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by

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INTRODUCTION

Investigation of protozoan effects on the spleen, ovaries, and testes of mature white mice may give some information as to physiological effects produced by bacteria-free protozoa on these tissues. Final conclusions are taken from histological examination of the spleen, ovaries, and testes that have been fixed in Helly's fluid, sectioned, mounted on slides, and stained with Heidenhain's iron hemotoxylin.

The present investigator made observations to determine whether there appeared an interference in endocrine function of the sex organs due possibly to various hormone neutralizing agents connected with the injected protozoa, as well as other serological responses such as antibody formation in the injected mice to the foreign bodies being introduced into them. Bacteria-free protozoa afford an ideal situation for investigation of introduced foreign bodies because only one type of organism is being introduced into the animals and any serological response will be specific for that one organism. If protozoan cultures are used that are not bacteria-free, serological and histological responses will not mean that the protozoa are entirely responsible for the observed effects but bacteria may be causing them.

Investigation of some splenic components may reveal a possible reticulo-endothelial involvement of the cells found in the spleen.

MATERIALS AND METHODS

The two strains of bacteria-free protozoa used in this work are classed as strains of the genus, Tetrahymena. These ciliates are known as Tetrahymena geleii strain W and Tetrahymena geleii strain GP. Tetrahymena cultures were the first among protozoa to be produced free of bacteria(A.Lwoff, 1923).

Serological methods are usually regarded as more exact than any other means of tracing relationships between organisms, but these methods have not been used extensively with protozoa. Earlier attempts to trace antigenic relationships have been criticized because the ciliate cultures were not bacteria-free. Members of the genus Tetrahymena from bacteria-free cultures, were investigated serologically by Robertson(1939a,b) and by Tanzer(1941). Both Robertson and Tanzer found rather close relationships between the strains of ciliates from immobilization tests in antisera. All in all the serological studies confirm the extensive serological and biochemical work by Kidder and his co-workers(1945a,b,c,d) which established and stabilized the nomenclature of the different strains of organisms in the genus, Tetrahymena.

Furgason(1940) formed a new genus and species to include several ciliates of uncertain taxonomic position and he has classified several "species" as strains of Tetrahymena geleii. It is now known that the old Glaucoma-Colpidium group of ciliates are strains of Tetrahymena geleii. The cultures of Tetrahymena geleii strain GP used in this experiment are included in the old Glaucoma-Colpidium group of ciliates.

Tetrahymena geleii strain W[1] was established in bacteria-free culture by C.L. Claff(1940) from Mill Pond, Woods Hole, Massachusetts.

Tetrahymena geleii strain GP[2] is a Hetherington strain(obtained from Elliott). According to Hall(1944) this culture was sent to Dr. A. Lwoff, who transmitted the ciliates to Robertson. Robertson mistakedly attributed the isolation of this strain to Hall and named the strain, GpH. After the work of Kidder and Dewey(1945) this strain became officially known as Tetrahymena geleii strain GP, being added as such by Hall(1945a) to the Kidder list(1945c) of strains of Tetrahymena geleii.

The stock cultures of Tetrahymena geleii strain W and Tetrahymena geleii strain GP were subcultured by transferring one loopful of each culture to 250 cc. Pyrex Erhlenmeyer flasks containing 125 cc. of sterile 2% Difco proteose-peptone solution made up with distilled water. The Erhlenmeyer flasks and all other glassware used in this investigation were washed in hot tap water and Calgonite, which contains Calgon(glassy sodium phosphate). Calgonite is a Fisher Scientific Company product. Then the glassware was rinsed several times in distilled water. The wire loop used was made from a twenty-six gauge platinum wire with an inside diameter of three millimeters. Sterile procedures were maintained throughout the subculturing. The organisms in the subcultures were allowed to attain a maximum growth

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[1]The cultures of Tetrahymena geleii strain W used in this experiment were obtained from Dr. G.W. Kidder, Amherst College, Mass.

[2]The Tetrahymena geleii strain GP cultures were obtained from Dr. H. Kirby, University of California, Berkeley, California.

phase before injection into the mice. This maximum growth phase was reached three to four days after subculturing. Then these cultures were used for the injection and with each succeeding day of injection the next older cultures were used in the experiment. The last cultures used were approximately fourteen to fifteen days old.

The mice used for this experiment were a non-homozygous laboratory strain of white mice obtained from the Research Laboratories in Philadelphia, Pennsylvania. A group of thirty-four mature white mice were used, half males and half females. One group was used for injection of Chalkley's solution as the control group. A second group was used for the injection of Tetrahymena geleii strain GP. A third group was injected with Tetrahymena geleii strain W. Each group contained six males and six females with the exception of the female groups of Tetrahymena geleii strains W and GP, which contained five each. All the mice were fed Purina Laboratory Chow and kept constantly supplied with water. All the cages were regularly cleaned.

Chalkley's solution[3] was the saline vehicle used for transport of the ciliates injected into the mice. It is non-toxic to the protozoa while mammalian physiological saline is toxic to them(Kidder, et al., 1945d).

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[3]Chalkley's solution: 80 mg. NaCl, 4 mg. $NaHCO_3$, 4 mg. KCl, 4 mg. $CaCl_2$, 2 mg. $CaH_4(PO_4)_2$, 2 mg. $Mg_3(PO_4)_2$, 1 H_2O, and 100 cc. H_2O.

Fifteen cubic centimeters of the suspensions of Tetrahymena geleii strains W and GP were transferred by sterile 25 cc. pipettes to sterile 15 cc. centrifuge tubes. These were centrifuged for one to two minutes at 235 RPM and the supernatant fluid drawn off the tubes with sterile 15 cc. pipettes. It was necessary to centrifuge four times before the organisms were built up to 0.1 cc. the first day of injection. Three centrifugations sufficed for the remainder of the injection period for the ciliates to build up to 0.15 cc. Only one flask of each strain of cells was used for centrifuging so as to cut down on possible bacterial contamination. Both strains were washed four times by centrifugation with Chalkley's solution the first day of injection and suspended in 1 cc. of Chalkley's solution. For the remaining nine days of injection the ciliates were washed three times and suspended in 0.5 cc of Chalkley's solution. Five-hundredths of a cc. of the respective cell suspension was injected into each mouse the first day and 0.04 cc. the remaining nine days. Ten daily injections were made and followed by half of the mice in each group being sacrificed by etherization to obtain the spleen, testes, and ovaries from each animal. The tissues were placed in vials to be run through a procedure discussed in Bensley and Bensley(1947) for fixing, imbedding, sectioning, mounting, and staining with Heidenhain's iron hemotoxylin(p.78). No serological tests were performed on the sera of these animals.

The remaining half of the mice injected for ten days and allowed to stay uninjected for an additional seven days were killed with ether to obtain the spleen, ovaries, and testes from each animal

for running through the same procedure as the tissues injected for ten days.

Standard in serological examination of free cells are agglutination and lysis tests. Immobilization can serve as a criterion for serological examination of ciliated cells(Landsteiner,1947). A simple serological test was performed on mouse serum for the animals injected for ten days and allowed to remain uninjected for an additional seven days. Immediately after etherization, the mice were bled and the pooled serum was placed in a refrigerator for twenty four hours to allow the red blood cells to settle out. Serological observations were made on sealed hanging-drop preparations containing equal parts of culture suspension and serum. One slide for Tetrahymena geleii strain W and strain GP were made with each serum from the pooled blood of the control and experimental animals. The preparations were examined under the high power objective of a microscope after intervals of twenty minutes and five and a half hours.

Three slides were made for each organ removed from the animals with an average of four sections to a slide. In the spleen, twenty fields per section and one hundred fields per spleen were counted for the average number of giant cells present. Nine slides were made from the spleens of animals injected with Chalkley's solution for ten days and, also, the spleens of animals injected with Chalkley's solution for ten days and allowed to remain uninjected an additional seven days. This same number of slides was made for both experimental

groups of animals injected with Tetrahymena geleii strain W and strain GP. The counts for spleens in a respective group were averaged together and these averages were tabulated in the results. All ovary sections on the slides were used to count the average number of follicles and corpora lutea present in the ovaries of the different groups. Final results showed the presence or absence, as well as, a difference in number of corpora lutea and follicles in the different groups of mice. The testis slides were examined for any difference in the interstitial cells of both experimental and control animals.

RESULTS

Table I is used to tabulate the average number of giant cells in the spleens of the different groups.

Table I

Average Numbers Of Splenic Giant Cells.

Injected Material	10 Day Males	10 Day Females	17 Day Males	17 Day Females
Chalkley's Solution, (Control)	1.84	1.42	1.27	1.44
Strain W Cells,(Chalkley's Solution,Vehicle)	1.97	1.80	3.20	2.87
Strain GP Cells,(Chalkley's Solution,Vehicle)	1.58	1.77	3.53	2.21

These results indicate that Tetrahymena geleii strain W and strain GP cells from bacteria-free cultures injected into mature white mice cause no appreciable or significant difference between the control and experimental animals injected for ten days nor any difference due to sex. In the animals injected for ten days and allowed to remain untreated for seven more days, there was a pronounced difference in the number of giant cells of the control group and the two experimental groups. Also, there was a pronounced difference due to sex in the number of giant cells observed for the two experimental groups.

Table II is used to tabulate the serological data obtained from the hanging-drop preparations.

Table II

Serological Investigation Of The Sera Of Control And Experimental Mice On Tetrahymena geleii Strains W And GP

	T. geleii strain W		T. geleii strain GP	
Sera Used	20 min.	5½ hrs.	20 min.	5½ hrs.
Anti-W Serum	No Immobilization.	No Immobilization.	Partial Immobilization.	Partial Immobilization.
Anti-GP Serum	No Immobilization.	No Immobilization.	Rounding Up	Rounding Up
Control Serum	No Immobilization.	No Results	No Immobilization.	Rounding Up

Serological investigation of the sera of mature white mice injected for ten days and allowed to remain untreated for seven more

days showed partial immobilization of strain GP in anti-W serum, rounding up of strain GP twenty minutes after preparation with anti-GP serum, rounding up of strain GP five and a half hours after preparation with control serum, and no immobilization or rounding up of strain W in any of the three sera after intervals of twenty minutes and five and a half hours. Immobilization in this investigation means the slowing down of organisms with noticeably slower ciliary beating. Rounding up means complete stoppage of movement of organisms and cilia. Although the majority of ciliates in a field showed the observed phenomena of immobilization or rounding up, not all of them showed these phenomena. Approximately fifty to seventy-five per cent per field responded to the tabulated results.

Micr0scopical observation of the ovaries showed no significant difference in the number of primordial, primary, and Graafian follicles of th0se control and experimental animals autopsied ten days after beginning injections and those autopsied that were injected for ten days and allowed to remain uninjected for seven more days. Corpora lutea were observed in all control animals and in the Tetrahymena geleii strain W animals that were injected for ten days and allowed to stand for seven additional days uninjected. No corpora lutea were observed in the Tetrahymena geleii strain W animals injected for ten days and autopsied, nor in any of the Tetrahymena geleii strain GP animals observed.

Microscopical observations of the testes of all control and experimental animals failed to show any difference in the interstitial cells.

Discussion

The only previous experimental work that has been done on observing the histological response of the endocrine organs of sex to injection of free-living protozoa into mammals was that done by Fennell(1944). The organism used by him for injection into mice was Paramecium caudatum cultured in hay infusion-lettuce medium which contained bacteria. Using immature white mice, the investigator found an interference with formation of the corpora lutea of the ovary which he has suggested may be due to some non-specific action of the anti-paramecium antibody. It was, furthermore, suggested that there was a non-specific inhibition of the formation of the luteinizing hormone of the anterior pituitary,i.e., an antihormone effect seemed to be produced.

Using mature white mice the present investigator found an inhibition to formation of corpora lutea in the ovaries of mice injected for ten days with Tetrahymena geleii strain GP; also, the same inhibition was found in the ovaries of mice injected for ten days with this organism and allowed to remain untreated for seven additional days. An interference in formation of corpora lutea in the ovaries of mice injected with Tetrahymena geleii strain W occurred only in the group injected for ten days. These results show that the histological response of the ovaries to injection of Paramecium caudatum and Tetrahymena geleii strain GP are apparently similar. Injection of Tetrahymena geleii strain W called forth an ovarian response similar to that found by Fennell for Paramecium caudatum

in that formation of corpora lutea seemed to be inhibited when the animals were autopsied at the termination of the period of injection. However, the group of mice injected for ten days and allowed to remain uninjected for seven additional days showed the presence of a very few corpora lutea. This indication that the apparent antihormone effect of the GP strain is longer lasting than the similar effect produced by the W strain, is not explainable on the basis of data available at the present time.

Also, no histological difference was observed in the testis of experimental and control animals by the present investigator. This is in general agreement with Fennell's observations of the absence of any histological effect on the testis of his experimental animals. However, he did state that the seminal vesicles seemed to be larger in the control than in the experimental animals.

Antibody tests on blood removed from mice injected for ten days and remaining uninjected for seven more days showed no lysis of Tetrahymena geleii strains W and GP. Only partial immobilization of GP cells in anti-W serum occurred. A five and a half hour preparation of the GP cells in anti-GP serum and untreated serum of the control animals rounded up. Robertson(1939a,b) in her work with ciliates belonging to the Glaucoma-Colpidium group stated that GpH, i.e., Tetrahymena geleii strain GP, "showed its rather poor tolerance of the conditions of experiment on heated normal sera". This information given by her is obscure, but it suggests that she found reactions similar to those of the present experiment wherein the cells of strain GP were partially immobilized in anti-W serum at

twenty minutes and at five and a half hours and were rounded up at five and a half hours in serum of control group. This latter reaction may indicate nothing more than the effect of an unfavorable medium; while the partial immobilization may have indicated that there are one or more antigens shared between the two strains of cells. The serological experiments were not extensive enough to clear up the question of shared antigens, but they do definitely show the presence of a specific GP antibody in the anti-GP serum. The serological tests with strain W cells showed no antibody formation and there was no observed effect of a shared antigen in strain W. Under the conditions of these serological tests, no unfavorable environment was created for strain W cells as was the case with strain GP cells.

Currently, it is believed that the reticulo-endothelial system is chiefly involved in antibody formation. Known phagocytic properties of the cells of this system furnish evidence to support this statement. Methods of experimentation give conclusive results. Removal of organs containing reticulo-endothelial cells or blockade of these cells by colloids results in loss of these phagocytic properties. Since the spleen contains reticulo-endothelial cells it is perhaps a center of antibody formation.

Stern and Willheim(1943) stated that the internal secretion of ovarian tissue implanted into normal rats was found impeded by the action of some antihormone factor but previous splenectomy prevented this restraining effect. This suggests that the reticulo-endothelium of the spleen may in some way combat this antihormone factor.

Summary

1. Cell suspensions in Chalkley's solution of Tetrahymena geleii strain W and strain GP injected into non-homozygous mature white mice for ten days do not produce any appreciable or significant difference in the number of giant cells in the spleens of the control and experimental animals nor any difference due to sex. In the animals injected for ten days and allowed to remain untreated for seven additional days, there was a pronounced difference in the number of giant cells of the control and experimental animals. Also, a pronounced sexual difference in the two experimental groups was observed.

2. Ovaries from mice injected with Tetrahymena geleii strain GP contained no observable corpora lutea. Ovaries from mice injected with Tetrahymena geleii strain W for ten days and allowed to remain uninjected for seven more days contained corpora lutea. Also, the ovaries of the control animals contained corpora lutea. No significant difference in the number of follicles of the ovaries was observable in the control and experimental animals.

3. Microscopical observations of the testes from control and experimental animals failed to reveal any appreciable difference in the interstitial cells of the organs.

4. A serological test revealed partial immobilization of strain GP in anti-W serum, rounding up of strain GP in anti-GP serum, and rounding up of strain GP in control serum five and a half hours after preparation of the slide. Strain W did not show any immobilization or rounding up in any of the three sera; namely, anti-W serum, anti-GP serum, and control serum.

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