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Human T-Lymphocyte Proliferation, Lymphokine Production, and Amebicidal Activity Elicited by the Galactose-Inhibitable Adherence Protein of *Entamoeba histolytica*

DENISE C. SCHAIN,[1] ROBERT A. SALATA,[2] AND JONATHAN I. RAVDIN[2]*

Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908,[1] and Department of Medicine, Case Western Reserve University and the Cleveland Veterans Affairs Medical Center, Cleveland, Ohio 44106[2]

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We studied human T-lymphocyte responses to the purified *Entamoeba histolytica* galactose-inhibitable adherence protein. Individuals having serum anti-adherence protein antibodies possess peripheral blood lymphocytes which demonstrate antigen-specific responses to the purified adherence protein (10 μg/ml) and whole soluble amebic antigen (100 μg/ml). This was determined by incorporation of [^{3}H]thymidine (53,080 and 73,114 dpm, respectively) and by increased production of interleukin-2 and gamma interferon (42.0 and 67.5 U/ml, respectively) ($P < 0.05$ for each in comparison with values for control lymphocyte responses). Lymphocytes from antiamebic antibody-positive subjects develop in vitro amebicidal activity only when incubated for 5 days with the purified adherence protein ($P = 0.02$). In conclusion, the *E. histolytica* galactose-inhibitable adherence protein elicits an in vitro amebicidal cell-mediated immune response, further supporting the potential for the use of this protein in a subunit amebiasis vaccine.

Amebic colitis and liver abscess result from infection with the enteric protozoan pathogen *Entamoeba histolytica*. The critical step in the pathogenesis of invasive amebiasis is adherence of the trophozoite form of the organism to host colonic mucins, intestinal epithelial cells, and responding polymorphonuclear leukocytes (9, 10, 21). Amebic adherence is mediated by a galactose-inhibitable adherence protein, which consists of a 170-kDa heavy and a 35-kDa light subunit (4, 7, 10). In vitro binding by the galactose-inhibitable adherence protein is required for cytolysis of mammalian target cells by *E. histolytica* trophozoites (10, 12). Subjects with amebic colitis, liver abscess, or asymptomatic infection by pathogenic *E. histolytica* strains produce serum antibodies to the adherence protein heavy subunit (5, 11). Recently, Petri and Ravdin reported that the purified adherence protein is a protective antigen in an experimental model of amebic liver abscess (6).

Studies of humans and experimental animal models indicate that cell-mediated rather than humoral mechanisms are primarily responsible for acquired immunity to amebic liver abscesses (15, 17, 19). Immunization of gerbils with the purified adherence protein is sufficient to induce an amebicidal cell-mediated immune response (19b). To evaluate the potential of the amebic adherence protein to serve as a protective T-cell antigen, we determined whether it contained conserved human T-cell epitopes. Lymphocyte responses to the immunoaffinity-purified adherence protein were studied by using peripheral blood mononuclear cells (PBMC) from subjects cured of invasive amebiasis or determined prospectively to possess serum anti-adherence protein antibodies. (This work was presented in abstract form at the National Meeting of the American Federation for Clinical Research, May 1990.)

Subjects included three patients determined to be cured of amebic liver abscesses by detection of a defect in the liver by ultrasound and/or computerized tomography, the presence of positive serum antiamebic antibodies to whole parasite antigen assayed by enzyme-linked immunosorbent assay (ELISA), and a salutory response to specific antiamebic therapy with metronidazole. Three additional subjects were identified among 50 Mexican migrant workers employed in central Virginia by assay of serum antibodies to the *E. histolytica* adherence protein by ELISA (11). A positive anti-adherence protein ELISA was defined as an absorbance value of >0.100 at a serum dilution of ≥1:1,000 (11). Six healthy age- and sex-matched control subjects were chosen and were confirmed not to have serum antiamebic antibodies.

The galactose-inhibitable adherence protein was purified by monoclonal antibody affinity chromatography, as reported previously (7). Protein concentration was determined by using the Pierce perchloric acid method as recommended by the manufacturer (Pierce Chemical Company, Rockford, Ill.). The purity of the adherence protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining and immunoblotting with anti-adherence protein-specific monoclonal antibodies (Fig. 1). Before use, purified adherence protein was aliquoted at standard protein concentrations and stored at −70°C.

Following the obtaining of informed consent, 120 ml of venous blood was drawn from subjects by using the sterile venipuncture technique. PBMC were obtained as described previously (17), PMBC membrane integrity was confirmed by trypan blue (0.4%) exclusion criteria, and PBMC were adjusted to a final concentration of 2×10^6 or 3.5×10^6 cells per ml in RPMI supplemented with 10% heat-inactivated pooled human AB type serum and 1% antibiotics (Penicillin-Streptomycin [penicillin, 10^4 U/ml; streptomycin, 10^4 μg/ml]; GIBCO Laboratories, Grand Island, N.Y.).

Lymphocyte proliferation was determined by [^{3}H]thymidine uptake, as reported previously (18). Concanavalin A (20 μg/ml), whole soluble amebic antigen (WSAA) (100 μg/ml),

* Corresponding author.

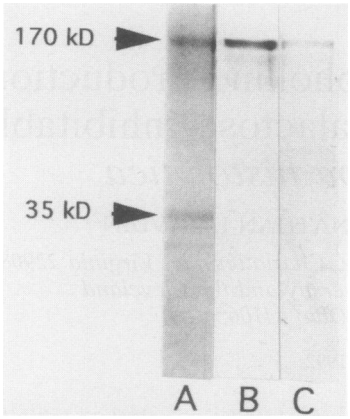

FIG. 1. Purity of the galactose-inhibitable adherence protein antigen preparation. Coomassie blue staining (lane A) of an SDS-PAGE gel containing 4 μg of adherence protein demonstrates single bands at 170 and 35 kDa, which are the mobilities of the heavy and light subunits, respectively (4). Immunoblotting with human immune sera at a 1:1,000 dilution (lane B) and four heavy-chain-specific anti-adherence protein mouse monoclonal antibodies (8C12, 1G7, 3F4, and 7F5, each at 0.04 mg/ml) (4, 7) (lane C) demonstrates that the only *E. histolytica* antigenic material present comigrates with the adherence protein heavy subunit.

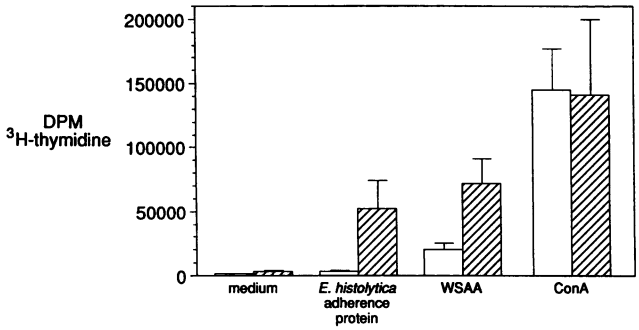

FIG. 2. Blastogenesis of PBMC from subjects with serum antibody to the *E. histolytica* adherence protein ($n = 6$) (▨) compared with that of PBMC from antibody-negative subjects ($n = 6$) (□). Blastogenesis was assayed with or without purified *E. histolytica* adherence protein (10 μg/ml), WSAA (100 μg/ml), or the mitogen concanavalin A (ConA) (20 μg/ml).

or immunoaffinity-purified adherence lectin (10 μg/ml) was utilized in culture media for a total volume of 0.2 ml per well. After 4 days of incubation at 37°C in 5% CO_2, plates were pulsed with [^{3}H]thymidine (thymidine-[*methyl*-^{3}H]; Dupont, NEN Products, Boston, Mass.) at 1 μCi per well and harvested 24 h later, and ^{3}H activity was determined with a scintillation counter (Beckman LS7500). The counts of [^{3}H]thymidine were expressed as disintegrations per minute (disintegrations per minute equal counts per minute corrected for machine efficiency). Preliminary dose-response studies demonstrated that 10 μg of adherence protein per ml resulted in an optimal lymphocyte proliferative response.

For the study of lymphokine production, PBMC (7.0×10^6 cells per well) from immune and control individuals were incubated in duplicate in a final volume of 2 ml for 24 h at 37°C with 5% CO_2 in the presence or the absence of WSAA (100 μg/ml), immunoaffinity-purified adherence lectin (10 μg/ml) or concanavalin A (20 μg/ml). After incubation, the supernatants were harvested and clarified by centrifugation at 10,000 × *g*, and interleukin 2 (IL-2) levels were measured in triplicate by a solid-phase immunoassay employing the multiple-antibody sandwich technique (Genzyme Corporation, Boston, Mass.). In order to quantitate IL-2 in test samples, known amounts of recombinant human IL-2 were assayed in parallel to generate a standard curve. Gamma interferon (IFN-γ) was measured in triplicate in PBMC supernatants by a commercially available radioimmunoassay (Centocor, Malvern, Pa.) with polystyrene beads coated with murine monoclonal antibody to human IFN-γ. Recombinant human IFN-γ was utilized to generate a standard curve.

PBMC amebicidal activity was assayed as described previously (13, 18). PBMC (7.0×10^6 cells per well in culture media) were incubated at 37°C in 5% CO_2 with or without adherence protein (10 μg/ml) for 5 days. PBMC (10^6 cells per ml) with or without trophozoites (10^4) were incubated at 37°C for 6 h in Medium 199 (GIBCO Laboratories) supplemented with cysteine, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), bovine serum albumin, 10% heat-inactivated pooled human AB type serum, and 1% antibiotics (Penicillin-Streptomycin). At the end of the incubation period, the pellet was suspended by gentle vortexing and the numbers of viable PBMC and amebae were determined by using trypan blue (0.4%) exclusion criteria.

Lymphocyte proliferation in response to the adherence protein was increased in five out of six antibody-positive subjects ($P < 0.01$). The mean lymphocyte proliferative response in the antibody-positive group was 53,081.1 ± 21,141.8 dpm, compared with a mean of 3,411.6 ± 1,439.0 dpm in controls ($P < 0.01$) (Fig. 2). When PBMC were incubated with WSAA, the mean proliferative response in lymphocytes from immune subjects was 73,114.6 ± 19,797.3 dpm, compared with 21,052.0 ± 5,414.6 cpm in those from controls ($P < 0.01$). The soluble amebic antigen preparation is known to be mitogenic for human lymphocytes (18). Equivalent mitogenic responses to concanavalin A (20 μg/ml) were observed in immune and control groups (Fig. 2).

Both IL-2 and IFN-γ were produced by lymphocytes from antibody-positive individuals in response to the adherence protein: 4.7 ± 3.2 U of IL-2 per ml compared with 1.6 ± 0.7 U of IL-2 per ml in the control group ($P < 0.05$) and 42.0 ± 22.6 U of IFN-γ per ml compared with 3.4 ± 2.7 U of IFN-γ per ml in controls ($P < 0.01$) (Fig. 3). Lymphokine production in response to concanavalin A (20 μg/ml) was equivalent in both groups (Fig. 3).

Amebicidal activity by PBMC from antibody-positive subjects was demonstrated only after 5 days of in vitro incubation with the purified adherence protein (10 μg/ml). Amebic viability was reduced to 51.6% of the control values following a 6-h incubation with adherence protein-stimulated PBMC ($P = 0.02$) (Table 1). PBMC incubated in medium alone for 5 days were not amebicidal (amebic viability, 88.7% ± 14.5%). PBMC from control subjects did not develop amebicidal activity, with or without 5 days of incubation with the adherence protein (Table 1).

The galactose-inhibitable adherence protein of *E. histolytica*, which is a highly conserved B-cell antigen (5, 8, 11), contains epitopes recognized by T cells obtained from subjects known to have had invasive amebiasis or presumed to have had it because of the presence of specific anti-adherence protein antibodies in sera (11). This single purified amebic antigen was sufficient to induce lymphocyte blastogenesis, IL-2 and IFN-γ production, and the development of

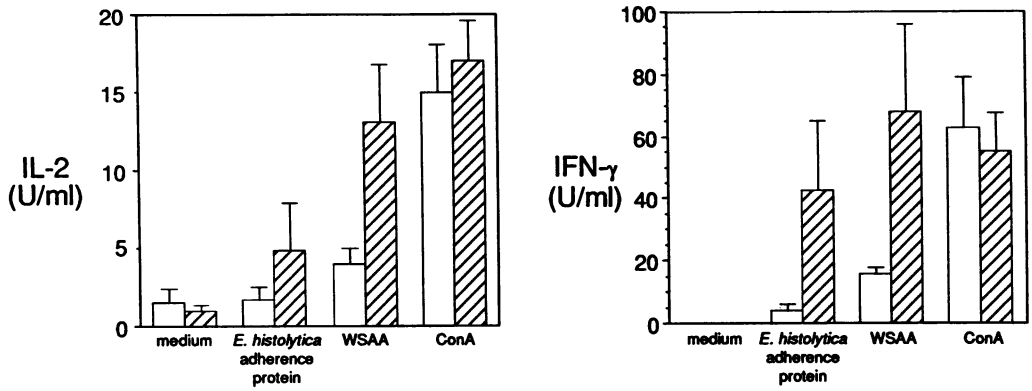

FIG. 3. Production of lymphokines (IL-2 and IFN-γ) by PBMC from antibody-positive subjects (n = 6) (▨) compared with that by PBMC from antibody-negative subjects (n = 6) (□) after 24 h of incubation (IL-2) and 48 h of incubation (INF-γ) in medium with or without purified *E. histolytica* adherence protein (10 μg/ml), WSAA (100 μg/ml), or concanavalin A (ConA) (20 μg/ml).

direct PBMC amebicidal activity. It is highly unlikely that the adherence protein preparation is contaminated with other significant amebic antigens, as indicated by immunoblotting with polyclonal immune sera and adherence protein-specific monoclonal antibodies. Lymphocyte responses to the purified antigen were comparable to those observed in this and previous studies with an optimal concentration of whole soluble amebic antigen (13, 16, 17).

Axenic *E. histolytica* trophozoites have the ability to kill human neutrophils, monocytes, lymphocytes, and monocyte-derived macrophages without any effect on parasite viability (2, 13, 17). In response to whole soluble amebic antigen, PBMC from hosts cured of invasive amebiasis produce lymphokines which activate monocyte-derived macrophages, enabling them to kill axenic trophozoites (17). Supernatants from PBMC stimulated with whole amebic antigen were demonstrated previously to contain high levels of IFN-γ (14). In addition, either purified native or recombinant IFN-γ is sufficient to activate human macrophage amebicidal activity (14). Excess anti-IFN-γ antibody partially inhibits the activation of macrophage amebicidal activity (16), suggesting that lymphokines other than IFN-γ are also operative. Therefore, the ability of the purified adherence protein to elicit production of IFN-γ by human lymphocytes is a critical finding for this potentially protective antigen.

Lymphocytes obtained from patients cured of amebic liver abscesses are directly amebicidal following in vitro incubation with whole soluble parasite antigen (18). Lymphocyte amebicidal activity is dependent upon direct contact and mediated by the OKT8-positive subset of cells (13, 18). It is unclear what mechanisms are used by amebic antigen-stimulated lymphocytes to recognize and bind to *E. histolytica* trophozoites. That the purified adherence protein is capable of inducing lymphocyte amebicidal activity suggests that it contains epitopes which induce clonal expression of a cytotoxic lymphocyte population. However, these studies do not address the possibility of genetic restriction in the T-cell recognition of the *E. histolytica* adherence protein, nor do they establish the protein to be an immunodominant T-cell antigen. However, Salata et al. reported that four of seven T-cell lines resulting from stimulation with whole soluble antigen recognized the purified adherence protein (19a). In an experimental model of amebic liver abscess, the vaccine efficacy of the adherence protein bore no relation to serum anti-adherence protein antibodies; importantly, immunized gerbils did develop amebicidal cell-mediated immune responses (19b).

During acute invasive amebiasis, there is evidence of antigen-specific immunosuppression of cell-mediated immune responses. Sera from these patients suppresses the T-cell response to whole soluble amebic antigen (14); an identical phenomenon has been noted in the gerbil model of amebic liver abscess (20). In addition, a subset of gerbils

TABLE 1. Amebicidal activity of PBMC following in vitro incubation with or without purified *E. histolytica* adherence protein

Presence of serum antibody to adherence protein (no. of subjects)	Incubation conditions for PBMC[a]	Viability[b] (no. of expts)	
		PBMC	Amebae
Yes (3)	Culture medium	59.4 ± 11.8[c] (14)	88.7 ± 14.5 (14)
	Culture medium with *E. histolytica* adherence protein	68.2 ± 9.8[c] (15)	51.6 ± 6.6[d] (15)
No (3)	Culture medium	38.8 ± 3.2[c] (18)	93.2 ± 6.0 (18)
	Culture medium with *E. histolytica* adherence protein	50.7 ± 3.1[c] (18)	94.9 ± 5.8 (18)

[a] PBMC were incubated for 5 days. The adherence protein concentration was 10 μg/ml.

[b] Mean ± standard error of the mean, expressed as a percentage of the viability of control PBMC or amebae incubated individually for an identical time period. PBMC incubated alone had a viability of 89.2%; amebae incubated alone had a viability of 94.1%. Viability was determined after a 6-h incubation of PBMC with amebae at a ratio of 100:1.

[c] $P \leq 0.004$ compared with the value for PBMC without amebae present.

[d] $P = 0.02$ compared with the value for amebic viability with PBMC incubated for 5 days in medium alone.

immunized with the galactose-inhibitable adherence protein developed liver abscesses that were larger than those found in control gerbils (6), suggesting that this protein may contain immunosuppressive epitopes. The recent cloning and sequencing of the heavy subunit of the adherence protein (3, 22) provides a new opportunity to define critical protection and suppressive T-cell epitopes. This is a crucial consideration in determining which portions of this molecule would be most effective in a subunit amebiasis vaccine.

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