Enzyme Immunoassay for Evaluation of *Toxoplasma gondii* Growth in Tissue Culture

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An enzyme-linked immunosorbent assay (ELISA) for the evaluation of growth kinetics of *Toxoplasma gondii* in tissue cultures was developed. Tissue culture microplates (96 wells) were seeded with Vero cells, infected with a virulent strain of *T. gondii*, and incubated for different time periods. The ELISA was performed with anti-*T. gondii* antibodies on the infected cells. The method was simple, rapid, and accurate, and very good correlations between the ELISA results and the percentage of *T. gondii* rosettes in infected cells, the number of free *T. gondii* in the supernatant, and the amount of *T. gondii* antigen in the supernatant were observed.

Toxoplasma gondii is a coccidian protozoan capable of infecting a wide range of vertebrate cells both in vivo and in vitro (4). Since 1972, in vitro cell infection has been utilized for parasite growth kinetics studies under various culture conditions (2), and subsequently, tissue cultures have been used to evaluate in vitro activities of many compounds active against *T. gondii* (3). This laboratory is presently involved in the study of the in vivo activity of anti-*T. gondii* chemotherapeutic compounds (A. Terragna, A. Canessa, and G. C. Orofino, Proc. Int. Congr. Chemother. 13th, Vienna, Austria, p. 76/20–76/23, 1983) and the comparison of results obtained in vivo with those an in vitro model.

The methods previously described to evaluate the in vitro growth of *T. gondii* include counting the percentage of *T. gondii* rosette-containing cells, which is indicative of intracellular parasitic reproduction (3), and the measurement of [³H]uracil incorporation, which is correlated with parasitic DNA replication (5). These methods are suitable but present some disadvantages. Microscopic evaluation of the percentage of rosettes in infected cells is time consuming, cannot be automated, and suffers the effects of subjective variations. Measurement of [³H]uracil incorporation requires the use of radioactive compounds.

The aim of this study was to evaluate whether an enzyme-linked immunosorbent assay (ELISA) (8) of infected cells is a reliable method to study *T. gondii* growth kinetics in tissue cultures. We have used this test for the evaluation of the activity of antiviral drugs in herpes simplex virus-infected cells, and results indicate that the assay is specific, sensitive, reproducible, and can be easily automated (G. Melioli and O. E. Varnier, in A. Mandelli, ed., *Therapy of Leukemias*, in press). In the present paper, we describe the correlation between the results of ELISA and two widely used reference parameters, i.e, the number of trophozoites in supernatant fluid and the percentage of rosette-containing cells in the monolayers.

MATERIALS AND METHODS

Organism. The RH strain of *T. gondii*, originally isolated by A. B. Sabin in 1941 from a child with encephalitis, was obtained by P. Tolentino and maintained by continuous mouse passages in our laboratories. The parasite-rich peritoneal fluid was diluted with medium 199 (GIBCO MB, Milan, Italy) centrifuged at 30 × *g* for 5 min to remove cells

and debris, treated for 30 min at 37°C with gentamicin (150 µg/ml) and cefotaxime (200 µg/ml), and washed three times with phosphate-buffered saline (PBS).

Tissue cultures. Vero cells (African green monkey kidney; strain ATCC CCL81) were maintained in medium 199 with 10% fetal bovine serum (FBS; Flow Italiana, Milan, Italy) at 37°C in 5% CO_2. For the assay, 15,000 cells per well were seeded onto 96-well tissue culture microplates (Flow Italiana). After 24 h, the monolayers were infected with different inputs of *T. gondii* (100,000, 50,000, 10,000, 1,000, and 500 trophozoites per well) in 100 µl of medium 199 with 2% FBS. After 4 h of adsorption at 37°C and extensive washings with PBS, 200 µl of fresh medium with 2% FBS was added to each well. After 0, 24, 48, 72, 96, 120, and 144 h of incubation, the supernatants were collected, the number of free *T. gondii* was microscopically counted, and the monolayers were fixed with methanol and stored at 4°C. The *T. gondii*-containing supernatants were also treated with five cycles of freezing and thawing to extract a soluble antigen (1) suitable for coating onto plastic microplates.

At the same time, Trac bottles with disks (Sterilin, no. 129 AX/1; Flow Italiana) were seeded with 40,000 Vero cells per tube in medium 199 with 10% FBS. After 24 h, cells were infected with trophozoites at the same multiplicity of infection (MOI) used for the microplate cultures. The monolayers were fixed as described above for the microplates and stained with a 1:15 dilution of Giemsa stain.

ELISA. An ELISA was performed to evaluate the amount of *T. gondii* antigen in the infected monolayers and in the supernatants. Antigens extracted from the supernatants as previously described were diluted in carbonate-bicarbonate buffer (pH 9.5) and adsorbed to flat-bottomed 96-well polystyrene micro-ELISA plates (type 129A; Dynatech PBI, Milan, Italy) overnight at 4°C.

The ELISA was performed as follows. The plates containing the infected monolayers and those with adsorbed antigens were washed three times with PBS–1% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) and filled with 200 µl of 0.5% bovine serum albumin (BSA; Sigma) per well in PBS for 30 min at room temperature. The BSA solution was then removed, and plates were washed once with PBS-Tween 20. A pretitrated dilution of a pool (100 µl per well) of anti-*T. gondii* high-titer human immunoglobulin G (IgG) antibodies in PBS-BSA with 4% polyethylene glycol 6000 (Merck Italia, Milan, Italy) (6) was added. After 30 min at room temperature and three washings with PBS-Tween 20, 100 µl of a

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TABLE 1. Rosette-containing cells at various incubation times

Input no. of trophozoites	% of rosette-containing cells at incubation time (h):						
	0	24	48	72	96	120	144
10^5	0	3.5	39	55	30	50	100
5×10^4	0	2	30	20	30	40	95
10^4	0	1.5	3	1	8	4.5	18
10^3	0	0	1	0.5	0	1	6
5×10^2	0	0	0	0	0	0.5	0.5

pretitrated dilution of anti-human IgG (Miles Italia, Milan, Italy) conjugated with alkaline phosphatase (type VII; Sigma) (8) in PBS-BSA-polyethylene glycol was added per well. After 60 min at room temperature and subsequent washings, 100 µl of p-nitrophenyl phosphate (Sigma) (1 mg/ml in diethanolamine buffer, pH 9.8) was added per well and incubated for 30 min at room temperature. The reaction was stopped with 50 µl of 3 N NaOH per well. Plates were washed by a multichannel microplate washing system (Skatron Multiwash; Flow Italiana), and the reagents were added by a multichannel dispenser (Titertek Autodrop; Flow Italiana). Photometric readings were performed at 405 nm by using an ELISA reader (Titertek Multiskan; Flow Italiana) connected to an Apple IIe personal computer (Apple Italiana, Milan, Italy) with a Titertek Multiskan interface (Flow Italiana).

The results are expressed as the mean of the adsorbance values of replications of the experiments minus the mean of the adsorbance values of the negative control wells. The controls included uninfected monolayers, antigens extracted from supernatants of uninfected cells (negative controls), and antigens from infected supernatants and infected monolayers incubated only with enzyme-conjugated anti-IgG.

Numbers of infected cells. The percentage of infected cells containing at least eight trophozoites per cell was calculated by examining microscopically at least 1,000 cells per cover slip.

Statistics. Mean ELISA adsorbance values and microscopic counts were calculated by using the geometric mean. Correlations between assays were performed by using linear regression analysis (7).

RESULTS

The percentages of *T. gondii* rosette-containing cells at different times of incubation are shown in Table 1. The increase in the numbers of infected cells was clearly a function of time, especially in wells infected with high MOIs. The numbers of extracellular trophozoites at different times of incubation are shown in Table 2. In this case, it was also evident that the increase in the numbers of trophozoites observed was a function of time and of MOI. It is worth noting that with low MOIs, the increase in the numbers of extracellular *T. gondii* was significant only after 48 h.
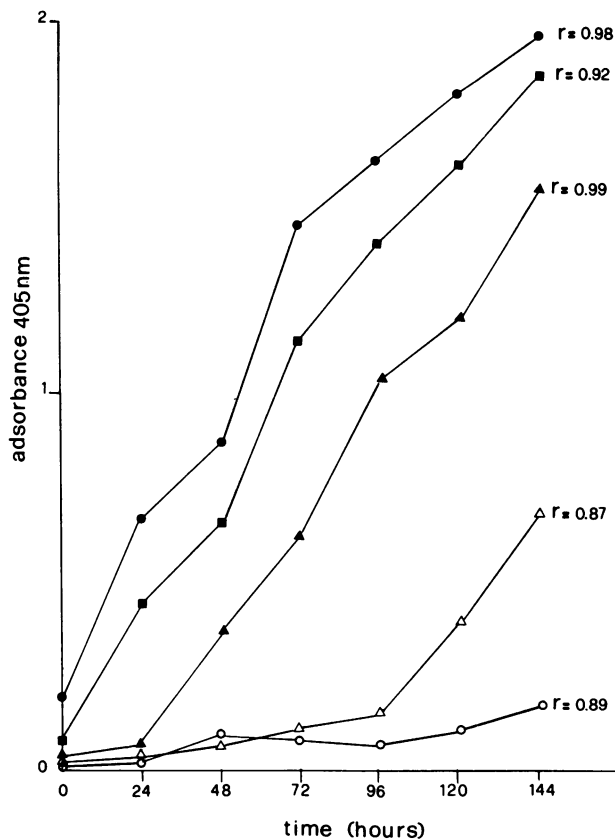

FIG. 1. ELISA with infected monolayers. Symbols (number of *T. gondii* per well): ●, 100,000; ■, 50,000; ▲, 10,000; △, 1,000; ○, 500. The infection and adsorbance values were evaluated after different incubation times.

The ELISA adsorbance values versus incubation times are shown in Fig. 1. There was a good correlation between adsorbance values on infected monolayers and *T. gondii* inputs at different incubation times. A significant correlation between *T. gondii* input and adsorbance values was present at all incubation times except time zero ($r = 0.73$, *P* was not significant). The adsorbance values obtained by performing the ELISA on the 1/10-diluted, infected tissue culture supernatants at different incubation times and with various *T. gondii* inputs are shown in Fig. 2. In both cases, low MOIs did not significantly increase adsorbance values during the first 120 h of incubation. High MOIs gave a more significant increase in the adsorbance values during the early phases of the infection. In the experimental model, a close correlation between adsorbance values of infected monolayers and corresponding supernatants was observed ($r = 0.96$; *P* < 0.001). Figure 3 shows the correlation between assays. The

TABLE 2. Extracellular trophozoites at various incubation times

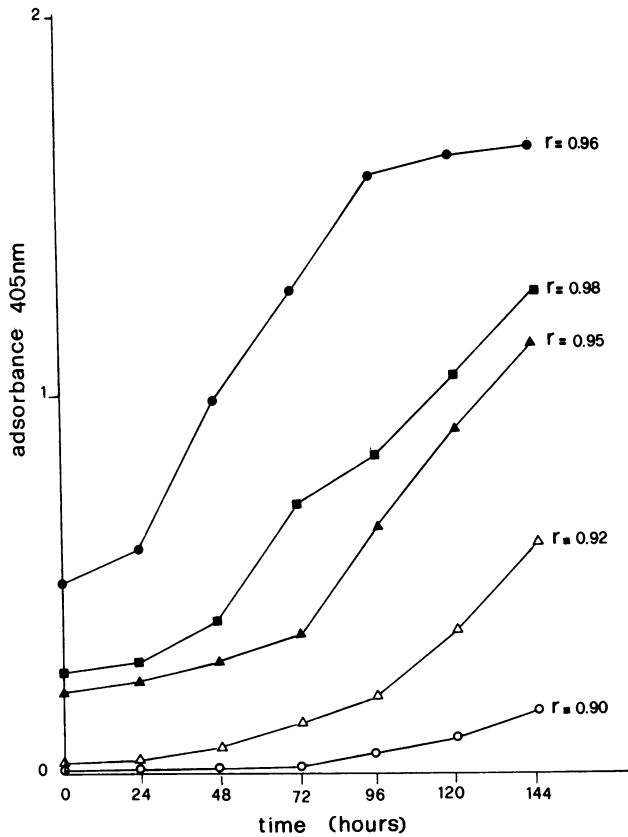| Input no. of trophozoites	No. of extracellular trophozoites at incubation time (h):						
	0	24	48	72	96	120	144
10^5	2×10^4	3×10^5	4.8×10^5	8×10^5	10^6	1.8×10^6	10^7
5×10^4	1.4×10^4	8×10^4	10^5	10^6	1.2×10^6	1.5×10^6	1.6×10^6
10^4	6×10^3	6.3×10^3	7×10^3	8.4×10^3	1.2×10^4	1.6×10^4	2×10^4
10^3	0	0	6×10^2	2×10^3	3.4×10^3	6.2×10^3	7×10^3
5×10^2	0	0	6×10^2	2×10^3	3.1×10^3	5.8×10^3	6.3×10^3

FIG. 2. ELISA with supernatants from *T. gondii*-infected cell cultures. The experimental conditions, *T. gondii* inputs, and incubation times were as for infected monolayers (see the text).

FIG. 3. Adsorbance values of the ELISA with infected monolayers versus adsorbance values of the ELISA with *T. gondii* antigen in the supernatant.

correlation between the ELISA of the supernatants and the numbers of free *T. gondii* was not significant in the first 72 h of incubation (data not shown). After this time, there was a greater correlation between the two tests ($r = 0.86$; $P < 0.05$). Figure 4 shows the correlation between adsorbance values and the logs of the numbers of free *T. gondii* in the supernatants. The experimental lower limit of the ELISA was 1,000 *T. gondii* per well. Finally, the percentage of rosette-containing cells and the adsorbance values of infected monolayers demonstrated an increasing correlation ($r = 0.82, 0.80, 0.90$, and 0.95, respectively) upon reduction of the infective dose from 100,000 to 1,000 trophozoites per well. Figure 5 shows the results of a typical experiment where adsorbance values were plotted versus the logs of the percentages of infected cells.

DISCUSSION

Variations in the percentages of infected cells and the amounts of free *T. gondii* in supernatant fluid in relation to time are widely accepted parameters representing the growth of *T. gondii* in tissue culture. The close correlation between the results of direct microscopic counting of *T. gondii* and the ELISA demonstrated that the ELISA performed with *T. gondii* antigens was able to accurately reproduce the growth kinetics of the protozoan. In addition, we found a significant correlation between ELISA results and *T. gondii* input and incubation time. Furthermore, the results of the antigen immunoassay in infected monolayers, the percentage of infected cells, and the number of trophozoites in the supernatant show a significant correlation. On the other hand,
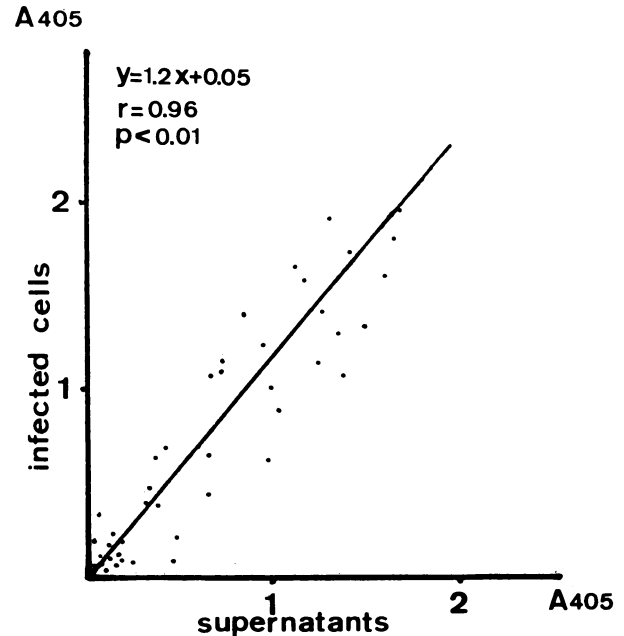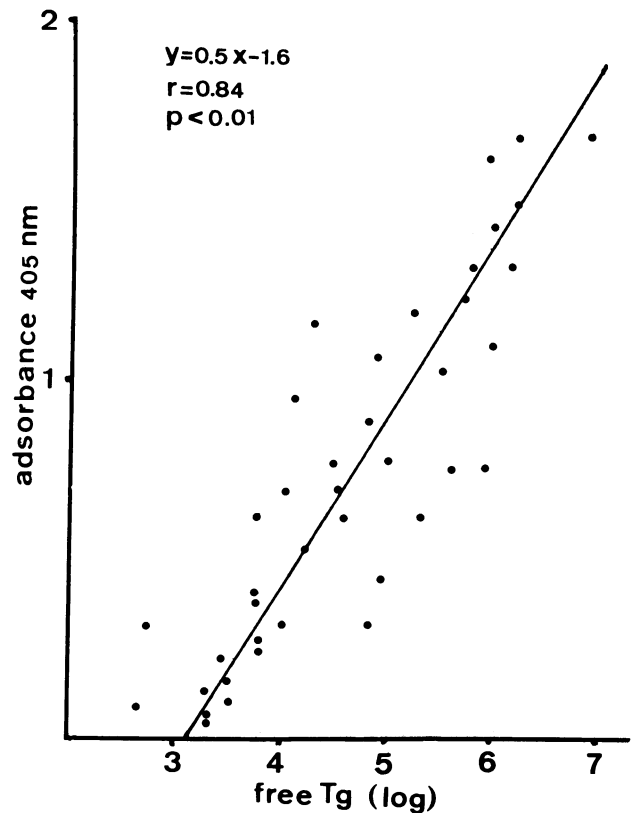

FIG. 4. Adsorbance values of the ELISA with antigen in the supernatants plotted versus the numbers of free *T. gondii* in the same group of supernatants. Significant adsorbance values were obtained when more then 1,000 free *T. gondii* were in the culture medium.
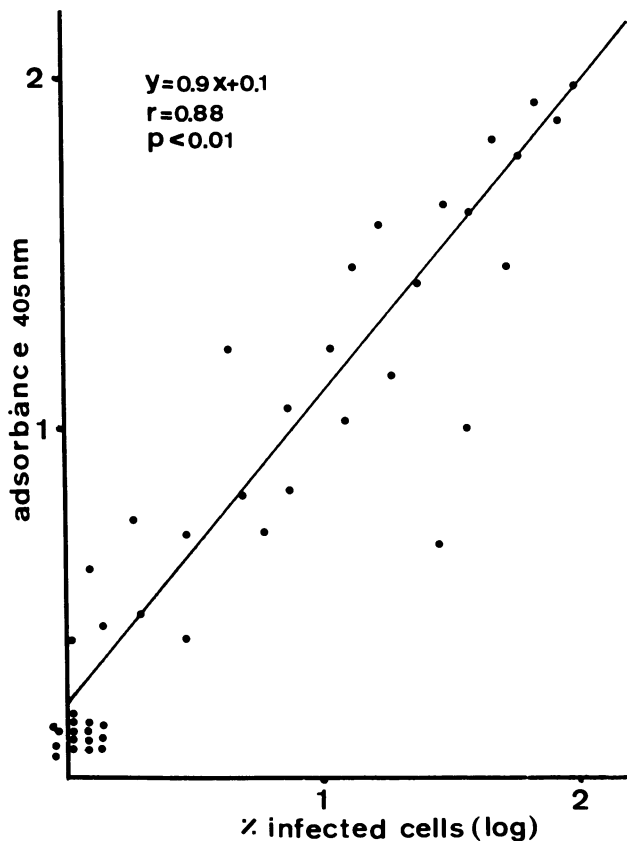
FIG. 5. Adsorbance values of the ELISA with infected mono-
layers plotted versus the logs of the percentages of infected cells.
The sensitivity of the assay was poor when low percentages of
rosette-containing cells were present in the monolayers.

only after 72 h after the infection was it possible to show a
significant correlation between free *T. gondii* and ELISA
results with antigen extract. This was probably due to
inaccuracies in counting extracellular *T. gondii* during the
early phases of the infection, when the number of trophozo-
ites was very small. The ELISA on infected monolayers did
not agree completely with the percentage of infected cells.
High MOIs at 72 and 96 h showed a reduction in the
percentage of infected cells, probably caused by the cyto-
pathic effect of high numbers of infecting protozoans (Table
1). Dead cells could not be included in the calculation of the
percentage of infected cells; consequently, the statistical
correlation suffered. In addition, with very long incubation
times, the number of uninfected cells decreased, inhibiting
the proliferation of the protozoans. These considerations
may explain the apparent irregular growth of *T. gondii* in our
experiments.

It should be mentioned that the negative control adsorb-
ance values were higher on uninfected monolayers than on

uninfected cell antigen-coated microplates. This resulted in a
reduction in the range of adsorbance values which could be
used in the assay. On the other hand, the test performed on
infected monolayers was more simple and offered a wider
range of adsorbance values (Fig. 1 and 2). Finally, at
between 96 and 144 h, there was a 10-fold increase of the
numbers of *T. gondii* at higher MOIs (Table 2), whereas
there was a very small increase in the corresponding adsorb-
ance values (Table 2). This was probably due to the great
number of *T. gondii* in comparison to the amount of anti-Tg
antibodies used for the ELISA. In other experiments (data
not shown) with a different preparation of anti-*T. gondii*
antibodies, similar numbers were detected with more preci-
sion.

Despite the problems described above, the ELISA per-
formed with monolayers was simple, rapid, and objective.
The assay was optimized in our laboratory especially for
long incubation times to obtain clear differences in adsorb-
ance values among wells containing different percentages of
infected cells. This made possible a precise quantitation of
the antigen in tissue cultures or in supernatants. Since the
assay was sensitive to variations in the amounts of antigen in
tissue culture, it could be utilized to study the influence of
different culture conditions (including the in vitro activity of
anti-*T. gondii* compounds) on the growth kinetics of *T.
gondii*.

ACKNOWLEDGMENTS

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