CHAPTER 4

RESULTS

1. Setting up the optimal conditions for indirect ELISA test for the detection of IgG anti-horse gamma globulin antibody

1.1 Determination of the optimal dilution of serum samples and rabbit anti-human IgG peroxidase conjugate.

Horse gamma globulin was diluted in coating buffer to 10 ug of protein per ml and 100 ul of this solution were used for coating the microtitre plate at 37°C for 1 hour. After the excess of horse gamma globulin was removed, the plate was washed with PBS-Tween 5 times, 3 minutes each time. One hundred microlitres of the positive and negative control sera diluted 1:200, 1:400, 1:800 and 1:1600 in PBS-Tween albumin were added in duplicate and the plate was incubated at 37°C for 1 hour. After 5 washes with PBS-Tween, 100 ul of rabbit anti-human IgG peroxidase conjugate diluted in PBS-Tween albumin to the dilutions of 1:5000, 1:10,000, 1:15,000 and 1:20,000 were added in duplicate and incubated at 37°C for 1 hour. The plate was washed with PBS-Tween 5 times and 100 ul of substrate solution were added and then incubated at room temperature

for 30 minutes in the dark. The reaction was stopped by adding 50 ul of 1 N H_2SO_4 to each well and the absorbance was read by spectrophotometer at 492 nm. Conjugate and substrate controls were also included in every condition tested.

The results are shown in (Table I Appendix II). It is evident that the optimal dilution of sample is 1:800 and the optimal conjugate dilution is 1:10,000 and these conditions were then selected for use in the subsequent experiments.

1.2 Determination of the optimal concentration of horse gamma globulin

Horse gamma globulin was diluted in coating buffer to the protein concentrations of 1, 5, 10 and 20 ug/ml. One hundred microlitres of each concentration were used to coat the microtitre plate at 37°C for 1 hour. After 5 washes with PBS-Tween, 100 ul of positive and negative control sera diluted 1:800 in PBS-Tween albumin were added and the plate was incubated at 37°C for 1 hour. Each antigen concentration was assayed in duplicate. After 5 washes with PBS-Tween, 100 ul of rabbit anti-human IgG peroxidase conjugate diluted 1:10,000 were added and incubated at 37°C for 1 hour. The plate was washed with PBS-Tween 5 times and 100 ul of substrate solution were

added and then incubated at room temperature for 30 minutes in the dark. The color development was stopped by the addition of 50 ul of 1 N H_2SO_4. The absorbance was measured with a spectrophotometer at 492 nm.

The results indicated that the optimal concentration of horse gamma globulin was 5 ug/ml as no further increase in reactivity was found when antigen concentration was increased to 10 and 20 ug/ml (Fig. 1, Appendix II). An antigen concentration of 5 ug/ml was therefore chosen for further work.

1.3 Determination of the optimal temperature for antigen incubation.

Microtitre plates were coated with 5 ug/ml of horse gamma globulin at various temperatures i.e. at 37°C for 1 hour, at room temperature for 1 hour and at 4°C overnight. The plates were then processed in the similar manner as that described above.

The results are shown in Fig. 2 (Appendix II). The optimal temperature for antigen incubation was chosen to be 37°C. Although antigen incubation at 4°C also gave the absorbance values as high as the incubation at 37°C, the antigen incubation time was shorter. Thus, this

condition was then selected for use in the subsequent experiments.

1.4 Determination of the optimal time for antigen incubation

This experiment was performed in the similar manner as that described in 1.3. The time for antigen incubation was varied from 0.5,1,2 and 3 hours.

The results are shown in Fig. 3 (Appendix II). It indicated that the optimal time for antigen incubation was 1 hour. This antigen incubation time was then selected for subsequent experiments.

1.4 Determination of the optimal temperature for serum incubation

Microtitre plates were coated with 100 ul of horse gamma globulin at the concentration of 5 ug/ml at 37°C for 1 hour. After washing the plates with PBST 5 times, 100 ul of positive and negative control sera diluted 1:800 in PBS-Tween albumin were added. Serum incubation was tested at 37°C for 1 hour, room temperature for 1 hour and 4°C overnight. The experiment was then performed in the same manner as described above.

The results are shown in Fig.4 (Appendix II). It indicated that the optimal temperature for serum incubation was at 37°C. Serum incubation at 4°C gave the highest absorbance value in the positive control but the negative control also showed the highest absorbance value. On the other hand, serum incubation at 37°C gave high absorbance value in positive control whereas negetive control gave low absorbance value. So, serum incubation at 37°C was chosen for the next experiments.

1.5 Determination of the optimal time for serum incubation.

The experiment was performed in the same manner as that described above except the serum incubation time was varied from 0.5,1,2 and 3 hours. at 37°C

The results are shown in Fig. 5 (Appendix II), which can be seen that the optimal time for serum incubation is 1 hour.

1.6 Determination of the optimal temperature for conjugate incubation

Plates were coated with 5 ug/ml of horse gamma globulin at 37°C for 1 hour. After 5 washes with PBS-Tween 100 ul of positive and negative control sera

diluted 1:800 in PBS-Tween albumin were added and the plates were incubated at 37°C for 1 hour. After washing with PBS-Tween 100 ul of rabbit anti-human IgG peroxidase conjugate diluted 1:10,000 in PBS-Tween albumin were tested at 37°C for 1 hour, at room temperature for 1 hour and at 4°C overnight. The plates were then washed 5 times with PBS-Tween and 100 ul of substrate solution were added. The plates were incubated at room temperature for 30 minutes in the dark. The reaction was stopped with 1 N H_2SO_4 and the absorbance was read spectrophotometrically at 492 nm.

The results are shown in Fig. 6 (Appendix II). The optimal temperature for conjugate incubation was chosen to be at 37°C and then used further in the subsequent experiments.

1.7 Determination of the optimal time for conjugate incubation

The experiment was performed in the same manner with that discribed above. The conjugate incubation time was investigated at 37°C for 0.5,1,2 and 3 hours.

The results are shown in Fig.7 (Appendix II), The optimal time for conjugate incubation was 1 hour which was chosen for further experiments.

1.8 Determination of the optimal time for substrate incubation

Previously selected conditions were used in this experiment and the time for substrate incubation was investigated at room temperature for 15,30,45 and 90 minutes.

The results are shown in Fig. 8 (Appendix II) which can be seen that optimal time for substrate incubation is 30 minutes.

The optimal conditions of ELISA test for the detection of IgG anti-horse gamma globulin was summarized in Table 5.

2. Setting up the optimal conditions for indirect ELISA test for the detection of IgE anti-horse gamma globulin antibody

2.1 Determination of the optimal dilution of serum samples and rabbit anti-human IgE peroxidase conjugate.

Horse gamma globulin was diluted in coating buffer to give a concentration of 20 ug protein per ml and 100 ul of this solution were used for coating the microtitre plate at 37°C for 1 hour. The plate was washed with

PBS-Tween 5 times, 3 minutes each time. The positive and negative control sera were diluted 1:25, 1:50, 1:100 and 1:200 in PBS-Tween albumin and 100 ul of each dilution were added in duplicae. The plate was incubated at 37°C for 1 hour and then washed 5 times. One hundred microlitres of rabbit anti-human IgE peroxidase conjugate diluted in PBS-Tween albumin to a dilution of 1:250, 1:500, 1:1,000 and 1:2,000 were added in duplicate and incubated at 37°C for 1 hour. After 5 washes with PBS-Tween 100 ul of substrate solution were added to each well and incubated at room temperature for 30 minutes in the dark. The reaction was stopped by the addition of 50 ul of 1 N H_2SO_4. Optical density was read by spectrophotometer at 492 nm. Conjugate and substrate controls were also included in every condition tested.

The resuts are shown in Table 2 (Appendix II)It is evident that the optimal dilution of sample is 1:50 and the optimal conjugate dilution is 1:500 and these conditions were then selected for use in the subsequent experiments.

2.2 Determination of the optimal concentration of horse gamma globulin

Horse gamma globulin was diluted in coating buffer to the protein concentrations of 5,10,20,40 ug/ml and 100

ul of each concentration were used for coating the microtitre plate. The plate was incubated at 37°C for 1 hour and then washed 5 times with PBS-Tween. One hundred microlitres of positive and negative control sera diluted 1:50 in PBS-Tween albumin were added and the plate was incubated at 37°C for 1 hour. Each antigen concentration was assayed in duplicate. After 5 washes with PBS-Tween 100 ul of rabbit anti-human IgE peroxidase conjugate diluted 1:500 were added and incubated at 37°C for 1 hour. After excess conjugate was removed and the plate was washed 5 times, 100 ul of substrate solution were added and incubated at room temperature for 30 minutes. The reaction was stopped with 1 N H_2SO_4 and the absorbance was read by spectrophotometer at 492 nm.

The result are shown in Fig. 9 (Appendix II). The optimal concentration of antigen was 40 ug/ml and it was chosen for use in the subsequent experiments.

2.3 Determination of the optimal temperature for antigen incubation

The experiment was performed in the similar manner as described above. The microtitre plates were coated with 40 ug/ml of horse gamma globulin at various temperatures i.e. at 37°C for 1 hour, at room temperature for 1 hour and at 4°C overnight.

The results indicated that the optimal temperature for antigen incubation was 37°C (Fig. 10, Appendix II). This antigen incubation time was then selected for the next experiments.

2.4 Determination of the optimal time for antigen incubation

Microtitre plates were coated with 100 ul of horse gamma globulin concentration 40 ug/ml. Antigen incubation time was tested at 37°C for 0.5,1,2 and 3 hours. The plates were then processed in the same manner as described above.

The results are shown in Fig. 11 (Appendix II). It was evident that the optimal time for antigen incubation was 2 hours which was chosen for further experiments.

2.5 Determination of the optimal temperature for serum incubation

Horse gamma globulin diluted in coating buffer to 40 ug/ml was used for coating the plates at 37°C for 2 hours. After 5 washes, 100 ul of positive and negative control sera diluted 1:50 were added. The serum incubation temperature was investigated at 37°C for 1 hour, at room

temperature for 1 hour and 4°C overnight. The experiment was then performed in the similar manner as described above.

The results are shown in Fig. 12 (Appendix II). It can be seen that the optimal temperature for serum incubation is 37°C and this condition was then selected for use in the subsequent experiments.

2.6 Determination of the optimal time for serum incubation

The experiment was performed in the same manner as that described above. The optimal time for serum incubation was tested at 0.5,1,2 and 3 hours.

The results are shown in Fig. 13 (Appendix II). The optimal time for serum incubation was 2 hours. This serum incubation time was then selected for subseequent experiments.

2.7 Determination of the optimal temperature for conjugate incubation

The microtitre plates were coated with coated with 100 ul of horse gamma globulin concentration 40 ug/ml at 37°C for 2 hours. After washing the plates with PBS-Tween 5 times, 100 ul of positive and negative control sera

diluted 1:50 in PBS-Tween albumin were added and incubated at 37°C for 2 hours. After 5 washes with PBS-Tween, 100 ul of rabbit anti-human IgE peroxidase conjugate diluted 1:500 in PBS-Tween albumin were investigated at 37°C for 1 hour, at room temperature for 1 hour and 4°C overnight. The plates were then processed in the same manner as that described obove.

The results are shown in Fig. 14 (Appendix II). It indicated that the optimal temperature for conjugate incubation was at 37°C as serum incubation at 37°C gave high absorbance value in the positive control and low absorbance value in the negative control. So, serum incubation at 37°C was chosen for the next experiments.

2.8 Determination of the optimal time for conjugate incubation

The experiment was carried out in the same manner as that described above. The conjugate incubation time was investigated at 37°C for 0.5, 1, 2 and 3 hours.

The results are shown in Fig. 15 (Appendix II). It can be seen that the optimal time for conjugate incubation is 2 hours.

2.9 Determination of the optimal time for substrate incubation

The experiment was carried out with the conditions selected previously. The optimal time for substrate incubation was tested at room temperature for 15, 30, 45 and 90 minutes.

The results are shown in Fig. 16 (Appendix II). The optimal time for substrate incubation was 45 minutes.

The optimal conditions of ELISA test for the detection of IgE anti-horse gamma globulin was summarized in Table 6.

3. Setting up the optimal conditions for IgM class capture ELISA test for the detection of IgM anti-horse gamma globulin

3.1 Determination of the optimal dilution of serum samples and rabbit anti-horse IgG peroxidase conjugate

The microtitre plate was coated with rabbit anti-human IgM diluted in coating buffer to 10 ug of protein per ml and 100 ul of this solution were added to each well. The plate was incubated at 37°C for 1 hour and then washed with PBS-Tween 5 times, 3 minutes each time. One hundred microlitres of the positive and negative

control sera diluted 1:5, 1:25, 1:50, 1:100 and in PBS-Tween albumin were added in duplicate and the plate was incubated at 37°C for 1 hour. After 5 washes with PBS-Tween, 100 ul of horse gamma globulin diluted in PBS-Tween albumin to give a concentration of 20 ug/ml were added to each well and the plate was incubated at 37°C for 1 hour. After washing with PBS-Tween, 100 ul of rabbit anti-horse IgG peroxidase conjugate diluted 1:250, 1:500, 1:1,000 and 1:2,000 in PBS-Tween albumin were added in duplicate and incubated at 37°C for 1 hour. The plate was washed 5 times with PBS-Tween and 100 ul of substrate solution were added to each well. After 30 minutes at room temperature in the dark, the reaction was stopped by adding 50 ul of 1 N H_2SO_4. The absorbance was measured with a spectrophotometer at 492 nm. Conjugate and substrate controls were also included in every condition tested.

The results are shown in Table 3 (Appendix II). It indicated that the optimal dilution of sample was 1:25 and the optimal conjugate dilution was 1:500 and these conditions were then selected for use in the next experiments.

3.2 Determination of the optimal concentration of anti-human IgM for coating the microtitre plate

Rabbit anti-human IgM was diluted in coating buffer to the protein concentrations of 5, 10,20 and 40

ug/ml and 100 ul of this solution were used for coating the microtitre plate. After 5 washes with PBS-Tween 100 ul of positive and negative control sera diluted 1:25 in PBS-Tween albumin were added and the plate was incubated at 37°C for 1 hour. Each rabbit anti-human IgM concentration was assayed in duplicate. The plate was then washed 5 times with PBS-Tween and 100 ul of horse gamma globulin diluted in PBS-Tween albumin to 20 ug/ml of protein per ml were added to each well and incubated at 37°C for 1 hour. After 5 washes with PBS-Tween, 100 ul of rabbit anti-horse IgG peroxidase conjugate diluted 1:500 were added and incubated at 37°C for 1 hour. The plate was washed with PBS-Tween 5 times and 100 ul of substrate solution were added and incubated at room temperature of 30 minutes. The reaction was stopped with 1 N H_2SO_4 and the absorbance was read by spectrophotometer at 492 nm.

The results are shown in Fig. 17 (Appendix II). It was evident that the optimal concentration of anti-human IgM for coating plate was 10 ug/ml and it was chosen for use in the subsequent experiments.

3.3 Determination of the optimal temperature for coating plate

The experiment was performed in the same manner as that described above. The microtitre plates were coated

with 10 ug/ml of rabbit anti-human IgM at various temperatures i.e. at 37°C for 1 hour, at room temperature for 1 hour, at 4°C overnight.

The results are shown in Fig. 18 (Appendix II). The optimal temperature for coating plate was 37°C. This condition was then selected for further experiments.

3.4 Determination of optimal time for coating plate

The experiment was carried out in the same manner as that described above. Rabbit anti-human IgM was diluted in coating buffer to 10 ug/ml for coating. The plates were incubated at 37°C for 0.5, 1, 2 and 3 hours.

The results indicated that the optimal time for coating plate was 2 hours (Fig. 19, Appendix II).

3.5 Determination of the optimal temperature for serum incubation

Rabbit anti-human IgM diluted in coating buffer to 10 ug/ml was used for coating the plates at 37°C for 2 hours. After 5 washes, 100 ul of positive and negative control sera diluted 1:25 were added. Serum incubation was test at 37°C for 1 hour, at room temperature for 1 hour and 4°C overnight. The experiment was then performed in the same manner as that described above.

The results are shown in Fig 20 (Appendix II). It indicated that the optimal temperature for serum incubation was 37°C.

3.6 Determination of the optimal time for serum incubation

The experiment was performed in the same manner as that described above. The serum incubation time was investigated at 0.5, 1, 2 and 3 hours at 37°C.

The results are shown in Fig. 21 (Appendix II). It indicated that the optimal time for serum incubation was 1 hours.

3.7 Determination of the optimal concentration of horse gamma globulin

This experiment was carried out in the same manner as that described above. The plates were coated with 10 ug/ml of rabbit anti-human IgM at 37°C for 2 hour. After 5 washes with PBS-Tween, 100 ul of positive and negative control sera diluted 1:25 were added and the plates were incubated at 37°C for 1 hour. After washing with PBS-Tween 5 times, 100 ul of horse gamma globulin diluted in PBS-Tween albumin to the protein concentration of 5, 10, 20 and 40 ug/ml. The plates were incubated at 37°C for 1 hour and then processed as in the routine assay.

The results are shown in Fig. 22 (Appendix II). The optimal concentration of antigen was 20 ug/ml.

3.8 Determination of the optimal temperature for antigen incubation

The experiment was performed in the similar manner as that described above. Horse gamma globulin diluted in PBS-Tween albumin to 20 ug/ml were investigated at 37°C for 1 hour, at room temperature for 1 hour and at 4°C overnight.

The results are shown in Fig. 23 (Appendix II). It indicated that the optimal temperature for antigen incubation was 37°C.

3.9 Determination of the optimal time for antigen incubation

The experiment was carried out in the similar manner as that described above. The antigen incubation time was tested at 37°C for 0.5, 1, 2 and 3 hours.

The results are shown in Fig. 24 (Appendix II). The optimal time for antigen incubation was 2 hours.

3.10 Determination of the optimal temperature for conjugate incubation

The microtitre plates were coated with 100 ul of rabbit anti-human IgM concentration 10 ug/ml at 37°C for 2 hours. After 5 washes with PBS-Tween, 100 ul of positive and negative control sera dilute 1:25 in PBS-Tween albumin were added and incubated at 37°C for 1 hour. The plates were washed 5 times with PBS-Tween and 100 ul of horse gamma globulin diluted in PBS-Tween albumin to 20 ug/ml were added to each well. The plates were incubated at 37°C for 2 hours. After 5 washes with PBS-Tween, 100 ul of rabbit anti-horse IgG peroxidase conjugate diluted 1:250 were investigated at 37°C for 1 hour, at room temperature for 1 hour and at 4°C overnight. The plates were then processed in the same manner as that described above.

The results are shown in Fig. 25 (Appendix II). It was evident that the optimal temperature for conjugate incubation was 37°C.

3.11 Determination of the optimal time for conjugate incubation

The experiment was carried out in the similar manner as that described above. The conjugate incubation time was tested at 37°C for 0.5, 1, 2 and 3 hours.

The results are shown in Fig 26 (Appendix II). The optimal time for conjugate incubation was 2 hours.

3.12 Determination of the optimal time for substrate incubation

The experiment was performed with the conditions selected previously. The optimal time for substrate incubation was investigated at room temperature for 15, 30, 45 and 60 minutes.

The results are shown in Fig. 27 (Appendix II). The optimal time for substrate incubation was 30 minutes.

The optimal conditions of ELISA test for the detection of IgM anti-horse gamma globulin was summarized in Table 7.

4. Standardization of the ELISA test

1. Precision analysis of the assay.

1.1 In a within plate precision analysis, the coefficients of variation (CV) of the positive serum were 4.21%, 5.75% and 6.26% for IgG, IgM and IgE antibody assays respectively and of the negative control serum were 3.76%, 5.82% and 7.05% respectively (Table 8, 9 and 10)

1.2 In a between plate precision analysis, the coefficient of variation (CV) of the positive serum were 8,18%, 8.8% and 9.50% for IgG, IgM and IgE antibody assays respectively and of the negative control serum were 7.85%, 9.18% and 9.37% respectively (Table 8, 9 and 10)

2. Specificity of the ELISA tests

An inhibition test was used for assuring the specificity of the IgG, IgM and IgE anti-horse gamma globulin antibodies. As shown in Figures 1, 2, and 3 IgG, IgM and IgE antibodies could be absorbed almost completely by horse gamma globulin. Antibody titers came down to negative levels in almost all instances. This confirmed the specificity of these ELISA test.

5. Detection of IgG anti-horse gamma globulin

According to the criteria described in Meterials and Methods, all positive results at titers of $\geq$ 1:800 were considered as having IgG anti-horse gamma globulin. On day 0,7 and 14, of the 104 patients,2,5 and 31 patients had IgG anti-horse gamma globulin respectively, an incidence of 1.9%, 4.8% and 29.8% respectively. Only 2 patients had pre-existing IgG anti-horse gamma globulin on day 0 and these 2 also had IgG antibody on day 7 and 14 but with higher titers. The titers on day 0 were 1:1600 and 1:3200, on day 7 were 1:3200 and 1:6400, and on day 14

were 1:12800 and 1:6400 respectively. None of these 2 patients with pre-existing antibody developed serum sickness. The geometric mean titers (GMT) of the positive sera on day 0 day 7 and day 14 were 1:2263 (range = 1:2161-1:2369) 1:4222 (range = 1:1496-1:11885) and 1:4376 (range = 1:1340-1:14288).

2 of the 104 patients developed serum sickness following ERIG administration. One had detectable IgG anti-horse gamma globulin on day 14 (titer 1:3200) while symptoms of serum sickness started on day 9 and the other had the antibody both on day 7 and 14 (titer 1:800 and 1:12800 respestively) while her symptoms started on day 6.

On day 7, only one of 2 serum sickness patients had IgG anti-horse gamma globulin with a titer of 1:800 (Figure 4). Similarly of the 102 asymptomatic patients, 4 (3.9%) had IgG antibody on day 7 with the geometric mean titers of 1:6400 (range = 1:3639-1:11246).

On day 14, 29 of the 102 asymptomatic patients (28.4%) had detectable IgG anti-horse gamma globulin with the geometric mean titer of 1:4263 (range = 1:1276-1:14190). The 2 serum sickness patients had titers of 1:3200 and 1:12800 (geometric mean titer 1:6400) (Figure 4). The one serum sickness patients who had IgG

antibody on day 7 of 1:800 developed an antibody titer of 1:12800 on day 14.

Because there were only 2 patients in our prospective study developed serum sickness, stored sera from a group of 27 serum sickness patients who were referred to our group for diagnosis and treatment of clinical serum sickness were also included in the study in order to make the serum sickness group large enough for statistical analysis. Twenty of these presented on day ±7 (range 5-10 days) after the administration of equine serum and 12 returned for follow-up on day 14 (60% follow up rate). Another 7 patients first presented with symptoms and sign of serum sickness on day ±14 (range 12-16 days). Therefore, including the 2 patients from the prospective study, a total of 29 serum sickness patients were included in this study.

As shown in Table 11, of the 102 asymptomatic patients, 4 (3.9%) had IgG anti-horse gamma globulin on day 7 with the geometric mean titer of 1:6400 (range = 1:3639-1:11246). 21 of the 22 (95.5%) serum sickness patients had IgG antibody with the geometric mean titer of 1:6192 (range = 1:843-1:45081) on day 7 (Figure 5).

On day 14, 29 of 102 (28.4%) asymptomatic patients had IgG anti-horse gamma globulin with the geometric mean titer of 1:4263 (range = 1:1276-1:14190). All of the 21

serum sickness patients (100%) had IgG antibody with the geometric mean titer of 1:7802 (range = 1:1914-1:31783). (Table 12, Figure 5)

The correlation between IgG anti-horse gamma globulin and serum sickness was statistically significant ($P<0.05$) by Fisher's exact test either on day 7 (Table 11) or day 14 (Table 12).

When the antibody titers of this special set of 14 serum sickness patients who had paired sera (day ± 7 and ± 14) were analysed, it was found that the IgG antibody on day ± 14 was not significantly higher than that on day ± 7 ($P > 0.05$) by paired t-test (table 13).

Since the titers of IgG anti-horse gamma globulin from the assay of 29 serum sickness and asymptomatic patients are overlapping as shown in Figure 4 and Figure 5 respectively, various cut off levels for the determination of IgG anti-horse gamma globulin were analysed. On day 7, when the titer of > 1:800 was used as the cut off level, the sensitivity, specificity, positive predictive value, negative predictive value and efficiency were 95.45%, 96.08%, 84.00%, 98.98% and 95.97% respectively (Table 14).

On day 14, when the titer of > 1:800 was used as the cut off level, the sensitivity, specificity, positive

predictive value, negative predictive value and efficiency were 100.00%, 71.56%, 42.00%, 100.00% and 76.42% respectively (Table 15).

6. The detection of IgM anti-horse gamma globulin

Any IgM anti-horse gamma globulin titers of $\geq$ 1:25 was considered positive as described in Materials and Methods. Of the 104 patients under this study, none had IgM antibody on day 0 but three each had antibodies on day 7 and 14 (Figure 6). Two of the three patients who had IgM antibody on day 7 were the same ones who had antibody on day 14. All of the 3 positive sera on day 7 and 2 of the 3 positive sera on day 14 had antibody titer of 1:25, whereas the third positive serum on day 14 had a titer of 1:50 (Figure 6). Of the 2 serum sickness patients, one had no IgM antibody either on day 7 or day 14 whereas the other one had antibody only on day 7 (titer 1:25).

On day 7, 2 of the 102 patients had IgM antibody with geometric titer of 1:25. One of the 2 serum sickness patients had IgM antibody on day 7 with a titer of 1:25.

On day 14, 3 of the 102 patients had IgM anti-horse gamma globulin. Two of these 3 patients already had the antibody on day 7 whereas the other one first had antibody on day 14 with a titer of 1:25. The IgM antibody

titers on day 14 of the 2 patients who had had antibodies already on day 7 remained at the same titers as on day 7 in one patient (titer 1:25), the other had rising titer (titer 1:25 on day 7, titer 1:50 on day 14). The geometric mean titer of IgM anti-horse gamma globulin of the entire group of 3 patients was 1:31. None of the 2 serum sickness patients had IgM antibody on day 14.

Similar to the evaluation of IgG anti-horse gamma globulin, the IgM antibody assay was also evaluated in an additional set of 27 serum sickness patients who were not systematically followed from day 0 as described earlier, resulting in a total of 29 serum sickness patients in this study.

As shown in Table 16 and Figure 6, of the 102 asymptomatic patients, 2 (2%) had IgM anti-horse gamma globulin on day 7 with the geometric mean titer of 1:25. Eight of 22 serum sickness patients (36.4%) had IgM antibody against horse gamma globulin with the geometric mean titer of 1:65 (range = 1:19-1:220) on day 7 (Table 16, Figure 7).

On day 14, 3 of the 102 asymptomatic patients (2.9%) had IgM anti-horse gamma globulin with the geometric mean titer of 1:31 (range = 1:21-1:46) (Table

17, Fiqure 6). Four of 21 serum sickness patients (19%) had IgM antibody with the geometric mean titer of 1:42 (range = 1:26-1:66) (Table 17, Figure 7).

The correlation between IgM anti-horse gamma globulin and serum sickness was statistically significant ($P<0.05$) by Fisher's exact test both on day 7 (Table 16) or day 14 (Table 17).

When the clinical symptoms of serum sickness were used as gold standard for definitive diagnosis, the sensitivity, specificity, positive predictive value, negative predictive value and efficiency of the IgM anti-horse gamma globulin assay using the titer of > 1:25 as the cut off level were 36.36%, 98.04%, 80.00%, 87.71% and 87.09% respectively on day 7 and 19.05%, 97.06%, 57.14%, 85.34% and 83.74% respectively on day 14 (Table 18, Table 19).

Of the 14 serum sickness patients who had paired sera (day $\pm$7 and $\pm$14), it was found that 5 had IgM antibody on day 7 (35.7%) with the geometric mean titer of 1:33 but the antibody was absent on day 14 whereas there was one patient who first developed IgM antibody on day 14 (titer 1:25) (Figure 8).

7. The correlation between IgG and IgM anti-horse gamma globulin.

When the specific IgM and IgG antibodies were analysed in 14 serum sickness patients who had paired sera. It was found that IgG antibody was present in 13 of these 14 patients (92.9%) on day ± 7 (rang 5-10) whereas IgM antibody was present only in 5 of these patients (35.7%). One week later (i.e, day ± 14), all IgM antibody disappeared and non emerged whereas 6 patients had higher IgG antibody titers, 6 had lower IgG antibody titers and titers remain unchanged in 2 (Figure 9). Only one patient first developed IgM antibody on day 14.

8. The detection of IgE anti-horse gamma globulin

According to the criteria described in Material and Methods, all positive results at titers of ⩾ 1:5 were considered as having IgE anti-horse gamma globulin. Of the 131 patients, 15 (11.5%) had positive IgE antibodies prior to receiving ERIG.

Of the 131 patients, 19 (14.5%) had positive IgE skin tests whereas 108 (82.4%) and 4 (3.05%) patients had negative and boderlined results respectively (Table 20). Of the 19 skin test positive patients, 12 (63.2%) had IgE anti-horse gamma globulin by ELISA test with

geometric mean titer of 1:6.5 (range = 1:3.5-1:12.2) whereas 3 of 108 (2.8%) skin test negative patients had positive results with geometric mean of 1:5 (Figure 10, Table 20). All 4 patients with boderlined skin test had negative IgE anti-horse gamma globulin antibody (Table 20).

For statistically analysis, patients with boderlined skin tests were included in the group of skin test negative patients. When the results of skin tests were used as gold standard for definitive diagnosis, the sensitivity, specificity, positive predictive value and negative predictive value of the IgE anti-horse gamma globulin assay by ELISA were 63.15%, 97.32%, 80.00% and 93.96% respectively.

By Chi-square analysis, the correlation was found between skin test and ELISA test for IgE anti-horse gamma globulin (Table 21).

Anaphylaxis or type I allergic reaction did not occur in any of the skin test-positive or IgE-positive individuals following ERIG administration. This indicates that there is no clinical relevance for in vivo and in vitro IgE test.

Table 5 Optimal conditions of ELISA test for the detection of IgG anti-horse gamma globulin.

Steps of ELISA test	Concentration or dilution	Temperature (°C)	Reaction time
Coating of horse gamma globulin	5 ug/ml	37°C	1 hr
Reacting with tested sera	1:800	37°C	1 hr
Reacting with conjugate	1:10,000	37°C	1 hr
Color development	-	25°C	30 mins

Table 6 Optimal conditions of ELISA test for the detection of IgE anti-horse gamma globulin.

Steps of ELISA test	Concentration or dilution	Temperature (°C)	Reaction time
Coating of horse gamma globulin	40 ug/ml	37°C	2 hr
Reacting with tested sera	1:50	37°C	2 hr
Reacting with conjugate	1:500	37°C	2 hr
Color development	-	25°C	45 mins

Table 7 Optimal conditions of ELISA test for the detection of IgM anti-horse gamma globulin.

Steps of ELISA test	Concentration or dilution	Temperature (°C)	Reaction time
Coating of anti-human IgM	10 ug/ml	37°C	2 hrs
Reacting with tested sera	1:25	37°C	1 hr
Reacting with horse gamma globulin	20 ug/ml	37°C	2 hrs
Reacting with anti-horse IgG peroxidase conjugate	1:500	37°C	2 hrs
Color development	-	25°C	30 mins

Table 8 Precision analysis of ELISA test for detection of IgG anti-horse gamma globulin.

	OD of test sera	
	Positive	Negative
Within-plate (n = 20)		
Mean	1.925	0.186
SD	0.081	0.007
% CV	4.21	3.76
Between-plate (n = 20)		
Mean	1.856	0.191
SD	0.152	0.015
% CV	8.18	7.85

Table 9 Precision analysis of ELISA test for detection of IgM anti-horse gamma globulin.

	OD of test sera	
	Positive	Negative
Within-plate (n = 20)		
Mean	0.540	0.189
SD	0.031	0.011
% CV	5.74	5.82
Between-plate (n = 20)		
Mean	0.585	0.196
SD	0.052	0.018
% CV	8.88	9.18

Table 10 Precision analysis of ELISA test for detection of IgE anti-horse gamma globulin.

	OD of test sera	
	Positive	Negative
Within-plate (n = 20)		
Mean	0.591	0.017
SD	0.037	0.012
% CV	6.26	7.05
Between-plate (n = 20)		
Mean	0.610	0.160
SD	0.058	0.015
% CV	9.5	9.37

Table 11 Correlation of day ±7 IgG anti-horse gamma globulin on day 7 in serum sickness.

IgG anti-horse gamma globulin	Serum sickness		Total
	Yes	No	
Posiive	21	4	25
Negative	1	98	99
Total	22	102	124

$P < 0.05$

Table 12 Correlation of day ±14 IgG anti-horse gamma globulin and serum sickness.

IgG anti-horse gamma globulin	Serum sickness		Total
	Yes	No	
Posiive	21	29	50
Negative	0	73	73
Total	21	102	123

$P < 0.05$

Table 13 Anti-horse gamma globulin IgG antibodies in 14 serum sickness patients who had paired sera on day ±7 and day ±14.

Patient No. (a)	Antibody titer on	
	Day ±7	Day ±14
1 (9)	1:800	1:3200
2 (10)	1:1600	1:800
3 (7)	1:3200	1:1600
4 (7)	1:3200	1:1600
5 (8)	1:3200	1:800
6 (7)	1:3200	1:51200
7 (7)	1:3200	1:204800
8 (8)	1:6400	1:6400
9 (9)	1:12800	1:12800
10 (5)	1:12800	1:25600
11 (7)	1:25600	1:12800
12 (7)	1:51200	1:25600
13 (7)	< 1:800	1:12800
14 (7)	1:800	1:3200
GMT	1:4099	1:7425

(a) The number of days from the time that equine serum was given until the day that the first serum was obtained for the investigation of serum sickness symptoms. Although the numbers range from 5 - 10 days, they were arbitarily grouped into ±7 days. They were asked to return exactly on day 14 for paired serum specimens. Anyone who first came on day 12 onward, they were grouped as ± 14 days and no attempts were made to obtain follow-up sera.

Table 14 The validity of indirect ELISA for the determination of IgG anti-horse gamma globulin on day 7 evaluated at various cut off level titiers, using the clinical symptom and signs of serum sickness as the gold standards.

cut off titer	sensitivity	specificity	positive predictive value	negative predictive value	efficiency
1:800	95.45	96.08	84.0	98.99	95.96
1:1600	72.73	96.08	80.0	94.23	91.93
1:3200	68.18	96.08	78.95	93.33	91.13
1:6400	36.36	97.06	72.27	87.61	86.29
1:12800	31.82	99.02	87.50	87.07	87.10
1:25600	22.72	100.00	100.00	85.71	86.29
1:51200	18.18	100.00	100.00	85.00	85.48
1:102400	9.09	100.00	100.00	83.60	83.87
1:204800	9.09	100.00	100.00	83.60	83.87
1:409600	4.54	100.00	100.00	83.60	83.87
1:819200	4.54	100.00	100.00	82.92	83.06

Table 15 The validity of indirect ELISA for the determination of IgG anti-horse gamma globulin on day 14 evaluated at various cut off titers, using the clinical symptom and signs of serum sickness as the gold standards.

cut off titer	sensitivity	specificity	positive predictive value	negative predictive value	efficiency
1:800	100.00	71.56	42.00	100.00	76.42
1:1600	90.47	71.56	39.58	92.40	74.79
1:3200	76.19	83.33	48.48	94.44	82.11
1:6400	66.66	90.19	58.33	92.92	86.17
1:12800	52.38	92.15	57.89	90.38	85.36
1:25600	23.80	98.03	71.42	86.20	85.36
1:51200	9.50	98.03	50.00	84.03	82.92
1:102400	4.70	99.01	50.00	83.47	82.92
1:204800	4.70	99.01	50.00	83.47	82.92
1:409600	0.00	100.00	0.00	82.92	82.92
1:819200	0.00	100.00	0.00	82.92	82.92

Table 16 Correlation of IgM anti-horse gamma globulin and serum sickness on day 7.

IgM anti-horse gamma globulin	Serum sickness		Total
	Yes	No	
Posiive	8	2	10
Negative	14	100	114
Total	22	102	124

$P < 0.05$

Table 17 Correlation of IgM anti-horse gamma globulin and serum sickness on day 14.

IgM anti-horse gamma globulin	Serum sickness		Total
	Yes	No	
Posiive	4	3	7
Negative	17	99	116
Total	21	102	123

$P < 0.05$

Table 18 The validity of indirect ELISA for the determination of IgM anti-horse gamma globulin on day 7 evaluated at various cut off level titers, using the clinical symptom and signs of serum sickness as the gold standards.

cut off titer	sensitivity	specificity	positive predictive value	negative predictive value	efficiency
1:25	36.36	98.04	80.00	87.71	87.09
1:50	22.73	100.00	100.00	85.71	82.29
1:100	9.09	100.00	100.00	83.60	83.87
1:200	9.09	100.00	100.00	83.60	83.87
1:400	4.54	100.00	100.00	82.92	83.06
1:800	4.54	100.00	100.00	82.92	83.06

Table 19 The validity of indirect ELISA for the determination of IgM anti-horse gamma globulin on day 14 evaluated at various cut off level titers, using the clinical symptom and signs of serum sickness as the gold standards.

cut off titer	sensitivity	specificity	positive predictive value	negative predictive value	efficiency
1:25	19.05	97.06	57.14	85.34	83.74
1:50	9.52	99.01	66.66	84.17	83.74
1:100	4.76	100.00	100.00	83.60	83.74
1:200	0.00	100.00	0.00	82.93	83.74
1:400	0.00	100.00	0.00	82.93	83.74
1:800	0.00	100.00	0.00	82.93	83.74

Table 20 Specific IgE antibody detected by ELISA and skin test.

ELISA test for IgE antibody	Skin test			Total
	positive	negative	boderline	
Posiive	12	3	0	15
Negative	7	105	4	116
Total	19	108	4	131

Table 21 Correlation of specific IgE antibody detected by ELISA and skin test.

ELISA test for IgE antibody	Skin test		Total
	positive	negative	
Posiive	12	3	15
Negative	7	109	116
Total	19	112	131

$P < 0.05$

Figure 1 Inhibition of IgG anti-horse gamma globulin by horse gamma globulin.

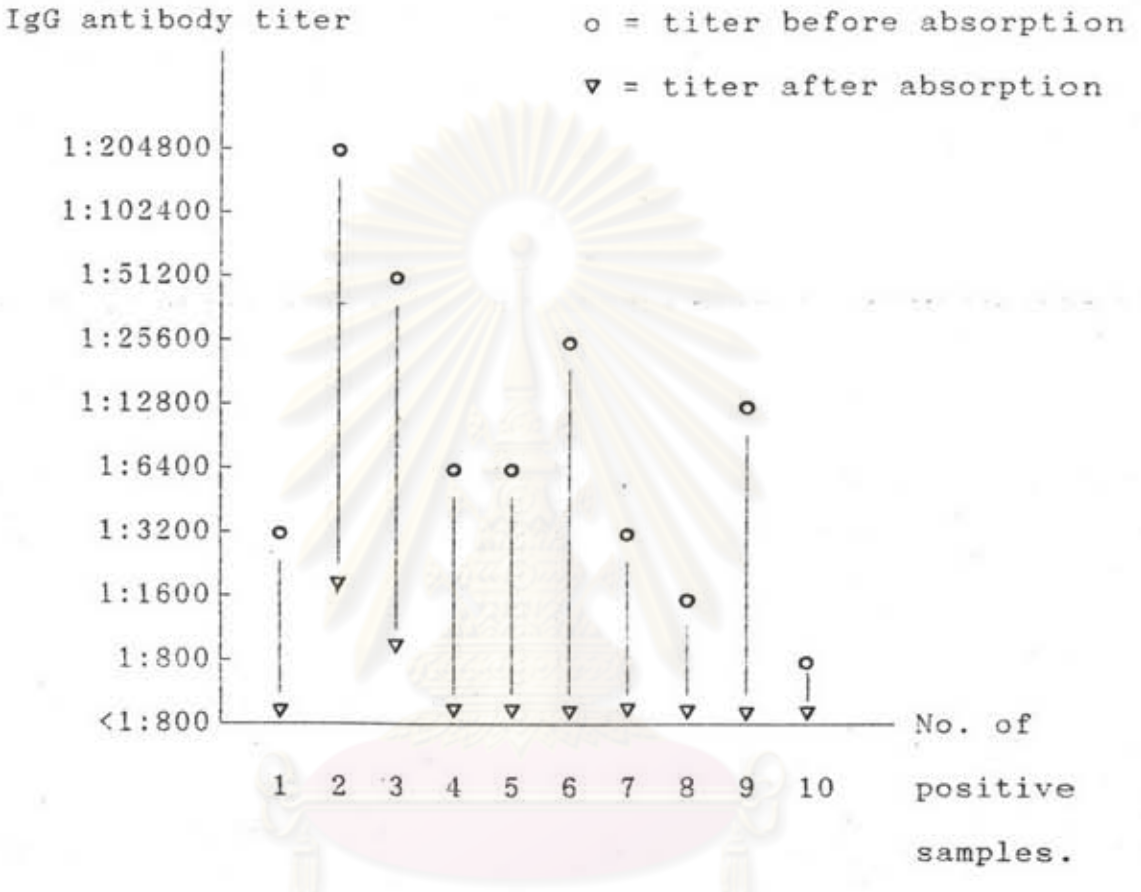


Figure 2 Inhibition of IgM anti-horse gamma globulin by horse gamma globulin.

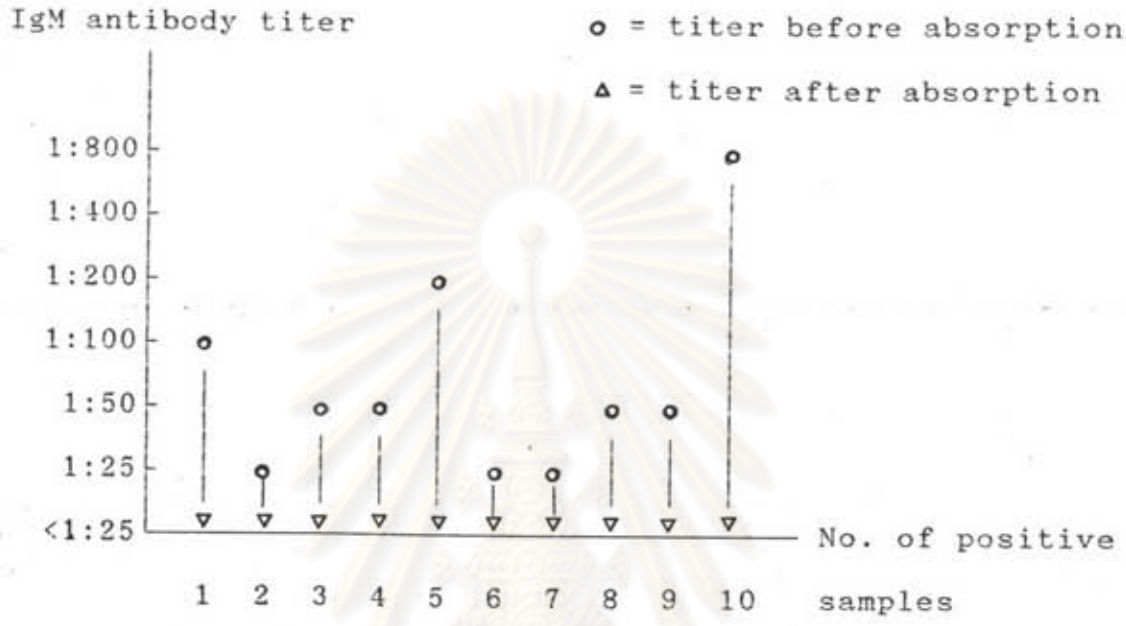


Figure 3 Inhibition of IgE anti-horse gamma globulin by horse gamma globulin.

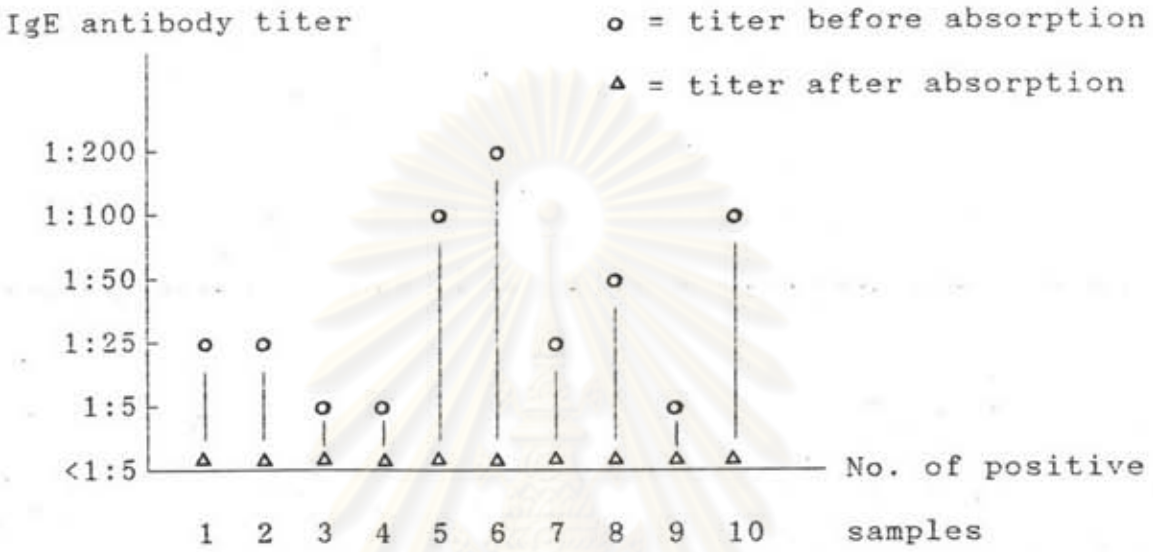
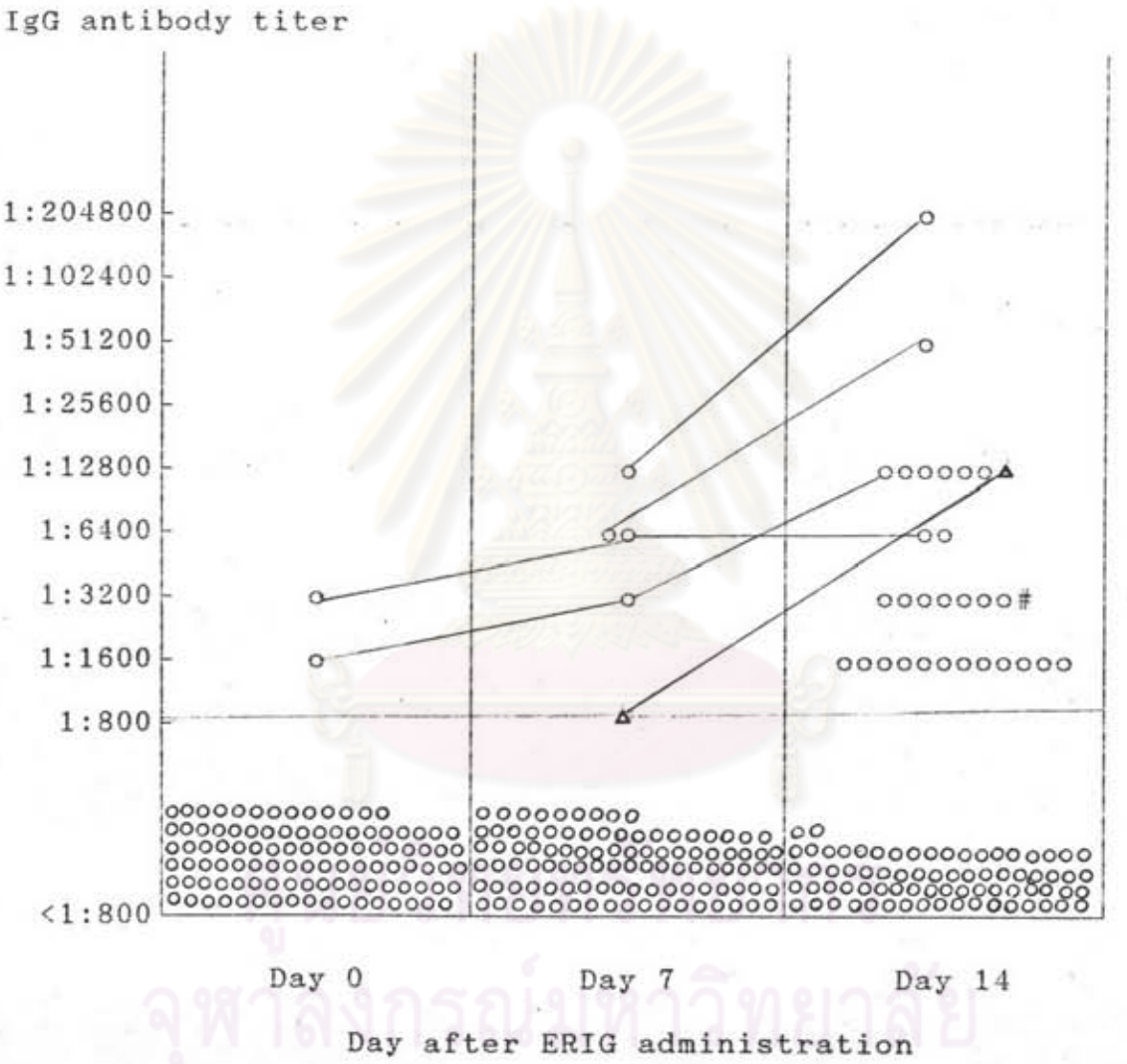


Figure 4 IgG anti-horse gamma globulin titers a total of 104 individuals before and 7 and 14 days after ERIG administration.

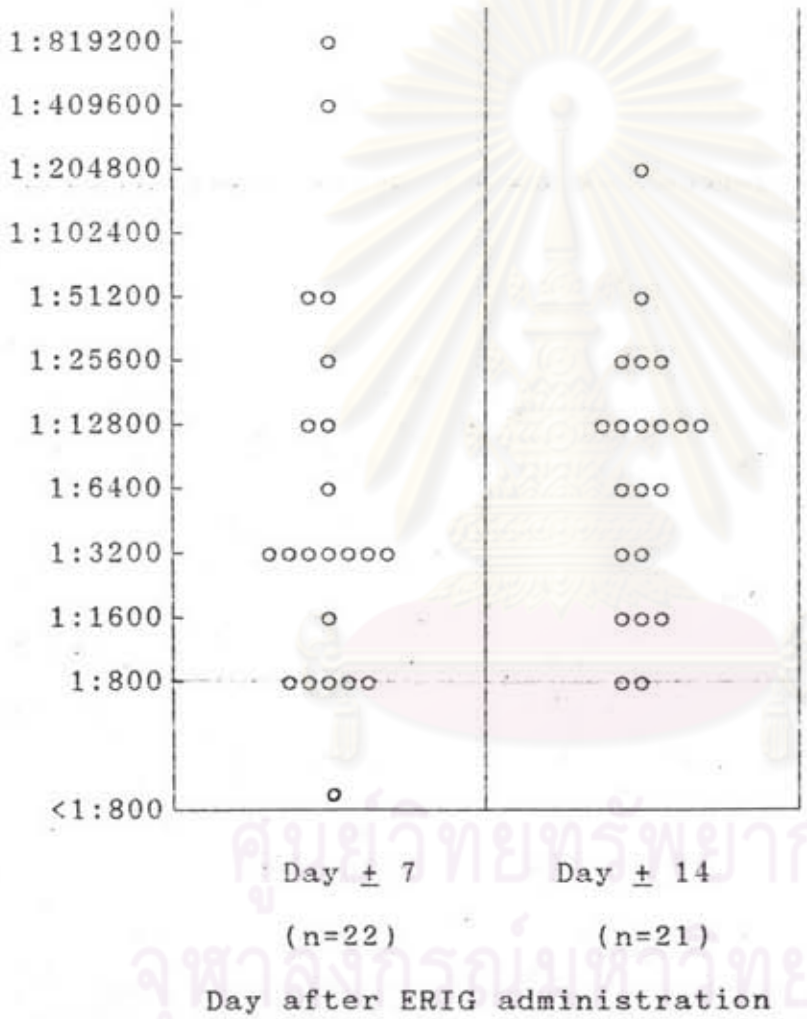


o Asymptomatic patients

Δ The first patient with serum sickness

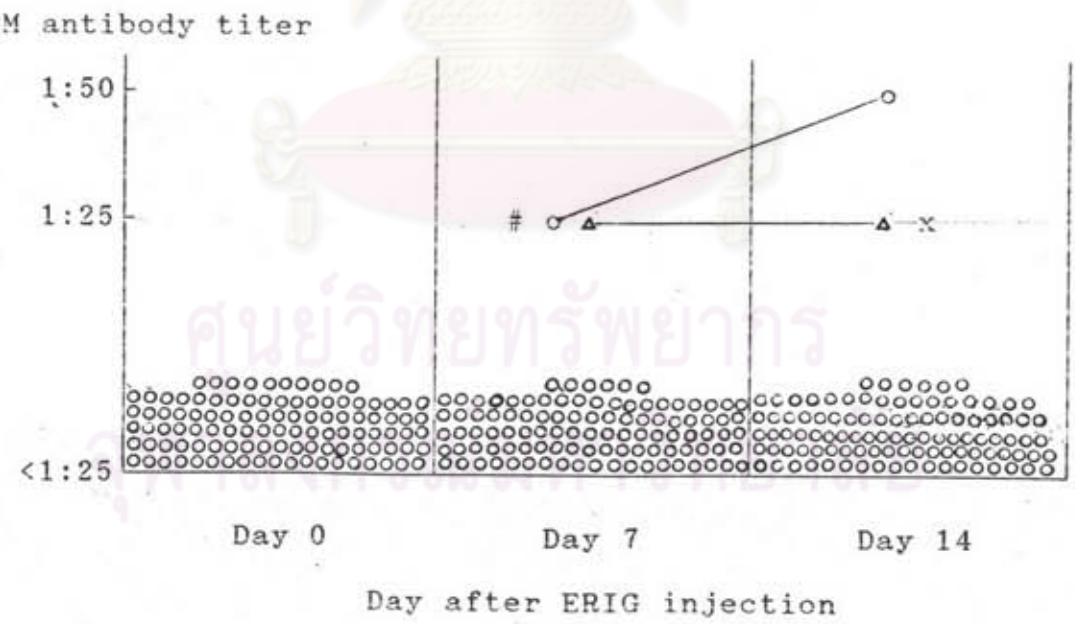
\# The second patient with serum sickness

Figure 5 IgG anti-horse gamma globulin titers in a total of 29 serum sickness patients* on day ±7 and ±14



NB : * of the total 29 patients, 2 were the patients that systematically followed from day 0 as described earlier and 27 were the serum sickness patients who were referred to our group for diagnosis and treatment of clinical serum sickness, of the 27 patient, 20 first showed up on day 5 - 10 (grouped as day ± 7), 12 also came on day 14 for the follow-up sera. In addition, another 7 patients first showed up on day 12-16, grouped into day ± 14.

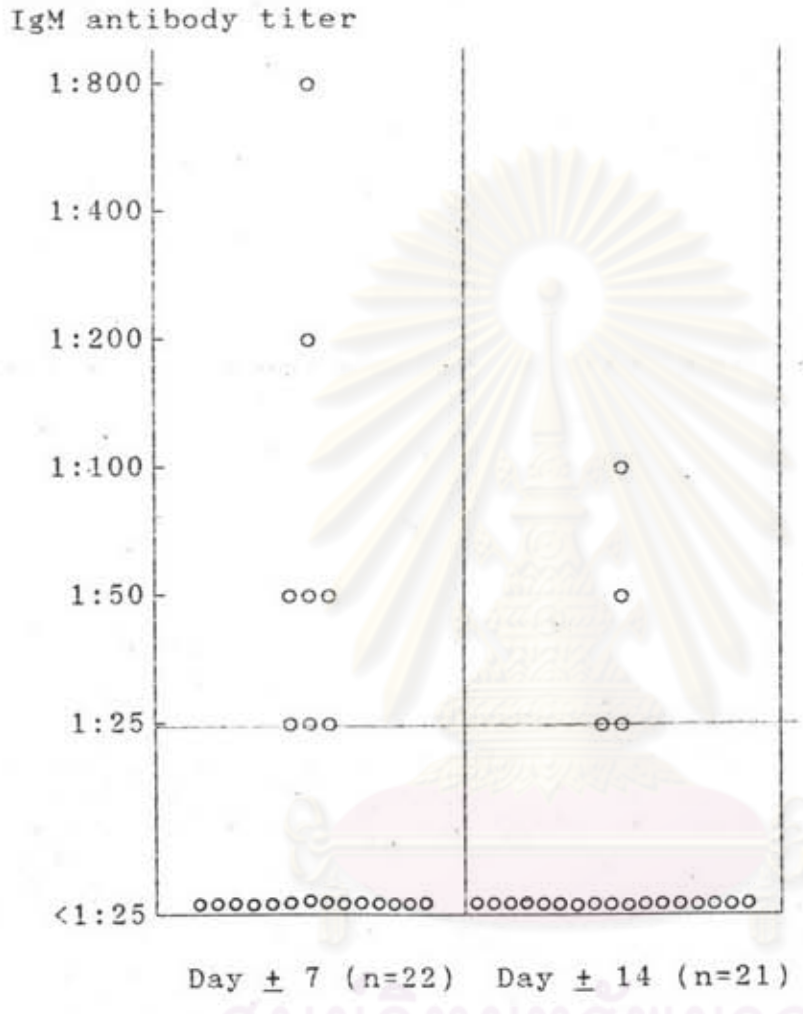
Figure 6 IgM anti-horse gamma globulin titers in a total of 104 patients before and 7 and 14 days after ERIG administration.



o
Δ
x
seropositive asymptomatic patients.

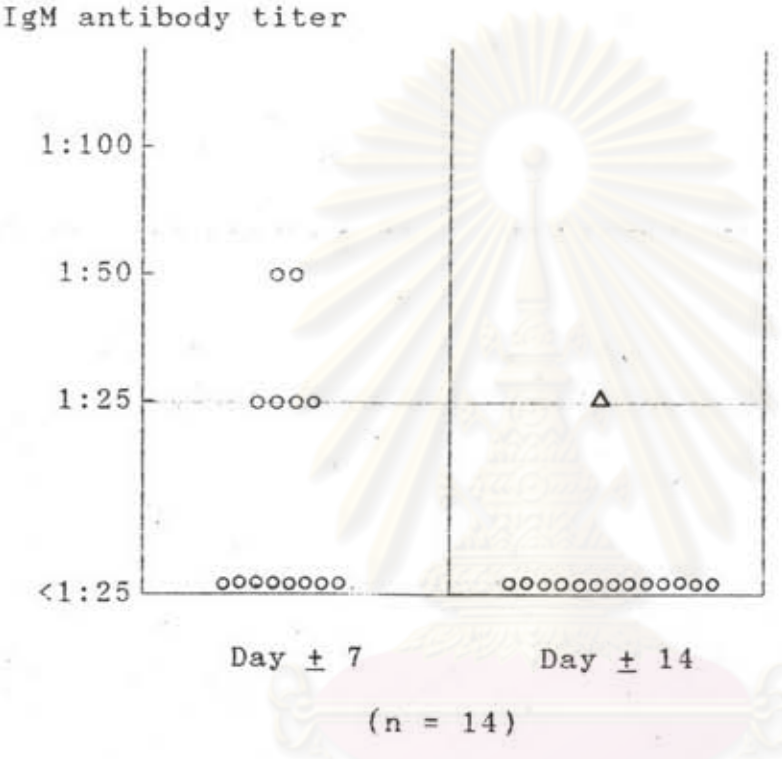
\# seropositive (serum sickness) patients

Figure 7 IgM anti-horse gamma globulin titers in a total of 29 serum sickness patients* on day ± 7 and ± 14



NB : * of the total 29 patients, 2 were the patients that systematically folloeed from day 0 as described earlier and 27 were the serum sickness patients who were referred to our group for diagnosis and treatment of clinical serum sickness. Off the 27 patients, 20 first showed up on day 5 - 10 (grouped as day ±7), 12 also came on day 14 for the follow-up sera. In addition, another 7 patients first showed up on day 12-16, grouped into day ± 14.

Figure 8 IgM anti-horse gamma globulin titers in a special set of 14 serum sickness patients who had paired sera (day ± 7 and ± 14).



o patients who had IgM antibody only on day 7

Δ patients who had IgM antibody only on day 14

Figure 9 Correlation between IgG and IgM anti-hores gamma globulin.

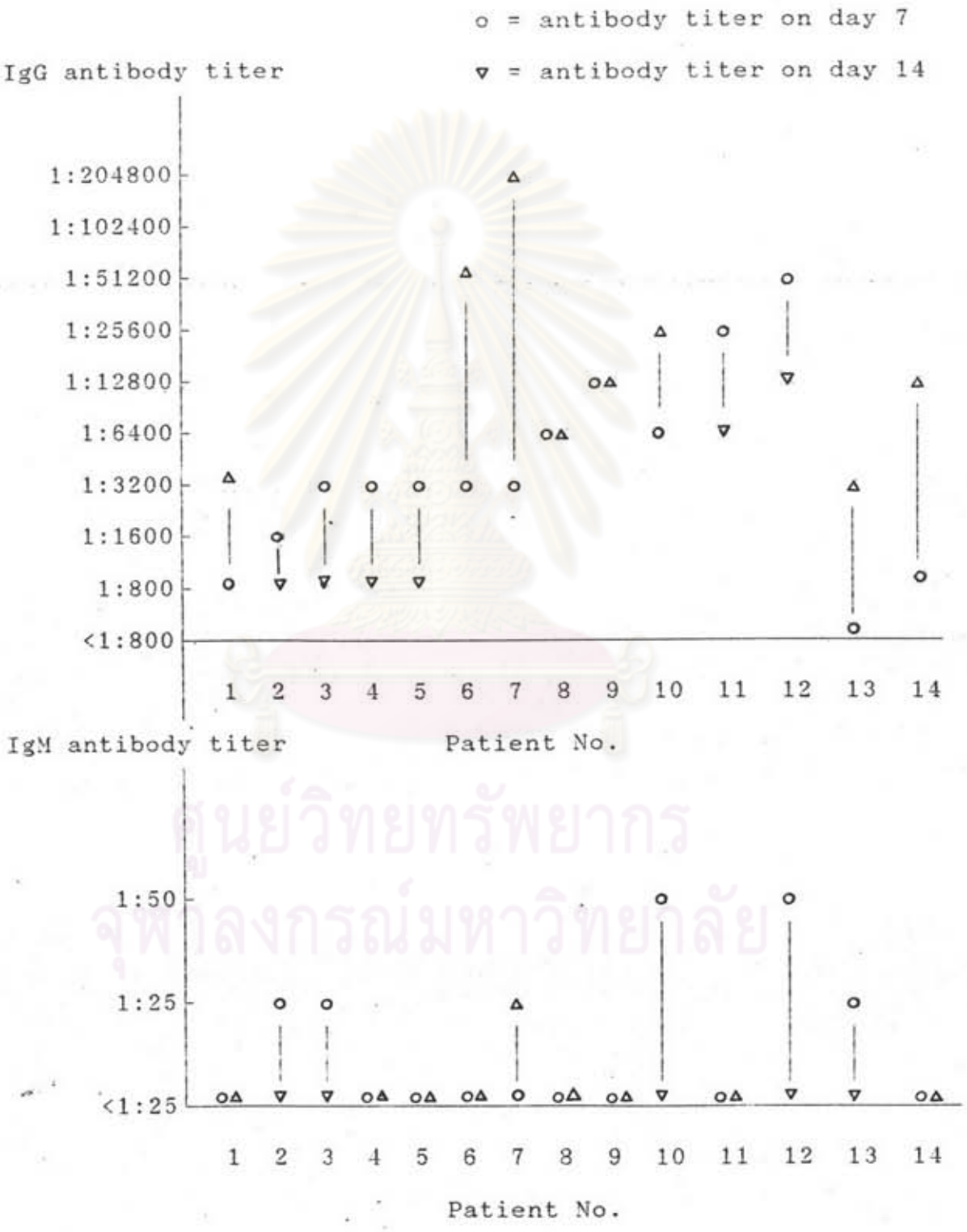


Figure 10 IgE anti-horse gamma globulin titers in a total of 131 individuals before ERIG administration.