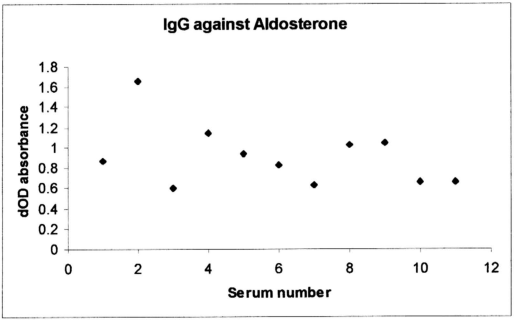
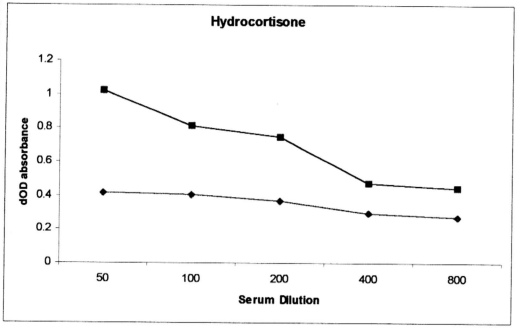


Figure 3.12: Natural IgG against Adreno-Cortical Hormones
A dOD > 0.2 was considered as positive
There was no IgG bound to androstenedione in all 11 NHS



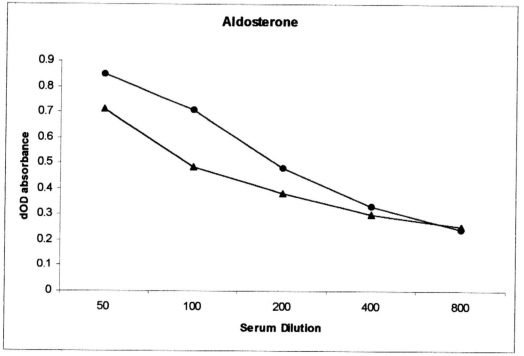
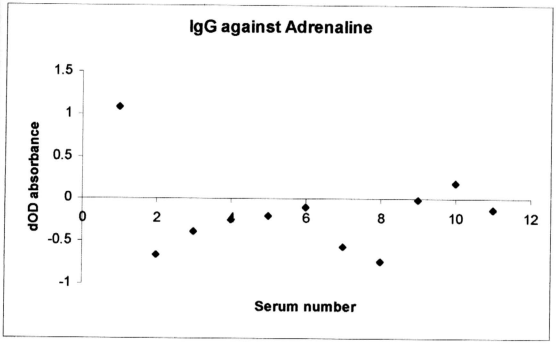


Figure 3.13: Dilution Curve for Natural IgG against Adreno-Cortical Hormones
A dOD > 0.2 was considered as positive
There was no IgG bound to androstenedione in all 11 NHS

Table 3.3b: NATURAL IgG AGAINST ADRENO-MEDULLARY HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
ADR	1.086	-0.656	-0.38	-0.248	-0.196	-0.1	-0.563	-0.734	-0.005	0.198	-0.125
NADR	0.385	2.824	1.845	0.17	0.457	0.932	2.66	2.618	0.12	2.178	0.449

(Each hormone was tested with different 11 NHS) Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbance between hormone positive and hormone negative microtiter wells. A dOD > 0.2 was considered as positive (red)
ADR = Adrenaline, NADR = Noradrenaline



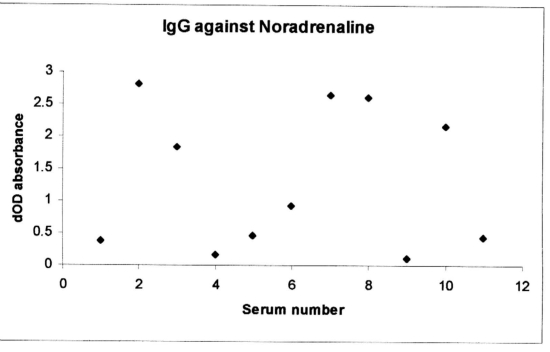


Figure 3.14: Natural IgG against Adreno-Medullary Hormones
A dOD > 0.2 was considered as positive. Negative
dOD in IgG anti-adrenaline diagram is an unexplained ELISA phenomenon

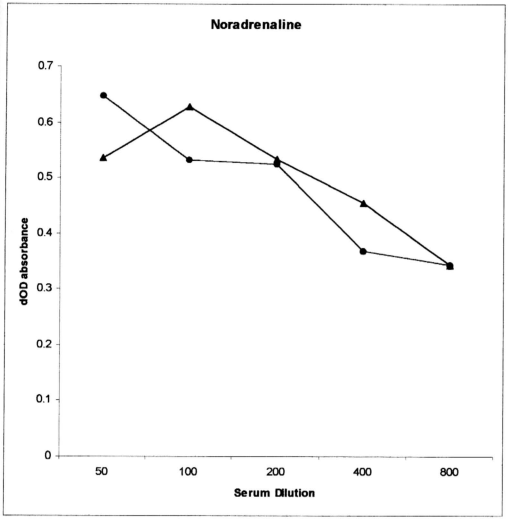


Figure 3.15 : Dilution Curve for Natural IgG against Noradrenaline
A dOD > 0.2 was considered as positive
There was no IgG bound to adrenaline in all 11 NHS

3.3c Other Steroid Hormones

IgG against some other steroid hormones were also studied. Table 3.3c shows the ELISA results and Figure 3.16, the same ELISA data graphically. All 11 NHS showed IgG reactivity with progesterone, while no binding was observed for hydroxy progesterone. Three out of 5 NHS had IgG to testosterone while β-estradiol was reactive in 2 out of 5 NHS.

Dilution curve of natural IgG binding to other steroid hormones was done. Two positive sera, for natural IgG was used in normal ELISA. IgG was still detectable with progesterone and testosterone at 1 : 800 dilution for both sera, while β-estradiol was measurable at 1 : 400 dilution for serum 1 and 1 : 800 dilution for serum 2. The dilution curves for two IgG positive sera to progesterone, testosterone and β-estradiol are shown in Figure 3.17. A final calorimetric ELISA microplate assay results are shown Appendix 10.

Table 3.3c: NATURAL IgG AGAINST OTHER STEROID HORMONES

Hormones	Serum										
	1	2	3	4	5	6	7	8	9	10	11
PROG	0.227	0.658	0.427	0.644	0.377	0.454	0.33	0.739	0.245	0.549	0.404
HYPR	-0.003	0.013	-0.002	-0.004	0.003	0.031	0.007	0.001	0.074	0.015	0.064
TEST	0.162	0.700	0.243	0.227	0.198						
β - ES	0.123	0.256	0.163	0.242	0.147						

Values are delta absorbance (dOD) i.e, the difference in the mean optical absorbance between hormone positive and hormone negative microtiter wells. A dOD > 0.2 was considered as positive (red)
PROG = Progesterone, HYPR = Hydroxyprogesterone, TEST = Testosterone, β-ES = Beta-Estradiol

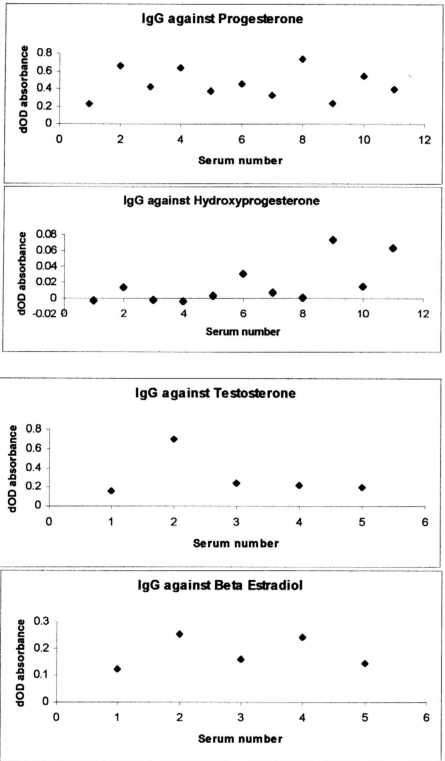
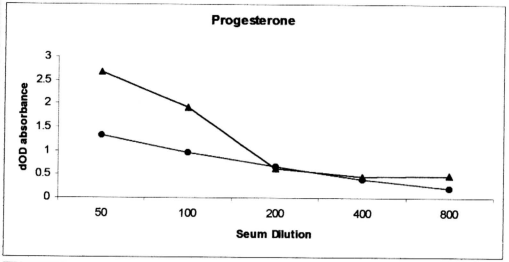
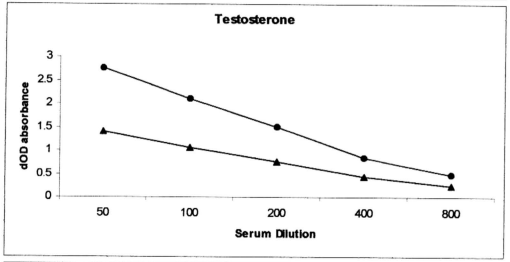


Figure 3.16: Natural IgG against Other Steroid Hormones
A dOD > 0.2 was considered as positive





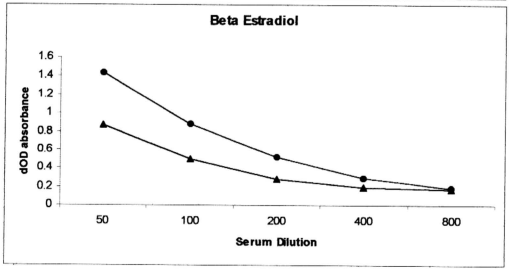


Figure 3.17: Dilution curve for Natural IgG against Other Steroid Hormones
A dOD > 0.2 was considered as positive

3.4 Fractionated Natural IgG Antibody to Hormones

Commercially fractionated IgG was used for tests in ELISA against adreno-cortical, medullary hormones and other steroid hormones. The hormone concentration for use in antigen coating of the microwells was the same for all the hormones. The dOD, was assigned positive when, dOD > 0.2.

The purified IgG used was the IgG, commercially extracted from many sera of healthy people. The concentration of purified IgG used was 10 mg/ml (diluted 1 : 50). During serum incubation we used the diluted IgG instead of NHS. The dilution curve values for purified IgG to hormones is showed in Table 3.4. It was observed that purified IgG still bound to hydrocortisone at 1: 800 dilution and aldosterone at 1: 400 dilution (Figure 3.18). Noradrenaline was only reactive with the IgG at 1 : 50 dilution (Figure 3.19).

Progesterone was still positive for purified IgG at 1 : 400 dilution, β-estradiol at 1 : 400 dilution, hydroxy progesterone at 1 : 50 dilution. Purified IgG did not bind to testosterone (Figure 3.20).

Table 3.4: Dilution Curve Values for Purified IgG to Hormones

Dilution	Co. Ho.		Me. Ho.	St. Ho.			
	HC	ALDO	NADR	PROG	HYPR	TEST	β- ES
1: 50	1.861	1.110	0.819	1.462	2.675	0.072	1.33
1 : 100	1.301	0.598	0.043	0.836	1.584	0.055	0.588
1 : 200	0.675	0.353	0.063	0.519	0.738	0.034	0.378
! : 400	0.524	0.203	0.038	0.384	0.565	0.026	0.252
1 : 800	0.428	0.1	0.022	0.156	0.270	0.011	0.108

Co. Ho. = Cortical Hormones, Me. Ho. = Medullary Hormones,

St. Ho. = Steroid Hormones.

A dOD > 0.2 was considered as positive. (Highlighted, values show the positive ELISA reactions when antibody bound to antigen).

HC = Hydrocortisone, ALDO = Aldosterone, NADR = Noradrenaline, PROG = Progesterone, HYPR = Hydroxyprogesterone, TEST = Testosterone, β-ES = Beta Estradiol.

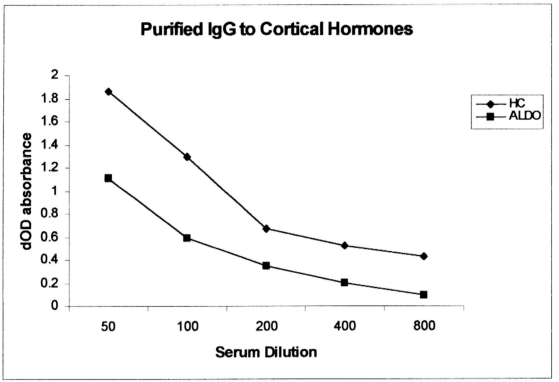


Figure 3.18: Dilution Curve for Purified IgG to Adreno-Cortical Hormones
A dOD > 0.2 was considered as positive
HC = Hydrocortisone, ALDO = Aldosterone

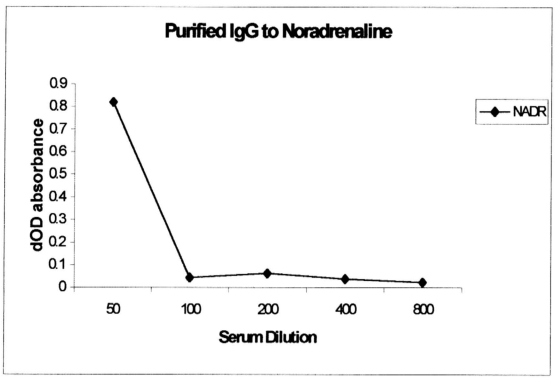


Figure 3. 19: Dilution Curve for Purified IgG to noradrenaline
A dOD > 0.2 was considered as positive. NADR = Noradrenaline

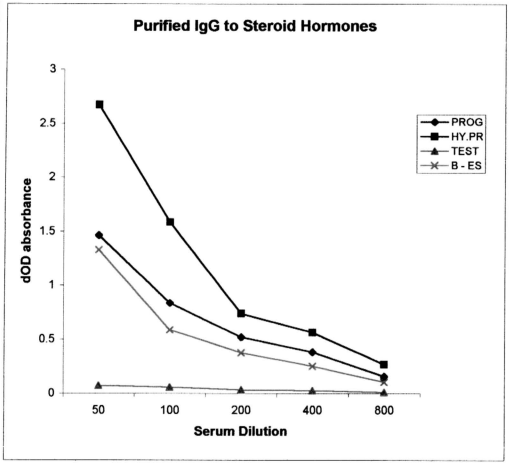


Figure 3. 20: Dilution Curve for Purified IgG to Other Steroid Hormones
A dOD > 0.2 was considered as positive. PROG = Progesterone
HY.PR = Hydroxyprogesterone, TEST = Testosterone,
B- ES = Beta Estradiol

3.5 Comparison Between IgG, IgA and IgM Natural Antibody to Adreno-Cortical, Medullary and Other Steroid Hormones

Results for natural IgA, IgG and IgM binding pattern to hormones were compared. Table 3.5 shows the summarized data of natural antibodies against adreno-cortical, medullary and other steroid hormones. It was observed that IgG was a predominant immunoglobulin in NHS that react with adreno-cortical hormones (hydrcortisone = 90%, aldosterone = 100%), adreno-medullary hormones (adrenaline = 18.2%, noradrenaline = 81.9%) and other steroid hormones (Progesterone = 100%, hydroxy progesterone = 0%, testosterone = 60%, beta estradiol = 40%).

IgA is also a major isotype, which bound to these molecules in NHS. The frequency of IgA binding to adreno-cortical hormones was very varied (hydrocortisone = 9%, aldosterone = 100%), adreno-medullary hormones (adrenaline = 63.7% and noradrenaline = 90%) and other steroid hormones (progesterone = 60%, hydroxy progesterone = 100%, testosterone = 40%, beta estradiol = 0%).

The binding of IgM was quite different. Adreno-cortical hormones showed a better binding (> 50%), whereas medullary hormones showed less binding (< 20%). The frequency of binding IgM to other steroid hormones ranged from 0% (hydroxy progesterone) to 100% (testosterone).

Table 3.5 : Summarized Data of Natural Antibodies against Adreno-Cortical, Medullary and Other Steroid Hormones

Hor. → / Ab ↓	Cortical hormones		Medullary hormones		Other steroid hormones			
	HC	ALDO	ADR	NADR	PROG	HYPR	TEST	β - ES
IgA	0/11	11/11	7/11	10/11	3/5	11/11	2/5	0/5
%	9%	100%	63.7%	90%	60%	100%	40%	0%
IgG	10/11	11/11	2/11	9/11	11/11	0/11	3/5	2/5
%	90%	100%	18.2%	81.9%	100%	0%	60%	40%
IgM	6/11	8/11	0/11	2/11	7/11	0/11	5/5	3/5
%	54.6%	72.8%	0%	18.2%	72.8%	0%	100%	40%

There was no natural antibody of any isotypes to androstenedione.

Hor. = Hormones, Ab = Antibody, HC = Hydrocortisone, ALDO = Aldosterone, NADR = Noradrenaline, ADR = Adrenaline, PROG = Progesterone, HY PR = Hydroxy progesterone, TEST = Testosterone, β-ES = Beta Estradiol

3.6 Comparison Between Natural Antibody Binding in Three Normal Human Serum to Adreno-Cortical, Medullary and Other Steroid Hormones

The hormones tested in the 3 different groups were aldosterone, noradrenaline, and progesterone. Noradrenaline was the dominant antigen and showed highest reactivity with IgA and IgG (Table 3.6; Figure 3.21). Aldosterone however, showed highest reactivity to natural IgM in the 3 NHS. Only IgG anti-progesterone was present in all the 3 NHS.

Table 3.6: Natural Antibody Binding to Different Hormones in Three Normal Human Sera

Ab ↓	Cortical Hormone (Aldosterone)			Medullary Hormone (Noradrenaline)			Steroid Hormone (Progesterone)		
	Serum 1	Serum 2	Serum 3	Serum 1	Serum 2	Serum 3	Serum 1	Serum 2	Serum
IgA	0.916	0.322	0.773	2.439	2.776	1.394	-	-	-
IgG	1.663	1.053	1.025	2.178	2.66	0.932	0.658	0.245	0.227
IgM	1.504	0.689	0.175	0.058	0.092	0.068	0.18	0.151	0.154

Ab = Antibody

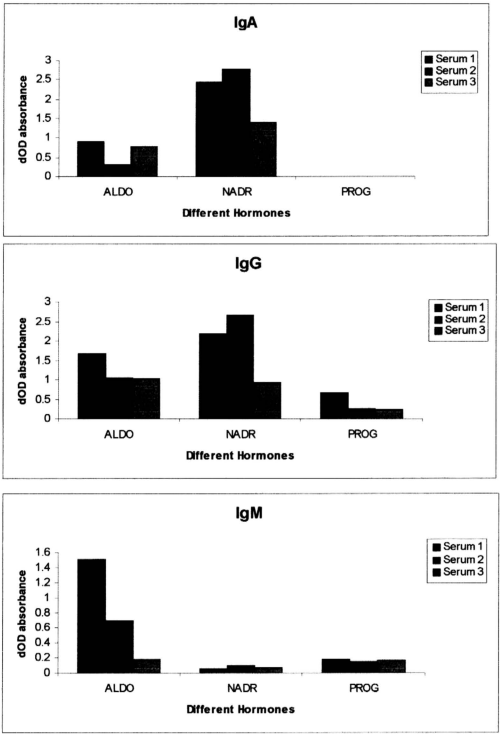


Figure 3.21 : Natural Antibody Binding to Different Hormones in 3 Normal Human Serum
ALDO = Aldosterone, NADR = Noradrenaline, PROG = Progesterone

3.7 Epitopes of Apolipoprotein E (Apo E) Precursor Recognized by Antibody in Normal Human Serum

The synthesized overlapping peptides (44 peptides, each 13 amino acids long) of Apo E precursor (317 amino acids) were reacted with healthy donor sera in a modified ELISA technique. IgA, IgG and IgM binding were investigated. The processed Apo E molecule have 299 amino acids and the sequence starts from peptide 2.

Epitopes of apo E recognized by natural antibody were analyzed in

- normal human serum
- cerebrospinal fluid
- normal cord blood
- sera from atherosclerotic patients

3.7a Epitopes of Apo E recognized by natural IgA

The Apo E peptidylated pins were reacted with five normal human sera (NHS) to identify the epitopes bound by IgA (Figure 3. 22a,b). The binding pattern in all the five NHS was different. The number of Apo E peptides bound by IgA in each serum was variable, but some common epitopes were found. Three NHS bound to 4 similar regions of the Apo E namely, peptides are 6, 23, 35 and 41. Peptides 20 and 24 were detected strongly positive twice. Besides these shared epitopes other antigenic regions of Apo E recognized by normal IgA in one NHS were peptides 2, 3, 25 and 43.

The binding pattern of natural IgA to Apo E of each NHS were combined for further analysis (Figure 3.23). The common epitopes for IgA were found to be located at peptides 6, 20, 23, 24, 35, and 41.

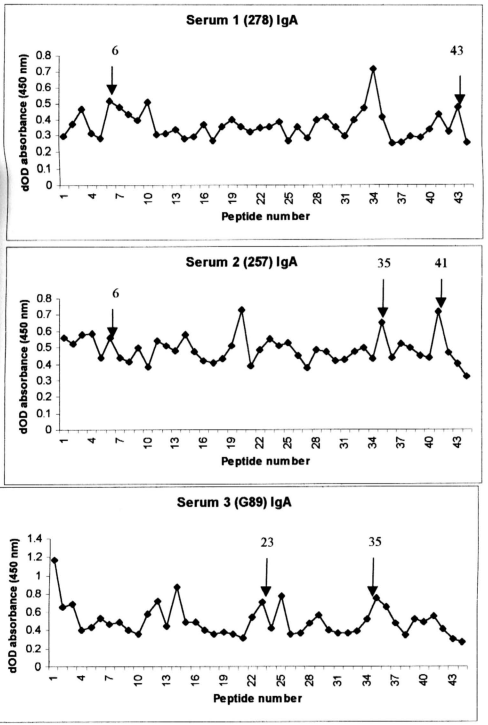


Figure 3.22a: Natural IgA Binding to Peptides of Apolipoprotein E precursor
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more NHS

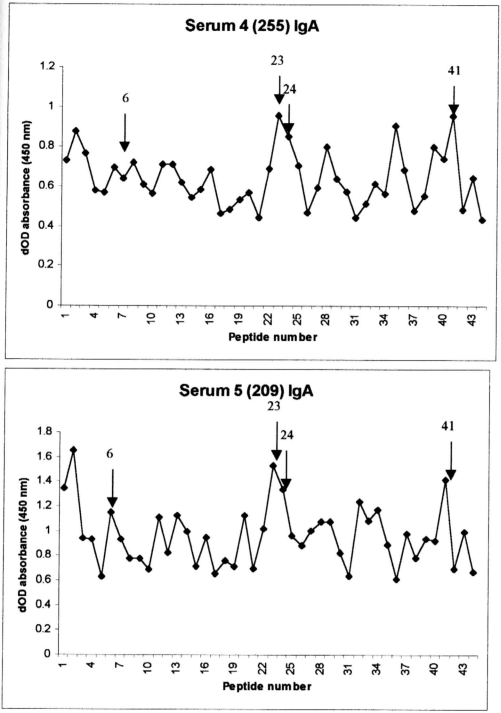


Figure 3.22b: Natural IgA Binding to Peptides of Apolipoprotein E Precursor
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more NHS

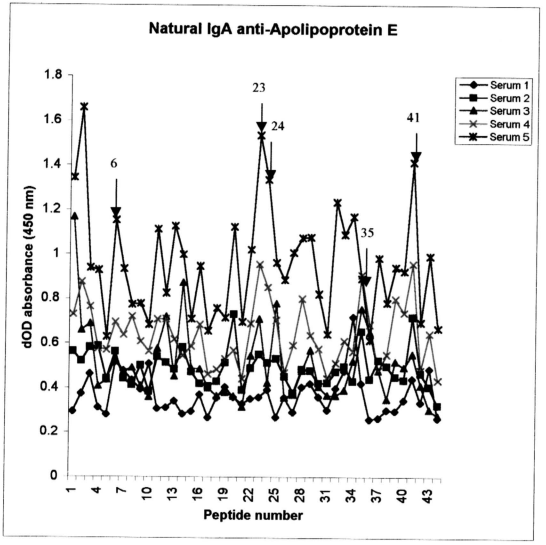


Figure 3.23: Natural IgA Binding to Peptides of Apolipoprotein E Precursor in 5NHS
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apoE
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate common epitopes (reactive with two or more NHS) of IgA in the 5 NHS

3.7b Epitopes of Apo E recognized by natural IgG

Five NHS were also analyzed for IgG against Apo E peptides (Figure 3.24a,b). Results showed that IgG in these 5 NHS bind different peptides, with some shared epitopes. Peptide 1, 2 and 23 were strongly reactive with IgG in all 4 NHS. Peptides 33 and 34 were bound by IgG in 3 sera. Peptides 8 and 35 were recognized by IgG in 2 sera. Other antigenic peptides that were occasionally bound were 13, 20, 24 and 29.

The combined IgG binding pattern of all 5 NHS to Apo E is shown in Figure 3.25. The peptides that are epitopes for IgG binding are 1, 2, 8, 23, 33, 34 and 35.

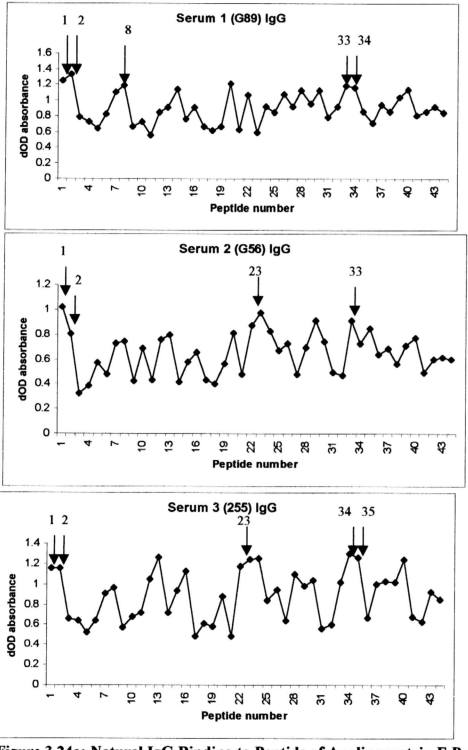
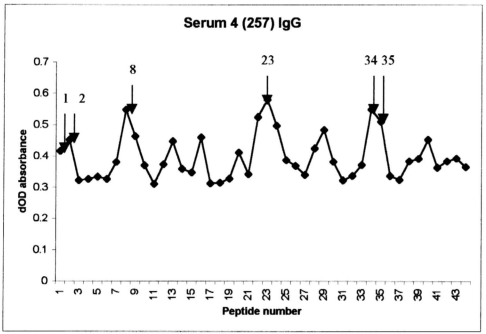


Figure 3.24a: Natural IgG Binding to Peptide of Apolipoprotein E Precursor
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E.
Y-axis: delta optical absorbance in apo E peptide ELISA.
Arrows indicate peptides that are reactive with two or more NHS



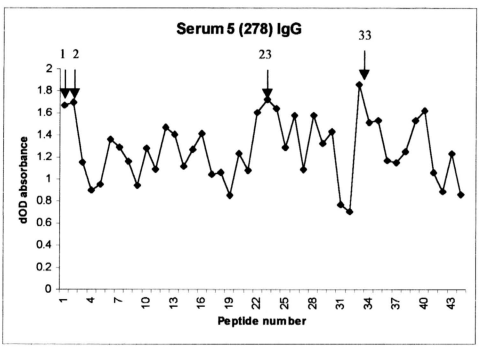


Figure 3.24b: Natural IgG Binding to Peptide of Apolipoprotein E Precursor
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more NHS

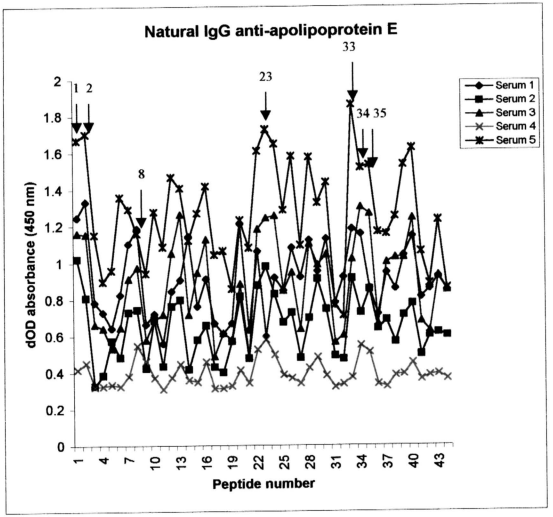


Figure 3. 25: Natural IgG binding pattern to apo E peptides in 5 NHS

X-axis: numbers are the 44 peptides (Each 13 aa) of the complete sequence of apo E

Y-axis: delta optical absorbance in apo E peptide ELISA

Arrows indicate common epitopes (reactive with two or more NHS) of IgA in the 5 NHS

3.7c Epitopes of Apo E recognized by natural IgM

Five NHS were used to detect the binding pattern of IgM (Figure 3.26 a,b). The highly reactive peptide with IgM in all the five sera was peptides number 6 and 33. Peptide 41 was recognized by 4 out of the 5 NHS. IgM recognized peptides 15, 22, and 32 in 2 out of the 5 normal sera. Peptides that were recognized only once are 20, 23, 34, and 42.

The results from all the 5 NHS were combined to show the combined binding pattern (Figure 3.27). The epitopes for IgM binding are 6, 15, 22, 32, 33, and 41.

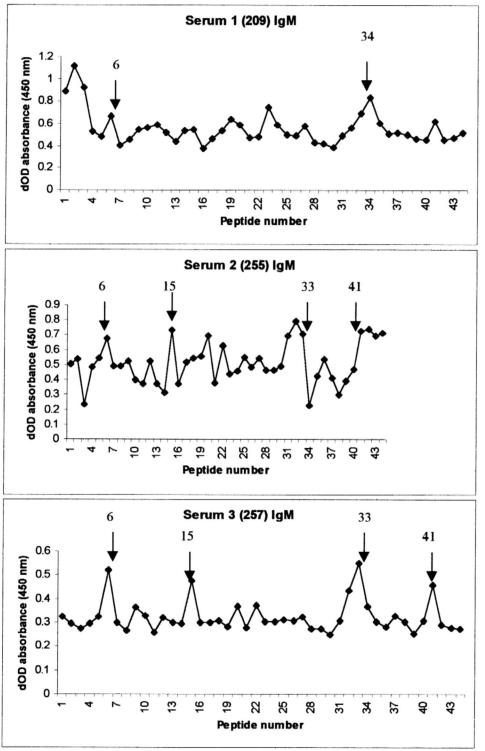
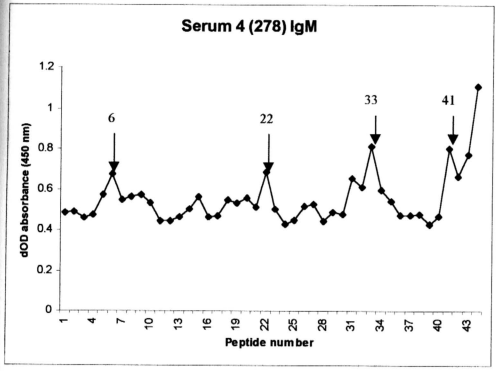


Figure 3.26a: Natural IgM Binding to Peptides of Apolipoprotein E Precursor
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E

Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more NHS



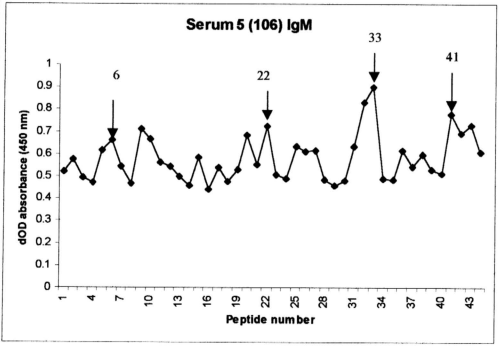


Figure 3.26b: Natural IgM Binding to Peptides of Apolipoprotein E Precursor
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E

Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more NHS

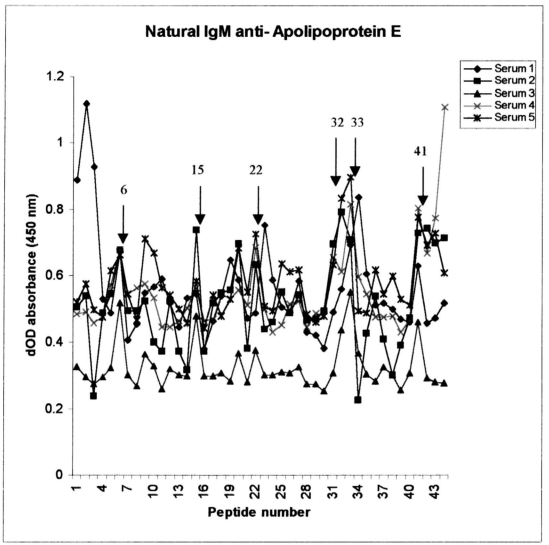


Figure 3.27: Natural IgM Binding to Peptides of Apolipoprotein E Precursor in 5 NHS
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate common epitopes (reactive with two or more NHS) of IgM in the 5 NHS

3.8 Comparison of IgA , IgG and IgM Epitopes of Apolipoprotein E Precursor

3.8a Isotype pattern in individual NHS

Three NHS 255, 257 and 278 were used to detect the binding pattern of natural IgA, IgG and IgM for the synthetic peptides of Apo E precursor (Figure 3.22 a,b; 24 a,b; 26 a,b & Figure 3.28). In serum 255, IgA and IgG bound to peptides 2, 23, 24, and 35. Peptide 41 was strongly reactive with IgA and IgM. Serum 257 shows strong binding to peptide 35 with IgA and IgG, whereas peptides 6, 14, and 41 were highly reactive with IgA and IgM. In serum 278 peptide 33 was bound by IgG and IgM and peptide 6 reacted with IgA and IgM. Common epitopes of Apo E in each serum are summarized in Table 3.7.

Serum 209 was used to detect the binding pattern of IgA and IgM to Apo E (Figure 3.29). This serum showed a highly reactive binding with IgA and IgM to peptides 6 and 23. Serum G89 was detected for IgA and IgG (Figure 3.29). This serum showed a different binding profile with both IgA and IgG. IgA mostly recognized peptides number 3, 12, 23, 25 and 35 and IgG reacted with peptides 1, 20, 28, 33 and 34.

Table 3.7: Common Epitopes of Apolipoprotein E in individual NHS

Serum Number	IgA	IgG	IgM
255	2, 23, 24, 35,41	2, 23, 24, 35	41
257	35, 6, 14, 41	35	6, 14, 41
278	6	33	33, 6

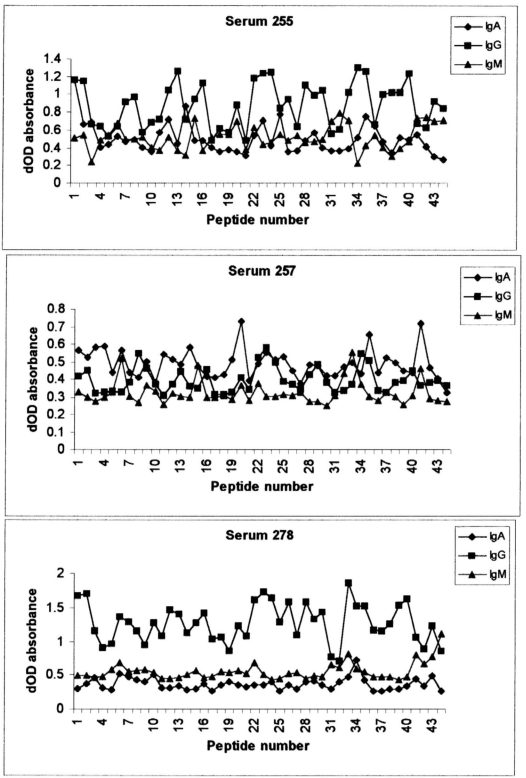
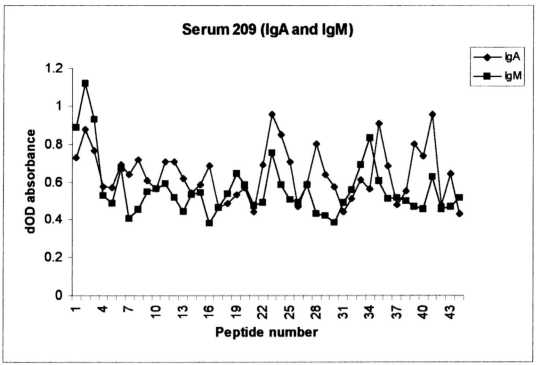


Figure 3.28: Comparison of natural IgA, IgG and IgM epitopes of Apolipoprotein E Precursor in individual Normal Human Serum



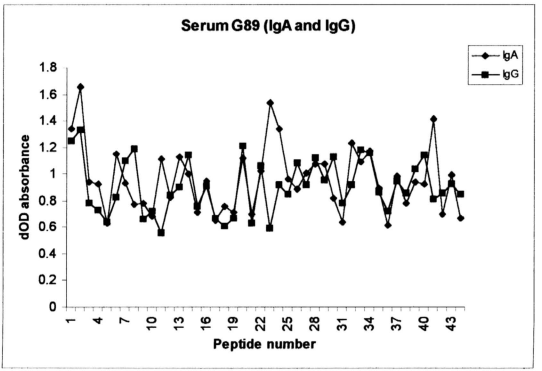


Figure 3.29: Comparison between Natural Antibody Isotypes to Apolipoprotein E In individual Normal Human Serum

3.8b Common apolipoprotein E epitopes of natural IgA, IgG and IgM

Epitopes that were bound by IgA, IgG, and IgM in NHS were mostly different, but some common epitopes for all isotypes were found. IgA bound to peptides 6 and 41 in 3 sera out of 5 NHS, while IgM bound to peptide 6 (5/5 NHS) and peptide 41 (4/5 NHS). IgG (3/5 NHS) and IgM (5/5 NHS) bound to peptide 33. Common Apolipoprotein E epitopes of the antibody isotypes in NHS are summarised in Table 3.8; Figure 3.30).

Results showed that antigenic sites of Apo E precursor molecule for natural IgA, IgG and IgM are present in amino end (e.g. Peptide 6), middle region (e.g. Peptide 33) as well as in carboxyl end (e.g. Peptide 41). Thus, the binding were observed significantly in all the regions of Apo E precursor molecule. Comparison of the reactive regions recognized by IgA, IgG, IgM and purified IgG in Apo E precursor is illustrated in Figure 3.30.

Table 3.8: Common Apolipoprotein E Epitopes of Natural Antibody Isotypes

Antibody	Common Epitopes
IgA	6, 20, 23, 24, 35, 41
IgG	1, 2, 8, 23, 33, 34, 35
IgM	6, 15, 22, 37, 33, 41
Purified IgG	1, 8, 23, 33, 34

[1]MKVLWAALLVTFLAGCQAKVEQAVETEPEPELRQQTEWQSGQRWELALG[49]

MKVLWAALLVTFL TEWQSGQRWELAL

LLVTFLAGCQAKV TEWQSGQRWELAL

MKVLWAALLVTFL

[50]RFWDYLRRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEE[98]

RFW DYLRRWVQTLS

RFWDYLRRWVQTLS

[99]QLTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEVQAMLGQS[147]

QLTPVAEETRARL

[148]TEELLVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAIEGAERGLSAIRE[197]

LASHLRKLRKRLL

LASHLRKLRKRLL

LASHLRKLRKRLL

KLRKRLLRDADDLQ

TEELLVRLASHLR

Figure 3.30: Comparison of reactive regions recognized by IgA (red), IgG (green), IgM (blue) and purified IgG (pink) to Apolipoprotein E precursor. The black letters are the complete apo E sequence

[198]RLGPLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRD[245]

AQAWGERLRARME MGSRTRD

AQAWGERLRARME MGSRTRD

AQAWGERLRARME

LRARMEEMGSRTR

LRARMEEMGSRTR

GQPLQERAQAWGE

[246]RLDEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQW[294]

RLDEVK

RLDEVK SWFEPLVEDMQRQ

SWFEPLVEDMQRQ

[295]AGLVEKVQAAVGTSAAPVPSDNH[317]

Figure 3.30 (continued): Comparison of reactive regions recognized by IgA (red), IgG (green), IgM (blue) and purified IgG (pink) to Apolipoprotein E precursor. The black letters are the complete apo E sequence

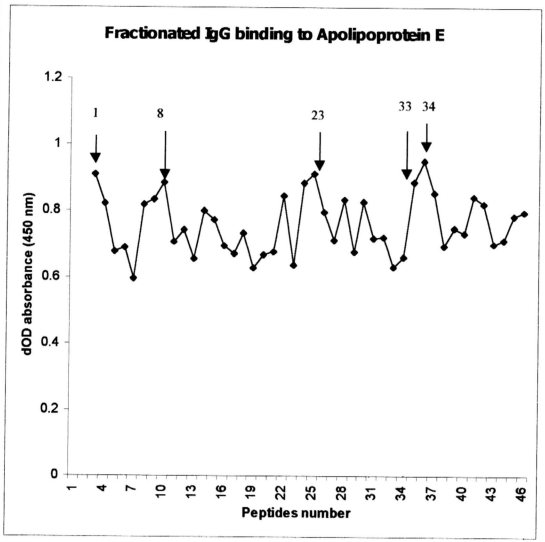
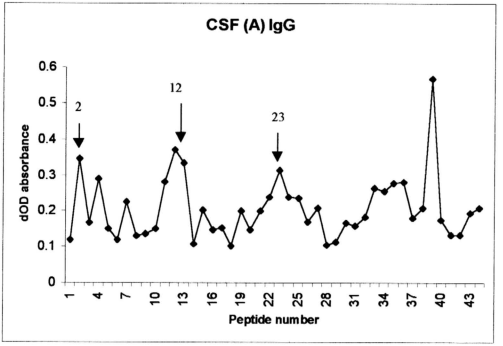


Figure 3.31: Binding to peptides of Apo E in Fractionated IgG from Normal Human Serum

X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of Apo E
Y-axis: delta optical absorbance in Apo E peptide ELISA
Arrows indicate major reactive peptide of
apo E



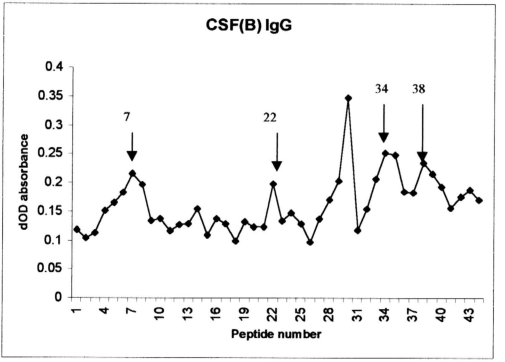


Figure 3.32: Natural IgG Binding to Peptides of Apo E in 2 Cerebrospinal Fluid Samples (CSF)

X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E

Y-axis: delta optical absorbance in apo E peptide ELISA

Arrows indicate major reactive peptides of apo E

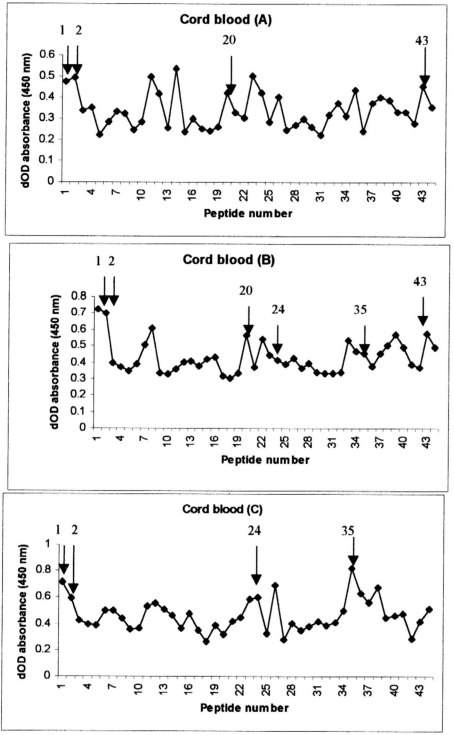


Figure 3.33: Natural IgG Binding to Peptides of Apolipoprotein E Precursor in 3 Cord Blood Samples

X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more NHS

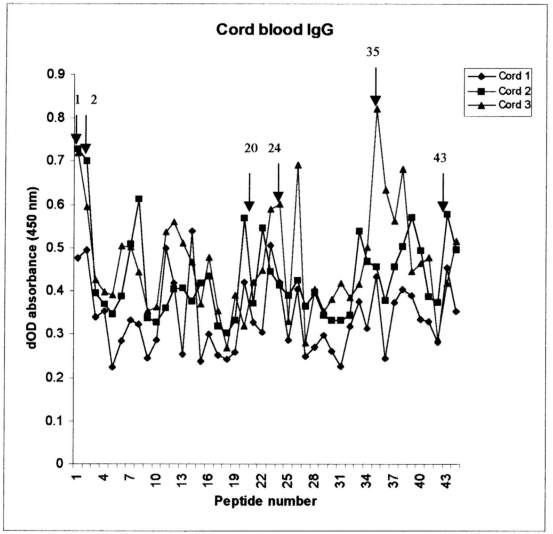


Figure 3.34: Natural IgG binding pattern of Apo E peptides in 3 cord blood samples
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: mean optical absorbance of cord blood samples to apo E peptides
Arrows indicate common epitopes (reactive with two or more cord blood) of IgG in the cord blood

3.12 Epitopes of Apolipoprotein E Recognized by Antibody in the Serum of Atherosclerotic Patients

Five sera from atherosclerosis patients were collected from University Hospital, Kuala Lumpur and used in this study to identify the binding pattern of IgA, IgG and IgM against Apo E precursor. The same sera were used to detect all the antibody isotypes i.e. IgA, IgG and IgM.

3.12a Epitopes of Apo E recognized by Serum IgA

The Apo E peptidylated pins were reacted with 5 atherosclerotic sera to identify the epitopes bound by IgA (Figure 3.35 a,b). The binding patterns in all the patient's sera were different. The number of Apo E peptides bound by IgA in each serum was variable, but some common epitopes were found. IgA in four sera consistently reacted with peptide 35. IgA strongly bound to peptide 23 in 3 sera. IgA recognized peptides 11, 16, and 41 in 2 out of 5 sera. Other peptides that were occasionally bound were 6, 8, 14, 25, and 34.

The combined IgA binding pattern in all five atherosclerotic samples is shown in Figure 3.36. The common epitopes for IgA were found to be located at peptides 11, 16, 23, 35, and 41.

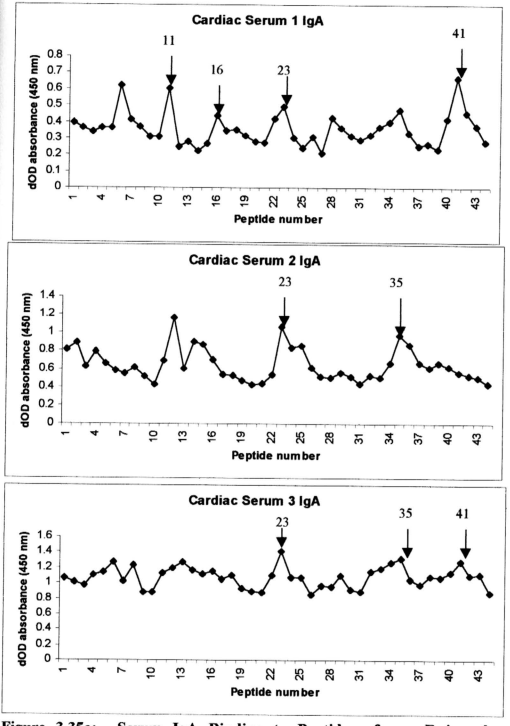
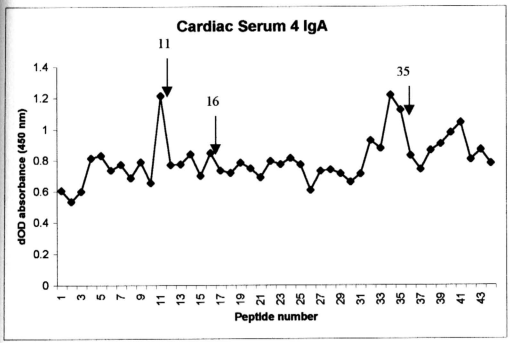


Figure 3.35a: Serum IgA Binding to Peptides of apo E in atherosclerotic patientsX-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E

Y-axis: delta optical absorbance in apo E peptide ELISA

Arrows indicate peptides that are reactive with two or more patients sera



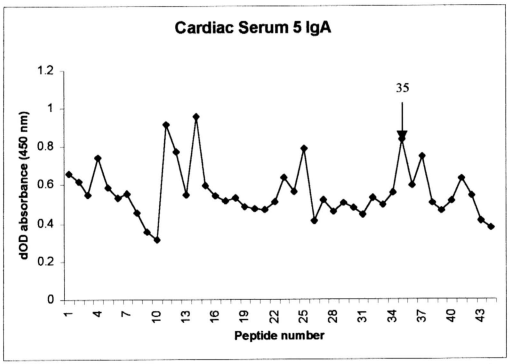


Figure 3.35b: Serum IgA Binding to Peptides of Apo E in atherosclerotic patients
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more patients sera

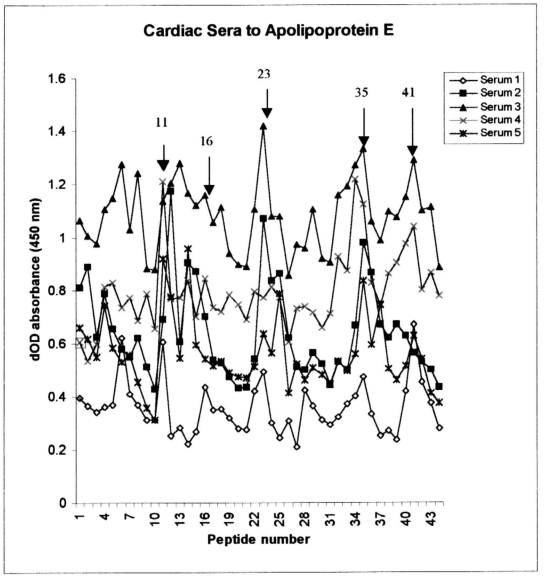


Figure 3.36: Serum IgA binding pattern to Apo E peptides in 5 atherosclerotic patients
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of Apo E
Y-axis: delta optical absorbance in Apo E peptide ELISA
Arrows indicate common epitopes (reactive with two or more sera) of IgA in 5 patients

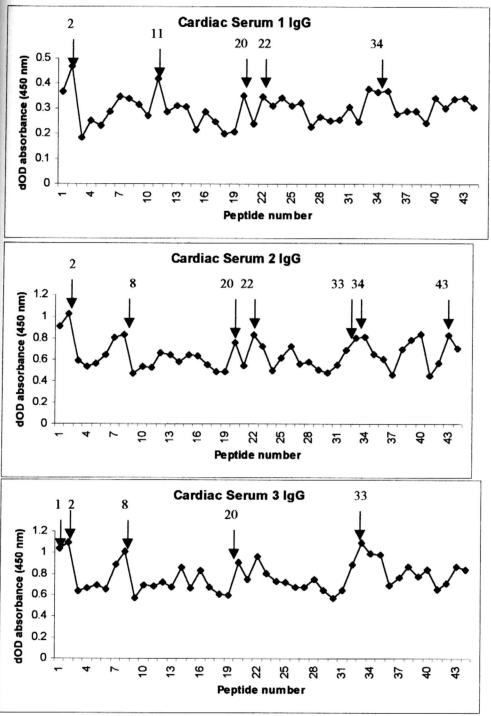
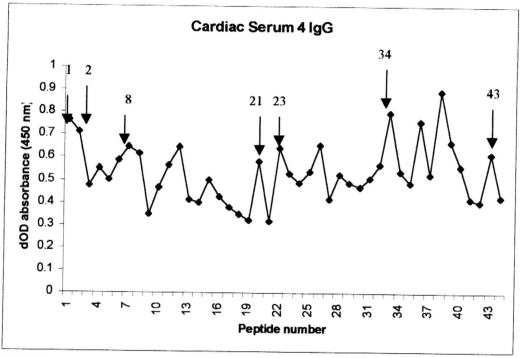


Figure 3.37a: Serum IgG Binding to Peptides of Apo E in atherosclerotic patients
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more patients sera



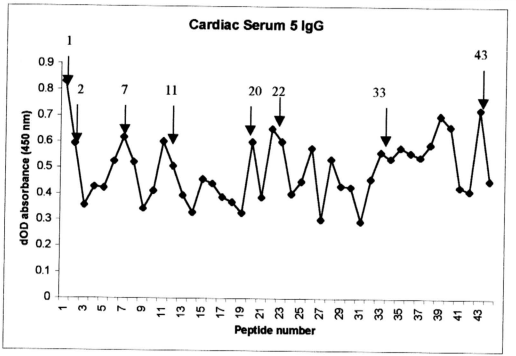


Figure 3.37b: Serum IgG Binding to Peptides of apo E in atherosclerotic patients
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more patients sera

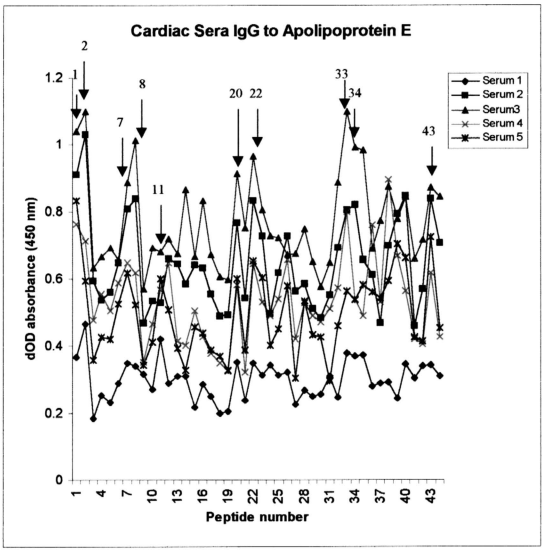


Figure 3.38: Serum IgG binding pattern to Apo E peptides in 5 Atherosclerotic patients

X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of Apo E

Y-axis: delta optical absorbance in Apo E peptide ELISA

Arrows indicate common epiopes (reactive with two or more sera) of IgA in 5 patients

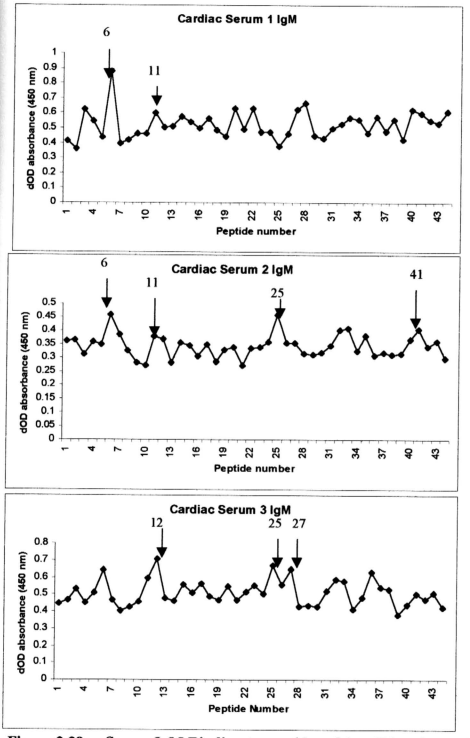
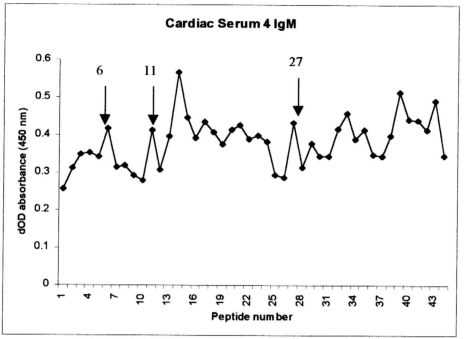


Figure 3.39a: Serum IgM Binding to peptides of Apo E in atherosclerotic patients
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of apo E
Y-axis: delta optical absorbance in apo E peptide ELISA
Arrows indicate peptides that are reactive with two or more patient's sera



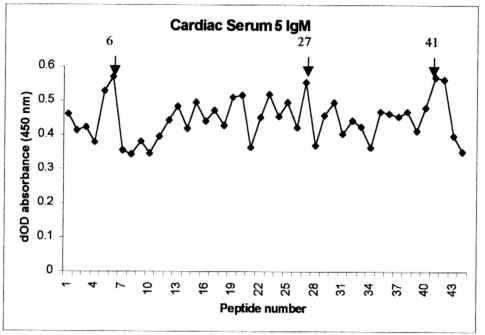


Figure 3.39b: Serum IgM binding to peptides of Apo E in athereosclerotic patients

X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of Apo E

Y-axis: delta optical absorbance in Apo E peptide ELISA

Arrows indicate peptides that are reactive with two or more patients sera

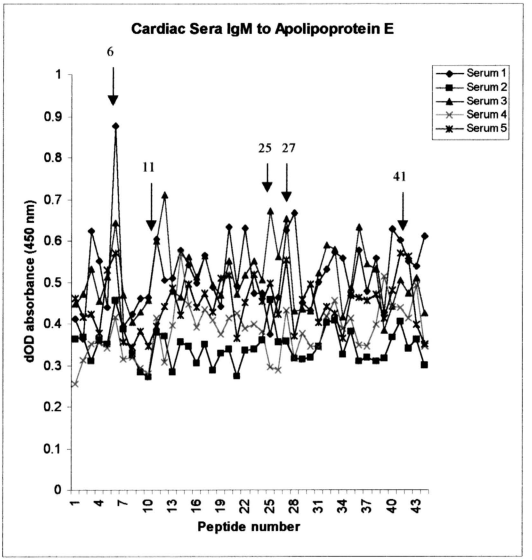


Figure 3.40: Serum IgM binding pattern to Apo E peptides in atherosclerotic patients
X-axis: numbers are the 44 peptides (each 13 aa) of the complete sequence of Apo E
Y-axis: delta optical absorbance in Apo E peptide ELISA
Arrows indicate common epitopes (reactive with two or more sera) of IgA in 5 patients

Table 3.10: Common Epitopes of Apo E in individual Atherosclerosis Serum

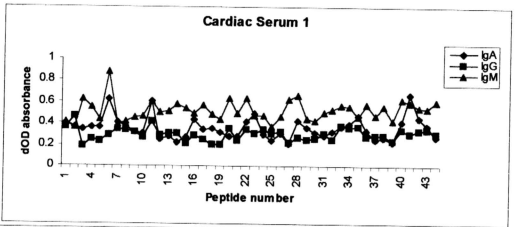
Serum	IgA	IgG	IgA
1	11	11,	11,
2		-	
3	8, , 33	8, 33	
4	11	-	11
5	-	-	-

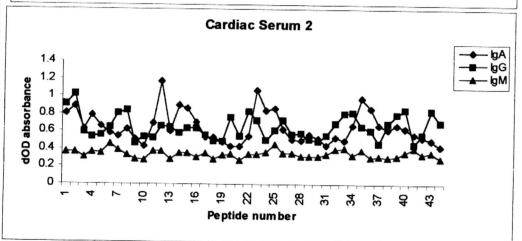
- = No common epitopes

Table 3.10: Common Epitopes of Apo E in individual Atherosclerosis Serum

Serum	IgA	IgG	IgA
1	11	11,	11,
2	35	-	35
3	8, 6, 35	8, 35	6
4	11	-	11
5	-	-	-

- = No common epitopes





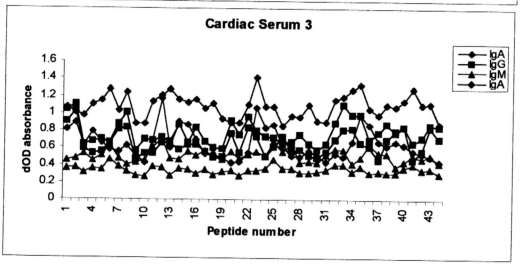


Figure 3.41a: Common Apo E epitopes of IgA, IgG and IgM in-atherosclerotic sera

[1]MKVLWAALLVTFLAGCQAKVEQAVETEPEPELRQQTEWQSGQRWELAL[48]

TEWQSGQRWELAL

TEWQSGQRWELAL

[49]GRFWDYLRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELE[97]

RFWDYLRWVQTLS SSQVTQELRALMD (2X)

RFWDYLRWVQTLS SSQVTQELRALMD

SSQVTQELRALMD (2X)

[98]EQLTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEVQAMLGQS[147]

VQYRGEVQAMLGQ

VQYRGEVQAMLGQ

[148]TEELLVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAIEGAERGLSAIRER[198]

[199]LGPLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRL[247]

MGSRTRDRL

MGSRTRDRL

MGSRTRDRL

[248]DEVKEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWA[295]

DEVK

DEVK

DEVK

[296]GLVEKVQAAVGTSAAPVPSDNH[317]

Figure 3. 42: Comparison of reactive regions of Apolipoprotein E recognized by IgA (red), IgG (green), IgM (blue) in individual Atherscleros is serum

3.14 Comparison Between the Epitope Binding Pattern of IgA, IgG and IgM in Normal Human and Atherosclerosis Sera

3.14a IgA in NHS and atherosclerotic serum

More or less similar profile was observed for IgA in NHS and atherosclerotic sera (Table 3. 12). IgA in both sera mainly bound to 6, 23, 25, 35, and 41. Dissimilar IgA epitopes of NHS and atherosclerotic sera are shown in Table 3.13.

3.14b IgG in NHS and atherosclerotic serum

Results showed that epitopes recognized by IgG in NHS and atherosclerosis sera are almost similar (Table 3. 12). In both sera, IgG bound to peptides 1, 2, 8, 20, 33, 34 and 35. IgG mainly bound to epitopes in the middle region and amino end of apo E. Dissimilar IgG epitopes of NHS and atherosclerotic sera are shown in Table 3.13.

3.14c IgM in NHS and atherosclerosis serum

Similar epitopes recognized by IgM in NHS and atherosclerosis sera are shown (Table 3. 12). IgM in both sera mostly bound to peptides 6, 20, 22 and 41. Dissimilar IgM epitopes of NHS and atherosclerotic sera are shown in Table 3.13.

Table 3.12 : Similar Epitopes of apo E recognized by antibody isotypes in NHS and atherosclerotic sera

Antibody Isotypes	Peptide Number	Normal Human Sera	Atherosclerosis Sera
IgA	6	3/5	2/5
	23	3/5	4/5
	25	3/5	1/5
	35	3/5	2/5
	41	1/5	1/5
IgG	1	4/5	3/5
	2	4/5	5/5
	8	2/5	2/5
	33	3/5	4/5
	34	3/5	2/5
	35	2/5	1/5
	20	1/5	5/5
IgM	6	5/5	4/5
	20	1/5	1/5
	22	2/5	1/5
	41	4/5	2/5

Table 3.13: Dissimilar Epitopes of apo E recognized by antibody isotypes in NHS and atherosclerotic sera

Antibody Isotypes	Peptide Number	Normal Human Sera	Atherosclerosis Sera
IgA	2	+	-
	3	+	-
	8	-	+
	11	+	-
	14	-	+
	16	+	-
	20	+	-
	24	+	-
	34	-	+
	43	+	-
IgG	7	-	+
	11	-	+
	12	-	+
	13	+	-
	22	-	+
	23	+	-
	24	+	-
	26	-	+
	29	+	-
	36	-	+
	38	-	+
	43	-	+
IgM	11	-	+
	12	-	+
	14	-	+
	15	+	-
	23	+	-
	25	-	+
	27	-	+
	28	-	+
	32	+	-
	33	+	-
	34	+	-
	36	-	+
	39	-	+
	42	+	-
	43	-	+

(+) (-); indicates antibody present in at least one normal human serum