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Investigation on the Immune Reactions of SARS-coV in BALB/c Suckling Mice

Introduction

The outbreaks of SARS in 2003 had caused global panic and attention. This communicable disease, which is induced by a new pathogenic agent, can produce atypical pneumonia in patients, and when serious, bring about symptoms such as immersive pneumonia and respiratory failure. It is more serious than the known atypical pneumonias caused by viruses or bacteria. It is, therefore, named Severe Acute Respiratory Syndrome (SARS)[1,2,3,4]. The World Health Organization issued the first global alert on March 12, 2003, and officially announced on April 16 that the pathogenic agent of SARS was a newly discovered coronavirus. The virus was then officially named the "SARS-coV". As the virus is a newly mutated one, there are no antibodies in humans for protection. Once infected, the virulence and pathogenicity could be serious. After some serious pathological changes in lungs such as fibrosis, the patient may die of respiratory failure. Cases were reported in Taiwan in March, and more in April. Some hospitals were forced to close, and several front-line medical and nursing staff in close contact with patients had died. For lack of and incorrect understanding of the disease, the

infection had caused upset and panic in the public. It had, however, been brought under control soon by the joint efforts of the government and the people.

The fear brought about SARS certainly is associated with its mortality. Early medical care and rescue of patients is a key to reducing mortality. Therefore, early, quick and accurate diagnosis is highly important. Though many laboratories are capable of detecting the existence of this virus, some of the testing methods are still under experiment, their specificity and sensitivity remain to be assessed. Some conventional testing methods are either time-consuming, or requiring more sophisticated laboratory facilities or skills, their restrictions in terms of time and operation may allow viruses to spread, and create problems for disease control. The present study intends to develop the use of enzyme-linked immunosorbent assay (ELISA) method on inactivated SARS-coV to test the changes of IgG and IgM in mice expected to produce antibody reactions, and at the same time, to assess the controls by neutralization test (NT), hoping to find out the best reaction conditions by this model for the testing of IgG in early SARS patients and IgM in their convalescence.

Materials and Methods

Preparation of SARS-coV

SARS-coV was cultured in large amount in Vero E6 cells. After some significant cytopathic effect (CPE) of cells, the virus fluid was freeze-thaw at -70℃ and 37℃ for three times, and centrifuged at 3,500 rpm for 30 minutes. The upper clear fluid was collected, quantified, and inactivated by cobalt 60 radiation. The inactivated virus fluid was tested twice by the neutralization test for stability, then placed at -70℃ for keeping. The inactivated virus was further concentrated and purified with the Centricon Plus-20 Centrifugal Filter

Devices. The virus fluid was placed in concentration tubes, centrifuged at 3,500 rpm for 10 minutes to remove protein of molecular weight smaller than 100 kd (such as BSA). The fluid on the filter membrane was the virus fluid after concentration. The filter and retentate cup were connected and placed upside down, centrifuged at a speed lower than 2,000 rpm for 10 minutes. The fluid in the retentate cup was the concentrated virus fluid.

Immunization of BALB/c Suckling Mice

Mice were coded M1-M6. Before initial immunization, blood of six mice was collected for negative controls. 0.2 ml $10^{7.5}$ $TCID_{50}$/50 μl of virus and Freund's adjuvant were mixed evenly in two-way syringe. Three BALB/c suckling mice each was immunized subcutaneously and abdominally (M1-M3 for subcutaneous immunization, and M4-M6 for abdominal immunization). On the 14^{th} and 28^{th} days, boosters were given and blood was collected for antibody testing. Time for follow-up boosters was decided by the changes of titers. Blood was collected again for testing on the 49^{th} day. Boosters were given again on the 56^{th} and 63^{rd} days. Blood was collected again on the 73^{rd} and 60^{th} days for testing (see Figure 1 for details). On the second booster in the process of immunization, the adjuvant was changed to lipopolysacchatride (from Salmonella Minnesota) 50 μg/mouse. Each blood specimen collected was placed for a while, and then centrifuged to collect serum. It was tested by NT and ELISA methods for changes in antibody titers.

Neutralization Test [5-8]

All serum specimens were tested by NT to decide their neutralization antibody titers. Serum specimens were diluted by 1:8 (added 700 μl of PBS to 100 μl of serum), placed in 56℃ water bath for heating for 30 minutes, put in the 96-microwell culture plate for two-fold dilution, added SARS-coV fluid,

placed in CO_2 incubator box at 36℃ for one hour. It was removed, added cell suspension ($2.5x10^4$ cell/100 μl), observed the cytopathic effect under microscopes everyday, and decided on its serum neutralization antibody titers on the 5th day.

ELISA IgM and IgG Testing

Virus ($10^{4.2}$ $TCID_{50}$/50 μl) was absorbed in 96 microwell immunoassay strips, placed at 4℃ overnight, washed four times with phosphate buffered saline-Tween20 (PBST), added 50 μl of diluted serum for testing, placed in a 37℃ incubator for one hour, washed four times and added 100 μl of 4,000-fold diluted horseradish peroxidase (HRP) conjugated goat anti-mouse IgG and IgM (Chemicon, California, USA), placed at 37℃ for one hour, washed and added 100 μl of TMB/E substrate (Chemicon, California, USA), placed by avoiding light under room temperature for 30 minutes, and terminated reaction by 2N sulphuric acid (H_2SO_4). At 450nm, OD was measured with EIA reader. In the testing of IgM and IgG, cell culture fluid without virus was used as controls.

Results

Reactions of BALB/c Suckling Mice to Virus

Mice either immunized subcutaneously or abdominally showed in terms of mobility and appearance no significant difference from regular mice, suggesting that SARS-coV did not induce disease in mice. However, with increase in boosters, mice M2, M4 and M5 died on the 4th booster (M5 on the night, and M2 and M4 the day after). The three mice showed before death symptoms of spasm, reduced mobility and backward hair. The other three mice showed on the 4th booster slightly reduced mobility and loss of appetite but soon recovered in a few days.

The Neutralization Test

NT reactions of all mice are shown in Figure 2. Before immunization, the neutralization antibody titers were all <1:8. Two weeks later, with the exception of two mice that showed slight increase (1:16), no changes were seen in other mice. Significant increase (1:64 for M1 and M4, and 1:128 for the rest) was noted after the first booster four weeks later. After the second booster on the 49th day, no significant changes in antibody titers were noted; and in some mice, the titers even declined, though that of the M1 continued to increase to 1:1024. Booster was given on the 56th day, and NT titers began to rise on the 63rd, 73rd and 80th days. Three mice had died. The NT titers of the rest three mice were 1:1024 (M1), and 1:512 (M3 and M6).

ELISA IgM and IgG

The checkerboard titration was used to decide the optimum dilution of serum specimens, SARS-coV and HRP-conjugated goat anti-mouse IgG and IgM as 1:100, $10^{4.2}$ $TCID_{50}$/50 μl and 1:4000. Specimens were decided positive when their test values were four-times higher than the negative controls and twice higher than the culture fluid controls. Figures 3 and 4 show the final IgM and IgG titers of blood collected at different times. Of the six mice, except that M6 showed slower IgG reaction, the rest mice showed significant increase in antibodies either IgM or IgG on the 14th day after the first immunization; and IgM titers were higher than IgG. Except M6, IgM could still be detected in the sera of the rest five mice even they were diluted 800 times. Results of blood collected at different times corresponded to the results of the neutralization test. The IgM and IgG reactions on the 49th day, for instance, showed decline or stagnation. After boosters, IgM and IgG antibodies significantly went up again on the 63rd, 73rd and 80th days.

When IgM and IgG antibodies were compared, they were somewhat different in titers. IgM appeared earlier than IgG, and was also higher in titer. On the last day of blood collection (the 80th day) for instance, the IgM in three mice was 2-8 times higher than the IgG; and the serum of M1 showed, even after 102,400 times dilution, positive reaction of ELISA IgM.

Discussion

There are several laboratory diagnostic methods for SARS-CoV detection. An RT-PCR method[7,8] can detect the virus RNA at the early stage of infection in a relatively short time. It was the major method used in Taiwan during the 2003 SARS outbreak. Although the method can detect the existence of the virus in an early stage of infection, it is likely to produce false positive results. On top of that, the collection of throat swabs needed for such testing can be hazardous for the collector is risking to be infected. Also, an ELISA[9] and a NT method are there for testing antibodies in the patient's blood sample. Since no monoclonal antibody has yet been developed, the current ELISA test focuses primarily on IgG. However, IgG appears late in the patient and is detectable only in a later stage of infection, thus this method is not applicable for early diagnosis. The NT method, on the other hand, is highly specific and uses live virus for the detection. Laboratory work involved is relatively dangerous and may only be carried out in a P3 level laboratory. The method is quite complicated and takes a longer time to perform; so it is not suitable for a situation where a large number of specimens are to be tested, or for an early screening job.

ELISA is a commonly used laboratory method for assessment of virus infection. It is sensitive and swift to get result. Subjective errors are minimized because the data are read through machines. It can also handle a large number of specimens all at once. Many kinds of quick reagents have

been developed for use[10]. The ELISA reagent we used in this laboratory for SARS testing is by courtesy of the US CDC. The operational procedures and final readings of the reagent are rather complicated. It cannot differentiate the IgM and IgG either. In the course of the present study, the procedure was slightly modified to assess the testing method by the expected outcomes of immune reactions and antibodies produced in mice immunized with the same virus in the hope that the method could be used for the testing of serum specimens of SARS patients.

The experiment procedure was so designed that it follows the model of animals producing immune reactions. Initially, the immune reactions in suckling mice were induced by repeated injections of highly concentrated inactivated viruses. Such boosters were to be temporarily held when the antibody concentration in mice had shown great increases. A secondary booster session began when the antibodies started to show some decline. Such procedure was planned for two purposes. Firstly, viruses could be destroyed if they were introduced at a time when the antibody concentration in mice was extremely high. More boosters would have been meaningless and the viruses wasted. Secondly, it was known that stronger immune reactions could be induced in mice if boosters were given later, particularly after antibody activities have shown some drops.

In the present study, we noticed by serum testing on the 28th day that antibody titers had increased significantly, and decided not to give more boosters to the six immunized mice starting the next day, for reasons mentioned above. Three weeks later (on the 49th day), blood was collected again for observation and comparison of antibody titers. As expected, antibodies had either stagnated or declined slightly. The NT antibody titer of M1 was still high, whereas the antibodies of M3 and M6 were still increasing

slightly. It was decided to wait for one more week till the 56th day, when the second stage booster (booster 3) was applied. Another booster (booster 4) was carried out a week later. Booster 4 resulted in the death of M2, M4 and M5. Readings from ELISA showed that the increase in the second antibody titers of the three dead mice on the 49th and 63rd days were much higher than those of the three surviving mice, particularly M5 that died in the night of the booster. Their IgM and IgG antibodies titers had increased in two weeks 64 times (from 1,600 to 102,400) and 16 times (from 1,600 to 25,600), respectively, suggesting that the sudden and excess immune reactions in mice could be the reasons for their untimely death. In NT antibody detections, with poorer sensitivities relative to ELISA, no such extensive changes were noticed. Though the rest three mice survived the ordeal, their appearance and mobility were rather abnormal after the booster given on the 63rd day. They recovered, however, a few days later. For fear that further boosters would eventually kill them, and their antibody reactions on the 80th day (either NT or ELISA) were satisfactory, booster was no longer given and our focus was placed on the development of monoclonal antibody.

Findings of the present study showed that both NT and ELISA could effectively detect antibodies in mice, and the changes in antibody titers were as expected. In NT, the neutralization antibodies in many mice did not appear yet on the 14th day of immunization, but increase significantly only after the 28th day. For ELISA, IgM increased significantly on the 14th day. Except M6, IgM in the other five mice still showed positive results even after the sera were diluted 800 times. Antibodies were still detectable on the 28th and 49th days even when the sera were diluted 3,200 times. As to the IgG antibodies, it appeared later than IgM, and the titers were not as high. By comparing the NT and ELISA, it was noted that ELISA could be more useful

in detecting antibodies at the early stage of the infection, and even when the specimens were greatly diluted in the first place (in the case of the antibody titers of the last serum in M1, for instance, the IgG ELISA and IgM ELISA were 50 and 100 times higher, respectively, than those of the NT data). ELISA was found to be with much higher sensitivity than NT. It could detect antibodies of relatively low concentrations; it could detect the existence of antibodies when other serological methods failed.

The non-specific reaction is the most disturbing problem with ELISA testing[11,12]. As the monoclonal antibody of SARS-coV has not been developed, it is not yet possible to design -coV reagents by the antigen or antibody-capture ELISA; such indirect reaction is used to detect the existence of antibodies. The purity of the pre-coated antigen should affect the specificity of the total reaction. The antigens used in the present study, which were processed with Centricon Plus-20 Centrifugal Filter Devices, were found to be capable of effectively reducing the non-specific reaction caused by BSA.

The ELISA method developed in the present study takes about 2.5 hours to perform. It effectively reduced the time required for SARS-coV antibody testing. The method is currently used for the testing and assessment of the sera of SARS patients, and, at the same time, for the development of positive values for screening using serum specimens of healthy groups. The quick test reagent so developed should be helpful in the mass screening of serum specimens in the future for early detection and treatment of SARS patients.

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References

1.Tan YM, Chow PK, Soo KC: Severe acute respiratory syndrome: clinical outcome after inpatient outbreak of SARS in Singapore. *BMJ* 2003, 326:1394.

2.Wong RS, Wu A, To KF, *et al*. Haematological manifestations in patients with severe acute respiratory syndrome: retrospective analysis. *BMJ* 2003, 326: 1358-1362.

3.Rainer TH, Cameron PA, Smit D, *et al.* Evaluation of WHO criteria for identifying patients with severe acute respiratory syndrome out of hospital: prospective observational study. *BMJ*. 2003,326:1354-1358.

4.Nicholls JM, Poon LL, Lee KC: Lung pathology of fatal severe acute respiratory syndrome. *Lancet*. 2003,361:1773-17788.

5.Schmidt N-J and Lennette E-H. Advances in the serodiagnosis of viral infections. *Prog Med Virol.* 1973,15:244-308.

6.Hsiung G-D. Diagnostic virology; An Illustrated Handbook. Revised and Enlarged Edition. New Haven and London, Yale University Press, 1973, pp. 62-64.

7.Peiris JS, Lai ST, Poon LL: Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 2003, 361:1319-1325.

8.Yang J, Wang ZH, Chen JJ, *et al.* Clinical detection of polymerase gene of SARS-associated coronavirus Di Yi Jun Yi Da Xue Xue Bao 2003, 23(5):424-427

9.WHO Multicentre Collaborative Networks for Severe Acute Respiratory Syndrome (SARS) diagnosis. *Wkly Epidemiol Rec* 2003, 78(15): 121-122.

10.Bidwell D-E, Buck A-A. The enzyme-linked immunosorbent assay. *Bull WHO* 1976, 54:129-139.

11.Frazier CL, Shope RE: Detection of antibodies to alphaviruses by Enzyme-linked immunosorbent assay. *J Clin Microbiol* 1979, 10: 583-585.

12.Chow L, Tseng TC. Detection of serum antibody to herpes simplex virus type I by Enzyme-linked immunosorbent assay. *Clin J Microbiol Immunol* 1981, 14:46-53.

Figure 1. Schedule of Immunization and Blood Collection of Six Mice
Day

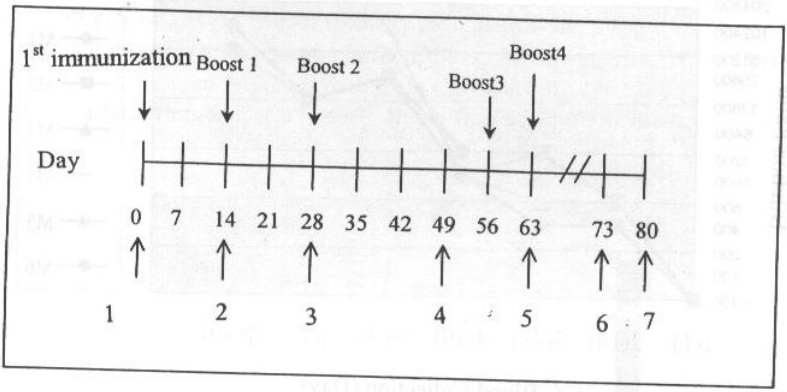

Figure 2. Neutralization Antibody Titers in Sera of Six Mice at Different Times

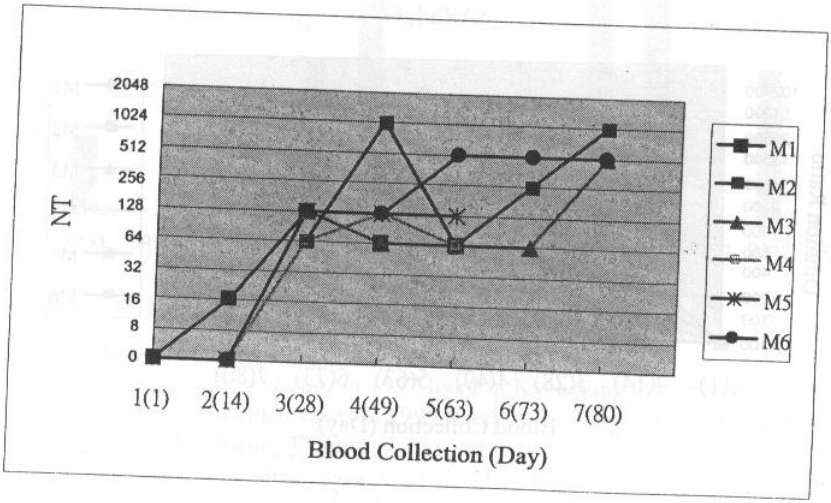

Figure 3. ELISA IgM Titers in sera of six Mice at Different Times

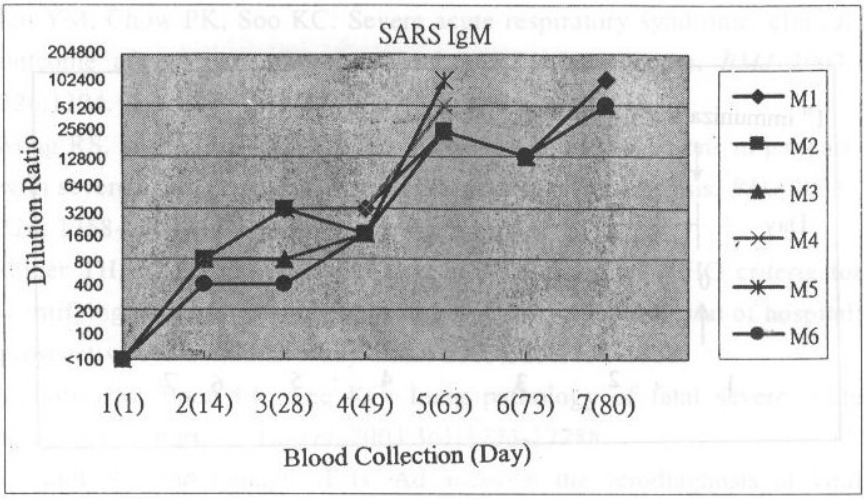

Figure 4. ELISA IgG Titers in sera of six Mice at Different Times