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Resistance in Mice against *Trichomonas foetus* Infection Induced by Immunization with Heat-Treated Homologous Antigens

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Introduction

Youmans and his collaborators (1955) succeeded in mice in the induction of resistance against homologous organisms when the animals were immunized with microsomes isolated from *Mycobacterium tuberculosis*. They analyzed mycobacterial antigens (Kanai *et al.*, 1960) and Youmans and Youmans (1965) reported that ribosomal vaccines prepared from a ribosomal fraction of *M. tuberculosis* were able to induce protective immunity in mice. Murakami *et al.* (1959a, b) also reported a similar potential of microsomes derived from tubercle bacilli and *Salmonella typhi*. Early in the 1960's, we started a series of study on immune responses in mice (Hayashi *et al.*, 1976, 1978; Ishikawa *et al.*, 1977; Ito *et al.*, 1975; Oka and Osaki, 1966; Oka *et al.*, 1976a, b, c; Osaki and Oka, 1963) induced by subcellular fractions of an extracellular protozoon, *Trichomonas foetus* of which intraperitoneal injection caused death in mice (Inoki and Hamada, 1953; Schnitzer *et al.*, 1950). These studies indicate that both immune lymphocytes to ribosomal antigens and macrophages activated by the immune lymphocytes participate in the effective protection of the host.

For the induction of the specific resistance, mice had to be immunized with ribosomes incorporated in complete adjuvant or sublethal dose of living parasites (Oka *et al.*, 1975a, 1976a, b, c). Resistance was not induced in mice by a immunization with ribosomes alone or together with incomplete adjuvant (Oka, *et al.*, 1975a, b, c). Besides, no appreciable protection was produced when ribosomes in incomplete adjuvant were used in the initial immunization and the antigens in complete adjuvant was used for a booster immunization. However, antibody production was remarkable under such immunization conditions (Oka *et al.*, 1976a, c). The present study was focussed on the immunity in mice produced by ribosomes with the use of adjuvant and also on the effectiveness of living and killed parasites working in this kind of immunity. To study the immunization stimuli which induced resistance in mice, ribosomes, cell homogenates, and whole cells of *T. foetus* were incubated at different temperatures and mice were immunized with these antigens in the presence and absence of the adjuvants.

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Materials and Methods

Animals: Thirty- to 40-day-old female ddY mice weighing 20 to 24 g (Tokushima Experimental Animal Laboratory) were used throughout the experiments. In principle, 10 mice were housed in a cage in an air-conditioned room and fed with mouse chows and water ad libitum.

Protozoa: *T. foetus* strain Inui was cultured in F-bouillon containing 10% bovine serum for 35~45 hr at 37 C. The cultured cells were harvested by centrifugation at the middle of the exponential growth stage and used for the isolation of ribosomes and also as immunizing antigens and challenge inoculants. The minimum lethal dose of *T. foetus* was $(2\sim3)\times10^7$ per mouse in intraperitoneal infections in ddY female mice.

Isolation of ribosomes: Isolation procedure and characteristics of ribosomes from *T. foetus* were described previously (Hayashi *et al.*, 1973a, b; Oka *et al.*, 1973). Briefly, the harvested cells were washed by centrifugation with 150 mM saline and suspended in sterilized 10 mM Tris-HCl buffer, pH 7.2 containing 10 mM $MgCl_2$, 60 mM KCl (TMK buffer), 1 mM spermidine and 2 μg/ml DNase (Worthington Biochemical) before the material was incubated for 20 min in an ice-water bath. Then, the cell suspension was freezed overnight at −80 C and then thawed at 30 C in a water bath in order to disintegrate the cells. The cell homogenate was centrifuged at 12,000×g for 20 min and the cell debris was removed. Brij-58 was added to the supernatant to dissolve remaining membrane structures (final concentration=0.5%). After centrifuging at 45,000 ×g for 45 min, the supernatant was collected carefully and centrifuged at 144,000×g for 2.5 hr to obtain ribosomal pellet.

Protozoan cell homogenate: Washed *T. foetus* cells were suspended in TMK buffer at a concentration of 4.0×10^8/ml and freeze-thawed. Less than 0.25% of the disintegrated organisms by this procedure were motile, however, such moving cells did not show multiplication.

Hyperchromicity of ribosomes: Thermal denaturation of the ribosomal preparation was measured by hyperchromicity in TMK buffer at 260 nm in a Hitachi-124 photospectrometer equipped with a thermocontroling apparatus (Komatsu Electronic Inc.). The temperature in sample cuvettes was increased by 0.5 C per min from 15 up to 55 C. Denaturation of ribosomes in heat-treated antigens was estimated from time changes of the optical density of ribosomes incubated at different temperatures from 20 to 55 C.

Effect of temperature on viability of protozoa: Effects of temperatures on the viability of protozoa were examined by measuring the rate of proliferation and the fate of organisms as follows. Culture tubes were incubated in a water bath at 25, 30, 35, 39, 40, and 45 C (±0.2 C) and the parasites were counted with a hemocytometer at a certain interval. The proliferation of the incubated parasites was calculated from the number of cell divisions per 100 hr at the exponential growth stage and the fate of incubated parasites was examined under the light microscope.

Immunizations and challenges: After incubating at various temperatures (0~100 C) in a water bath for 30 min, *T. foetus* antigens, ribosomes, cell homogenates and whole cells were diluted properly and were emulsified with an equal volume of complete (CFA) or incomplete Freund's adjuvant (IFA). In immunizations with adjuvant, mice were inoculated intraperitoneally with 0.2 ml of the antigen-CFA or -IFA emulsion while in the absence of adjuvant, the animals were given 0.5 ml of the antigen suspension alone intraperitoneally. The mice were challenged intraperitoneally with 4.0×10^7 living *T. foetus* cells 3 weeks after immunizations with CFA- or IFA-incorporated antigens. In groups immunized with antigen alone, mice were challenged intra-

peritoneally with 3.5×10^7 living parasites. Resistance produced by immunization was estimated by both survival rates on day 30 after challenge and by the mean survival days of the dead mice within 30 days after the challenge.

Statistical analyses: Analyses of the survival rates and the mean survival days were performed by chi square test and Student's t test, respectively.

Results

Hyperchromicity of T. foetus ribosomes: An absorbance-temperature profile of *T. foetus* ribosomes is illustrated in Fig. 1. The Tm value calculated from this profile was about 51.2 C. Time course of denaturation of heat-treated ribosomes used for immunization was evaluated by optical density at 260 nm (Fig. 2). At 35 C and below, optical absorbance of ribosomes did not increase but at 40 C and more, a sudden change in the structure of ribosomes was presumed. Temperatures around 42.5 C was presumed to be the critical point which cause structural change of *T. foetus* ribosomes.

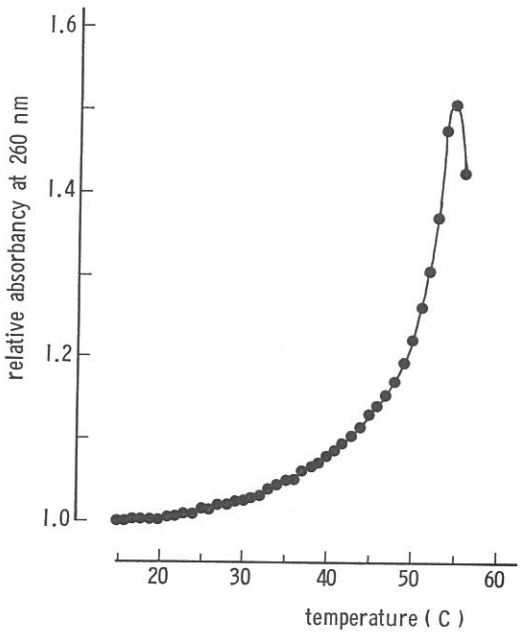

Fig. 1 Absorbance-temperature profile of *Trichomonas foetus* ribosomes.

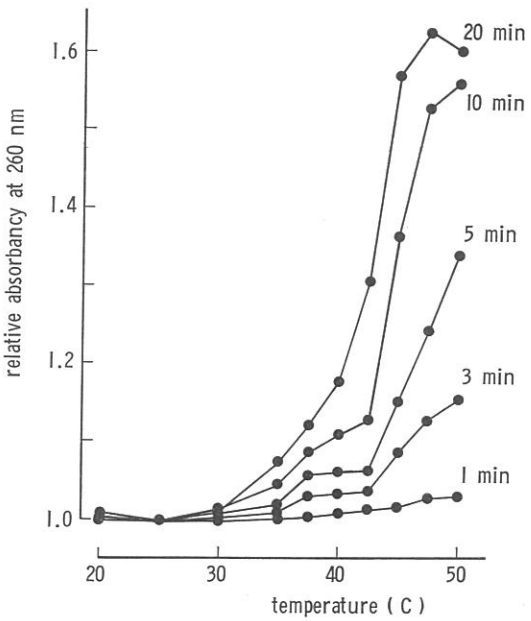

Fig. 2 Effect of duration of heating on *Trichomonas foetus* ribosomes at various temperatures.

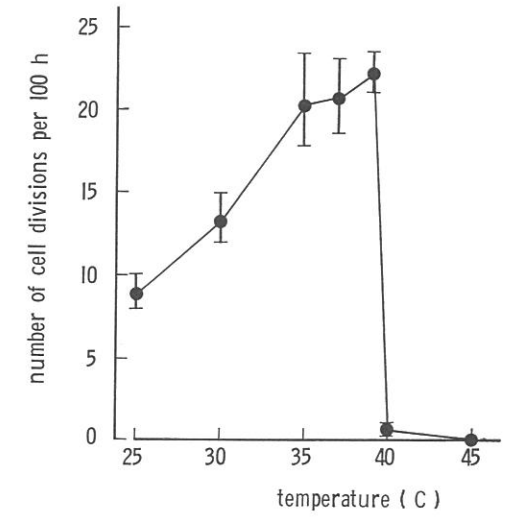

Fig. 3 Growth rate of *Trichomonas foetus* cells cultured at various temperatures.

Effect of temperature on viability of T. foetus cells: Effect of temperature on pro-

Table 1 Effect of heat treatment on protective immunogenicity of *Trichomonas foetus* ribosomes with complete Freund's adjuvant

Incubation temperature (C)	Number of examinations	Number of mice		Mean survival days of the dead mice (mean ± SD)
		Inoculated	Survived	
0	4	39	22*(4)[a)]	9.5±6.9
30	1	10	6*	6.3±1.0
35	1	10	10*(2)[a)]	——
40	3	30	12*(4)[a)]	7.1±2.0
45	2	22	14*(2)[a)]	7.0±1.4
60	3	30	17*(2)[a)]	8.8±2.0*
80	3	30	14*(6)[a)]	8.0±3.3
100	2	20	10*(1)[a)]	8.5±3.7
CFA-TMK buffer	4	46	4	6.6±2.5
Nontreatment	4	44	0	4.1±0.4

Antigen dose: 0.65 to 1.0 mg ribosomes per mouse
a): Number of mice manifesting peritoneal fluid
*: $p<0.005$; significantly higher than the value for mice inoculated with CFA-TMK buffer

Table 2 Effect of heat treatment on protective immunogenicity of *Trichomonas foetus* cell homogenate in the absence of adjuvant

Incubation temperature (C)	Number of mice		Mean survival days of the dead mice (mean ± SD)
	Inoculated	Survived	
0	21	4*(2)[a)]	7.4±3.1*
15	20	1 (1)[a)]	7.8±3.9*
30	20	2 (1)[a)]	7.3±4.9
35	20	0	8.0±5.3*
40	20	0	8.3±5.6*
45	21	1 (1)[a)]	8.3±5.5*
60	20	1	8.0±5.3*
80	21	3	6.7±3.0*
100	21	2 (1)[a)]	7.6±3.6*
Buffer alone	20	0	5.2±0.9
Nontreatment	21	0	4.8±0.7

Data are calculated from two separate experiments.
Antigen dose: 4.0×10^7 cell per mouse
a): Number of mice manifesting peritoneal fluid
*: $p<0.05$; significantly higher than the value for mice inoculated with buffer

liferation of the protozoa is shown in Fig. 3. A linear acceleration of the division rate was observed between 25 and 39 C. On the other hand, almost no division occurred at 40 C but the parasites were all alive and at 45 C, no living cells were detected under the microscope.

Effect of heat treatment on protective antigenicity of T. foetus ribosomes: Antigenic activity of ribosomes heated at varying temperatures from 0 to 100 C is shown in Table 1. Throughout four consecutive experiments, the survival rate of the test mice was between 40~60% in all of the groups

Table 3 Effect of heat treatment on protective immunogenicity of *Trichomonas foetus* cell homogenate with incomplete Freund's adjuvant

Incubation temperature (C)	Number of mice		Mean survival days of the dead mice (mean ± SD)
	Inoculated	Survived	
0	10	10*(1)[a)]	——
30	10	10*	——
40	10	8†(2)[a)]	9.0±2.0
60	10	8†(1)[a)]	5.5±0.5
80	10	7†(1)[a)]	11.0±4.3
100	10	10*(2)[a)]	——
IFA–TMK buffer	10	2	8.9±4.6
Nontreatment	10	2	5.1±0.3

Antigen dose: 4.0×10^7 cells per mouse
a): Number of mice manifesting peritoneal fluid
*: $p<0.005$, †: $p<0.05$; significantly higher than the value for mice inoculated with IFA–TMK buffer

Table 4 Effect of heat treatment on protective immunogenicity of *Trichomonas foetus* cell homogenate with complete Freund's adjuvant

Incubation temperature (C)	Number of mice		Mean survival days of the dead mice (mean ± SD)
	Inoculated	Survived	
0	10	9*	6.0
15	10	10*	——
30	10	8*	7.5±0.7
35	10	8*(2)[a)]	6.0±0.0
40	10	10*(2)[a)]	——
45	10	9*(1)[a)]	4.0
60	10	10*(3)[a)]	——
80	11	8*(3)[a)]	10.3±8.4
100	10	10*(6)[a)]	——
CFA–TMK buffer	10	1	5.9±1.5
Nontreatment	10	0	5.2±0.4

Antigen dose: 4.0×10^7 cells per mouse
a): Number of mice manifesting peritoneal fluid
*: $p<0.005$; significantly higher than the value for mice inoculated with CFA–TMK buffer

immunized with heat-treated ribosomes. These values were not different from those of mice immunized with native (incubated at 0 C) ribosomes and were significantly higher than that of control mice given with CFA-TMK buffer. Although significant difference between test mice and control mice was not found in the statistics, small prolonging of the mean survival days was observed in the dead mice from the test group. In each experimental group, some of the survived mice retained appreciable amount of ascites.

Effect of heat treatment on protective immunogenicity of T. foetus cell homogenate: In two consecutive experiments in which mice were immunized with cell homogenate equivalent to 4.0×10^7 parasites per mouse,

Table 5 Effect of heat treatment on protective immunogenicity of *Trichomonas foetus* cell antigen in the absence of adjuvant

Incubation temperature (C)	Number of mice		Mean survival days of the dead mice (mean ± SD)
	Inoculated	Survived	
0	10	4†(2)[a)]	12.8±6.4†
37	10	8*(5)[a)]	12.5±6.4
40	10	7*(5)[a)]	8.7±3.1
45	10	3 (3)[a)]	5.1±1.1†
60	10	1	4.8±0.7*
80	10	0	4.2±0.4†
100	10	0	5.2±1.1*
Bouillon	10	0	3.9±0.3
Nontreatment	10	0	3.9±0.3

Antigen dose: 5.0×10^6 cells per mouse
a): Number of mice manifesting peritonel fluid
*: $p<0.005$, †: $p<0.05$; significantly higher than the value for mice inoculated with bouillon

Table 6 Protections produced in mice immunized by heated *Trichomonas foetus* cell antigen (5.0×10^6 cells per mouse) given with complete Freund's adjuvant

Incubation temperature (C)	Number of mice		Mean survival days of the dead mice (mean ± SD)
	Inoculated	Survived	
0	8	8*(3)[a)]	——
40	10	8*(3)[a)]	14.0±2.8
45	10	8*(2)[a)]	7.5±0.7
60	10	6*(1)[a)]	10.0±6.4
80	10	2	13.4±4.0†
100	10	1	14.0±4.0*
CFA–bouillon	10	0	8.0±3.2
Nontreatment	13	0	4.5±0.7

a): Number of mice manifesting peritoneal fluid
*: $p<0.005$, †: $p<0.05$; significantly higher than the value for mice inoculated with CFA–bouillon

no protection was observed (Table 2). Then, the immunogenic activities of *T. foetus* cell homogenate in the presence of IFA and CFA were examined. The survival rates of the groups immunized with heat-treated cell homogenate were higher than 70% (Tables 3, 4). The resulted rate was significantly high as compared with those (10 and 20%) of the sham-immunized (IFA- or CFA-TMK buffer) controls.

Effect of heat treatment on protective immunogenicity of T. foetus cells: Table 5 shows the specific protection in mice injected with heat-treated 5.0×10^6 parasites in the absence of adjuvant. High survival rates were obtained in mice injected with the parasites after being incubated for 30 min at low temperatures (0~40 C), even though the parasites were not killed by incubation (Fig. 3). Mean survival days of the dead mice in the test group were slightly prolonged.

As Table 6 indicates, high resistances were induced in mice immunized with the heat-

Table 7 Protections produced in mice immunized by heated *Trichomonas foetus* cell antigen (4.0×10^7 cells per mouse) given with complete Freund's abjuvant

Incubation temperature (C)	Number of mice		Mean survival days of the dead mice (mean ± SD)
	Inoculated	Survived	
0	3	3*	—
30	3	3*	—
40	1	1†	—
45	10	10*	—
60	10	10*(1)[a]	—
80	11	10*(1)[a]	7.0
100	10	10*	—
CFA–bouillon	10	1	8.0±1.4
Nontreatment	10	0	5.1±0.3

a): Number of mice manifesting peritoneal fluid
*: $p<0.005$, †: $p<0.05$; significantly higher than the value for mice inoculated with CFA–bouillon

Table 8 Protections produced in mice immunized by heated *Trichomonas foetus* cell antigen given with incomplete Freund's adjuvant

Incubation temperature (C)	Antigen dose (cells)	Number of mice		Mean survival days of the dead mice (mean ± SD)
		Inoculated	Survived	
0	4.0×10^6	6	2 (1)[a]	17.8±5.5†
45	4.0×10^6	10	5*(3)[a]	19.8±4.0*
60	4.0×10^7	10	7*(3)[a]	21.3±8.1
100	4.0×10^7	10	4†(1)[a]	18.0±4.4*
IFA–bouillon	—	10	0	6.6±1.3
Nontreatment	—	5	0	5.5±0.0

a): Number of mice manifesting peritoneal fluid
*: $p<0.005$, †: $p<0.05$; significantly higher than the value for mice inoculated IFA–bouillon

treated parasites together with CFA. Survival rate was 60~100% when the parasites were heated at 0~60 C. While 20 and 10% of mice given with parasites heated at 80 and 100 C with CFA survived, respectively.

In the subsequent experiments in the presence of CFA, mice were immunized with 4.0×10^7 heat-treated parasites (Table 7) and the strongest protection was induced in these groups. Mice immunized with 4.0×10^7 heat-treated (60~100 C) parasites together with IFA showed high grades of protection (70 and 40%, respectively) in comparison with controls inoculated with IFA-TMK buffer emulsion (Table 8). But, per cent survival in those mice was slightly lower than that in mice immunized together with CFA (Tables 7, 8). Mice immunized with 4.0×10^6 heat-treated (0 and 45 C) parasites together with IFA also showed slightly lower survival rates (33 and 50%, respectively) than those immunized together with CFA (Tables 6, 8).

Discussion

Sublethal infection with living *T. foetus* induced protection in mice, and similar immunity could be acquired in mice by the

use of isolated ribosomes as immunizing antigen (Oka *et al.*, 1975a). But, in immunizations with heat-killed parasites in mice, antibody production was rather prominent than immune protection (Oka *et al.*, 1963, 1976b). The present work was carried out in order to study if the stimulus by heat-denatured subcellular component was able to induce protective immunity in mice.

First, the heat resistance of ribosomes was examined by thermal hyperchromicity test. The Tm value for *T. foetus* ribosomes obtained from thermal denaturation curve was 51.2 C (Fig. 1). The Tm value was markedly low in contrast to that of other bacterial ribosomes (Altenburg and Saunders, 1971; Friedman, 1971; Pace and Campbell, 1967). Pace and Campbell (1967) reported that the Tm value was about 70 C in certain microorganisms of which maximal growth temperature was similar to that of *T. foetus* (39 C) (Fig. 3) and regardless of low maximal growth temperature the Tm value of *Vibrio marinus* ribosomes was 69 C. Besides, it appeared that irreversible denaturation of *T. foetus* ribosomes occurred at about 42 to 43 C (Fig. 2) and this value was lower than the temperature causing irreversible denaturation of *Escherichia coli* ribosomes (Bodley, 1969).

Effects of heat treatment on antigenicity of *T. foetus* ribosomes were examined. Minor difference was found in the produced immunity in mice immunized with heated ribosomes regardless of the degree of effected temperature (Table 1). This indicated that the antigenic determinants of ribosomes which were essential for the induction of protective immunity in mice were stable to heat treatment in contrast to extreme thermal unstability of the ribosomal structure. On the other hand, antigenic activity of ribosomes was heat-stable. Thus, antigenic determinants are likely to be localized rather in ribosomal RNA than in ribosomal proteins. But in our preliminary experiments, it was found that trypsin treatment caused a greater loss of ribosomal antigenicity than RNase treatment. Youmans and Youmans (1969, 1971) reported the immunogenicity of ribosomal RNA. Johnson (1973) and Tewari *et al.* (1978) reported that the protective antigenicity was attributable to ribosomal proteins in *Salmonella typhimurium* and *Haemophilus influenzae*. Further clarification of both types of ribosomal antigens will be required in the present study.

The reason why heat-killed *T. foetus* cells could not induce any protection in mice in spite of high heat-stability of the antigenicity of their ribosomes was examined. Immunizations with whole cells incubated at low temperatures (0~40 C) and living cell immunizations could induce resistance in mice (Table 5) but the vaccination effect was reduced by disintegration of the cell structure by freeze-thawing instead of by heating (Table 2). These results suggest that one of conditions required for induction of protective immunity in mice was the subsistence of the cell. But, this inducing stimulus was replaced not only by CFA but also by IFA (Tables 3, 4, 7, 8). Protective immunity in mice immunized with ribosomes was contrarily elicited by the addition of CFA but not IFA (Oka *et al.*, 1975a, b, 1976a). Therefore, three different stimuli were considered to be essential to the induction of protective immune responses in mice: The first is a physical factor(s) that is given by infection *per se* with living organisms or by the injection of IFA. Secondarily, the manifestation of protective immunogenicity of ribosomes seemingly required a certain chemical stimulus such as killed BCG contained in CFA. The feature of the chemical adjuvant was seemingly associated with cellular components of *T. foetus* other than ribosomes and the activity was considerably heat-stable (Tables 3, 8). In addition to the two stimuli discussed above, the antigenicity attributable to ribosomes was equally essential.

Several investigators reported respective results concerning with the ribosomal vaccine (Johnson, 1973; Misfeldt and Johnson, 1976; Swendsen and Johnson, 1976; Tewari *et al.*, 1977; Venneman and Berry, 1971). In our previous study on immunizations with killed parasites and a combination of ribosomes and IFA (Oka *et al.*, 1976c), protection was not induced but a certain antibody production was stimulated. In the present study, ribosomal antigens, alone or incorporated with IFA, failed to induce protective immune responses in mice and this fact may suggest different adjuvant stimuli induced a variety of different immune responses in mice. Physical and chemical properties of the used adjuvant may also hold a important key to the induction of either cellular or humoral immunity, or either protective or non-protective cellular immunity.

Summary

The mechanism of protection against *Trichomonas foetus*, an extracellular parasitic protozoon, has been studied in mice. The results indicated that host animals had to be immunized with living parasites or isolated ribosomes incorporated with complete Freund's adjuvant (CFA) in order to induce effective resistance to the protozoan infection. Killed cells, ribosomes alone, cellular components excluding ribosomes, and CFA alone were not effective as antigens. Induction of resistance was studied in mice immunized with whole cells, cell homogenate, and purified ribosomes of *T. foetus* which were subjected to heat-treatment at various temperatures. The complete and incomplete Freund's adjuvants were added to the prepared antigens. Results obtained suggested that the following three stimulations were required for the induction of protection in mice. 1) Antigenic stimulus exerted by ribosomes. 2) A certain adjuvant stimulus originating from protozoan cell components other than ribosomes. 3) Some other stimuli provided by living cell activities, which was also induced by mineral oil in the Freund's adjuvants. Experiment without use of adjuvant showed that heat treatment caused loss of activities 3) in the protozoa.

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Trichomonas foetus 感染に対するマウスの抵抗性の誘導
—— 特に，抗原の熱処理が免疫原性に及ぼす影響 ——

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細胞外寄生性原虫 *Trichomonas foetus* の一定量以上の腹腔内接種は，マウスに致死的である．このような致死感染に対する免疫抵抗性をマウスに誘導する条件の分析を試みた．その結果，特異抵抗性獲得の条件は，生虫で，あるいは Freund の complete adjuvant と共に *T. foetus* ライボソームで免疫することであった．死虫，ライボソーム単独あるいはライボソーム以外の細胞成分は“防御抗原”としての能力に欠けた．そこで，生虫，細胞ホモジネート及びライボソームを種々の温度で処理し，complete 又は incomplete adjuvant と共に免疫することにより，防御免疫誘導に必要な抗原側の具備条件を求めた．その結果，1) *T. foetus* ライボソームに局在する抗原物質，2) ライボソーム以外で BCG と類似のアジュバント活性を示す物質，及び 3) 生虫感染でのみ得られた物理的刺激，の3条件が必要であることが示唆された．なお，第3の物理的活性は Freund の adjuvant 中のミネラル油で代用が可能であった．今回の研究では，原虫免疫の際，加熱によって失われる刺激は，生原虫のみが与え得た上述の第3の刺激条件であることが分かった．