STUDIES ON TOXOPLASMOSIS V

COMPLEMENTAL OBSERVATIONS ON THE TISSUE CULTURE METHOD, ESPECIALLY ON THE EFFECT OF THE NUTRIENT FLUID UPON THE INVASION AND MULTIPLICATION OF THE ORGANISMS

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(Received for publication, Nov. 5, 1962)

INTRODUCTION

It may safely be said that the tissue culture of *Toxoplasma gondii* is one of the reliable methods for the isolation and maintenance of the organisms as was already reported by JACOBS et al. discussing the first case of isolation from an ocular patient by the use of a tissue culture, and by SCHUHOVÁ discussing the maintenance of the organisms.

Moreover, further studies on this method may possibly reveal something more potent as immunizing agent. The organisms harvested by using this technique are obviously more suitable for purification than those from the experimental animals, and also may scarcely be affected by the immunological or self-defensive responses which occur in the inoculated animals.

In the preceding paper[3], the author described several factors which may have some intimate relationship with the heavy multiplication of the organisms. In that study, not so much attention was paid to the effect of the nutrient fluid. However, for the cultivation of a small number of organisms in materials, it is very likely that the constitution of the nutrient fluid may exert some serious effects on the development of the organisms; moreover, the possible elimination of protein substances contained in the nutrients is very necessary, which may result in ease of purification of the organisms or the use of the culture fluid as the various antigens. The present report deals with these problems.

MATERIALS AND METHODS

1. Preparation and maintenance of cells

Throughout the experiments, only HeLa cells prepared so as to attain a nearly complete

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monolayer sheet on the 3rd day of the culture, were employed. The making of cultures was followed by employment of the stationary method in the use of 40×65 mm glass bottles for the cell sheet preparation, which contained 10 ml of the nutrients.

For the growth of cells, HL (Hanks' balanced salt solution plus 0.5% lactalbumin hydrolysate) with 20% of inactivated bovine sera was used and the same with 1% of sera for the maintenance of cells after inoculation of the organisms. PH value of the nutrients was adjusted to 7.0~7.2 with normal solution of sodium bicarbonate by using an electric pH meter. Penicillin and streptomycin were generally added to the concentration of 200 u and 200γ per ml respectively.

2. Inoculation and observations

RH strain which had been maintained by intraperitoneal passages in mice since its receipt from Prof. TSUNEMATSU, Institute for Infectious Disease, Tokyo University, was employed.

The organisms from the mice peritoneal fluid on the 3rd day of infection were washed with the culture fluid several times. After this, the desired number of the organisms suspended in 5 ml of the maintenance media (culture fluid) were inoculated on the cell sheet, from which the growth nutrients had already been removed. The replacement of the culture fluid of 10 ml was followed after overnight sensitization at 37°C. In this study invasion (adsorption) and multiplication were estimated from increase or decrease of the number of the organisms which appeared in the nutrient fluid; counting was done by the haemocytometer.

EXPERIMENTS

I. Effect of the Culture Fluids upon the Invasion (Adsorption) and Multiplication of the Organisms.

A. Invasion

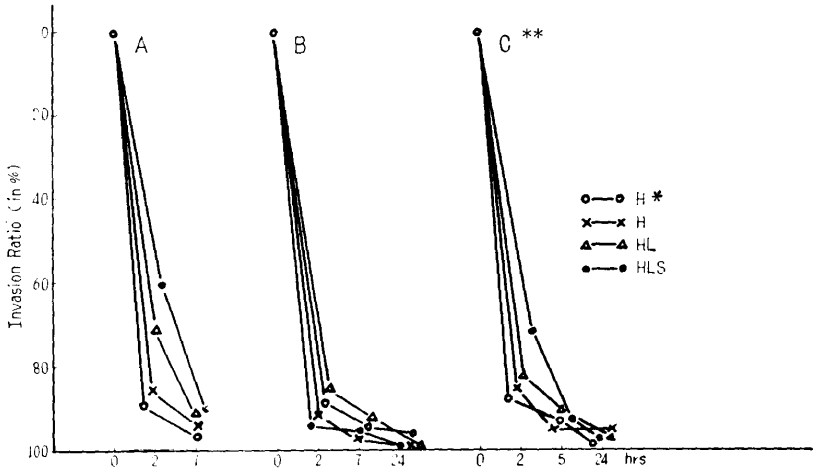
As the organisms outside the live cells tend to be destroyed considerably in a short time, suitable culture fluid must be employed for the inoculations. Seven kinds of media were tested; YLE (Earle's balanced salt solution plus 0.5% lactalbumin hydrolysate and 0.1% yeast extract), YLES (1% bovine serum in YLE), H (Hanks' balanced salt solution), H* [Modified Hanks', viz. divalent salts ($CaCl_2$ and $MgSO_4$) were taken out], HS (1% bovine serum in H),

TABLE 1 *Infectivity of T. gondii suspended in Culture Media*
(By the Mice Inoculation Test)

CULTURE MEDIA		1	2	4	6	12	24 hrs
Exp. 1	H	●10 ●10	○ ○	○ ○	○ ○	○ ○	○ ○
	HLS	●9 ●9	●9 ●9	●10 ○	●10 ○	○ ○	○ ○
Exp. 2	H	●7 ●8	●8 ●9	●10 ●11	●11 ●12	○ ○	○ ○
	HLS	●8 ●8	●8 ●8	●9 ○	●9 ●9	●10 ●10	●11 ●11

Notes: 1) In Exp. 1, the materials were so prepared as to reach the proportion of 200 individuals per ml, and 2,000 in Exp. 2.
2) The mice were intraperitoneally inoculated in the doses of 0.5 ml in each; viz. 100 individuals in Exp. 1 and 1,000 in Exp. 2.

FIG. 1 *Effect due to the Varieties of the Culture Media on the Invasion into the Cell*

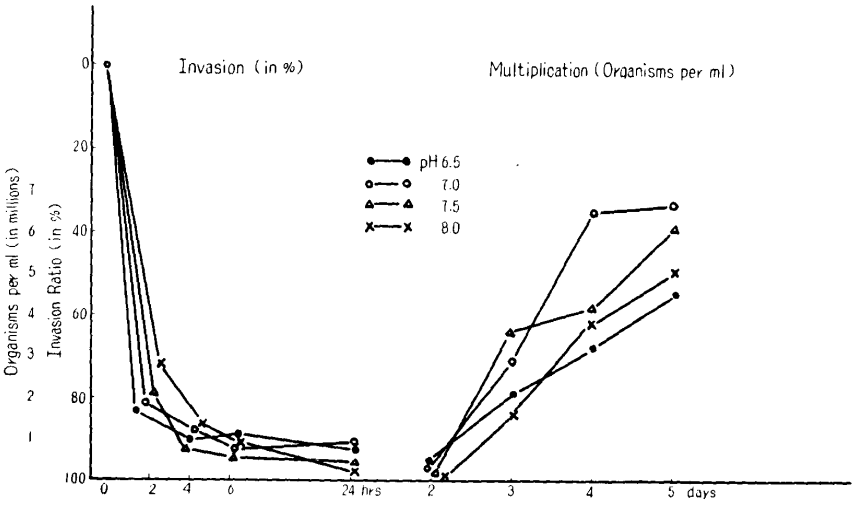


Notes: 1) No. of organisms inoculated was 2.5×10^6/ml (organism: cell≒3:1) in A, 2×10^6/ml (organism: cell≒1:1) in B and 5.4×10^6/ml (organism: cell≒1:2) in C.

2) H···Hanks' balanced salt solution, H*···H devoid of Mg, Ca ions, HL··· H plus 0.5% lactalbumin hydrolysate, HLS···HL plus 1% bovine serum.

3) ** Invasion curves due to YLE (Earle's balanced salt solution plus 0.5% lactalbumin hydrolysate and 0.1% yeast extract) and YLES (YLE plus 1% bovine serum) had nearly coincided with that of H.

FIG. 2 *Effect of pH Value on the Invasion into the Cell and the Multiplication of the Organisms*



HL (0.5% lactalbumin hydrolysate in H) and HLS (HL plus 1% bovine serum).

Through the experiments as shown in fig. 1 it may be said that in the early stage of the

inoculation, media HLS or HL are generally not very good ones for the invasion of the organisms into cell. Namely, in media H or H*, more than 90% of the organisms will finish the invasion by 5~7 hours after inoculation; however in the other media, the similar invasion ratios may not be seen until later stages. Though it is not clear for media YLE or YLES because of only 1 experiment, the possibility may be supposed similar to HL or HLS.

At any rate, high invasion ratios above 95% could be observed at 24 hours after inoculation, in all media tested.

In this connection, it must be remembered that the organisms in such media absolutely without protein substances are likely to lose the infectivity in rather a short period; this can be seen from table 1.

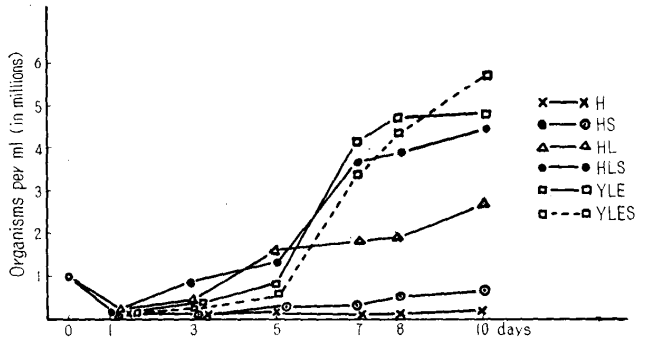
Hydrogen ion concentrations ranging from 6.5 to 8.0 did not reveal any differences in the invasion ratio; this is indicated in fig. 2.

B. Multiplication

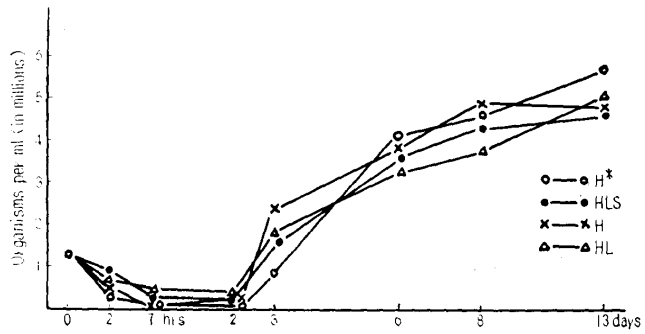
1. Culture nutrients

Despite the good invasions observed in H or H* media, the multiplication of the organisms in these media is so poor as not to be countable by the haemocytometer. The multiplication curves in each medium are indicated in fig. 3 A. In meida YLE, YLES and HLS, almost

FIG. 3 *Effect of Culture Media on the Multiplication of T. gondii*



A. Growth Curves in Each Culture Medium



B. Growth Curves, when Each Culture Medium, Employed for the Inoculation, was Changed into HLS at 7 Hours after Inoculation

similar growth curves were observed, whilst medium HL revealed middle-graded growth. Medium HS showed somewhat better results than those in media H or H*.

On the other hand, whenever each culture fluid was replaced by HLS at 7 hours after inoculation, good multiplication was observed, not influenced by the variations of the invasion ratios due to each medium; this is indicated in fig. 3 B. Accordingly, even if one employ such media as H or H* because of their superiorities with respect to the invasion ratios, it is recommended to change the culture fluid to HLS, YLE or YLES at an early stage.

2. PH value of the nutrients

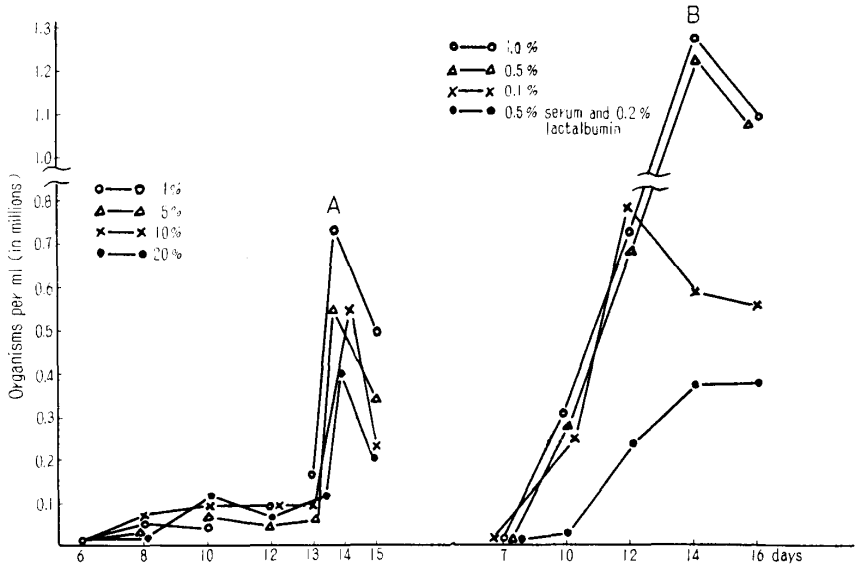
Even the pH values which are not so strong alkaline or acid, i.e. pH 8.0 and 6.5 revealed rather pretty bad effects on the HeLa cell (granular degeneration, destruction and falling off) and caused poor multiplication, although these pH values did not exert any significant effect on the invasion ratio as has already been described.

The optimum value of pH for multiplication was demonstrated to be neutral (fig. 2).

3. Effects of serum concentrations and antibiotics

Test were performed on bottles inoculated with small amounts of organisms, where the effects due to the serum concentrations may be observed more clearly than in these inoculated with heavy doses of the organisms. From the data exhibited in fig. 4, the presence of 0.5~1% of the bovine sera in medium HLS may be good enough to cause heavy multiplications. Addition of the higher percentages of sera was proved not only be unnecessary but even harmful, contrary to the author's expectations.

FIG. 4 · *Effect of the Concentration of Bovine Sera in HLS Medium*

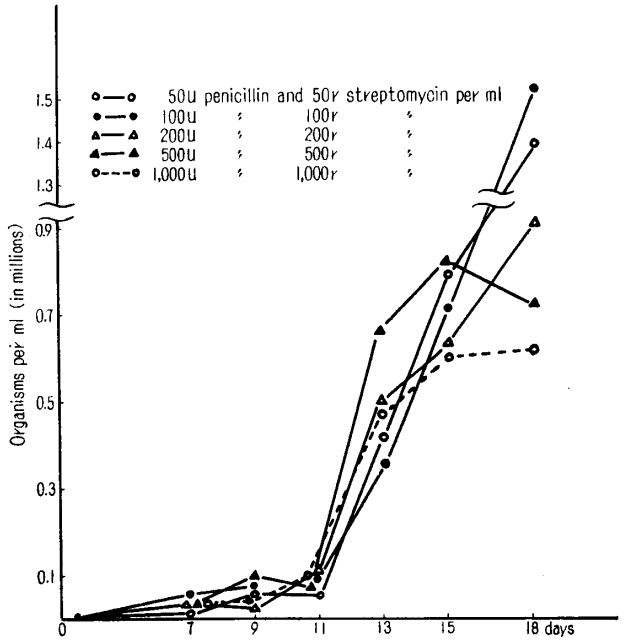


Notes: Number of organisms inoculated···4.25×10^3 per bottle in the left, 2.3×10^4 per bottle in the right experiment

For the isolation of the parasites, especially from the organ materials contaminated with the other organisms, the use of antibiotics as heavy doses as possible is highly preferable.

However, addition of more than 100 u of penicillin with 100 γ of streptmycin will inhibit the growth of the parasites probably because of their harmful effects on cells; this inhibition is shown in fig. 5.

FIG. 5 *Effect of Antibiotics (Penicillin and Streptomycin)*

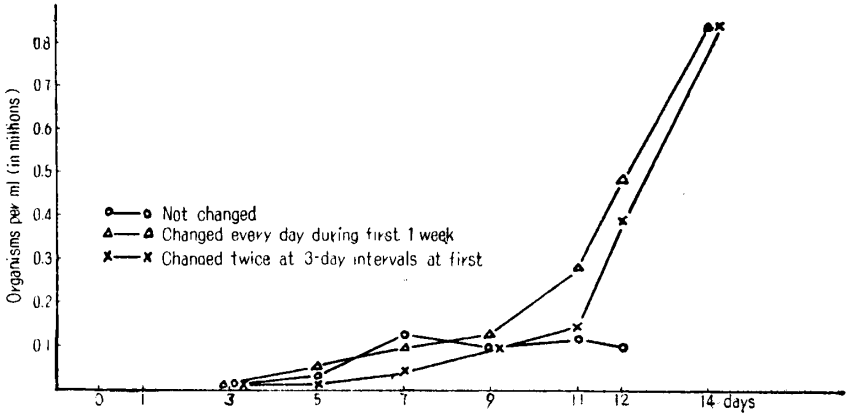


Notes: No. of organisms inoculated···1×10^4 per bottle
(organism : cell≒1 : 210)

4. Effects of the replacement of the culture fluid

Inoculation of the heavy doses of the organisms always resulted in luxurious growths during 7~8 days without changing the culture fluid at all. However, to cultivate the minimum number of organisms in materials, the replacement of the culture fluid several times is absolutely important because of the necessity of maintaining the activity of the cells for

FIG. 6 *Effect of Replacement of the Nutrient Fluid*

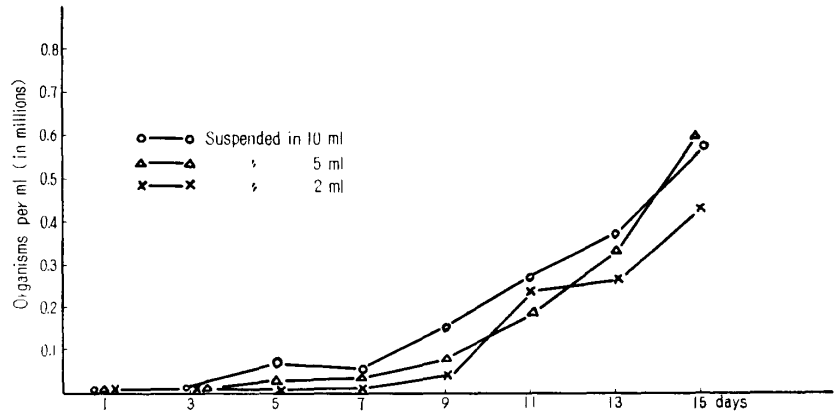


a pretty long period, which seems to be one of the most important factors in the multiplication of the organisms. In the bottle group in which the culture fluids were never changed, non-specific degeneration and destruction of the cell sheets had always occurred at 8~10 days after inoculation; clear proof of multiplication of the organisms could not be recognized. These data are indicated in fig. 6.

5. Effect of volumes of inocula suspensions

The comparatively higher concentration of the organisms may certainly promote the invasion of the cell because of the abundance of chances of encounter between cells and organisms. Accordingly, it is very likely that the materials for inoculation must be prepared in as minor volumes of suspension as possible. However, as indicated in fig. 7, any differences of the multiplication could scarcely be recognized among each of 3 groups which were inoculated with a constant number of organisms 1×10^4 suspended in 2, 5 and 10 ml respectively when the first exchange of the fluid was made after 24 hours following inoculation. This may be due to the fact that *T. gondii* have a pretty large body weight and easily sink down to the cell layer in a comparatively short time, which gives them an almost uniform chance of invasion into the cells.

FIG. 7 *Effect of the Suspended Volumes of the Organisms*



Notes: No. of the organisms inoculated···1×10^4 per bottle
(organism : cells = 1 : 1,000)

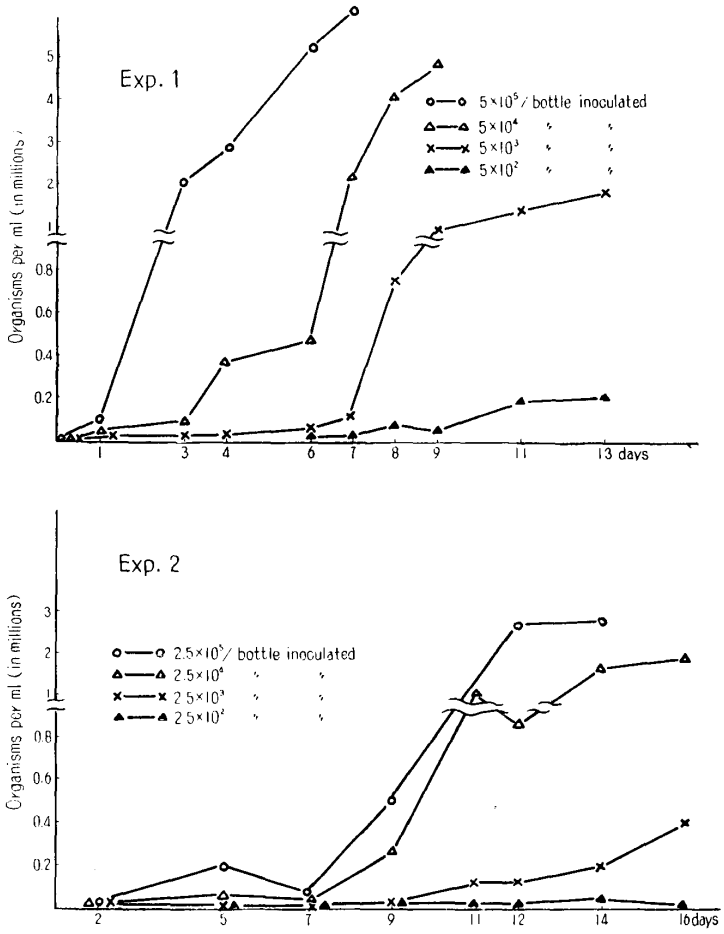
For these reasons, the volume of the suspension may not constitute a serious factor so far as a suitable nutrient fluid good for the maintenance of the activity of the organisms was employed.

II. The Ability of the Tissue Culture Method for the Isolation of the Organisms

As a method of isolation, the tissue culture has already been recognized to be one of the best although it is not so sensitive as the mice inoculation. However, it has not yet been fully ascertained to what extent that method is advantageous for the isolation of *T. gondii* from the infected materials.

Experiments were performed by inoculating serial minor doses of the organisms from infected mice. As shown by the data graphed in fig. 8, it had become clear that the multiplication could be fairly well observed in the experiment group which had received at least several thousands of organisms, viz., numbers of 2.5×10^3 and 5×10^3 per bottle. However, in the groups which had received the organism to the number of some hundreds, the author could not recognize any clear proof of multiplication.

FIG. 8 *The Suitability of the Tissue Culture for the Detection of the Organisms*



In the present paper, the multiplication of the parasites was estimated by counting the free organisms in the culture fluid, so it should be emphasized that the multiplication of the organisms was recognized at first only when it had reached to a number larger than ten thousands per ml; sub-cultivation of the concentrated materials from the first generation was not conducted.

DISCUSSION

The tissue culture method is generally accepted to be not so sensitive for the

isolation and multiplication of *T. gondii* compared with the mice inoculation method. However, that method in vitro has many superior points which could not be expected in the use of experimental animals. For example there are: production of large doses of organisms that can easily be separated and concentrated, and are not badly influenced by the host animals, and easy production of a large quantity of complement-fixing antigen[4] from the culture fluid etc. The isolation of *T. gondii* by mice inoculation does not always yield positive results, even if extremely many organisms are detected in the original materials.

Furthermore, in the course of the adaptation of *T. gondii* to the mice, gradual or sudden disappearance of the organisms from the inoculated mice is sometimes experienced by many workers. The author also lost two strains from hare and squirrel which seemed nearly be adapted to mice. Prof. MIYAZAKI, Kyushu University, reported too at the meeting of the Research Committee on toxoplasmosis that all 10 strains which had been isolated from human beings by him had gradually disappeared from the mice in the course of subinoculation. In addition, accidental death or contamination by other organisms are not rarely observed in the experimental animals. Accordingly the tissue culture method must increasingly be used as a routine one because it may scarcely be disturbed by the difficulties above mentioned. In fact, numbers of the organism equal to those from a mouse peritoneal fluid ($3\sim4\times10^8$) can be easily multiplied by using the roux flask and the procedure seems to be rather inexpensive.

In addition, this method is by no means as insensitive as an isolation one. In the bottle which had received several thousand organisms, heavy multiplication had always occurred when the culture was carried out under suitable conditions as described above.

In the culture fluid without protein substances such as serum or lactalbumin, it must be emphasized that *T. gondii* tends to die in rather short period. So, the change of the culture fluid to HLS or related ones which contain protein substances at 5 or 7 hours after inoculation is very important, even if a culture fluid free from these substances, such as Hanks' balanced salt solution was employed for the inoculation because of their superiority in promoting invasion into the cells. Commonly, in tissue culture, the inoculation will be carried out so as to bring the organisms into good contact with the cells; the organisms suspended in small volumes are spread on a cell layer, from which the nutrient fluid had already been discarded, and incubated for several hours. However, in case of toxoplasma, special procedures which are generally considered to promote invasion, may be considered unnecessary because *T. gondii* will precipitate on the cell sheet in a comparatively short time by their high gravity and will cause almost the same degree of growth, without relation to the suspended volume of the inocula.

As to the serum concentrations of the culture fluid, SUZUKI and his co-worker (1958) reported that the culture fluid of pH 7.2 composed of Hanks' balanced salt solution, 20 % bovine serum produced the best growth; they used the roller tube culture of 12 days egg embryonal lung, liver, muscle and monkey kidney cells. According to the present worker's data on HeLa cell and RH strain of toxoplasma, the best results were always observed in the low content of bovine serum such as 0.5 or 1%; such experience was recorded in the author's previous report. Though it is difficult to compare these data obtained by separate investigators, due to the difference of constitution of the culture fluid, it is desirable to use the culture fluid contained as lower concentration of protein substances as possible. These substances may disturb the purification procedures and may often become the cause of anti-complemental activity, from the viewpoint of the utilization of the multiplied organisms and culture filtrates.

SUMMARY

Following the previous report, complemental observations were carried out to ascertain the suitable cultural conditions including the effects of the nutrient fluid upon the invasion and multiplication, the ultimate aim being to promote the utility of tissue culture in isolation of the organisms. Throughout the experiments HeLa cells and *T. gondii* strain RH were used.

Data obtained are summarized as follows:

1. Good invasion occured very frequently when Hanks' balanced salt solution and solutions devoid of divalent ions such as Mg, Ca, were employed. However, there seems to be no significant differences in the ratio of invasion due to the culture fluids tested—H (Hanks), HL (Hanks plus lactalbumin hydrolysate), HLS (HL plus serum), YLE (yeast extract, lactalbumin hydrolysate and Earle's balanced salt solution) and YLES (YLE plus serum), and organism numbers more than 90% may invade the host cell 24 hours after inoculation (fig. 1).

2. The organisms which were suspended in the culture fluid devoid of protein substances tend to die in rather short period and could not multiply well in cells when cultured by these proteinless nutrients (Fig. 3 A, Table 1). Accordingly, the nutrients must be replaced by ones containing protein after 5~7 hours when the media such as Hanks were employed in inoculation (Fig. 3B).

3. Addition of bovine sera to the concentration of 0.5~1.0% seems to be quite enough to cause heavy growth of *T. gondii* if the medium HL is used for the culture nutrient.

4. PH values ranging from 6.5 to 8.0 do seem not to show any effects on the invasion ratio of the organisms. However, in the more acid or alkaline sides (6.0, 8.0) poor multiplication was always observed and especially in acid side, elution of

the soluble complement-fixing antigenic substances was also scarce (Fig. 2).

5. The volume of the suspension of the inocula seemed to have no significant relations with the yield of the organisms when the inoculated bottles had been left overnight for the invasion (Fig. 7).

6. To cultivate a small number of the organisms in material, frequent changes of the nutrient fluid every day or every other day during the first week are very necessary (Fig. 6).

7. Clear proofs of the multiplication of the organism numbers of $2.5 \sim 5.0 \times 10^3$ in the materials were observed by counting method when the tests were performed with due consideration of the above described points (Fig. 8).

ACKNOWLEDGEMENTS

The author is indebted to Prof. HIRATO, the chief of the Department for his helpful advices and many suggestions during the course of this study. The present study was supported in part by a Grant in Aid from the Ministry of Agriculture and Forestry, for which the author would express his thanks.

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