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The Use of Irradiated and Formalin-fixed *Trichomonas vaginalis* to Examine Protective Immune Responses in the Mouse Intraperitoneal Model

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Abstract

Gamma irradiated *Trichomonas vaginalis* was used to initiate a protective immune response in mice by intraperitoneal inoculation using host mortality as the measure of virulence. Irradiated trichomonads of a virulent strain gave some immune protection but no more so than the use of formalin-fixed, virulent trichomonads. A formalin-fixed virulent strain combined with complete Freund's adjuvant (CFA) gave complete protection. Metabolically produced antigens do not appear to be important in conferring protective immunity in these experiments. A common laboratory strain of *T. vaginalis* (ATCC 30001) was used as an avirulent control. It gave no protection against a virulent strain. Combining CFA with ATCC 30001 (avirulent) gave partial protection indicating that a protective antigen is present, but needed an immune stimulant to be detected. IgG analysis corresponded to the mortality results with the avirulent strain being the weakest responder and the strongest being the formalin-fixed virulent strain with CFA. Western blot analysis indicated a band of about 31-kDa that was present using the protocols that showed from partial to complete protection. This band was not present using the avirulent strain but appeared with the addition of CFA. These results indicated that a 31-kDa protein is present in the avirulent strain but it requires an immune stimulant to be revealed. Whether this antigen confers protective immunity or not in the mouse intraperitoneal model is an open question.

Introduction

Trichomonas vaginalis, a flagellated protozoan, produces a fulminant vulvovaginitis in women. It is one of the most common venereal disease agents and is transmitted by males, who for the most part, may not have any symptoms themselves. The general clinical aspects, chemotherapy, and immunology have recently been reviewed by Krieger and Alderete (1999). The drug metronidazole has been very effective against this organism, but cases resistant to treatment and drug resistant strains have been found. Immunotherapy has been considered, but protective immunity to this parasite appears to be poor or non-existent .However, evidence for both humoral and cell-mediated immune responses have been found. This has given rise to the possibility of a vaccine or at least for an immunodiagnostic test that can be used for epidemiological purposes.

Immunological studies on *T. vaginalis* have used the mouse as the primary animal model. Three routes have been used for antigen exposure to develop an immune response and each has its positive and negative aspects: Intraperitoneal (IP) and intramuscular (IM) injections and vaginal inoculation are the routes used (Warren et al. 1960; Honigberg, 1978; Abraham et al., 1996). IM models are preferred to IP methods for antigen inoculation since IM uses ulcer development as a measure of pathology and does not cause host death as does IP. Vaginal models in mice, although more closely related to the parasites normal site of infection, are the most complex models. They require sterol injections and/or manipulation of *Lactobacillus* flora to make the vaginal vault receptive to maintaining *T. vaginalis* populations (McGrory and Garber, 1992; Abraham et al., 1996). Unfortunately, although more clinically realistic, the vaginal model suffers from the same problem as the human vagina. Direct exposure to antigen does not seem to stimulate an apparent protective immunity. However, IM injections combined with a vaginal model have been shown to provide protection reducing parasite populations in mouse vaginas (Abraham et al., 1996). It has been shown that circulating antibodies may diffuse through the vaginal mucosa and give some degree of protection, therefore giving hope for developing an anti-trichomonad vaccine. The more easily accomplished IP route has also been used

for studying immune responses but has been most effective in studying strain virulence (Honigberg, 1978). Death of the host and time of death, usually in days, have been measurements used to determine relative virulence of a strain in the IP model.

The primary objective of this study was to use irradiated *T. vaginalis* to stimulate a protective immune response using the IP model. Protective antigens may be metabolically produced rather then being part of the pathogen's cellular structure. As a rule, reproduction is halted but the organisms continue to metabolize. In some cases, reproduction has not been affected, but the pathology usually produced has been attenuated. It has been shown with a number of protozoan parasites that irradiated cells can produce a more efficient immune response than crude cellular extracts (Hostetler., 1987). The IP route was considered to be the best choice for these experiments based on ease of manipulation of the model and the fact that the environment of the peritoneal cavity would serve as a nutritionally supportive locale for irradiated trichomonad survival. Previous studies on the survival of *T. vaginalis* with gamma radiation showed a dosage whereby trichomonads would remain metabolically active but unable to reproduce (Daly et al., 1991). Different treatment protocols for preparation of antigen source were used for comparison and as controls. IgG response was measured for each of these treatments, and Western blot analysis was used to detect protein(s) that might be protective.

Materials and Methods

The experimental design include the following: (1) selection of a virulent and avirulent strain of *T. vaginalis* based on IP inoculation, (2) gamma irradiation of *T. vaginalis*, (3) determination of the length of time irradiated trichomonads would survive in vitro, (3) determination of the number and quantity of sensitizing antigen doses needed to illicit a protective immune response, (4) IgG analysis to correlate with the mortality or protection seen with challenges to the respective inoculation protocols, and (5) Western blot analysis to determine protein bands associated with protective immunity.

A. *Trichomonas vaginalis* strains and Growth Medium. *T. vaginalis* strain ATCC 30001 was used as the avirulent agent, and two isolates from clinical cases of trichomoniasis, designated C_1 and C_2, were used as the virulent strains. Trichomonads were grown in TYM medium (Diamond, 1957) with 10% heat inactivated (56° C) Seitz-filtered human serum. Serum was obtained from the clinical laboratories of the University of Arkansas for Medical Sciences Hospital.

B. Experimental animals. Female BALB/c mice were obtained from the National Center for Toxicological Research (Jefferson, AR) or purchased from SASCO Incorporated (Omaha, NE). Mice, 3 or 24 weeks of age, were used in some experiments to determine if age affects the immune response.

C. Preparation of Inocula. *T. vaginalis* strains were grown to a concentration of 10^4/ml and passaged three times at this population level to insure that cells were in the logarithmic phase of growth. Cells were harvested from 100 ml of medium at 500xg in 50 ml centrifuge tubes. The cells were resuspended and washed 3x in sterile phosphate buffered-saline (PBS - pH 7.2). The final pellet was suspended in buffer and adjusted to the final cell concentrations to be used. *T. vaginalis* were irradiated with gamma radiation from a Cesium^{137} irradiator by the methods of Daly et al. (1991). Total cell counts were made in a Neubauer hemocytometer, and viable counts were with the semi-soft agar technique of Ivy (1961) as modified by Matthews and Daly (1974).

D. Trypan Blue Dye Exclusion. Fifty µl of cells to be tested was removed from irradiated and control (nonirradiated cells) culture tubes. Trypan blue dye was added to a final concentration of 0.4%. After mixing, the two suspensions were loaded into separate counting chambers, and the number of total cells and the number of cells excluding the dye were determined. Final values were from three counts.

E. Inoculation of Mice. Cell concentrations were adjusted to contain a predetermined number in 200 µl for intraperitoneal inoculation using tuberculin syringes with 26g needles. Twenty gauge needles were used with formalin-killed cells with complete Freund's adjuvant (CFA). Immunizing inocula were prepared by resuspending a pellet of cells in 0.5% formalin at 4°C for 1 hr. Irradiated cells were suspended in fresh, sterile TYM medium without serum.

F. Procurement of blood and anti-Trichomonad antibody assay. Mice were anesthetized with methoxyfurane and bled from the retroorbital plexis with heparinized capillary tubes. The tubes were centrifuged, the plasma removed, and the plasma stored at minus 20°C until use. Dilutions of plasma were done two-fold starting with 1/10 increasing to 1/256. These were placed in slide wells. Fluorescein isothiocynate conjugated with goat anti-mouse IgG (Cappel Laboratories, West Chester, PA), diluted 1/20 in PBS, was then added to the wells and incubated for 30 minutes at 37°C. The sample wells were counterstained with Evans blue dye and allowed to dry. Three controls were used - (a) normal mouse serum, (b) immune mouse serum, (c) and mouse serum with a known high titer to *T. vaginalis*. Examination of the wells was with a Zeiss flourescence microscope at X400 magnification. The antibody titer was defined as the highest dilution of serum that resulted in flourescence of the cells.

G. SDS-PAGE and Western Blot. *Preparation of trichomonad*

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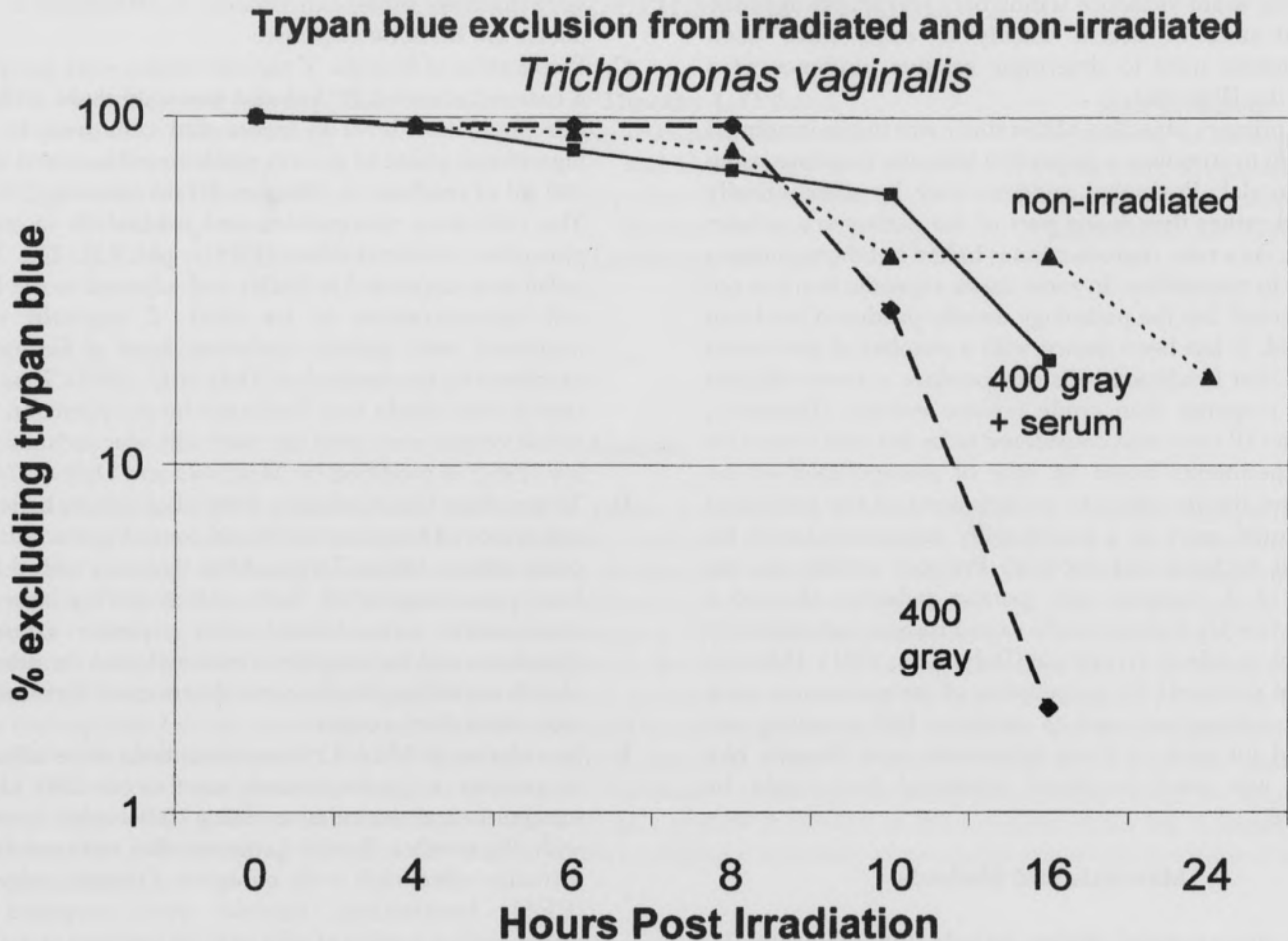

Fig. 1. Metabolic activity over time as demonstrated by trypan blue exclusion from irradiated or non-irradiated C_1 *Trichomonas vaginalis* at 37°C. The 16 hour measurements are the final data points shown because there were no viable cells at 24 hours. Cells were irradiated in TYM medium with and without serum (one experiment).

protein extracts: Cells were harvested from 2 liters of TYM medium containing 10^9 cells by a single speed Sorvol centrifuge. After washing the cells, a solution of 1% Triton x-100 was added to dissolve a portion of the pellet. The suspension was centrifuged at X2000g at 4°C to pellet the lipid component, which was discarded. The supernatant was dialyzed against 4mM tris buffer (pH 6.8) at 4°C overnight and then frozen at -70°C until used. 10^9 trichomonads yields approximately 15 mg of protein. *SDS electrophoresis*: Dialyzed extract was diluted 1:1 with tris buffer, boiled for 5 minutes, and then chilled on ice until used. A 12% polyacrilamide gel with a 5% stacking gel with one wide well and one narrow well was prepared, and 1.6 ml of extract was placed into the wide well. High and low molecular weight standards were loaded into the narrow well. An initial current of 70 mAmps was applied until the samples were out of the well at which time the current was increased to 120 mAmps. The current was discontinued when the dye front reached the bottom edge of the gel. *Transblot of proteins*: The proteins in the gel were transblotted onto nitrocellulose overnight at 30 volts. The nitrocellulose was removed from the transblot cell and a strip containing the molecular weight standards and a section of the well of the sample was stained with amido black. *Western Blot*: The nitrocellulose that remained unbound to protein was blocked by incubation in "blotto" (Carnation Instant Milk, 5%) at 4°C for 4 hours. Strips containing the protein samples were cut from the nitrocellulose and incubated at 4°C overnight with serum from the various treatment groups that had been diluted 1:150 or 1:500

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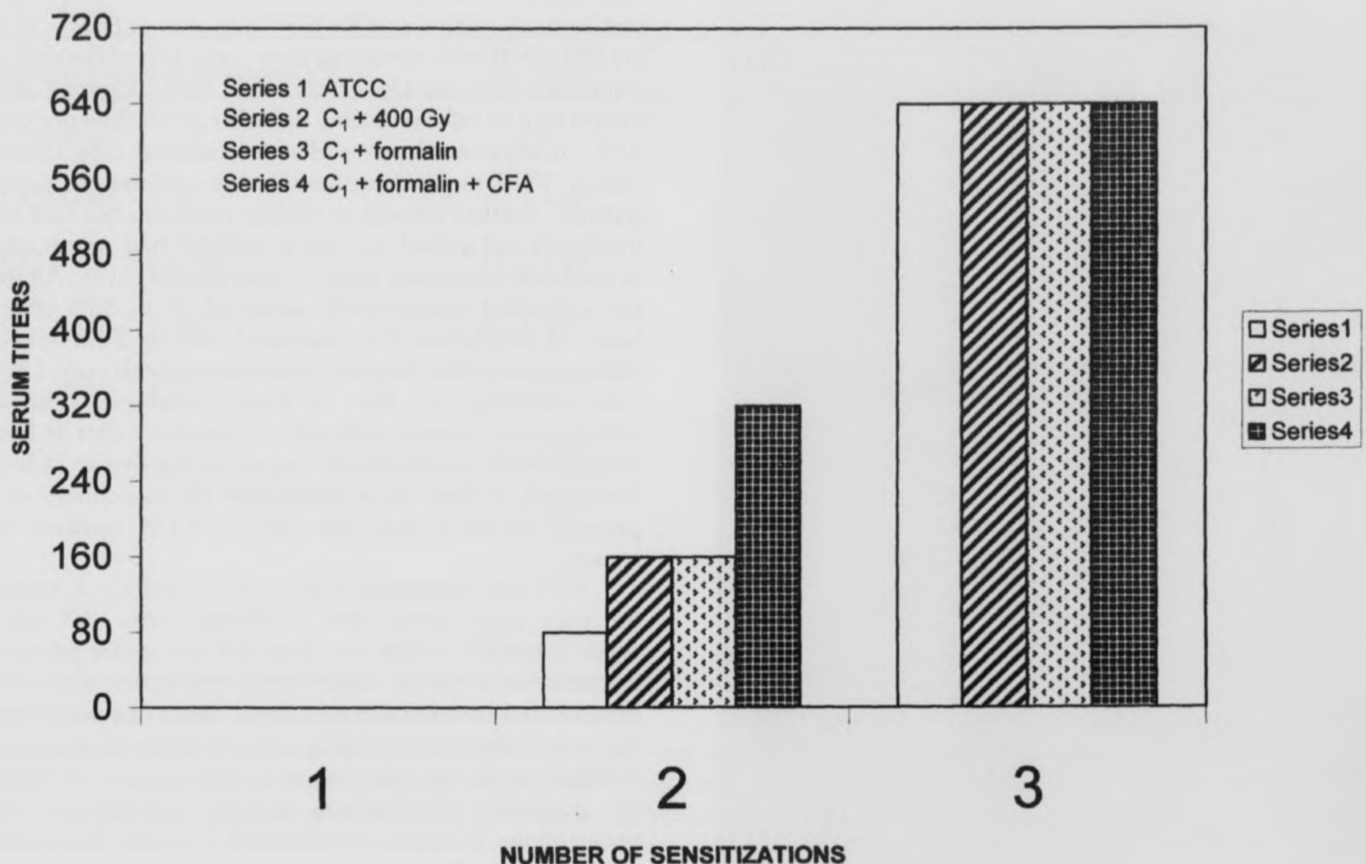

Fig. 2. Development of serum IgG titers in 24 week-old mice to *Trichomonas vaginalis* by four sensitization protocols. Titers were determined by indirect immunoflourescence using *T. vaginalis* C_1 as the antigen source. Each group represents pooled plasma samples from 5 mice.

in blotto. The strips were washed in fresh blotto at 4°C at 15 minute intervals to remove primary mouse antibody. Secondary antibody (rabbit anti-mouse) was diluted 1:600 in blotto, added to the strips, and incubated at 4°C for 2 hours. Strips were then washed in blotto and 20 µl of I^{125} labeled *Staphylococcus aureus* protein A (ICN Laboratories, Irvine, CA) was added which was first diluted 1:40 in PBS). The strips were then incubated at 4°C for 1 hour, washed once in blotto and then exposed to x-ray film for 8 hours at -70°C.

Results

ATCC strain 30001 of *Trichomonas vaginalis* did not produce mortality or outward signs of sickness or distress three weeks post-inoculation when injected with a range of 10^6 to 10^7 cells (Table 1). Necropsy results showed no pathology in the peritoneal cavity. C_1 was isolated from a patient from University Hospital and caused death in 2 to 5.8 days with dosages ranging from 10^6 to 2 x 10^7 (Table 1). ATCC 30001 was then used as the avirulent control in these experiments and C_1 as the virulent strain. As C_1 was maintained in culture it began to lose virulence after several months passage in TYM medium. When the average mortality in mice of C_1 reached 28 days, it was decided to replace this strain with a newly isolated one designated C_2. Loss of virulence during cultivation is well known with *T. vaginalis* (Honigberg, 1978). Different dosages of C_1 (or C_2) ranging from 10^3 to 10^7 cells showed a threshold for producing death in mice at 10^6 cells. This inoculation size was selected since a larger inoculum might overwhelm any acquired immune response seen using mortality of mice as a criterion.

In order to determine if irradiated trichomonads could

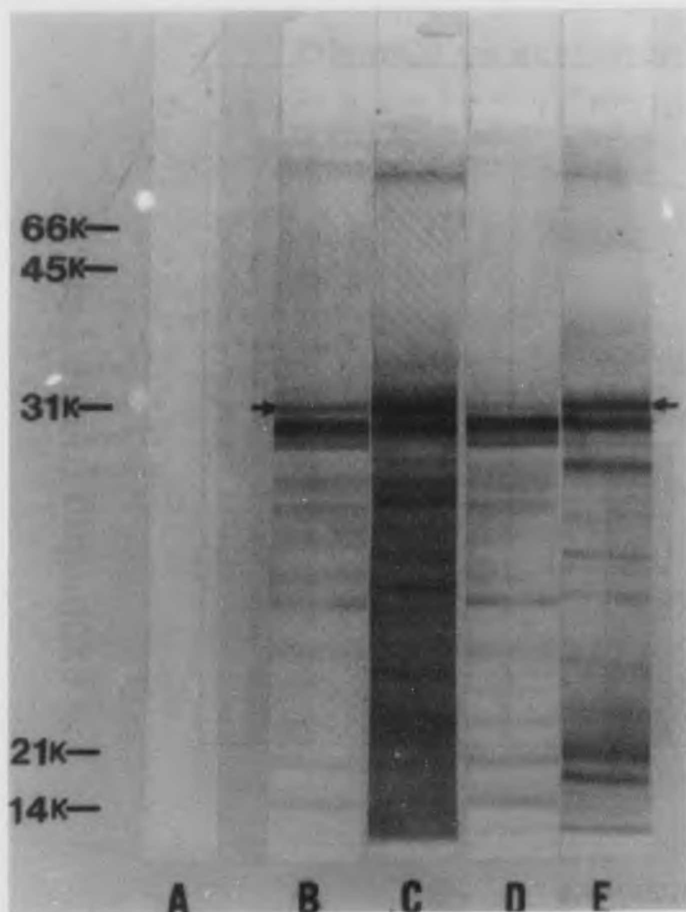

Fig. 3. Western blot analysis of serum from different mouse treatment groups sensitized with *Trichomonas vaginalis* antigen preparations. A protein extract from *T. vaginalis* C_2 was separated according to molecular weight by SDS-Page. The sensitization protocols used to obtain the sera for each lane were (A) Normal mouse serum (control – no antigen inoculated); (B) Formalin-fixed *T. vaginalis* ATCC 30001+ CFA (partial protection); (C) Formalin-fixed *T. vaginalis* C_2 + CFA (complete protection); (D) Irradiated *T. vaginalis* C_2 (partial protection); (E) Formalin-fixed *T. vaginalis* C_2 (partial protection).

produce a protective immunity through IP inoculation, a radiation dose had to be found where the organisms could survive, but not cause mortality in mice. The ability of *T. vaginalis* to produce mortality after radiation was determined by exposing 10^6 C_1 trichomonads in TYM medium to 100, 200, 400, and 800 Gy, respectively (Table 2). Mortalities of mice averaged 3.6, 5.8, and 5.8 days after 0, 100, and 200 Gy. No survival was seen in mice inoculated with trichomonads exposed to 400 and 800 Gy. Since very high dosages of radiation can cause cellular disruption and disintegration of *T. vaginalis*, a trypan blue dye assessment was made to determine if the cells would still remain metabolically active and for how long after exposure to 400 Gy (400 Gy is not a sterilizing dose since 13% of the cells can reproduce after 500 Gy – Daly et al., 1991). Figure 1 shows the percent of cells excluding dye after given time periods at 37°C incubation. One set of non-irradiated cells (control) was in TYM medium without serum additive (to prevent growth), another set was in similar medium, but had been irradiated and a third set, also irradiated, had serum added to duplicate conditions inside the peritoneal cavity. All three sets remained metabolically active at 70 to 80% after 12 hours of incubation. The irradiated cells in TYM medium without serum then began to lose activity with only 1.8% of cells excluding dye after 16 hours; irradiated cells with added serum followed with only 2.5% activity after 16 hours. Neither of the irradiated sets was excreting dye at 24 hours. As a result of these experiments 400 Gy was chosen as the primary radiation dose for cells in TYM medium with serum.

After one sensitizing dose of irradiated C_1 *T. vaginalis,* the mice (n = 5/set) were challenged with 10^6 cells of nonirradiated C_1. This one dose did not confer protection because those mice died earlier (3.4 ± 0.5 days) than unsensitized controls (4.0 ± 0.0 days). Since one sensitization did not produce protection, a series of three inoculations of irradiated cells was administered, once a week, to increase the possibility of obtaining protective immunity. These preparations included formalinized C_2 with the immune stimulant complete Freunds adjuvant (CFA), formalinized C_2 without CFA, live avirulent ATCC 30001, formalized ATCC 30001 with CFA, and a sublethal dose (10^4) of C_2 (Table 3). Three sensitizing doses of irradiated cells did produce some measure of protective immunity in the mice that received irradiated cells as did the formalin-fixed C_2 cells and the formalin-fixed avirulent cells with CFA. The best protection was conferred with the use of virulent C_2 formalinized cells with added CFA.

IgG titers of adult mice showed that antibody response using irradiated cells correlated with the mortality studies (Fig. 2). CFA stimulated a faster response but the titer was the same as all other titers, which did not differ after three weeks. The avirulant ATCC 30001 control showed the least stimulatory effect as would be expected from the mortality studies, but the serum titer was also the same as the others at the end of three weeks. Passive transfer of immunity was tried with serum from these mice, but it was unsuccessful. Three passages of sublethal dosages of 10^3, 10^4, and 10^5 cells of C_1 were also used, but these showed no IgG titer after the first week, 1:160 titer for only the 10^5 dose after weeks 2 and 3, and 1:80 for the lower dosages for week 3 only. The data indicate that the amount of antigen administered is important in the IgG response.

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Table 1. The effect of different intraperitoneal doses of *Trichomonas vaginalis* strains ATCC 30001 and C_1 in five BALB/c mice (N = 5/dose).

Inoculum (No.of trichomonads)*	Inoculum	Percent Survival	Days to Death
PBS (control)	PBS	100	-----
ATCC 30001 1 x 10^6	C_1 1 x10^3	100	----
ATCC 30001 2.5 x 10^6	C_1 1 x10^4	100	----
ATCC 30001 5 x 10^6	C_1 1 x10^5	100	----
ATCC 30001 1 x 10^7	C_1 1 x10^6	0	5.8
	C_1 4 x10^6	0	4.0
	C_1 7 x10^6	0	4.0
	C_1 1 x10^7	0	4.0
	C_1 2 x10^7	0	2.0

*100% survival

Table 2. The mean time to death of female female Balb/c mice after intraperitoneal injection of *Trichomonas vaginalis* C_1 that were irradiated with different doses of ionizing radiation. Inoculum size was 10^6 for all groups, five mice in each group.

Radiation dose (Gray)	No. mice surviving/group	Mean time to death (days ± SD)
0	0/5	3.6 ± 1.9
100	0/5	5.8 ± 1.4
200	0/5	5.6 ± 1.3
400	5/5	----'
800	5/5	----

The Western blot analysis of serum from mouse treatment groups with different levels of protection can be seen in Figure 3. There were a large number of different molecular weight proteins recognized by serum from all the mouse treatment groups. There were, however, some differences in the size of the proteins that were demonstrated by this procedure. In the three treatment groups sensitized with either virulent strain (C_1 or C_2) that exhibited partial or complete protection (gamma irradiated, formalin-killed, and formalin-killed plus CFA), there were IgG antibodies to a *T. vaginalis* protein with an approximate molecular weight of 31,000 (arrow, Fig. 3). This band did not appear in the serum from animals that were sensitized with the avirulent strain of ATCC 30001 (Hostetler, 1987). However, when serum from mice that were sensitized with the avirulent strain plus CFA was used as the antibody source, this 31-kDa protein was also seen (arrow, Fig. 3). Thus in all cases where protective immunity was demonstrated, antibodies to this protein were seen.

Table 3. The percent survival of BALB/C different aged mice after intraperitoneal challenge with live C_1 or C_2 virulent strains of *Trichomonas vaginalis* after sensitizing the mice with different protocols. Ten mice were used for each group except where noted. Three separate sensitizing inoculations for each group were done a week apart. All sensitizations were accomplished with 10^6 cells unless noted.

Sensitization Protocol	Percent Surviving	Challenge
	Young[b] (3 weeks-old)	Adult[c] (24 weeks-old)
ATCC 30001 (live avirulent)	0	0[a]
ATCC 30001 formalin-fixed + complete Freund's adjuvant	60	---
Sublethal (10^4 cells), Virulent strain	0	0
Virulent strain, Formalin-fixed	20	60
Virulent strain, Gamma radiated (400 Gy)	20	40
Virulent strain, Formalin-fixed + complete Freund's adjuvant	100	100[d]

[a] 5 mice used
[b] C1 (2 x 10^3 cells) used to challenge
[c] C2 (1 x 10^6 cells) used to challenge
[d] 20 mice used

Discussion

Radiation was first used to attenuate a parasitic protozoan for vaccination purposes by Wallace and Sanders in 1966 (Hostetler, 1987) using the murine parasite, *Trypanosoma lewisi.* Since then there have been a number of successful immunizations using this technique with important parasitic protozoa such as *Leishmania, Plasmodium, Toxoplasma*, and *Eimeria* (Daly et al., 1991), including a study in which protective immunity in human volunteers was developed using irradiated sporozoites of *Plasmodium falciparum* (Egan et al., 1993). In the present study, however, the use of irradiated *Trichomonas vaginalis* in the mouse IP model did not offer any advantage over other methods of antigen preparation used in the study. It was originally hypothesized that perhaps metabolic or other short-lived antigens might produce a better immune response than fixed or otherwise killed trichomonads. Failure to improve on killed antigenic preparations is due either to the lack of metabolically-produced protective immunogens or the inability of the trichomonads to survive long enough in the peritoneal cavity to produce enough antigens to stimulate a protective response. *In vitro*, the cells were only able to survive 16 hours as indicated by trypan blue exclusion, but it is not known what the survival is *in vivo* in the peritoneum. The use of a much greater inoculum size of irradiated trichomonads might resolve this. However, the ability of the avirulent, formalin–killed ATCC strain to confer some protective immunity, when used with CFA, supports the idea of a structural antigen more so than a metabolic one. Also, the intraperitoneal route of antigen administration used in this study is artificial since the natural site of infection and where the most obvious pathology is produced is the human vagina. It would be of interest to see if radioattenuated *T. vaginalis* could stimulate an immune response, protective or otherwise, in the mouse vagina (or in human volunteers) by inoculation directly into the vagina or by the IM route.

The protective immunity, if any, to *Trichomonas vaginalis* in human females is poorly understood. IgG, IgA, and IgM to *T. vaginalis* have all been found in both vaginal washings and serum samples from patients or mouse models (Su,

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1982; Alderete, 1984; Cogne et al., 1985; Wos and Watt, 1986; Bhatt et al., 1992; Paintla et al., 2002) as has IgE (Su, 1982). In this study the only immunoglobulin response measured was IgG in mouse serum. The IgG response correlated well with the degree of protection obtained with the different protocols and may indicate a protective role, but IgA and IgM were not examined and these may be involved in the protective immune responses seen. Cell-mediated immunity (CMI) may also play a role. A mouse footpad assay for delayed type hypersensitivity has shown strong responses for formalin-fixed virulent strains, with and without CFA, and irradiated virulent cells (Hostetler, 1987). CMI responses to *T. vaginalis* such as phagocytosis by neutrophils have been found (Rein et al., 1980; Mason and Forman, 1982), The cytokines TNF-α, IL2, LTB4, αINF, and IL8 have been found to be stimulated by *T. vaginalis* as well (Shaio and Lin, 1995 ; Shaio et al., 1995; Paintla et al., 2002). A role for complement has also been found (Alderete and Kasmala, 1986; Caterina et al., 1999). Regardless, in spite of evidence from the above cited studies, the immune response in the vagina does not seem to confer obvious protective immunity. Complicating possible protective immunity is that the trichomonads can absorb host proteins that could act as a protective barrier against antibodies (or CMI mechanisms) directed to surface receptors (Peterson and Alderete, 1982). The variation, as observed in this study with virulence, and more specifically with protein-epitopes (Alderete et al., 1987; Dailey and Alderete, 1991; Fiori et al., 1992) may also be a mechanism for immune avoidance.

The protective immune responses seen so far have not been proven to be linked to any specific antigens conferring protection although associations have been inferred. A number of studies have focused on the most quantitatively significant antigens (Addis et al., 1999) and those that might be associated with virulence such as *T. vaginalis* proteinases (Alderete et al., 1991a, 1991b), cell membrane surface proteins (Alderete, 1987; Alderete, 1999), and heat shock antigens (Bozner, 1997; Davis-Haymer et al., 2000). Most of these antigens have been high molecular weight proteins (>100,000) but smaller immunogens have been found. Of special interest is a 30-kDa proteinase detected by Arroyo and Alderete (1995). Inhibition of this proteinase and another proteinase of 65-kDa resulted in decreased levels of *T. vaginalis* cytoadherence and totally abolished contact-dependant cytotoxicity. It may be that the 31-kDa protein in the present study and the 30-kDa proteinase are the same. Live ATTC 30001 did not stimulate a visible antibody response to this 31-kDA band. Although when stimulated with CFA, the ATCC strain revealed this band showing that the protein was present but in much smaller amounts. This 31-kDa protein may be coincidental with immune protection or may be associated with a loss of virulence and not related to protective immunity. Isolation of this protein in sufficient quantity for immunization studies is needed to determine if it plays a any role in protective immunity.

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