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Antigen-Specific Proliferation Responses of Peripheral Blood Lymphocytes to *Trichomonas vaginalis* Antigen in Patients with *Trichomonas* Vaginitis

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This report describes the development of an assay system which overcomes the difficulty of detecting immune responses of patients with *Trichomonas* vaginitis by making use of peripheral blood leukocytes obtained from such patients. When peripheral blood leukocytes from the patient were stimulated in microcultures with the soluble antigen extracted from *Trichomonas vaginalis*, significant degrees of proliferation ensued, as measured by the incorporation of [*methyl*-^{3}H]thymidine 4 to 5 days after initiation. The antigen-induced proliferation response of peripheral blood leukocytes is specific for *T. vaginalis* antigen. The *T. vaginalis*-specific [*methyl*-^{3}H]thymidine incorporation is mediated by Leu-1-positive cells, namely, T lymphocytes, in the peripheral blood leukocyte population. This assay system should prove useful for the analysis of the immune response to the protozoa in patients with *Trichomonas* vaginitis.

Trichomonas vaginitis is generally believed to be caused by *Trichomonas vaginalis*. It is often observed, however, that some patients complain of severe itching or irritation in the genital region but seem to have no symptoms other than the genital discharge (1, 5, 7, 10). The real etiology of *Trichomonas* vaginitis still remains to be analyzed. To examine the antigenicity of *T. vaginalis*, the soluble antigen was extracted from protozoa cultured in vitro. The soluble antigen has been shown to elicit an anti-*Trichomonas* antibody in mice (13). Although patients with *Trichomonas* vaginitis raise a significant level of antibody specific to the antigen in a hemagglutination assay, the responsiveness of the antibody in patients is not so strong (unpublished data). The immunological study of *Trichomonas* vaginitis has been hampered by the lack of a reproducible assay system for the disease. An antigen-specific T-lymphocyte proliferation assay giving significant degrees of stimulation has been established in rabbits, guinea pigs, and mice (2, 8, 9). In humans, proliferation responses of peripheral blood leukocytes (PBL) to various antigens (e.g., *Toxoplasma* antigen, etc.) have been reported (3, 11, 12). In this study, the responsiveness of PBL from patients with *Trichomonas* vaginitis to the *Trichomonas* antigen was examined by means of a [*methyl*-^{3}H]thymidine (^{3}H-TdR) incorporation system. Lymphocytes obtained from the patients incorporated substantial amounts of tritiated thymidine when stimulated with the antigen in vitro. This *Trichomonas*-specific proliferation is shown to be dependent upon the presence of Leu-1-positive cells, or T lymphocytes.

MATERIALS AND METHODS

Isolation of lymphocyte population. PBL from patients with *Trichomonas* vaginitis and from healthy controls were isolated by Lymphoprep (Nyegoard Co., Oslo, Norway) density centrifugation. After the separation, the PBL were washed three times with RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) and were then suspended in RPMI 1640 supplemented with heat-inactivated 10% fetal calf serum (GIBCO), 50 μg of kanamycin (Meiji Pharmaceutical Co., Tokyo, Japan) per ml, and 5×10^{-5} M 2-mercaptoethanol (culture medium). The patients with *Trichomonas* vaginitis were diagnosed by microscopic examination of wet smears, and PBL were collected from the patients who were receiving anti-*Trichomonas* reagent(s) chemotherapy (Trichomycin or Flagyl [metronidazole]).

Antigens. The preparation method of the soluble extract from the in vitro-cultured *T. vaginalis* line (ATCC 3001) has been described previously (13). Briefly, *T. vaginalis* cultured in the medium described above was extensively washed with 0.05 M phosphate-buffered saline (PBS) at 3,000 rpm for 15 min. After being washed five times, the protozoa were sonicated with a Sonicator (Tominaga Industry Co., Tokyo, Japan) for 60 s on ice, and the sonicated material was supercentrifuged at 10,000 rpm for 60 min. The supernatant was dialyzed in PBS for 3 days. After dialysis,

the material was again supercentrifuged and sterilized by being passed through a Millipore membrane (0.22 μm; catalog no. GSWP 04700; Millipore Corp., Bedford, Mass.). The protein concentration was measured by the method of Folin and Ciocalteu (4), and then the soluble antigen was adjusted to a concentration of 5 mg/ml and stocked at 4°C until use. Purified protein derivative of tuberculin (PPD) was purchased from Japan BCG Co. (Tokyo, Japan).

Cell cultures. One hundred microliters of culture medium containing 5×10^4 to 2×10^5 PBL was placed in each well of a sterile, U-bottomed, microculture plate (1-63302; Nunclon, Roskilde, Denmark). One hundred microliters of soluble *T. vaginalis* or PPD antigen was added to give a total volume of 200 μl and a final antigen concentration of 1 to 1,000 μg of *T. vaginalis* per ml or 2 or 20 μg of PPD per ml. Cultures were maintained at 37°C under a humidified atmosphere of 5% CO_2–95% air for 3 to 6 days. At 16 to 24 h before harvesting, 1 μCi of ^{3}H-TdR (specific activity, 27 Ci/mmol; Radiochemical Centre, Amersham, England) was added. Cultures were harvested onto filter paper strips with a semiautomated harvester (Abe Kagaku Co., Chiba, Japan), and the strips were washed with distilled water. ^{3}H-TdR incorporation was measured with a liquid scintillation counter (Packard Co., Downers Grove, Ind.). All determinations were done in triplicate, and the data were expressed either as mean counts per minute (cpm) ± standard error of the mean (SEM), as the difference in cpm between antigen-stimulated and control cultures (Δcpm), or as the ratio between cpm obtained from cultures with and without antigen (stimulation ratio).

Depletion of T lymphocytes with monoclonal anti-Leu-1 antibody plus complement. Anti-Leu-1 antibody (Becton, Dickinson & Co., Sunnyvale, Calif.) was used to remove the T lymphocytes from the PBL. Two micrograms of anti-Leu-1 antibody in 100 μl of PBS (0.01 M, pH 7.0) was reacted with 5×10^6 PBL for 20 min at room temperature. The cells were then incubated for 30 min at 37°C with 1 ml of rabbit complement (1:3 diluted). After anti-Leu-1 antibody plus complement treatment, the cells were washed three times with RPMI 1640. The control group of PBL was treated with complement alone.

Sephadex chromatography of *T. vaginalis* crude extract. Columns of freshly prepared Sephadex were usually made for each run. Twenty grams of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was mixed with 1,000 ml of 0.01 M PBS (pH 7.8) and allowed to swell under vacuum for 3 days. The slurry was poured into a 50-mm-diameter glass column. The height of the gel bed after settling was approximately 80 cm. Antigen (5 mg in 2 ml) was then applied to the column. The eluting fluid was PBS (pH 7.8), and the elution rate was approximately 40 ml/h. The fraction volume was 9 ml. Each fraction was concentrated into the original input volume and then sterilized by being passed through a Millipore membrane. Eluted column fractions were detected by their optical density at 280 nm.

Statistics. Stimulation with antigen (*T. vaginalis* antigen or PPD) or without antigen was assessed by measuring the incorporation of a pulse of tritiated thymidine, expressed either as the mean cpm for triplicate determinations ± the SEM (cpm ± SEM) or as the difference in cpm between cultures with and without antigen (Δcpm). The significance of the differences between the patients with *Trichomonas* vaginitis and the healthy controls in a lymphocyte-transformation response was determined by the Mann-Whitney test.

RESULTS

Response of PBL to *T. vaginalis* antigen. PBL from patients with *Trichomonas* vaginitis were found to be highly responsive to in vitro stimulation with *T. vaginalis* soluble antigen, as measured by incorporation of ^{3}H-TdR (Fig. 1). In 18 consecutive experiments in which PBL were collected from 10 patients with *Trichomonas* vaginitis and from 8 healthy controls, the mean of the response (Δcpm) of 10^5 PBL from patients

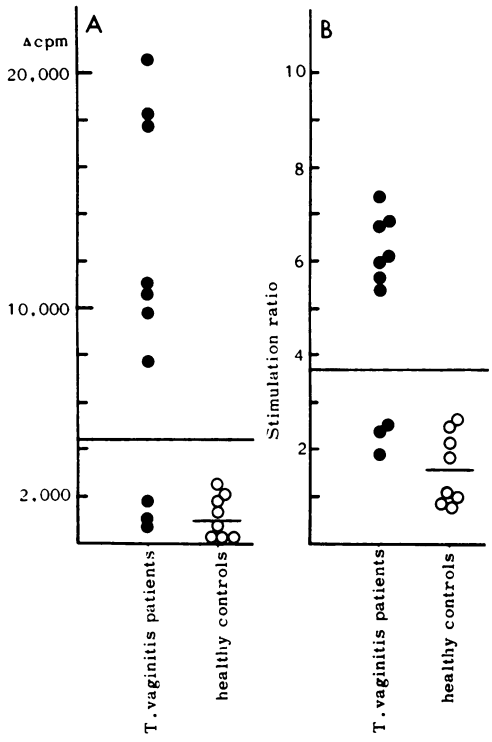

FIG. 1. PBL proliferation responses to *T. vaginalis* antigen expressed as antigen-specific response (Δcpm) (A) and stimulation ratio (B). 10^5 PBL from patients with *Trichomonas* vaginitis or from healthy controls were stimulated in vitro with 100 μg of *T. vaginalis* antigen per ml. The magnitude of the stimulation was assessed 5 days later by measuring the incorporation of a pulse of ^{3}H-TdR (cpm). Antigen-specific response (Δcpm) and stimulation ratio were estimated as either the difference or the ratio between cpm obtained from cultures with and without antigen. The short bar denotes the mean response in controls, and the solid line means plus 3 standard deviations. Unstimulated responses (cpm) are 2,100 ± 1,100 for patients with *Trichomonas* vaginitis and 2,300 ± 1,300 for healthy controls.

to 100 μg of the antigen per ml was 9,879 (the range of response was 20,510 to 940), whereas that of the PBL from healthy controls was 1,080 (the range of response was 2,800 to 0). Cultures from 7 of 10 infected patients had a positive stimulation ratio (mean, 5.1; range, 7.3 to 1.9), whereas no cultures from controls had a significant positive stimulation ratio (mean, 1.6; range, 2.6 to 0.9). Differences in the lymphocyte transformation response between infected patients and healthy controls were significant if expressed either as antigen-specific increase in cpm (Δcpm) ($P < 0.002$) or as stimulation ratio (cpm obtained from cultures with antigen/cpm obtained from cultures without antigen) ($P < 0.002$). Thus, *T. vaginalis*-specific stimulation was only detectable with the PBL from patients with *Trichomonas* vaginitis. The significant responses (Δcpm, >6,000; stimulation ratio, >4) can therefore be considered as indicative of *T. vaginalis* infection, although a negative response does not exclude the infection diagnosed by microscopic examination. The negative response of some patients is discussed later.

The details of the responses of PBL from either patients or controls to in vitro challenge with the *T. vaginalis* antigen are shown in Fig. 2 and Table 1. As few as 5×10^4 cells (Fig. 2) and as little as 3 μg of the antigen per ml (Table 1) gave significant stimulation to the PBL of the patients. Maximum responses were usually obtained with an antigen concentration of 100 μg/ml, and an antigen concentration of more than 300 μg/ml suppressed responses (Table 1). Usually the greatest responses occurred with 2

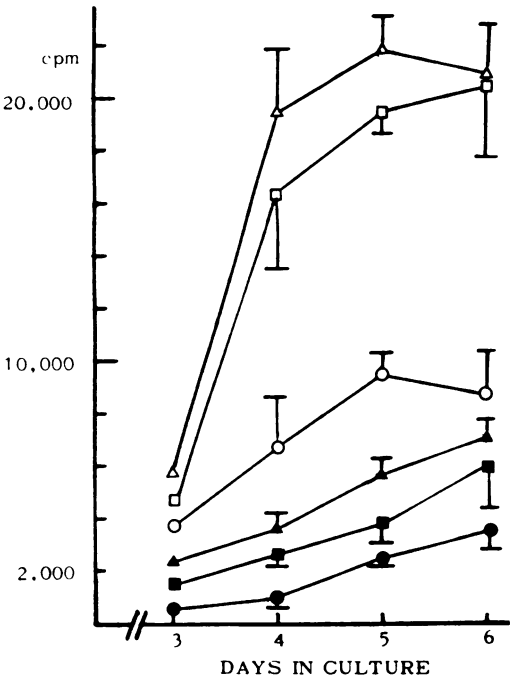

FIG. 2. 2×10^5 (△, ▲), 1×10^5 (□, ■), and 3×10^4 (○, ●) PBL from a patient with *Trichomonas* vaginitis were cultured either with 100 μg of *T. vaginalis* antigen per ml (open symbols) or without antigen (closed symbols) for various lengths of time. Stimulation was measured by the degree of incorporation of a pulse of ^{3}H-TdR (Δcpm). The bracer above each bar represents 1 SEM for triplicate determinations.

TABLE 1. Proliferation response of PBL from a patient with *Trichomonas* vaginitis and from a control as a function of *T. vaginalis* antigen dose[a]

Antigen	Dose (μg/ml)	Proliferation response			
		PBL from patient with *Trichomonas* vaginitis		PBL from healthy control	
		cpm	Δcpm	cpm	Δcpm
Medium alone		650 ± 50		2,700 ± 170	
T. vaginalis	1,000	3,640 ± 250	2,990	2,540 ± 390	<0
	300	14,820 ± 670	14,170	3,190 ± 650	490
	100	25,660 ± 8,680	25,010	3,830 ± 790	1,130
	30	18,050 ± 3,190	17,400	3,070 ± 770	370
	10	7,570 ± 380	6,920	1,980 ± 840	<0
	3	2,260 ± 50	1,610	2,230 ± 1,100	<0
	1	540 ± 70	<0	2,640 ± 1,010	<0
PPD	20	40,780 ± 6,080	40,130	32,910 ± 7,990	30,210

[a] 10^5 PBL from a patient with *Trichomonas* vaginitis or from a healthy control were stimulated in vitro with various concentrations of *T. vaginalis* soluble extract or with 20 μg of PPD per ml. The magnitude of the stimulation was assessed 5 days later by measuring the incorporation of a pulse of ^{3}H-TdR (cpm). The data expressed here as mean cpm for triplicate determinants ± the SEM (cpm ± SEM) and as Δcpm were estimated as the difference between cultures with and without antigen. Both patient and control were found to be tuberculous reaction positive.

$\times 10^5$ cells per well (Fig. 2); larger cell numbers showed either a plateau or a decrease in ^{3}H-TdR incorporation by the antigen-stimulated cultures and often a continued rise in ^{3}H-TdR incorporation by the control cultures.

The kinetics of the antigen response are shown in Fig. 2. In general, the response peaked at 5 days, but occasionally the peak was reached at 6 days (10^5 PBL, in this particular experiment). The data presented in Fig. 1 and Table 1 also indicate that the *T. vaginalis* soluble extract did not contain any mitogenic factor stimulating PBL, because the healthy controls responded to PPD as well as did the patients with *Trichomonas* vaginitis, in spite of the failure of the controls to respond to the *T. vaginalis* antigen in wide ranges of antigen doses.

T-cell dependence of *T. vaginalis* antigen-induced proliferation. The T-cell dependence of the antigen-induced proliferation response of the PBL from the patients was demonstrated by pretreating the population with anti-Leu-1 antibody plus complement (Table 2). The response of the PBL from a patient with *Trichomonas* vaginitis to both the soluble extract of *T. vaginalis* and PPD was almost completely eliminated by this treatment at *T. vaginalis* antigen concentrations of 100 and 10 μg/ml. The response of the PBL from a healthy control to PPD was also eliminated by the same pretreatment; in contrast, the PBL did not show significant incorporation of ^{3}H-TdR with the *Trichomonas* antigen. This experiment demonstrated that *T. vaginalis*-induced proliferation responses of PBL are mainly mediated by Leu-1-positive cells, or T cells, in the PBL population.

Fractionation of crude *Trichomonas* antigen by Sephadex chromatography. To purify the antigenic molecules from the crude antigen, we chromatographed the soluble *Trichomonas* antigen on Sephadex G-200; eluted column fractions were tested for their capacity to stimulate the ^{3}H-TdR incorporation of PBL from patients with *Trichomonas* vaginitis. The elution pattern detected with optical density is depicted in Fig. 3. Two main peaks (molecular weights, 160,000 and 12,500) and three small peaks (molecular weights, 100,000, 74,000, and 34,000) were eluted and fractionated into five fractions. These fractions were assayed for ability to stimulate ^{3}H-TdR incorporation of PBL. Fractions II, III, and IV appeared to be potent antigens to the proliferation response of PBL (Table 3). Typical ^{3}H-TdR incorporation patterns of PBL are listed in Table 3. PBL from patient no. 1 were stimulated by both fractions II and IV (fraction II, 10,460 cpm; fraction IV, 8,780 cpm). PBL from patient no. 2 were stimulated by fractions II and III, whereas only fraction II could stimulate PBL from patient no. 3. Thus, fraction II was found to be the most potent antigen for the proliferation response of PBL in each case, although fractions III and IV could stimulate PBL to uptake ^{3}H-TdR in certain cases. The time of maximum response did not shift with the various concentrations of the fractioned antigens over a 100-fold range (data not shown).

DISCUSSION

The data presented in this study clearly demonstrate that *Trichomonas* antigen-specific proliferation responses can be observed in PBL from *Trichomonas* vaginitis patients. The ^{3}H-TdR incorporation of PBL was eliminated by treatment with monoclonal anti-Leu-1 antibody plus complement. Anti-Leu-1 antibody is specific for the common determinant on human T cells. Thus, this assay system should prove useful for the analysis of immune responses,

TABLE 2. Elimination of antigen-induced proliferation by pretreatment of PBL from a patient with *Trichomonas* vaginitis with Anti-Leu-1 antibody plus complement[a]

Antigen	Dose (μg/ml)	Proliferation response (cpm)			
		PBL from patient with *T. vaginalis*		PBL from healthy control	
		C alone	Anti-Leu-1 plus C	C alone	Anti-Leu-1 plus C
Medium alone		4,860 ± 330	1,010 ± 420	6,620 ± 490	1,030 ± 140
T. vaginalis	100	29,590 ± 4,830	2,860 ± 270	6,990 ± 720	2,870 ± 430
	10	8,330 ± 680	2,590 ± 410	7,230 ± 550	2,430 ± 40
PPD	20	44,570 ± 7,110	3,420 ± 160	59,660 ± 8,490	4,910 ± 1,050
	2	24,720 ± 990	2,160 ± 220	28,010 ± 980	3,270 ± 180

[a] 2×10^5 PBL from a patient with *T. vaginalis* or from a healthy control were treated with either anti-Leu-1 antibody plus rabbit complement or complement alone as described in the text. The treated cells were cultured in vitro for 5 days. Stimulation with antigen (either *T. vaginalis* or PPD) was assessed by measuring the incorporation of a pulse of ^{3}H-TdR, expressed as the mean cpm for triplicate determinations ± the SEM (cpm ± SEM). C, complement.

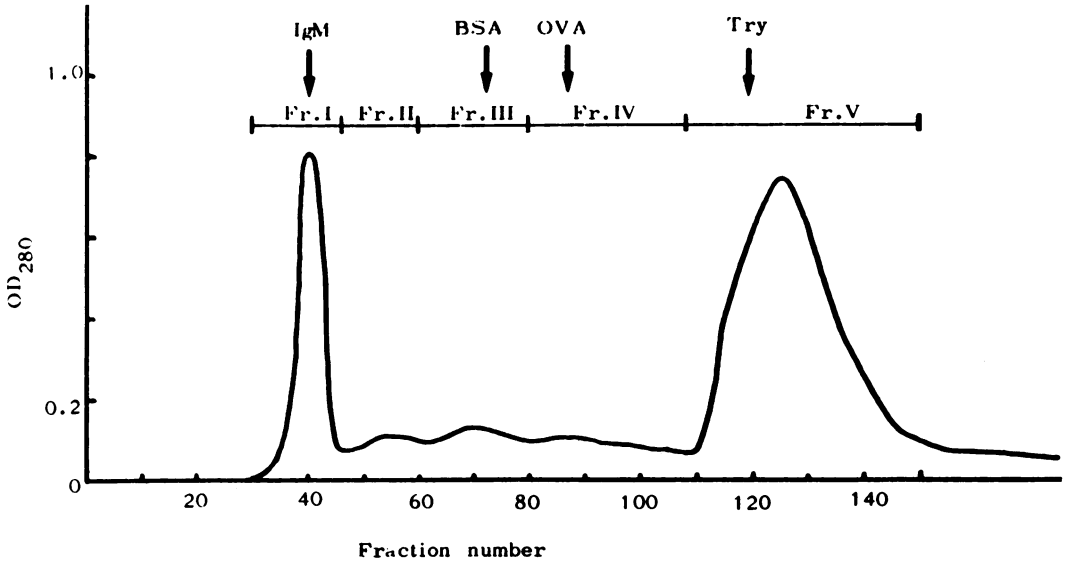

FIG. 3. Gel filtration of *T. vaginalis* soluble extract on a Sephadex G-200 column. The soluble extract was chromatographed on Sephadex G-200 (5 by 80 cm) equilibrated with 0.01 M PBS (pH 7.8). The flow rate was 40 ml and the fraction size was 9 ml. Each fraction was concentrated into the input volume and then sterilized by being passed through a Millipore membrane. Fr., Fraction; BSA, bovine serum albumin; OVA, ovalbumin; Try, trypsin; OD_{280}, optical density at 280 nm.

especially T-cell functions, to *Trichomonas* protozoa in patients with *Trichomonas* vaginitis, as well as for the diagnosis of the disease. PBL from some infected patients failed to respond to the antigen. Anti-*T. vaginalis* immunoglobulin E (IgE) antibody responses are regulated by at least two genes in mice, as we have previously reported (13). One of the immune response genes regulating the magnitude of the IgE antibody response is located in the *I* region of *H-2*. If the *T. vaginalis*-specific proliferation response of PBL is also genetically regulated in humans, it may be possible to assume that the failure of PBL from the patients to respond to the antigen is attributable to the genetical unresponsiveness of the patients.

In the study of antigen purification, PBL were stimulated mainly by fraction II antigen and to

TABLE 3. Ability of fractioned antigen to stimulate the proliferation response of PBL from patients with *Trichomonas* vaginitis

Antigen[a]	Proliferation response of PBL[b]					
	Patient no. 1		Patient no. 2		Patient no. 3	
	cpm	Δcpm	cpm	Δcpm	cpm	Δcpm
Medium alone	1,740 ± 210		2,410 ± 390		4,870 ± 560	
Unfractioned *T. vaginalis*	13,260 ± 2,050	11,520	18,740 ± 2,310	16,330	28,300 ± 2,790	23,430
Fractioned *T. vaginalis*						
I	1,990 ± 40	250	2,950 ± 100	540	5,080 ± 740	210
II	10,460 ± 1,120	8,720	14,630 ± 940	12,220	19,460 ± 1,150	14,590
III	2,530 ± 190	790	11,380 ± 2,110	8,970	7,970 ± 1,040	3,100
IV	8,780 ± 1,010	7,040	3,020 ± 880	610	4,220 ± 520	<0
V	1,470 ± 620	<0	2,190 ± 170	<0	2,700 ± 80	<0
PPD	19,890 ± 2,430	18,150	9,420 ± 110	7,010	38,440 ± 4,250	33,570

[a] The concentration of *T. vaginalis* soluble extract was 100 μg/ml, and each fraction was adjusted to the original input concentration (100 μg/ml). PPD concentration was 20 μg/ml.

[b] 2×10^5 PBL were cultured for 5 days with or without antigen. Stimulation was measured by the degree of incorporation of a pulse of ^{3}H-TdR (cpm).

some extent by fractions III and IV (Table 3). PBL from patient no. 1 responded to fractions II and IV and failed to respond to fraction III. In contrast, PBL from patient no. 2 were stimulated by both fractions II and III. PBL from patient no. 3 responded only to fraction II and not to fractions I, III, IV, or V. It is assumed that *Trichomonas*-specific proliferative T lymphocytes recognize various antigenic determinants of *T. vaginalis*. In addition, murine and human anti-*Trichomonas* IgG and IgM antibodies affix on fraction III antigen (50,000-molecular-weight molecule [estimated by sodium dodecyl sulfate-acrylamide gel electrophoresis]), whereas murine IgE antibody binds with fraction II (75,000 molecular weight) (A. Yano et al., manuscript in preparation). Although the biological meaning of the *Trichomonas*-specific proliferative T cells is still unknown, such T cells may be involved in antibody formation as helper T cells or in delayed-type hypersensitivity as effector T cells. If *Trichomonas* vaginitis is caused by either immediate or delayed-type hypersensitivity to the protozoan antigen(s), the etiology of the disease can be analyzed easily by using the purified antigen. On the other hand, if the antibody response has a prophylactic function in the disease, development of vaccine may be possible. Recently, Milovanović et al. (6) reported that vaccination with *Lactobacillus acidophilus* improved clinical symptoms in patients with *Trichomonas* vaginitis, although the mechanisms of the vaccination are still unknown. The cross-reactivity of the *Lactobacillus* antigen and the *T. vaginalis* antigen for T-cell stimulation can be easily examined by this assay. The system introduced by this study and the development of the antibody assay system shed some light on the analysis of the real etiology of *Trichomonas* vaginitis, including the analysis of the genetic make-up of the disease, and on the development of a vaccine for the disease.

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