INFECTION AND IMMUNITY, Nov. 1990, p. 3553–3557
0019-9567/90/113553-05$02.00/0

Vol. 58, No. 11

Reduced Lymphocyte Responses to Mitogens in Natural and Experimental Trichomoniasis

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Received 9 May 1990/Accepted 20 August 1990

Proliferative responses to mitogens were determined by using peripheral blood mononuclear cells from women with active trichomoniasis, with serological evidence of past infection with *Trichomonas vaginalis*, and with no evidence of current or past infection. Even after the human immunodeficiency virus antibody status of the patients was taken into account, cells from women with active trichomoniasis showed reduced responses to phytohemagglutinin, concanavalin A, pokeweed mitogen, and bacterial lipopolysaccharide. Similar findings were obtained by using spleen cells from mice inoculated subcutaneously with live trichomonads. Reduction in proliferative responses by these cells could be detected 3 days after inoculation. There was some evidence to suggest that more-pathogenic strains of the parasite induced a greater degree of immunosuppression. The responses of spleen cells from mice inoculated with trichomonad-free culture supernatants were within normal limits, indicating that live trichomonads were needed to induce suppression. Support for this was gained from studies with cells from women who were treated successfully. Cells from these women rapidly regained normal lymphoproliferative function. Interleukin-2 (IL-2) production by spleen cells from infected mice was determined from measurements of mitochondrial activity in an IL-2-dependent T-cell line following incubation with stimulated spleen cell culture supernatants. These tests demonstrated lower IL-2 activity in supernatants from cell cultures from infected mice than in those from uninfected mice. The reduction in IL-2 activity did not, however, appear to correlate with the degree of reduction of mitogen-induced lymphoproliferation. Suppression of T-cell-mediated immunity may be one of the mechanisms by which *T. vaginalis* is able to evade host responses to infection.

Although both cellular and humoral responses to the urogenital protozoan *Trichomonas vaginalis* can be detected in natural human infection (5, 10, 13, 18, 20) and in animal models of disease (11), there is little evidence of protective immunity. Trichomoniasis is frequently seen in women arriving at the hospital for delivery who have few outward signs or symptoms of infection (12, 14). Whether these cases represent chronic infections, with parasite numbers controlled by host factors, or infections with less-virulent strains of the parasite is not known, but even in these patients immune responses are ineffective in eliminating parasites from the genital tract.

A number of factors may be important in this, including low immunogenicity of the parasite and the noninvasive nature of the infection. Many protozoa, however, use specific mechanisms to reduce the effectiveness of the immune responses to them. These mechanisms may include either stimulation or suppression of lymphocyte proliferation (3, 6).

In previous studies we found no evidence that trichomonal proteins were mitogenic for T cells either in natural human infection or in laboratory-infected mice (11, 13). In this report we present data showing reduced lymphoproliferative responses to mitogens by cells from patients with acute trichomoniasis but normal responses from patients with serological evidence of past infection but no evidence of current infection. These data are supported by experiments demonstrating spleen cell proliferation with cells from mice artificially infected with trichomonads isolated from these women.

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MATERIALS AND METHODS

Trichomonad isolates. Individual isolates of *T. vaginalis* were obtained from women attending a genitourinary clinic in Harare. Methods of isolation and culture of isolates in Diamond medium have been detailed elsewhere (10, 13). Once an isolate had become established, it was suspended in Diamond medium containing 5% dimethyl sulfoxide, split into portions, and cryopreserved at −70°C. When required for an experiment, a portion was removed from the freezer, thawed rapidly in warm (37°C) water, and inoculated into fresh Diamond medium containing 10% fetal bovine serum (FBS). Cultures reestablished in this way were normally used within 8 days (i.e., four passages) of being removed from frozen stocks.

The pathogenicity of each isolate was determined on the basis of the results of mouse subcutaneous assays, carried out as described before (11, 13). Isolates producing a lesion larger than 100 mm^2 in area in the mouse assay were designated high-pathogenicity isolates, and those producing lesions of less than 70 mm^2 in mice were designated low-pathogenicity isolates. The remainder were more variable in their pathogenicity for mice.

Lymphocyte proliferation tests. At the time of collection of the vaginal swab samples, peripheral blood samples (10 ml) were also collected; 8 ml of the sample was put into sterile heparin (2 U/ml), and the remainder was allowed to clot for serum. Sera were stored at −20°C until testing for anti-trichomonal antibodies could be carried out by the indirect fluorescent-antibody assay (10). Mononuclear (MN) cells were separated by centrifugation (500 × *g*, 15 min) of blood diluted with an equal volume of sterile phosphate-buffered saline (pH 7.3) on lymphocyte separation medium (Flow Laboratories). The cells were aspirated from the centrifuge tubes, washed twice in culture medium (RPMI 1640; Flow),

and suspended at 2×10^6 methylene blue dye-excluding MN cells in complete culture medium (RPMI 1640 containing 2 mM glutamine, 10 mM sodium bicarbondate, 20 mM HEPES buffer [*N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.3], 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% FBS).

Four mitogens were used for stimulation assays: phytohemagglutinin (PHA [Miles Laboratories] at a final dilution of 1:512), concanavalin A (ConA; Sigma; 6 μg/ml), pokeweed mitogen (PWM; Sigma; 4 μg/ml), and *Escherichia coli* lipopolysaccharide (LPS; Difco; 2.5 μg/ml). Each mitogen was made up in complete culture medium as a stock solution, filter sterilized, and stored at −20°C until used. In addition, a crude *T. vaginalis* antigen was prepared by repeated freeze-thawing of a suspension (5×10^6/ml) of culture trichomonads in complete culture medium. Cellular debris was sedimented by centrifugation, and the supernatant was filter sterilized, divided into portions, and stored for use at −20°C.

Assays were carried in sterile 96-well microtiter plates (Flow) with 50 μl of the MN cell suspension mixed with 150 μl of mitogen diluted in complete medium to the appropriate concentration. Control wells contained MN cell suspension with 150 μl of complete culture medium (negative control) and MN cell suspension with 150 μl of a 1:256 dilution of the crude antigen in complete culture medium (positive control). All experiments were carried out in triplicate.

After 2 days of incubation at 37°C in a 10% CO_2 atmosphere, cells were labeled by the addition of tritiated thymidine (Amersham; 1 μCi per well in 20 μl of complete culture medium) for a further 18 h of incubation. Cells were harvested onto glass fiber disks by using a semiautomated cell harvester for scintillation counting. For analysis, the stimulation index was calculated as the counts per minute (cpm) for each test divided by the mean cpm for the triplicate negative controls with the same MN.

As human immunodeficiency virus (HIV) infection is prevalent in Zimbabwe, all sera were also tested for antibody to HIV by an enzyme-linked immunosorbent assay (Abbott).

Five of the women treated for trichomoniasis with metronidazole reported for follow-up examination 10 to 14 days after initiation of therapy. Blood and vaginal swab specimens were taken and processed as above.

Mouse spleen assays. Male or female BALB/c mice were inoculated subcutaneously with 0.5 ml of a suspension of *T. vaginalis* in Diamond medium containing 0.5% agar, as described previously (11). At intervals up to 6 days later, mice were killed by cervical dislocation, and the length and breadth of the subcutaneous (s.c.) trichomonal lesion was measured. The spleen of each mouse was removed by aseptic technique, and the spleens and then the mice were weighed. Spleen cells were obtained by gently teasing the spleens apart in culture medium as described previously (11). Spleen cell suspensions were made up in complete culture medium at 2×10^6 methylene blue dye-excluding cells per ml.

Proliferation assays were carried out as for human MN cells, except that PWM was not used and the positive control was omitted. Negative controls were either uninfected mice or mice that had been inoculated 6 days previously with 0.5 ml of Diamond medium with agar but without trichomonads. All assays were carried out in triplicate, and the stimulation index was calculated as above.

Further experiments were done with mice inoculated s.c. with 0.5 ml of culture supernatant. Twenty-four-hour cultures of *T. vaginalis* in Diamond medium containing 10% FBS were centrifuged, and the supernatant was collected and filter sterilized. Immediately before use, the supernatant was mixed with an equal volume of Diamond medium without FBS but containing 1% agar at 45°C. A 0.5-ml portion of this was used for inoculation as above.

An additional five mice were given metronidazole (May & Baker; 2.5 mg intraperitoneally) 2 days before inoculation with *T. vaginalis* and on days 2 and 4 postinfection. Six days after inoculation, the spleens were removed and spleen cells were tested for reactivity to mitogens as described above.

IL-2 activity assays. Spleen cells from mice infected 6 days previously were stimulated with ConA (5 μg/ml) in complete culture medium. After 36 h of incubation, supernatants were collected and tested for interleukin-2 (IL-2) activity with an IL-2-dependent cell line (CTLL-2) and a colorimetric assay system (19). Briefly, 2×10^4 CTLL cells in 50 μl of modified Eagle medium containing 5% FBS were incubated in sterile flat-bottomed 96-well microtiter plates with 50 μl of supernatant from stimulated spleen cell cultures. Mitochondrial activity in the CTLL cells was determined by adding 20 μl of MTT (3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide, 5 mg/ml; Sigma). After 4 h of incubation at 37°C, the reaction was stopped by the addition of 100 μl of 10% sodium dodecyl sulfate and incubation overnight. The A_{620} of individual wells was determined by spectrophotometry.

At least three mice were used for each experiment, and all supernatants were tested in triplicate. Control wells contained CTLL cells with culture medium alone, CTLL cells with supernatants from unstimulated spleen cell cultures, and CTLL cells with supernatants of stimulated cells from uninfected mice.

RESULTS

Human experiments. Of 33 women examined, 8 (24%) had active trichomoniasis, and a further 11 (33%) had antitrichomonal antibody at a titer of 1:8 or greater, indicating past infection with *T. vaginalis*. There was no significant difference in the uptake of [^{3}H]thymidine in unstimulated cultures of cells from the three experimental groups (mean ± standard deviation [SD], 534 ± 161 cpm for negatives, 603 ± 186 cpm for indirect fluorescent-antibody positives, and 683 ± 189 cpm for *T. vaginalis* positives). There was, however, a significant reduction in the responsiveness to mitogens of cells from women with evidence of active trichomoniasis compared with cells from antibody-negative women or women with serological evidence of past infection (Fig. 1). This contrasted with the response to trichomonal antigen; cells from women with active trichomoniasis showed a greater response than cells from women with no evidence of infection, while cells from women who were seropositive but from whom *T. vaginalis* could not be cultured showed the highest degree of proliferation (Fig. 1).

All five women treated with metronidazole showed parasitological cure. The lymphoproliferative responses to mitogen all increased to within the normal range in the posttreatment blood samples (Table 1).

Thirteen women (39%) were HIV antibody positive by enzyme immunoassay. Cells from HIV-positive women showed a marked variation in the response to mitogens, but even among the HIV antibody-negative women there was evidence of reduced responsiveness to mitogens by cells from those with active trichomoniasis (Table 2).

Mouse studies. Mice infected subcutaneously with *T. vaginalis* showed enlarged spleens, with the spleen-body weight ratio (milligrams to grams) of infected animals (5.44 ± 1.24)

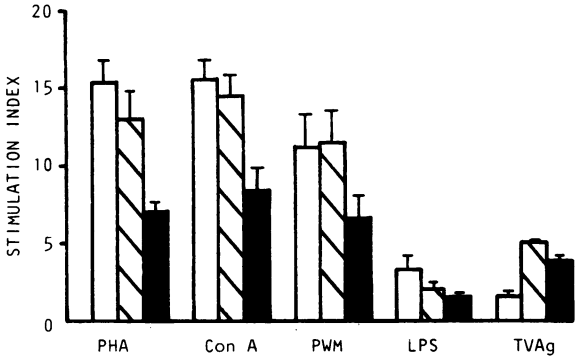

FIG. 1. Lymphocyte responses to mitogens and trichomonal antigen (TVAg) in human trichomoniasis. Mean (± SEM) stimulation index of peripheral MN cells from uninfected women (open bars), women with serological evidence of past infection (hatched bars), and women with current infection (solid bars).

being significantly greater than that of uninfected animals (4.36 ± 1.3; $t = 12.0$; $P < 0.05$). Spleen cells from infected mice showed reduced responses to each of the mitogens tested (Fig. 2), and this reduction was noticeable within 3 days postinfection (Fig. 3). There was an indication that more-pathogenic isolates produced a greater degree of immunosuppression than less-pathogenic isolates (Fig. 4), although differences were not statistically significant, and there was no significant correlation between lesion area and percent reduction of spleen cell activity.

Metronidazole-treated mice had no subcutaneous lesions, and spleen cells showed normal response to mitogens, with stimulation indices of 12.0 ± 1.6, 13.9 ± 2.1, and 3.4 ± 0.8 (mean ± standard error of the mean [SEM]) to PHA, ConA, and LPS, respectively.

Mice inoculated with culture supernatants showed proliferative responses within the normal range (stimulation indices [mean ± SEM]: PHA, 12.3 ± 2.3; ConA, 14.7 ± 1.9; LPS, 4.6 ± 0.9). Supernatants from spleen cell cultures from infected mice stimulated with ConA showed lower levels of IL-2 activity than supernatants from control cells (Fig. 5).

DISCUSSION

Inhibition of lymphocyte proliferative function is known to occur in a number of invasive infections with viral, bacterial, or parasitic organisms (2, 4, 6, 7, 15, 17). Here we present evidence that infection with *T. vaginalis*, a protozoan that normally invades only the superficial genital epithelium, also inhibits mitogen-induced and, to a lesser degree, antigen-induced lymphocyte blastogenesis. One half of the women with active trichomoniasis examined in this study also had evidence of concurrent HIV infection, and this infection is associated with impaired lymphocyte function (8). Our data indicate, however, that even HIV antibody-negative women with active trichomoniasis exhibit lower responses to mitogen than uninfected women.

TABLE 1. Effect of metronidazole treatment on lymphoproliferative responses in acute trichomoniasis[a]

Stimulant	Mean stimulation index ± SD ($n = 5$)	
	Before treatment	After treatment
PHA	4.7 ± 2.9	12.2 ± 4.2
ConA	6.0 ± 4.2	13.2 ± 2.5
PWM	5.1 ± 1.7	8.7 ± 0.9
LPS	1.1 ± 1.1	2.6 ± 0.3
T. vaginalis antigen	3.6 ± 1.4	4.8 ± 1.0

[a] See text for details.

TABLE 2. Proliferative response of lymphocytes from *T. vaginalis*-infected women: effect of concurrent HIV infection

Stimulant	Mean stimulation index ± SD	
	HIV antibody positive ($n = 4$)	HIV antibody negative ($n = 4$)
PHA	7.2 ± 2.3	6.9 ± 1.8
ConA	5.2 ± 2.8	11.7 ± 2.5
PWM	5.9 ± 4.6	7.3 ± 4.1
LPS	1.7 ± 0.6	1.3 ± 0.4
T. vaginalis antigen	2.0 ± 0.7	2.0 ± 0.7

Trichomoniasis is a commonly occurring sexually transmitted disease in Zimbabwe (12, 14), and it appears unlikely that all women harboring *T. vaginalis* in their genital tract are immunodepressed. These studies were carried out at a referral genitourinary clinic, where patients usually arrive with symptoms of acute discharge and dysuria. It was considered unethical to monitor changes in lymphocyte competence in these women without offering treatment for their infection. Thus, we were not able to correlate lymphocyte function status with duration of infection. The evidence we have that normal functional responses return very rapidly after treatment suggests, however, that the immunodepression may be a transient response to acute infection similar to that recorded in human and animal chlamydial disease (4, 9). Either subcutaneous or intraperitoneal infections of mice, if left untreated, progress towards severe infection that frequently results in death of the animal. The mouse model is therefore very dissimilar to human disease, and so the effects of chronic infection cannot be readily assessed.

The mechanism of induction of suppression in infectious diseases is not well understood, and soluble products (16), cell antigens (2), live organisms (4, 9), and whole killed organisms (6) have all been shown to induce depressed proliferative responses. The treatment of some mice in this study with metronidazole enabled us to examine the effect of organisms that failed to induce infection. The normal proliferative responses of spleen cells from these mice suggest that immunodepression in trichomoniasis results from prod-

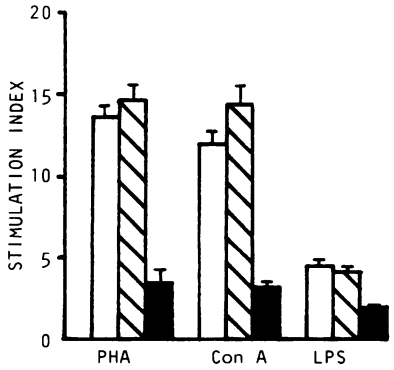

FIG. 2. Depression of responses of murine spleen cells to mitogens in experimental trichomoniasis. Mean (± SEM) stimulation index of spleen cells from mice inoculated with *T. vaginalis* (solid bars), mice inoculated with agar (hatched bars), and uninfected mice (open bars).

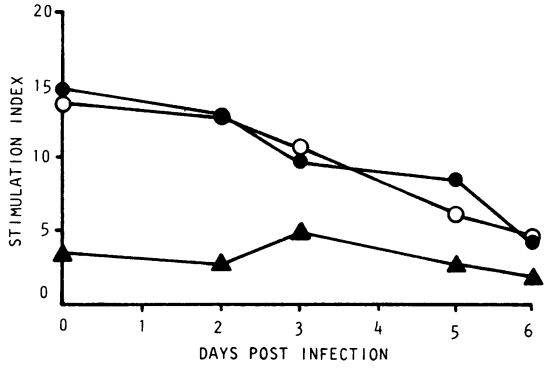

FIG. 3. Kinetics of immunodepression in *T. vaginalis*-infected mice. Spleen cell responses (mean stimulation index) to PHA (●), ConA (○), and LPS (▲) at different times postinfection.

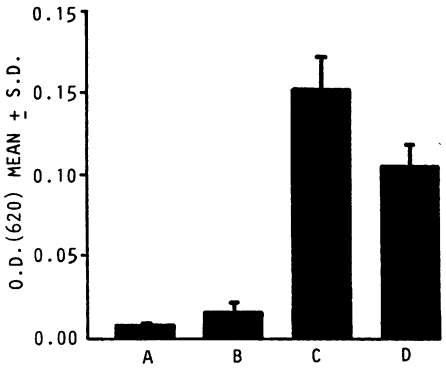

FIG. 5. IL-2 activity of supernatants from spleen cell cultures. Activity was measured photometrically as described in the text. Supernatants were obtained from (A) culture medium alone, (B) unstimulated spleen cell cultures, (C) uninfected mouse spleen cells stimulated with ConA, and (D) spleen cells from *T. vaginalis*-infected mice stimulated with ConA.

ucts released by live organisms rather than trichomonal structural antigens. Trichomonads are known to shed immunogenic proteins into the culture medium (1), and some of these may modulate immune responses. The injection of trichomonad-free culture supernatants into mice failed to bring about depressed lymphoproliferative responses, although experiments are under way to examine the effect of culture conditions on shedding of immunoactive proteins by trichomonads.

We were unable to demonstrate a correlation between suppression of the proliferative response and the size of the lesion produced in the mice, although in general the more pathogenic the strain, the greater the degree of suppression. In other studies, detectable suppression appears to correlate with the virulence of the bacteria (6). It has previously been shown that antigens from trichomonad isolates showing different pathogenicity levels in mice exhibit different patterns of stimulation of proliferation of mouse spleen cells (11), although this was not apparently the case in human studies (10). There was marked variation in the sizes of lesions produced in mice in the present study, and the mouse assay may therefore be a relatively insensitive marker of virulence. More consistent markers of virulence, such as cytotoxicity assays, could not be done at the time these experiments were carried out, but may be useful in investigating the relationship between virulence and immunosuppression.

Marked reduction in IL-2 and IL-2 receptor activity has been noted in noninvasive bacterial disease (16), although in some infections IL-2 activity may remain intact despite decreased lymphoproliferative responses to mitogens (4, 15). In mice infected with *T. vaginalis*, there was only a 25% reduction in the IL-2 concentration in culture supernatants but a 70% reduction in lymphoproliferation in response to ConA. It should be noted that in a closed culture system such as used here, external concentrations will be a result of the dynamic relationship between production and utilization. Decreased levels of IL-2 in the supernatants may therefore have resulted from either decreased production or increased utilization. Measurement of IL-2 receptor activity might have given some insight into which of these was responsible for the observed effect, but we were not able to carry out such tests.

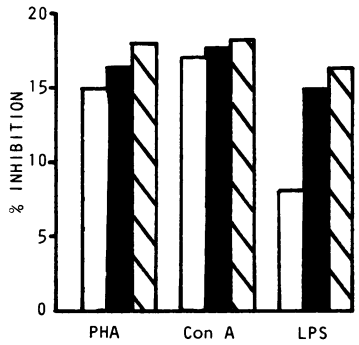

FIG. 4. Inhibition of spleen cell mitogenic responses in mice infected with different strains of *T. vaginalis*. The strains produced lesions with areas of >100 mm^2 (hatched bars), 70 to 100 mm^2 (solid bars), and <70 mm^2 (open bars).

The clinical significance of immunosuppression is not easily determined. *T. vaginalis* infections frequently follow an initial acute course, followed by a long period of chronic infection during which there may be periodic recurrence of acute symptoms (5, 14). A combination of cellular and humoral reactions may contribute to control of parasite numbers in the genital tract and reduce symptoms to subclinical levels. Periodic disturbances in immune competence, perhaps induced by parasite-derived products, may then be responsible for the increase in parasite numbers leading to increased epithelial damage and inflammatory response. These suggestions are currently being investigated.

ACKNOWLEDGMENTS

The permission of the MOH, City Health Department, Harare, to obtain specimens from women attending the Mbare Genitourinary Center is appreciated. The assistance of the nursing staff at this clinic is also gratefully acknowledged.

Financial support was given by the University of Zimbabwe Research Board.

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