The observation that the SAL effect was blocked by PEN only in 10/16 animals may be explained by the central stimulating effect of salicylates. Such an effect has been reported in salicylate poisoning(8). It was also indirectly observed in the present experiments, in that 6 of the SAL treated animals, but none of 25 controls showed signs of incomplete or shortened anesthesia.

Summary. The previously established specific action of salicylic acid on the adrenal-pituitary system has been investigated as to duration, effect of repeated doses of salicylic acid, effect of pretreatment with cortisone and possible mechanism of action. The effect of a single injection of salicylic acid (300 mg/kg) lasted more than 16 hours, at which time still appreciable bloodlevels of salicylic acid were present. Repeated injections of salicylic acid did not impair the responsiveness of the adrenals. Pretreatment with cortisone reduced, but did not abolish the effect of salicylic acid. Complete anesthesia with pentobarbital blocked the effect of salicylic acid. The results are interpreted as an effect of salicylic acid on the hypothalamus with subsequent stimulation of the pituitary.

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Intracellular Multiplication of *Toxoplasma gondii* in Adult Mammalian Macrophages Cultivated *in vitro*.* (21117)

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Toxoplasma gondii, an obligate intracellular parasite, can easily be propagated in cultures of different types of tissues of either embryonic or adult origin and the parasites have been maintained in serial passages in tissue cultures without detectable loss of virulence for mice(1,2). The organisms invade and probably multiply in cells derived from all 3 embryonic layers and there is no evidence for any cellular specificity. In experimental toxoplasmosis, especially after intraperitoneal infection, the parasites are mainly located in macrophages. These cells can be maintained in tissue cultures and provide a system for *in vitro* study of intracellular parasites(3). After preliminary experiments had shown that Toxoplasma do multiply in macrophage cultures, the ability of Toxoplasma to multiply *in vitro* within macrophages derived from susceptible and resistant animals was studied.

Materials and methods. Animals. Swiss white mice (Schwentker strain) were raised and supplied by the Department of Bacteriology and Immunology, Harvard Medical School. Rats, guinea pigs and rabbits were obtained from outside sources. The animals

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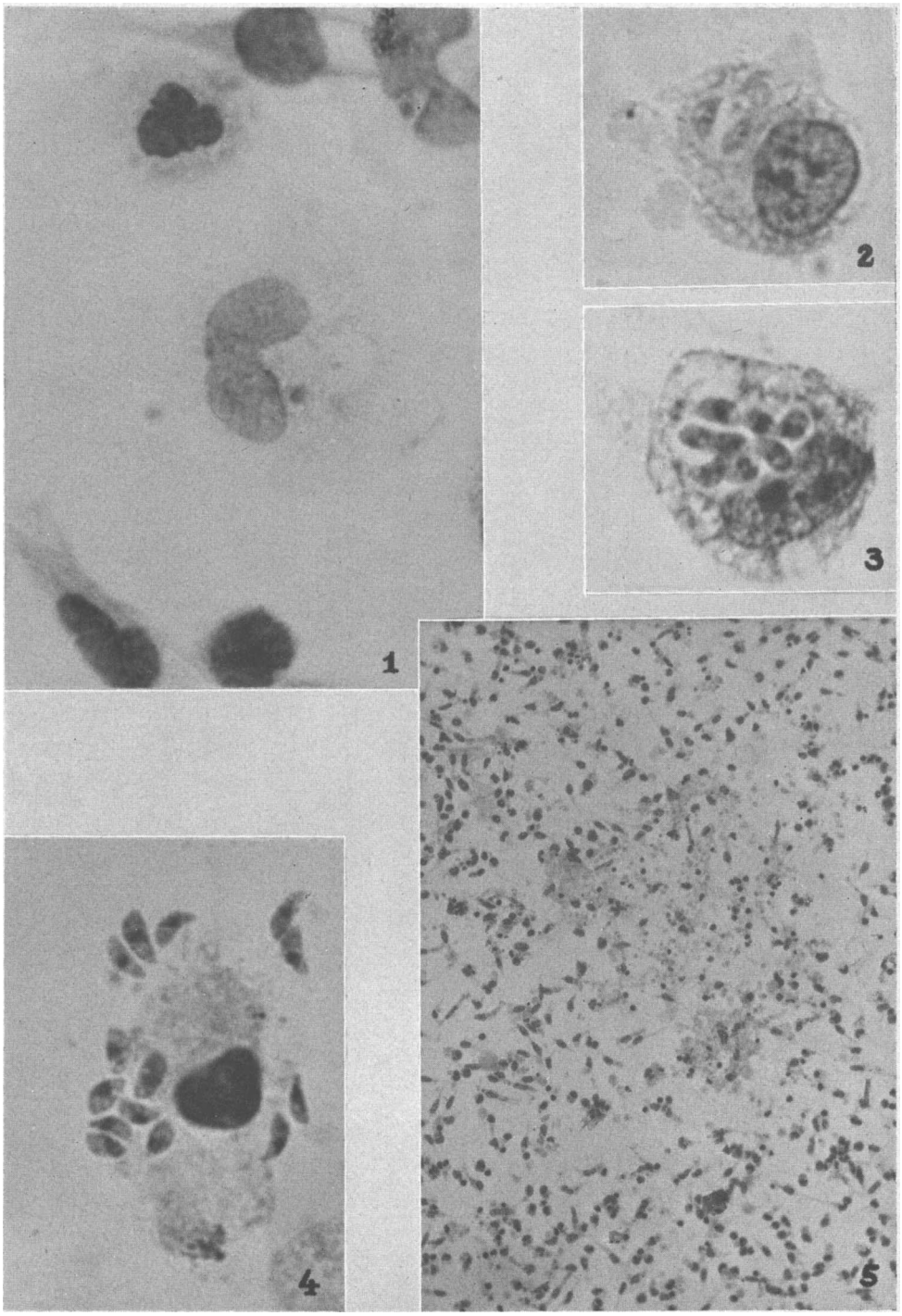
were fed with pellets and water, and the guinea pigs and rabbits supplied with cabbage *ad lib*.

Toxoplasma. The RH strain, originally isolated from a child by Sabin(4), was used throughout this study. It was obtained from 2 independent sources: one strain from Dr. Q. M. Geiman (Department of Tropical Health, Harvard School of Public Health, Boston), and the other through the courtesy of Dr. J. K. Frenkel (Department of Pathology and Oncology, University of Kansas, Kansas City.) The strains were maintained in mice. The content of the peritoneal cavity of a moribund mouse was collected and 0.2 ml of varying dilutions was injected intraperitoneally into fresh mice; 0.2 ml of a 10^{-7} dilution usually contained enough Toxoplasma to kill the mice within 7-14 days. To obtain a comparable infective dose of Toxoplasma for the infection of the macrophage cultures, mice were killed 4-5 days after infection with ether and were injected in the peritoneal cavity with 2-3 ml of Gey's balanced salt solution containing 1:20000 heparin (La Motte Chemical Products Co., Baltimore). The mixture of ascitic fluid and physiologic solution was withdrawn aseptically and squeezed twice with a syringe through a 27-gauge needle to free the intracellular Toxoplasma. This suspension was centrifuged for 3 minutes at 500 r.p.m. to separate the remaining cells from the suspension of free Toxoplasma. The supernatant fluid was drawn off and the Toxoplasma were counted in a hemocytometer using physiologic saline solution as a diluent. Similarly, for the virulence studies 10-fold dilutions of the supernatant fluids of exudates containing a known number of Toxoplasma were injected intraperitoneally into groups of animals. The *macrophage cultures* were prepared and maintained as described earlier(3). Five to 7 days after the intraperitoneal injection of the chemotactic agent (glycogen or 12% sodium caseinate) the exudate was collected aseptically in Gey's balanced salt solution containing 1:20000 heparin. The number of cells in the suspension was determined with the hemocytometer. Aliquots of 9 ml of cell suspensions were prepared to contain 2000-2500 cells per mm^3 and poured into small Petri dishes, which had 10 small coverslips on the bottom. Usually the Toxoplasma were mixed with the cells at this time: to 9 ml of cell suspension one ml of Toxoplasma suspension, prepared in the manner described above, was added. The number of Toxoplasma in the suspension was adjusted to approximate in the final mixture a proportion of one Toxoplasma per 7 macrophages. In some experiments, the size of the inoculum was either increased or decreased. The Petri dishes were incubated at 37°C for one to 2 hours in an air-tight box filled with alveolar air. Each coverslip was then removed, washed in balanced salt solution, covered with a thin film of formvar, and finally placed in a small screw capped vial. Each vial contained 0.5 ml of culture medium with 30% serum (guinea pigs, rat, or rabbit) in Gey's solution. To inhibit the growth of contaminants, streptomycin was added in a final concentration of 2 μg/ml and penicillin 50 u/ml. These concentrations of antibiotics have been reported not to inhibit the multiplication of Toxoplasma(5). To adjust the pH to 7.2 the tubes received 5% CO_2 in air. The tubes were closed airtight and placed in the incubator in almost a horizontal position. Coverslips were taken out immediately after the period of sedimentation and phagocytosis, and at about 12-hour intervals. The cultures were fixed in absolute methyl alcohol for 10 minutes, then the formvar was removed mechanically from the back of the slide. After drying, the slides were placed in ethylene dichloride for one hour to dissolve or alter the remaining formvar. This procedure was found to be essential to obtain good staining of the macrophages and the Toxoplasma. The preparation was then washed in water and stained in hematoxylin for 2 hours, and mounted on slides with permount and covered with a coverslip.

Immunization. Rats and guinea pigs were immunized by subcutaneous injection of living Toxoplasma obtained from the peritoneal fluid of infected mice. One to 3 injections of 0.5 ml each were given at intervals of 3 weeks. For the first injection the peritoneal fluid was diluted 100-fold, for the second 10-fold and it was used undiluted for the third injection.



Various stages of intracellular multiplication of Toxoplasma within macrophage cultures from mice, guinea pigs and rats. Hematoxylin.

FIG. 1. Mouse cells, one hr. Indication of disappearance of one of the parasites from the cell. ×1800.

FIG. 2-4. Mouse cells, 67 hr. Macrophages with increasing numbers of parasites and rupture of the cell. ×1800.

FIG. 5. Mouse cells, 51 hr. Macrophage destruction around a ruptured cell. ×250.

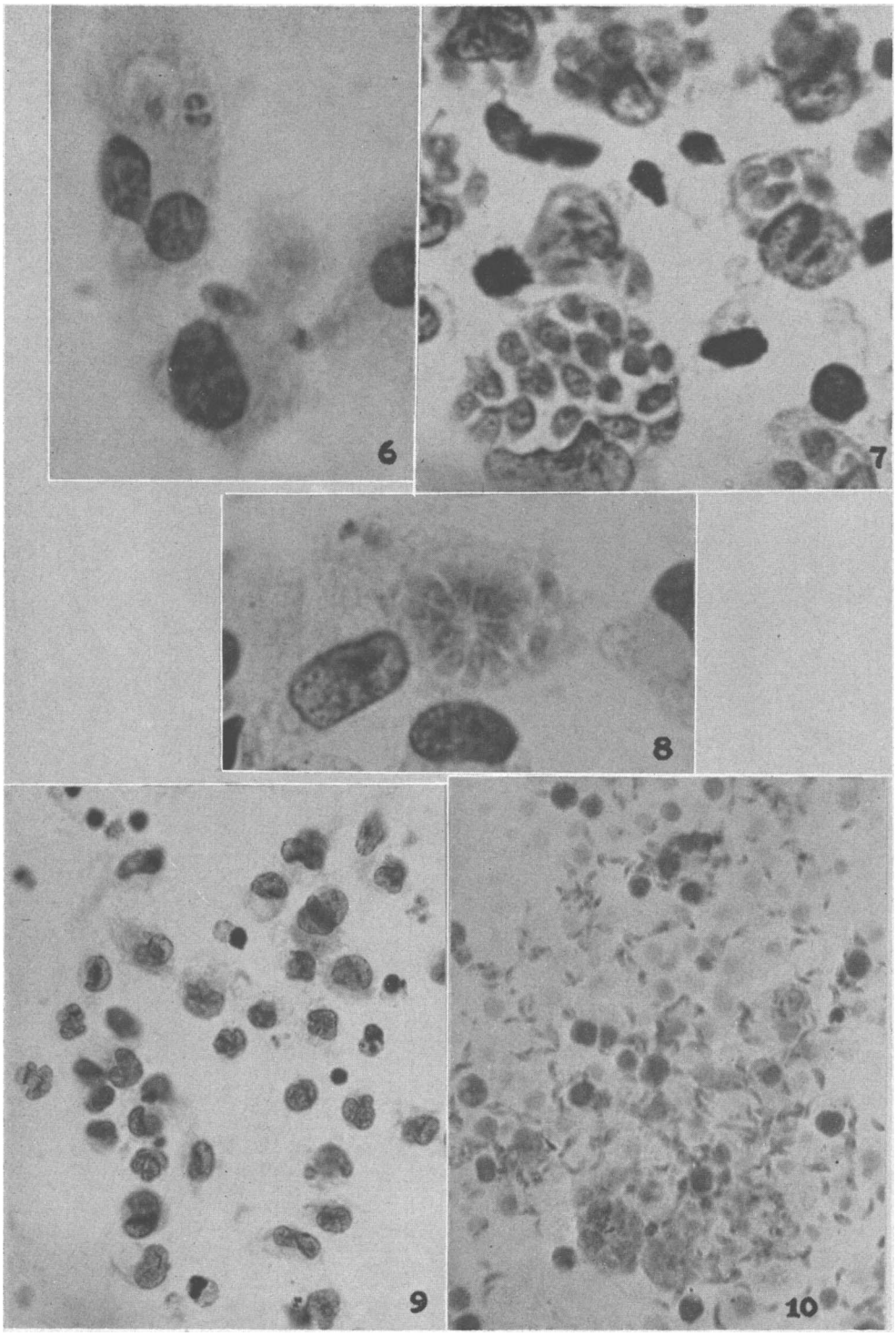


FIG. 6. Guinea pig cells, one hr. Upper cell with 3 parasites losing their staining properties. ×1800.

FIG. 7. Rat cells, 19 hr after heavy infection. Cells containing 4-20 or more parasites. ×1800.

FIG. 8. Rat cells, 51 hr. "Rosette" formation. ×1800.

FIG. 9 and 10. Macrophages from immunized and normal rat cultured for 72 hr in a medium containing immune and normal serum, respectively. Difference in the number of Toxoplasma in the cultures indicates the inhibitory property of the macrophages from the immunized rat. (Fig. 9.) ×600.

Simultaneously with the immunization, control animals were injected with 0.5 ml of a suspension of macrophages obtained from peritoneal washings of normal mice. As a criterion for the presence of antibody in sera obtained from immunized rats and guinea pigs, the dye-test for cytoplasm modifying antibody(6) was used.

Experimental observations. 1. *Multiplication of Toxoplasma in mouse macrophages.* At the beginning of cultivation the proportion of cells containing Toxoplasma and the number of parasites per infected cell depended on the size of the inoculum (Fig. 1). The first observed change within about 8 hours was an almost complete disappearance of stainable intracellular parasites. During this phase the cytoplasm of many parasites remained unstained and at the site of the nucleus a small structure, stained deeply with hematoxylin, was visible. A few hours later Toxoplasma reappeared within the cells and steadily increased in number. At 36 hours and later cells were found containing 1, 2, 3, 4, 6, 8 or more parasites, in general a multiple of 2 (Fig. 2 and 3). In some instances a vacuole was clearly visible around the Toxoplasma. Mouse monocytes contained rarely more than 16 parasites. In this stage, the nucleus of the cell was pushed towards the periphery and finally, the cell ruptured and released the Toxoplasma (Fig. 4). Because the culture was covered with formvar the parasites could not escape into the fluid but were held on the slides and were therefore phagocytized by other macrophages where they multiplied again. This process of cell rupture and reinfection finally resulted in the destruction of all the macrophages. In the early phases of cellular rupture, it could usually be observed that the release of Toxoplasma led to damage of adjacent cells although no parasites could be seen within these cells (Fig. 5). The release of free Toxoplasma depended on the size of the inoculum. Thus, in cultures in which the ratio of Toxoplasma to macrophages was 3:5 in the infection period, Toxoplasma was released from the cells after 19 hours of cultivation; with a ratio of 1:10 they only appeared after 43 hours. If the inoculum was very small (1:20), no increase of Toxoplasma was observed over a period of 6 days.

TABLE I. Per Cent of Intracellular Toxoplasma in Macrophages from Mice, Guinea Pigs, Rats and Rabbits after Increasing Length of Incubation.

	Min. of incubation		
	15	30	45
Mouse	38	70	67
Guinea pig	82	93	96
Rat	63	70	77
Rabbit	77	100	100

2. *Multiplication of Toxoplasma in macrophages of different species.* When cultures of macrophages from mice, guinea pigs, rats, and rabbits were set up side by side, a number of differences were observed in the rate of uptake of Toxoplasma by the cells and of multiplication of the parasites and in the extent of destruction of macrophages resulting from the increasing number of intracellular parasites. Because humoral factors possibly present in peritoneal exudates and influencing the interaction between macrophages and Toxoplasma had to be excluded, the procedure of preparing the cultures had to be modified. The macrophages were first allowed to settle on the slides during an hour's incubation, which were then washed in physiological solution, placed in new Petri dishes and covered with a suspension of Toxoplasma. After a second period of incubation for one hour the cultures were finally washed and covered with formvar and cultivated as described. From Table I it is clear that first the parasites were taken up more rapidly and more completely by macrophages from rats, guinea pigs, and rabbits, than from mice. Secondly, the disappearance of intracellular Toxoplasma in the first few hours of cultivation was observed consistently and to a more complete extent in the cultures of macrophages from guinea pigs,

rats, and rabbits than in those from mice (Fig. 6). Thirdly, in cultures from the guinea pigs, rats, and rabbits a longer time elapsed until the parasites began to reappear compared with those of mouse macrophages. Likewise intracellular multiplication was much slower in macrophages from rats, guinea pigs, and rabbits. In consequence, the cultures were destroyed to a far less extent and only after prolonged cultivation. It was also observed that mouse cells seemed far more susceptible to intracellularly accumulating parasites than the cells from other animals. This resulted in a more rapid destruction of the cultures of mouse cells, whereas a high number of Toxoplasma accumulated within cells of rats and guinea pigs (Fig. 7 and 8). Comparing the susceptibility of these species to the intraperitoneal infection with Toxoplasma, it was found that mice and guinea pigs were killed by approximately 5 to 10 parasites whereas rats survived the injection of 6 x 10^6 Toxoplasma. Mice were also susceptible to small numbers of parasites injected subcutaneously whereas guinea pigs were much more resistant to this route of infection.

3. *Multiplication of Toxoplasma within macrophages from immunized animals, and the role of antiserum.* Because of the high virulence of the RH-strain for mice, immunization could not be achieved and the experiments were done with macrophages from rats and guinea pigs previously immunized with living Toxoplasma. Sera from normal animals never showed a titer of cytoplasm modifying antibodies above 1:8, whereas the sera from immunized animals had titers from 1:2000 to 1:8000. These titers correspond well with those given in the literature(6). To separate inhibitory properties residing within the macrophages from those exhibited by the serum, macrophages from normal and immunized animals were cultivated separately in normal and in immune serum. In addition, in some experiments, the macrophages were washed with balanced salt solution before they were infected with Toxoplasma to remove as completely as possible any antibody absorbed on the surface of the cells. Extent of multiplication of Toxoplasma in such cultures, as judged by the accumulation of intracellular and free parasites, is summarized in Table II. Macrophages from immune animals cultivated with immune serum exhibited the greatest inhibitory property, and after 4 days of cultivation only a few intracellular Toxoplasma could be found (Fig. 9 and 10) at a time when in the cultures of macrophages from normal animals free and intracellular Toxoplasma were abundant. Less inhibition was exerted by either immune cells with normal serum or vice versa. Washing the macrophages before exposing them to the Toxoplasma did not abolish their limited inhibitory power. Pycnotic nuclei, presumably from dying Toxoplasma, were frequently observed within macrophages from immunized animals cultivated with immune serum.

TABLE II. Degree of Intracellular Multiplication of Toxoplasma within Macrophages Derived from Immunized Animals and Influence of Immune Serum.*

Cells: Serum:	Immune "	Immune Normal	Normal Immune	Normal "
Exp.† 37	1	2	2	4
43	1	2	2	4
61	1	3	2	4
76	1	2	2	4
81	1	2	3	4
84	1	2	2	4
91	1	2	3	4
94	1	2	2	4

* Multiplication is judged by the presence of free and intracellular parasites and is rated on an arbitrary scale, 4 indicating maximal and 1 minimal multiplication.

† Exp. 37-84 were done using rat macrophages, 91 and 94 guinea pig cells.

Discussion. The results reported indicate that intracellular reproduction of Toxoplasma in macrophage cultures occurs in 3 phases: penetration and phagocytosis, multiplication, and release of parasites followed by infection of new cells. There was good evidence that macrophages do phagocytize Toxoplasma which themselves penetrate actively into nonphagocytic parenchymal cells. The phagocytic activity of the macrophages was found different with cells from mice, rats, and guinea pigs. It was consistently observed that a few hours after infection only very few cells contained stainable Toxoplasma although a great number of parasites were present immediately after infection. The significance of this disappearance is not clear. Sabin and Feldman(6)

described similar changes of the staining properties under the influence of heat, cold, and antibody, suggesting that such changes are manifestation of degeneration. Differences of behavior of Toxoplasma within macrophages from various species, as time of reappearance of stainable forms and susceptibility of the cells to the accumulating parasites, suggest that the interaction between macrophages and Toxoplasma is a determining factor in the mechanism of native resistance. This view has been raised by Frenkel(7) and is supported by evidence presented in this paper. Finally, our experiments indicate that immunization results in restriction of intracellular multiplication of Toxoplasma by the macrophages as well as by a humoral factor. It should be added that it is difficult to prove conclusively that the inhibition exerted by cells was not due to remaining antibody adhering to the cells, although washing of the macrophages did not abolish this inhibition. However, the fact that macrophages from immune animals combined with immune serum were much more inhibitory than the immune serum alone with normal macrophages, supports the assumption of a cellular factor in immunity against Toxoplasma. This duality had been postulated much earlier by Levaditi (8) and again by Frenkel(7). This property of macrophages from immune animals to inhibit intracellular proliferation of Toxoplasma is no unique observation. Thus, destruction of malarial parasites by spleen macrophages in immunized animals has been described long ago(9), and similarly, inhibition of intracellular multiplication of tubercle bacilli by macrophages from animals vaccinated with BCG has been demonstrated in tissue cultures(10), but the mechanism of cellular inhibition in any of these infections remains unknown.

Summary. *Toxoplasma gondii* has been propagated successfully in macrophage cultures of adult laboratory animals. The pattern of the interaction between the macrophages cultured *in vitro* from different animal species could be correlated with the susceptibility of the species to the infection with Toxoplasma. Macrophages cultured *in vitro* from animals actively immunized by injection with living Toxoplasma had an inhibitory effect on the intracellular multiplication of these parasites. This effect was increased by the presence of immune serum which had a high titer of cytoplasm-modifying antibodies.

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