NEUTRALIZATION OF TOXOPLASMS WITH SPECIAL REFERENCE TO THEIR INTRA- AND EXTRACELLULAR LOCALIZATION*

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INTRODUCTION

Neutralization of *Toxoplasma gondii* was first reported by SABIN & OLITZKY (1937). Their findings were extended by SABIN (1941) and SABIN & RUCHMAN (1942). The neutralizing antibody was originally found in the serum of *Macaccus rhesus* inoculated with *T. gondii*, and subsequently in the serum of infected man. However, the neutralizing antibody was not detected in the serum of infected rabbits, mice, cats or dogs. It was also described that the neutralizing antibody is so labile even at a temperature of about 5°C, that it may disappear after one to 2 weeks of storage in an ordinary refrigerator. Later, a new serological test for toxoplasmosis, the so-called dye test, was introduced by SABIN & FELDMAN (1948). The antibody detected by this test was called cytoplasm-modifying antibody and was considered by them to correspond to the neutralizing antibody. The dye test was found to be completed with the aid of fresh human serum, which was designated accessory factor.

In a previous paper from this department, YANAGAWA & HIRATO (1963) reported the antitoxoplasmic effect of immune swine serum which was revealed in the culture of swine leukocytes. The inactivated immune swine serum showed a remarkable antitoxoplasmic effect without any additional thermolabile factor. Recently, LYCKE et al. (1965) in their experiment using HeLa cells also proved the antitoxoplasmic effect of heated immune serum.

While undertaking the previous work, (YANAGAWA & HIRATO, 1963) it was realized by R.Y., one of the authors, that distinguishing intra- and extracellular localization of toxoplasms was very necessary for the neutralization experiment. Previously, toxoplasms used in experiments of neutralization were collected from the ascitic fluids of infected mice. It is evident in such material that there existed many ascitic cells which harbored toxoplasms intracellularly. These parasites would be out of reach of immune serum because they were located inside

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cells. Similar findings are well known in many diseases caused by viruses and intracellularly parasitized bacteria. The result of neutralization of toxoplasma might be quite confusing if one neglects the existence of intracellular parasites. Giving careful consideration on this particular point, an attempt was made by the authors to investigate in vitro neutralization of toxoplasma. Mice were used as experimental animals. The results which were derived from the above consideration are described below.

MATERIALS AND METHODS

Toxoplasma: RH strain, 1837th~1973rd mouse passages

Mouse: NIH and ddY mice, all 5 weeks old

Immune serum: Two rabbits were immunized. They were initially inoculated with avirulent Beverley strain, and then repeatedly with RH strain, until they gave 1:20,000 cytoplasm-modifying antibody titer. Then they were bled, the sera were pooled and stored at −20°C. The serum was inactivated by heating at 56°C for 30 minutes before use. The control rabbit serum used was the pooled serum of 3 normal rabbits. They were treated likewise.

Human serum: Human sera, obtained from a blood clot prior to 10 hours after bleeding, were stored at −20°C. These sera are listed in table 1. All proved negative in 1:2 dilution in the dye test. They were used mostly within 16 days.

TABLE 1 *Human sera used*

SERUM NO.	SUITABILITY AS ACCESSORY FACTOR EXPRESSED WITH % OF UNSTAINED TOXOPLASMA*1	LENGTH USED (days)*2
A B-10	9	16
B-22	13	75
B- 7	11	3
A-21	7	8
O- 8	13	10
O-15	34	16
O-23	41	10
A-10	19	3

*1 This was tested by the dye test as described in "Materials and methods".
*2 Stored at −20°C

Dye test: The procedure originally reported by SABIN & FELDMAN (1948) was followed. For testing the suitability of fresh human serum as an accessory factor, the same human serum was used at the same time as the test serum and accessory factor. The percentage of unstained toxoplasma was determined for each human serum; more than 10%

was thought to indicate the accessory factor was unsuitable.

Neutralization test: The ascitic fluid, obtained from the mice infected with toxoplasma 3 days before, was centrifuged at 1,500 rpm for 5 minutes. The sediment was suspended in cold saline solution or cold serum. To clarify the number of intra- and extracellular toxoplasms, stained preparations were made from the toxoplasmic suspension and were examined microscopically. Tenfold serial dilution of the parasitic suspension in the same diluent was then made for neutralization test. Immune serum, 1 : 10 dilution, was mixed with each serial dilution of the toxoplasma suspension in equal volume. The mixtures were placed in a water bath for 1 hour at 37°C. The control normal serum was tested likewise.

A half ml of each mixture of serum and toxoplasma suspension was injected into a mouse either peritoneally or subcutaneously. Four mice were used for each mixture. The inoculated mice were observed every 24 hours for 3 weeks. When death occurred, detection of toxoplasma was made by examining a stained preparation from the mouse. Survivors were examined serologically by bleeding them after a 3 week observation period and testing their sera (1 : 16 dilution) by the dye test.

Toxoplasma counting: Extracellular toxoplasms per ml were counted by using haemocytometer. Intracellular toxoplasms were calculated as follows.

$$\text{icp/ml} = \text{ecp/ml} \times \frac{\text{icp}}{\text{ecp}}$$

icp: intracellular toxoplasms
ecp: extracellular toxoplasms

For obtaining $\frac{\text{icp}}{\text{ecp}}$ value, the number of intra- and extracellular parasites were counted in more than 10 microscopic fields of the Giemsa-stained preparation.

Phase contrast microscopy and cinematography: Nikon phase contrast microscope was used. Magnification, ×400 (10×40); light source, 4.8 volt, 5 ampere; exposure, 2 seconds; interval, 5 seconds. One drop of toxoplasma suspension made in human serum or other diluent was placed and covered with a coverslip on a glass slide, then, maintaining a 37°C temperature, photographed.

Estimation of cell nucleotide: Cells collected from the ascitic fluid of 10 mice, which previously received 2 ml of 0.01% glycogen saline solution for 3 successive days then received nothing for 3 days, were equally divided into 3 parts. After sedimentation at 2,000 rpm for 5 minutes, each cell residue was washed twice with saline solution. One was suspended in human serum, the second was in inactivated human serum, and the third was in distilled water. The volume of the suspension was 10 ml. They were thoroughly mixed and incubated at 37°C for 1 hour, and shaken occasionally. After the incubation, they were sedimented by centrifugation, and washed twice with saline solution. To the final sediment, 7 ml of 10% perchloric acid was added, left 30 minutes at 0°C, and shaken several times by hand. Each sample was filtered through filter paper and examined spectrophotometrically.

RESULTS

1 Neutralization test

The survival ratio and the days survived were different in the mice inoculated intraperi-

TABLE 2 *Survival difference between the mice inoculated subcutaneously and intraperitoneally*

INOCULATED TOXOPLASMS IN NUMBER*1	SUBCUTANEOUS INOCULATION		INTRAPERITONEAL INOCULATION	
	Survival ratio	Days survived	Survival ratio	Days survived
5×10^{6}	5/5*2	7～ 8	8/8	4～ 5
5×10^{5}	5/5	8	8/8	5～ 6
5×10^{4}	5/5	8～ 9	8/8	6～ 7
5×10^{3}	5/5	9～10	8/8	7～ 8
5×10^{2}	6/6	10～11	8/8	8～ 9
5×10^{1}	2/6	11～13	8/8	8～10
5×10^{0}	0/5		3/8	9～10
5×10^{-1}*3	0/5		0/8	

*1 Diluted with cold saline

*2 Numerator denotes number of mice that died of toxoplasmosis, while denominator indicates number of mice inoculated.

*3 Means 1/10 dilution of 5×10^{0}.

toneally and subcutaneously (tab. 2). LD_{50} was calculated as 73 by subcutaneous inoculation while it was only 7 by intraperitoneal inoculation.

Care was taken in the following experiments to differentiate intra- and extracellular parasites. Infected mouse ascites used as toxoplasma material unavoidably included mouse

TABLE 3 *Neutralization of toxoplasma with inactivated serum*

INOCULATION ROUTE	INACTIVATED SERUM (1 : 10)	SURVIVAL OF MOUSE AFTER INOCULATING WITH GIVEN NUMBER OF TOXOPLASMS			
		Total	6420	642	64	6.4
		Extracellular*1	6000	600	60	6
		Intracellular	420	42	4	0.4
Subcutaneous	Immune		4/4*2 (10～11)*3	2/4 (11)	0/4	0/4
	Normal		4/4 (9)	4/4 (10～11)	2/4 (10～11)	0/4
Intraperitoneal	Immune		4/4 (9～10)	3/4 (10～11)	2/4 (10)	1/4 (10)
	Normal		4/4 (7～9)	4/4 (8～9)	4/4 (10)	0/4

*1 Number of intra- and extracellular toxoplasms were calculated as described in the text.

*2 Numerator denotes number of mice that died of toxoplasmosis, while denominator indicates number of mice inoculated.

*3 Days survived are given in parentheses.

ascitic cells which contained intracellular toxoplasms.

Table 3 shows the neutralizing effect of the inactivated immune serum, without adding the human serum, accessory factor. Particular attention was paid to the results obtained with subcutaneous inoculation. LD_{50} was 642 when toxoplasms were mixed with immune serum, whereas it was 64 when the parasites were mixed with normal serum. This may suggest that 64 in 642 escaped the effect of immune serum. The same table shows that the intracellularly located toxoplasms were calculated as 42, which is close to the above 64.

There is a possibility, from these results, that almost all of the extracellular toxoplasms, but not the intracellular toxoplasms, were neutralized by the heat-inactivated immune serum alone. Confusion might appear if one neglects the existence of the intracellular parasites which might escape the effect of immune serum.

When intraperitoneal inoculation was applied, as shown also in table 3, the effect of immune serum was not clear. This may be due to the mice having a very high sensitivity to toxoplasma by this inoculation route, as proved by LD_{50} which was very small in number.

TABLE 4-A *Neutralization test: inactivated immune serum was mixed with toxoplasms suspended in two types of human sera*

TOXOPLASMS WERE SUSPENDED IN	ADDED INACTIVATED SERUM	SURVIVAL OF MOUSE AFTER INOCULATING WITH GIVEN NUMBER OF TOXOPLASMS*1			
		5000	500	50	5
Human serum AB-10*2	Immune	2/4*3 (9~11)*4	0/4	0/4	0/4
	Normal	NT*5	4/4 (9~11)	2/4 (10~11)	0/4
Human serum O-15*2	Immune	3/4 (9~13)	0/4	0/4	0/4
	Normal	NT	3/4 (10~12)	0/4	0/4
Saline (Control)	Immune	4/4 (9)	4/4 (9~11)	1/4 (11)	0/4
	Normal	NT	4/4 (9~10)	4/4 (9~11)	0/4

*1 Indicates total number of toxoplasms inoculated after mixing with inactivated immune and normal sera. Intra- and extracellular toxoplasms contained in the total are given in table 4-B.

*2 Dye test proved that 9% and 34% of toxoplasma were unstained when human sera AB-10 and O-15 were tested respectively (tab. 1).

*3 Numerator denotes number of mice died of toxoplasmosis, while denominator indicates number of mice inoculated.

*4 Days survived are given in parentheses.

*5 Not tested.

The effect of added human serum, known essentially in the dye test as accessory factor, in the neutralization system was then tested. The results were shown in table 4-A. Two types of human serum were used; one was AB-10 which was suitable as the accessory factor and another was O-15 which was not suitable as the accessory factor because of allowing 34% unstained toxoplasms by the dye test, as shown in table 1.

Toxoplasma used for this neutralization test was mouse ascitic fluid containing a large number of the cells parasitized intracellularly. Table 4-B shows the number of intracellular toxoplasms contained in the total number of parasites inoculated.

TABLE 4-B *Number of intra- and extracellular toxoplasms in the toxoplasmic inoculum used in experiment of table* 4-A

TOXOPLASMS WERE SUSPENDED IN	LOCATION OF TOXOPLASMA	TOTAL NUMBER OF TOXOPLASMA INOCULATED			
		5000	500	50	5
Human serum AB-10	Extracell.	4980	498	49.8	4.9
	Intracell.	20	2	0.2	0.0
Human serum O-15	Extracell.	4980	498	49.8	4.9
	Intracell.	20	2	0.2	0.0
Saline (Control)	Extracell.	3000	300	30	3
	Intracell.	2000	200	20	2

As shown in table 4-B, the toxoplasma located intracellularly was originally 40% but it was greatly reduced to 0.4% after mixing with human serum. It was presumed from this data that intracellular toxoplasms were released from the parasitized cells by the action of human serum.

The neutralization effect of immune serum was not clear but was very noticeable, particularly when human serum AB-10 was added. Less than 50 toxoplasms suspended in human serum AB-10 were enough to kill 50% of the mice when they were mixed with normal serum. When mixed with immune serum, however, the number of toxoplasms required to kill 50% mice were increased to 5,000. Of the 5,000 toxoplasms, actually only 20 were located intracellularly as shown in table 4-B; which was close to the above mentioned number of toxoplasms required to kill 50% of the mice when mixed with normal serum.

Human serum O-15 was thought to have a very small amount of antibody expressed by giving 34% unstained toxoplasms in the dye test. Perhaps for this reason, toxoplasms suspended in human serum O-15 were already neutralized before mixing with normal serum. As a matter of course, the neutralizing effect of immune rabbit serum was not clearly demonstrated compared to normal serum. However, human serum O-15, though not suitable as accessory factor, exhibited a tendency to increase extracellular toxoplasma by reducing the intracellular ones, resulting in increased neutralizing effect of immune serum.

Thus, it is evident that only human serum suitable as accessory factor can bring the contrast of the neutralization effect between immune and normal sera.

2 Direct observation of the release of toxoplasma from mouse ascitic cells by the action of human serum

Table 5 shows that the number of intracellular toxoplasms was greatly reduced when the infected mouse ascitic cells were mixed with fresh human serum, but not with saline and guinea pig serum. This characteristic activity of human serum was cinematographically

TABLE 5 *Percentage of intracellular toxoplasma after the toxoplasmic material was suspended in human serum, guinea pig serum, and saline solution*

TOXOPLASMS WERE SUSPENDED IN	37°C INCUBATION FOR 1 HOUR	INTRACELLULARLY LOCATED TOXOPLASMS (%) IN THE MOUSE ASCITIC MATERIAL OBTAINED ON THE POSTINFECTION HOURS OF		
		36	48	60
Human serum	Yes	5.4%	3.7%	3.1%
Guinea pig serum	"	56.2	44.3	40.2
Saline	"	57.7	54.8	37.8
Saline	No	69.8	61.1	48.4

studied using the phase contrast microscope. As shown in figure 1, release of toxoplasms from mouse cells was clearly observed when they came into contact with human serum. Prior to the release of toxoplasms, changes in the mouse cells were noticed which were swelling, becoming transparent and granular. Cells that did not contain toxoplasms showed the same degeneration. The degeneration suggests that mouse ascitic cells were lysed. Release of toxoplasms from the degenerated cell was thus considered initiated by the cell destruction. However, it was also noticeable that intracellular toxoplasms themselves, moved outside cells by their accord within a very short period of time after the cell destruction.

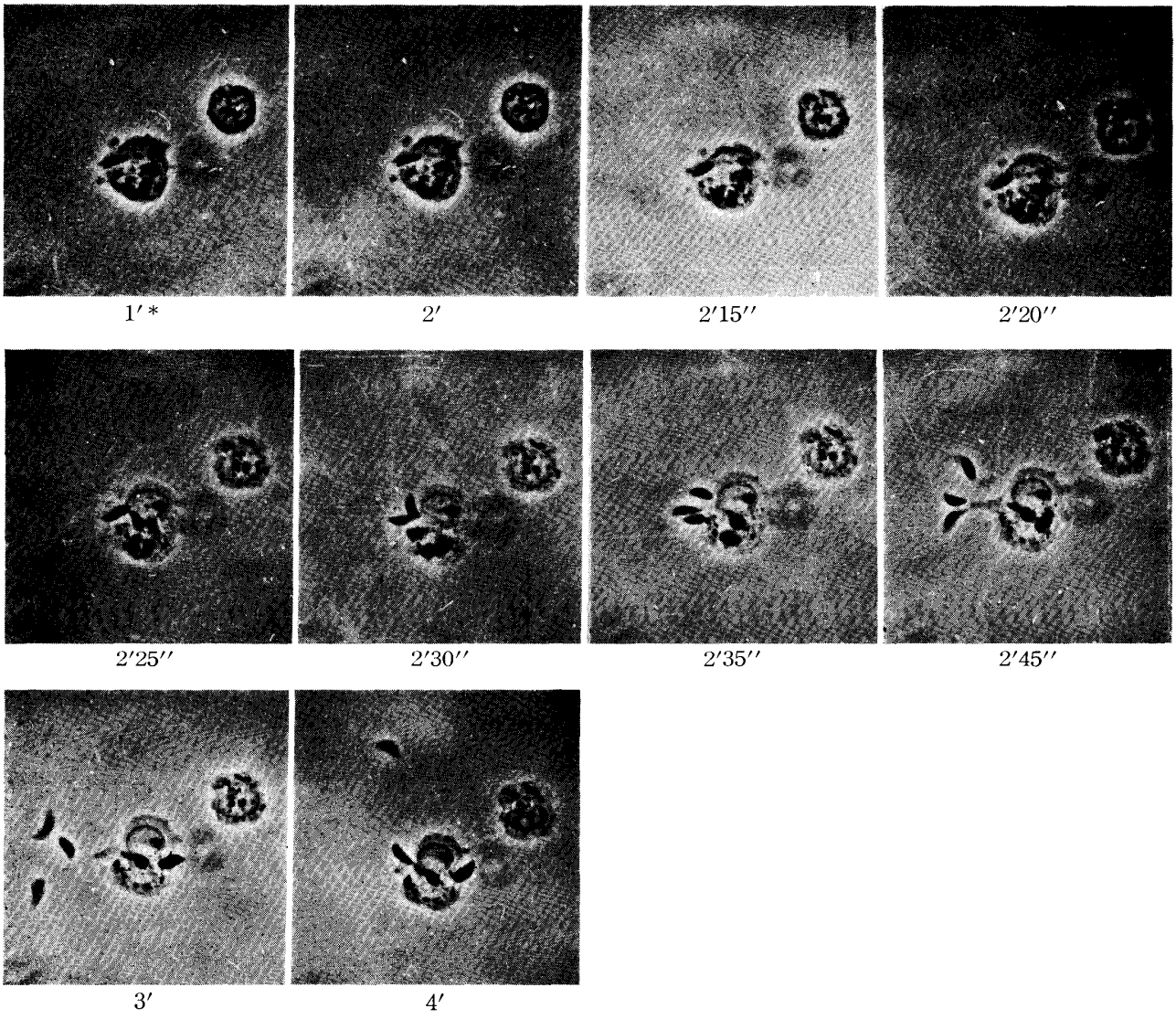
The above findings were then studied from the Giemsa-stained preparation. Since this action of human serum was revealed within the first several minutes, it was necessary to stop the reaction before mounting the specimens on the slide glass. A preliminary experiment showed that the activity of human serum was reduced by dilution; no more activity was detectable when diluted to 1 : 64. Therefore, the test sample was diluted 100 times with saline solution to stop the reaction just prior to making the stained preparation.

Figure 2 shows that the activity of human serum to release toxoplasms from mouse ascitic cells was demonstrated within 2 minutes. All of the fresh human sera, but not inactivated sera, showed the same activity regardless their suitableness as the accessory factor.

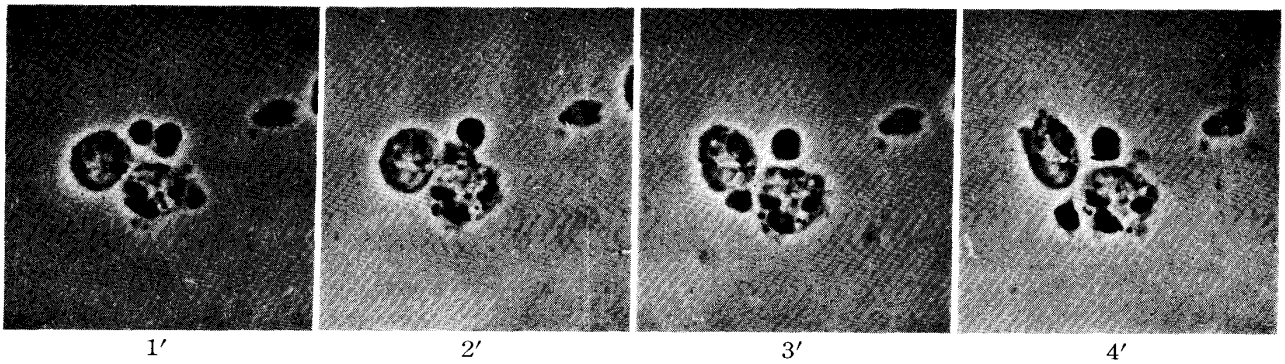
The lysis of the mouse cells by fresh human serum was further proved in the next experiment. Ascitic cells collected from normal mice as described in "Materials and methods" were mixed respectively with fresh human serum, inactivated serum and distilled water for 1 hour at 37°C. Nucleotide in the cells was estimated after the treatment. The nucleotide content of the mouse cells was markedly reduced after the treatment with fresh human serum; and nearly equal to that treated with distilled water, whereas it was not reduced by human inactivated serum (fig. 3). These findings are proof that fresh human serum alone is

FIGURE 1 *Release of intracellular toxoplasms by treating with human serum*

1) Cells treated with human fresh serum

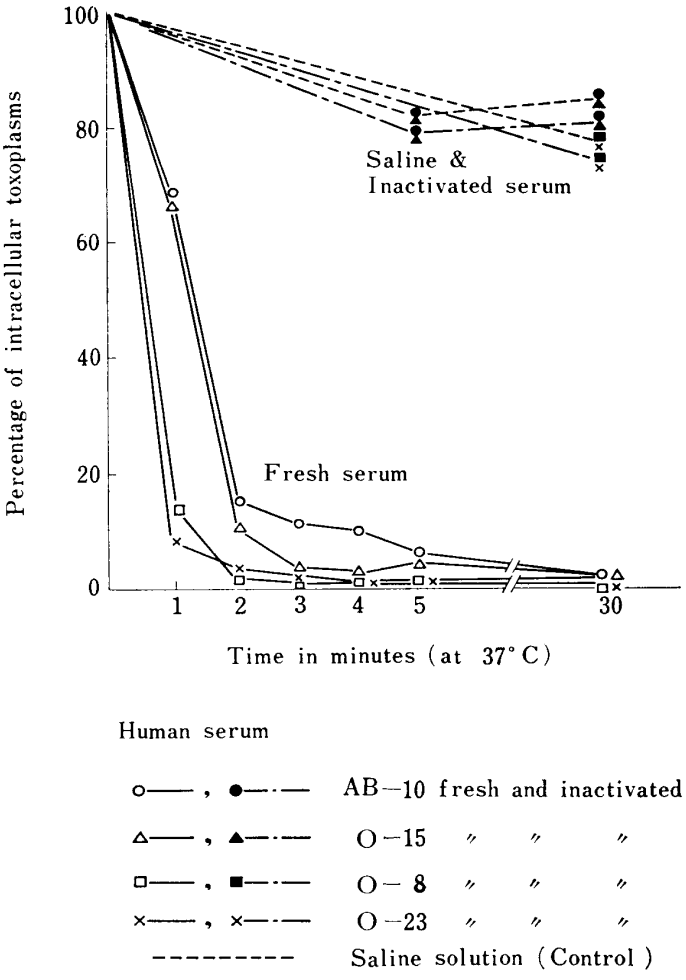


2) Cells treated with human inactivated serum



* Time in minutes after the treatment

FIGURE 2 *Effect of human serum decreasing intracellular toxoplasms*



destructive to mouse ascitic cells.

Table 6 shows the result of an additional experiment in which mouse red cells were treated with fresh human serum. Complete hemolysis occurred. Inactivated human serum did not cause hemolysis. The result shows that not only ascitic cells but also red cells of mice are lysed by fresh human serum.

FIGURE 3 *Decrease of nucleotide content of mouse ascites cells when treated with human serum*

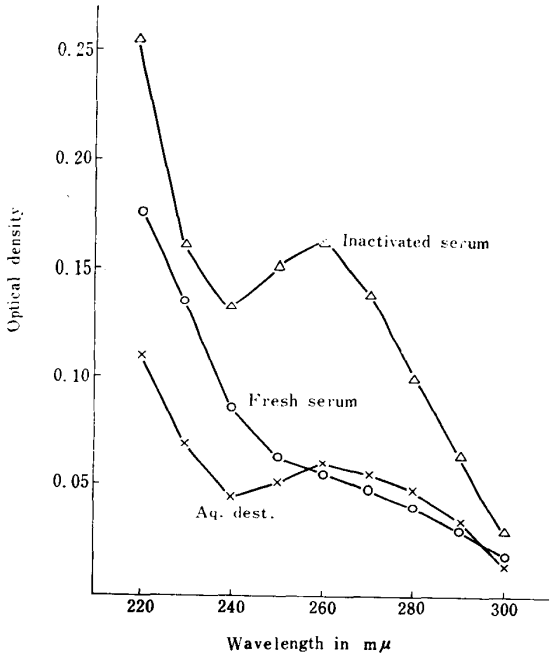


TABLE 6 *Lysis of mouse red cells by human serum*

HUMAN SERUM	HEMOLYSIS*1
Fresh O-31*2	+
" O-13*3	+
Inactivated O-31	—
" O-13	—
Saline Control	—

*1 + Complete hemolysis; — No hemolysis
*2 Suitable as accessory factor
*3 Not suitable as accessory factor

DISCUSSION

Neutralization of toxoplasms by inactivated immune serum has been shown in vitro by YANAGAWA & HIRATO (1963) and LYCKE et al. (1965). On the other hand, neutralization of toxoplasms using mice was not yet clear. The reason for the difficulty in neutralizing toxoplasma using mice was investigated by the authors analysing first the route of inoculation, and then the intra- and extracellular localization of toxoplasms in the inoculum.

The subcutaneous inoculation route was found to favor the whole experiment.

Intraperitoneal inoculation was found not suitable because this route gave a high susceptibility of mice to toxoplasma.

Toxoplasma suspension used in the neutralization tests was obtained from the ascitic fluid of infected mice. Such fluid contains mouse ascitic cells, and many of them have toxoplasms in their cytoplasm. Since such intracellular toxoplasms would be protected from the action of immun serum, the existence of such mouse cells in toxoplasma suspension should be carefully investigated to determine whether they have any relation to the result of the neutralization test.

As shown in the experimental results, free extracellular toxoplasms were neutralized by inactivated immune serum alone. This is in agreement with the previous finding of YANAGAWA & HIRATO (1963). It was also clearly shown in the present study that intracellular toxoplasms were not affected by immune serum and accordingly, when injected, was fatal to the mice.

Therefore, it is now evident that confusion in neutralization test is inevitable when infected mouse ascitic cells are used as the starting material of toxoplasmic inoculum. It always contains cells containing intracellular toxoplasms.

By the addition of human serum, so-called accessory factor, neutralization of toxoplasma was accelerated. This was analysed and the authors came to the conclusion that the neutralization accelerating phenomenon was due to cytolytic action of human serum against mouse ascitic cells. Ascitic cells derived from normal mice were destroyed by human serum. It seemed that the site of the destruction occurred at the cell membrane and that the intracellularly located toxoplasms were released. Observation under the phase contrast microscope (HIRAI et al., 1966) showed that intracytoplasmic toxoplasms were actively motile and moved outside of cells particularly when the host cell became degenerative.

The cytolytic activity was not proved with guinea pig serum. Although we did not test very many animal sera, difference in animal species would be a big factor in this lytic phenomenon. It was found that mouse red cells were also lysed by human serum. Therefore, the authors are inclined to believe this phenomenon to be a manifestation of Forssman-antigen antibody reaction.

From this point of view, the findings of SABIN (1941) and SABIN & RUCHMAN (1942) seem to be interesting; they mentioned that toxoplasma neutralizing effect was proved with the infected serum of human and monkey, but not with those of rabbit, mice, cats, and dogs. Presumably, the human and monkey sera they used acted simultaneously as the specific antitoxoplasmic antibody and as the lytic agent against mouse ascitic cells in which toxoplasms were contained.

It could be said in the neutralization of toxoplasma that most important role is played by the specific immune serum, heat stable antibody, in accordance with the previous finding of YANAGAWA & HIRATO (1963) using a tissue culture system

of swine immune serum and swine leukocytes. While the role of heat labile accessory factor (human serum) is not essential in the neutralization, it is neccessary to allow the toxoplasma to come into contact with immune serum due to its lytic action upon the parasitized mouse cells.

The accessory factor is known to be essential for completing the dye test. Cytoplasm-modifying antibody detected by the dye test has been considered to correspond to the neutralizing antibody (SABIN & FELDMAN, 1948). Differentiation of the role of the accessory factor (human serum) between the dye test and the neutralization test should be the subject of future study.

SUMMARY

Neutralization in vitro of toxoplasms was investigated paying special attention to their intra- and extracellular localization. Toxoplasms were obtained from the ascites of infected mice. To detect the neutralization effect, mice were used as experimental animals, the subcutaneous route of inoculation proved to be successful.

Inactivated immune rabbit serum neutralized extracellular, but not intracellular toxoplasms.

Addition of fresh human serum to the neutralization system decreased the number of intracellular toxoplasms resulting in a considerable acceleration of neutralizing effect. This finding was investigated and the authors found that this was due to the cytolytic action of human serum on mouse ascitic cells containing toxoplasma intracellularly.

The lysis of mouse ascitic cells by human serum was proved by microscopic examinations and also by demonstrating the loss of cell nucleotide.

It is to be emphasized that toxoplasms could be neutralized by heat stable specific antibody, in accordance with the previous report of YANAGAWA & HIRATO (1963).

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