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ANTITOXOPLASMIC EFFECT OF IMMUNE SWINE  
SERUM REVEALED IN  
THE CULTURE OF SWINE LEUKOCYTES\*

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INTRODUCTION

Animals recovered from the infection of *Toxoplasma gondii* are known to be immune against the parasites. SABIN and OLITZKY found a neutralizing antibody in the serum of *Macacrus rhesus* inoculated with *T. gondii*. The finding was extended by SABIN, SABIN and RUCHMAN. They described a method of titration of the neutralizing antibody, and noted that this antibody is so labile even at a temperature of about 5°C, that it could disappear after one to 2 weeks of storage in an ordinary refrigerator. Also they observed that no neutralizing antibody was found in the fresh sera of normal rabbits, mice, cats, or dogs, nor did these animals develop any appreciable antibody after recovery from experimental infection with toxoplasms. SABIN and FELDMAN found that the cytoplasm of the parasites lost their affinity to methylen blue when they are exposed to the homologous immune serum. The reaction was found to occur with the aid of human serum, so-called accessory factor. This type of antibody, called cytoplasm-modifying antibody, was considered by them to correspond to the neutralizing antibody. It was found in rats by RUCHMAN and FOWLER, and in pigeons by JACOBS et al. that parasitemia following the inoculation of *T. gondii* usually disappeared when the cytoplasm-modifying antibody developed in the blood. These findings suggest that immunity in toxoplasmosis is largely dependent upon humoral factors.

Little is known, however, in toxoplasmosis, about the possibility of passive immunity. According to JACOB's statement, passive immunization of rabbit against toxoplasmosis was negative. LEVADITI who could not prove antiparasitic effect in infected rabbit serum, considered that immunity against toxoplasms would be cellular in character.

VISCHER and SUTER showed that macrophages cultured *in vitro* from rats actively immunized by injection with live toxoplasms had an inhibitory effect on

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the intracellular multiplication of these parasites. This effect was increased by the presence of immune serum showing a high titer of cytoplasm-modifying antibody.

*In vitro* experiment such as done by VISCHER and SUTER will be useful for the study of the mechanisms of immunity in toxoplasmosis. With this eventual aim, an attempt has been made by the present authors to study *in vitro* the effect of homologous immune serum on toxoplasms, employing leukocytes and sera from pigs. Pigs are known to be susceptible to oral inoculation of toxoplasms, survive the infection, and easily become immune to toxoplasmosis. This paper deals with the results of the experiments.

#### MATERIALS AND METHODS

*T. gondii* strain RH, originally isolated from a child by SABIN, was provided in 1957 by Y. TSUNEMATSU, Institute of Infectious Diseases, University of Tokyo. In the present study 1665 to 1720th passages of this strain through mice were used.

Two castrated Yorkshire piglets, purchased when 1.5 to 2 months old, were fed respectively 3 flayed mice inoculated peritoneally 3 days previously with approximately half a million parasites. Another piglet of the same age was kept as a normal control. The inoculated animals had fever several days after the feeding, when parasitemia was proved in one of them. Cytoplasm-modifying antibodies appeared soon after the febrile stage and the titer rose thereafter. Thirty-three days after the initial oral infection, these piglets were given boosters subcutaneously with peritoneal fluid from 1 or 2 infected mice, 3 times at 7 day intervals, in order to obtain highly immunized serum. Samples of serum from these immunized pigs were obtained by cardiac puncture on various days following immunization. The sera were inactivated at 56°C for 30 minutes and stored at -20°C. Normal serum was obtained from a normal pig in a slaughter house; it was inactivated and stored likewise. Cytoplasm-modifying antibody titer of the normal serum sample was less than 1:2.

For the cultivation of swine leukocytes, the procedure reported by SUTER, and VISCHER and SUTER was applied with a slight modification. Twenty ml of the blood obtained from normal and immune pigs by cardiac puncture were mixed with 4 ml of 3% sodium citrate. The citrated blood was centrifuged at 2,000 r.p.m. for 10 minutes. Supernatant plasma and the fragment of buffy coat was withdrawn, to the mixture of the two were added 2 or 3 drops of phytohemagglutinin (Difco). After leaving 5 minutes in room temperature, the mixture of the buffy coat and plasma was slightly centrifuged, 500 to 700 r.p.m. for 3 minutes, for separating excessive red cells. The upper leukocytes portion was withdrawn and washed twice by centrifuging with phosphate buffer solution (pH 7.0). The washed leukocytes were resuspended in 2 ml of the same solution. Leukocytes in the suspension were counted, and resuspended in HANKS's balanced salt solution to which was added equal amount of serum from immunized or normal pigs. The number of leukocytes in the medium was adjusted using a hemocytometer to obtain 3 to  $5 \times 10^6$  per ml. PH of the serum medium was previously adjusted, using CO<sub>2</sub> gas, to 7.0. To the medium were added for the final concentration 200 unit penicillin and 200  $\mu$ g streptomycin per ml. Thus, leukocytes from immunized and normal pigs were suspended separately in immune and normal swine serum media. They were placed

in ice water before toxoplasms were inoculated to them. Mice infected with toxoplasms 3 days in advance as described above were injected in the peritoneal cavity with 2 ml of saline solution containing 1 : 20,000 heparin (Novo Industri A/S). The mixture of ascitic fluid and saline solution was withdrawn aseptically. This suspension was washed twice with HANKS's solution to which was added 1% normal swine serum. Pipetting was done more than 30 times to free the intracellular toxoplasms. The washed toxoplasms were counted in a hemocytometer, the number was adjusted to approximate in the final culture a proportion of one toxoplasma per 4 leukocytes. To aliquots of 9 parts of cell suspension in either immune or normal serum medium were added 1 part of toxoplasma suspension. The mixture, after thorough mixing by pipetting, was poured into small petri dishes, which had 5 or more small coverslips on the bottom. The petri dishes were incubated at 37°C for 2 hours. Each coverslip was then removed, washed in phosphate buffer solution, covered with a thin film of formvar, and finally placed in a small screw-capped tube containing 1 ml of the corresponding serum medium. The tubes were closed airtight and stood vertically in the incubator. They were gently shaken 3 times a day in order to diffuse the nutrient fluid, which was renewed every other day. Control cells of each group which were not inoculated with toxoplasms were also cultivated likewise. Coverslips were taken out at the 6th hour of incubation and then at 24 hour intervals. The cultures were fixed in absolute methyl alcohol for 10 minutes. After drying, the coverslips were placed in ethylene dichloride for at least one hour to dissolve the formvar. The preparation was then washed in water, stained in Giemsa solution, and mounted on slide. Four hundred leukocytes, which were separated from each other and were not in clusters, were examined in the fields of various parts of each coverslip. Toxoplasms parasitised in these cells were counted.

## RESULTS

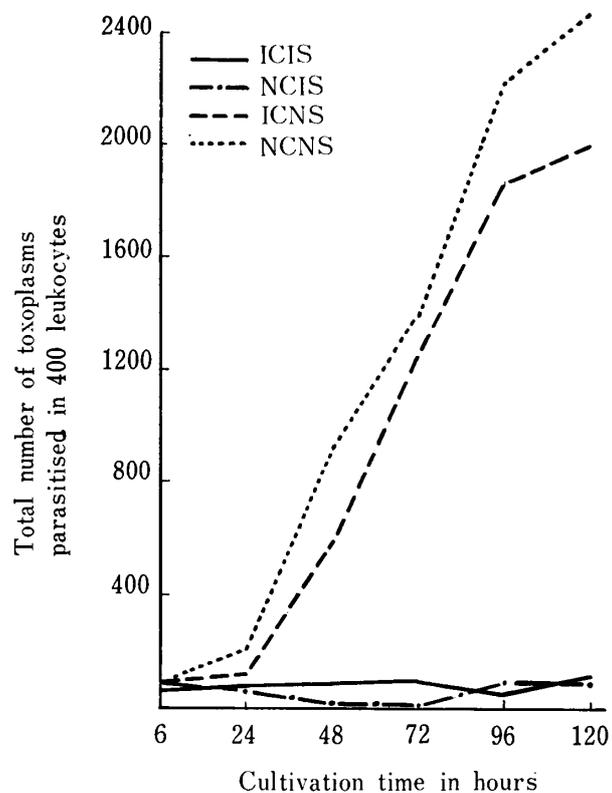
As shown in fig. 1, multiplication of toxoplasms was inhibited when leukocytes were brought into contact with the parasites in the presence of hyper-immune serum. The inhibition continued throughout 120 hours, to the termination of this experiment. Inoculated toxoplasms were found not penetrated into the cytoplasm of leukocytes. There was little difference in the inhibition of the multiplication of toxoplasms by immune serum between the culture of the leukocytes from immunized pig and that from normal pig.

Multiplication of toxoplasms was obvious, on the other hand, when leukocytes were caused to come into contact with toxoplasms in the presence of normal serum. The parasites multiplied with the passage of elapsed time. The rate of the multiplication was, however, a little slower in the culture of the leukocytes from immunized pig than in that from normal pig, as shown in the same figure.

The growth of leukocytes was obvious in immune serum, while the growth was poor in normal serum, due to the multiplication of toxoplasms (figs. 3 to 6).

The proportion of the infected leukocytes (including leukocytes to which toxoplasms adhered) was, at the 6th hour after infection, 12 and 10% in the leukocytes from immunized and normal pigs respectively, both cultivated in normal serum. The proportion was increased and, at the 120th hour, attained to 48 and 57% respectively. In immune serum, on the contrary, the proportion of the infected leukocytes at the 6th hour, which was 9 and 11%

FIG. 1. *Multiplication of Toxoplasms Which Were Inoculated to the Cultures of Leukocytes*



Notes :

- Leukocytes from immunized pig (IC) cultivated in immune serum (IS).
- · — · Leukocytes from normal pig (NC) cultivated in immune serum (IS).
- Leukocytes from immunized pig (IC) cultivated in normal serum (NS).
- ..... Leukocytes from normal pig (NC) cultivated in normal serum (NS).

Cytoplasm-modifying antibody titer of the immune serum was 1:1024.

Immunized pig which gave leukocytes showed the same antibody titer.

respectively in the cultures from immunized and normal pigs, did not increase but was decreased to 7 and 5% after 120 hours cultivation time.

Differential count with the blood smears from used pigs indicated that the great majority of the leukocytes consisted of lymphocytes (ranging from 52 to 78%) and granulocytes (25 to 45%). Monocytes were only 0.5 to 1%. It was found early in the cultivation time that infection of toxoplasms occurred in normal serum to both lymphocytes and granulocytes.

Experiments were also carried out using other combinations of serum and leukocytes obtained at various stages of immunization. The immune sera used were those showing the following cytoplasm-modifying antibody titers; 1:1024, 1:256, and 1:32. The last one was obtained early on the 13th day of the oral infection. The leukocytes used were obtained from an immunized pig showing antibody titer ranging from 1:512 to 1:1024. Cultivation time was prolonged to 168 hours. In accordance with the results graphed in fig. 1, inhibition

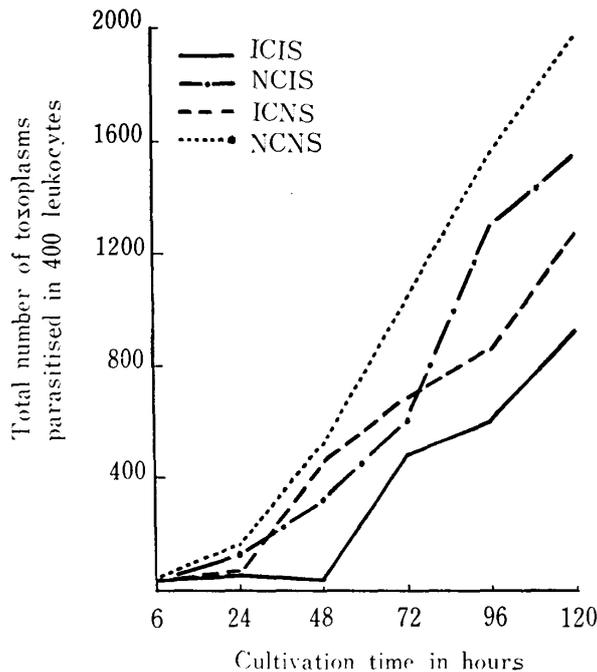
of the multiplication of toxoplasms was always observed when the leukocytes, both from immunized and normal pigs, were cultivated in immune serum. The inhibitory effect was demonstrated even in the serum obtained on the 13th post-infection day. On the contrary, toxoplasms multiplied obviously in the leukocytes, both from immunized and normal pig, cultivated in normal serum.

The samples of immune sera used in the present study were all inactivated before use at 56°C for 30 minutes. Heat-stability of the antitoxoplasmic effect of immune serum was thus proved.

The experimental results of the present study were obtained, as mentioned under "experimental methods", primarily by observing the leukocytes separated from each other. When the leukocytes in clusters were examined, in which case exact counting of toxoplasms in each cell was difficult, the finding was nearly similar to that of the above.

Control cultures made from the toxoplasmic inocula alone showed that there actually were many mouse peritoneal cells associated with the inocula. The encumbrance of the

FIG. 2. *Multiplication of Toxoplasms Which Were Inoculated to the Cultures of Leukocytes; Incubation was done, for initial 2 Hours, in Normal Serum*



Notes:

- 1) Cultivation of the leukocytes was, after the initial 2 hours, made separately in normal and immune sera.
- 2) The serum and leukocytes were obtained one week earlier from the same pigs used in the experiment shown in fig. 1. No difference of the serum antibody titer was found between the experiments shown in figs. 1 and 2.
- 3) For other explanation see the footnote of fig. 1.

associated mouse peritoneal cells was minimized in the subsequent experiments by using a proportion of one toxoplasma per 10 leukocytes. Also toxoplasmic inoculum was slightly centrifuged, 500 r.p.m. for 3 minutes, for separating toxoplasms from the greater part of mouse cells. The results of the experiments thus conducted showed little difference with those graphed in fig. 1.

Another experiment was performed in order to examine the effect of immune serum on the multiplication of toxoplasms which had already penetrated intracellularly. Penetration of toxoplasma was allowed by exposing leukocytes to toxoplasms preliminarily in normal serum medium, in petri dishes. The petri dishes were incubated at 37°C for 2 hours. Then the coverslips in the bottom of the dishes were separately cultivated in immune and normal serum media. As shown in fig. 2, multiplication of toxoplasms occurred in all 4 cultures, regardless of the immune state of leukocytes and sera. The rate of the multiplication was, however, somewhat slow in the cultures of the leukocytes from immunized pig cultivated in immune serum.

#### DISCUSSION

Multiplication of toxoplasms was inhibited when the organisms were caused to make contact with leukocytes in the presence of immune serum. On the other hand, toxoplasms multiplied obviously when the contact was brought about for initial 2 hours in the presence of normal serum. Successive cultivation of the infected leukocytes in immune serum did not appreciably inhibit the intracellular multiplication of toxoplasms. The difference of the above two types of experimental results shows that toxoplasms exposed to immune serum lose their ability of multiplication, probably owing to the loss of their penetrability. Further experiments are needed to confirm the possibility of the loss of penetrability.

The results of the present study do not entirely coincide with those reported by VISCHER and SUTER, the only preceding work along the same direction. The main different points of the present findings are as follows: (1) inhibition of the multiplication of toxoplasms by immune serum was observed regardless of the "immune state" of leukocytes, and (2) leukocytes from immunized pigs showed little inhibitory effect when cultivated in normal serum. Of course the materials used were different from each other. Especially the difference of the animal species should be noted. Furthermore, the type of leukocytes was not the same. VISCHER and SUTER used peritoneal macrophages, while the cells used by the present authors were those of the buffy coat and mainly consisted of lymphocytes and granulocytes. So the results of the present study could not be readily compared with those described by VISCHER and SUTER. The present results, however, contrary to those of VISCHER and SUTER, lead the workers to stress the role of circulating immune substances. In parallel with the present work, NIKI followed the work of VISCHER and SUTER using rat macrophages and dog serum. The results agreed with those of the present work showing the important role of immune serum.

The immune sera used in the present study were heated before use for 30 minutes at 56°C. Unheated serum could not be used for the cultivation of leukocytes because of their considerable cytotoxicity. The antitoxoplasmic effect of immune serum thus proved to be heat-stable. This is a marked difference from a heat-labile neutralizing antibody reported by SABIN and OLITZKY. Both antibodies have, however, a resemblance in that they appear soon after the infection, within 2 weeks. The *in vitro* antitoxoplasmic effect of immune serum shown in the present study manifested itself without particular participation of added complement. Accordingly, the mechanisms of this effect seems to be different from that of cytoplasm-modifying antibody, which requires the aid of added complement and is considered by SABIN and FELDMAN to correspond to neutralizing antibody. Further investigation is needed to explain the difference among neutralizing antibody, cytoplasm-modifying antibody, and antitoxoplasmic effect of immune serum shown in the present study.

There is a possibility of utilizing the antitoxoplasmic effect of immune serum *in vitro* shown in the present study as a new method of sero-diagnosis of toxoplasmosis. Especially the test will be interesting since it is expected to indicate existence of a specific protecting antibody. Experiments are being extended to establish a useful method of titration of the antitoxoplasmic antibody.

#### SUMMARY

Samples of buffy coat obtained from pigs actively immunized with *Toxoplasma gondii* and from a normal pig were cultivated separately in immune and normal swine sera, and the multiplication of toxoplasms in these cultures was studied. In the presence of immune serum, multiplication of toxoplasms was inhibited. The inhibition by immune serum was observed in the cultures of leukocytes derived from both immunized and normal pigs. On the contrary, in the presence of normal serum, toxoplasms multiplied abundantly in the cultures of leukocytes derived from immunized and normal pigs. These findings suggest that circulating immune substances will play a leading role in immunity against toxoplasmosis. The antitoxoplasmic effect of immune serum hardly reached to toxoplasms which had previously penetrated inside leukocytes. The antitoxoplasmic effect shown in the present study proved to be heat-stable, thus was considered different from the neutralizing antibody reported by SABIN and OLITZKY. However, the difference between the two is not yet elucidated.

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Fig. 3

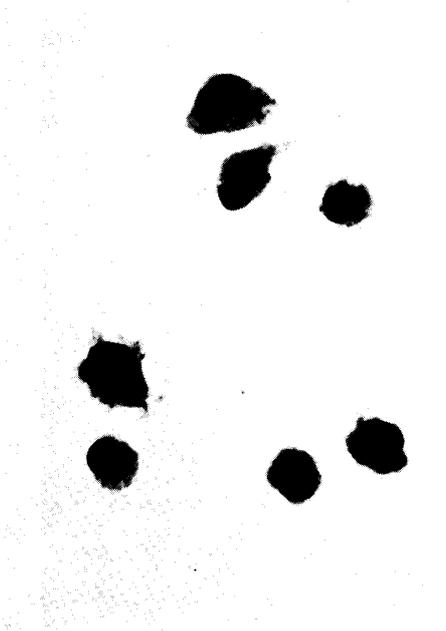


Fig. 4

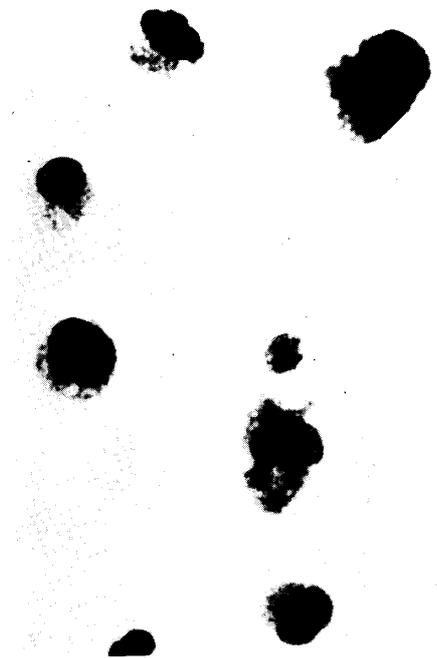
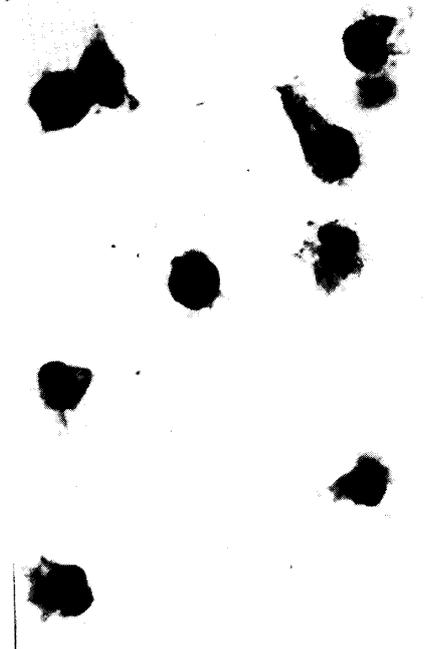


Fig. 5



Fig. 6



All photographs are taken at 96th hour after inoculation and cultivation,  $\times 600$

Fig. 3 Leukocytes from normal pig cultivated in immune serum

Fig. 4 The same as the above (Uninoculated control)

Fig. 5 Leukocytes from immunized pig cultivated in normal serum

Fig. 6 The same as the above (Uninoculated control)