FULL PAPER *Immunology*

Protective Effect of Humus Extract Against *Trypanosoma brucei* Infection in Mice

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ABSTRACT. Humic substances are formed during the decomposition of organic matter in humus, and are found in many natural environments in which organic materials and microorganisms are present. Oral administration of humus extract to mice successfully induced effective protection against experimental challenge by the two subspecies, *Trypanosoma brucei brucei* and *T. brucei gambiense*. Mortality was most reduced among mice who received a 3% humus extract for 21 days in drinking water *ad libitum*. Spleen cells from humus-administered mice exhibited significant non-specific cytotoxic activity against L1210 mouse leukemia target cells. Also, spleen cells produced significantly higher amounts of Interferon-γ when stimulated *in vitro* with Concanavalin A than cells from normal controls. These results clearly show that administration to mice of humus extract induced effective resistance against *Trypanosoma* infection. Enhancement of the innate immune system may be involved in host defense against trypanosomiasis.

KEY WORDS: humic substance, Interferon-γ, lymphocyte activation, protection, *Trypanosoma brucei*.

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Trypanosoma brucei is a causative agent of Nagana in cattle and African sleeping sickness in human. It is a bloodstream hemoflagellate protozoan parasite which is transmitted by the tsetse fly [14], and is distributed in sub-Saharan Africa. Affected animals suffer weakness, severe anemia and cachexia, resulting death [4, 19]. In spite of many years of effort, efficient prophylactic vaccines have not been developed due to the antigenic variation of trypanosomes. They change their variant surface glycoprotein to evade the immune system of the host [2–4, 19]. Furthermore, despite mass surveillance campaigns, the vectors have not been fully eliminated, and acquisition of resistance to insecticides is a concern [14]. The drugs used for treating African trypanosomiasis also have restrictions due to their associated host toxicity. It is therefore worthwhile to seek new natural and synthetic compounds that can effectively induce host resistance against trypanosomes.

Humic substances are formed during the decomposition of organic matter in humus. They can be found in many natural environments in which organic materials and microorganisms are or have been present [24]. Their chemical composition, structure and functional groups can vary according to their origin and age and the conditions of the humification process (humidity, aeration, temperature, mineral microenvironment, etc.). Natural humification products, such as humus, peat, sapropel and mumie, have been used to develop pharmacologic agents having diverse applications in medical practice [23, 25]. These agents have been successfully used as anti-inflammatory agents, since they have local anti-inflammatory and analgesic properties [7, 16, 21]. Use of coal-derived humic acid and fulvic acid as antimicrobials has also been investigated [20], and anti-HIV activity of oxihumate [22] and synthetic humic acid analogues [18] has been reported. The potential of humic substances in the treatment of animal diseases has scarcely been investigated, however. Accordingly, the present study was conducted to study the protective effect of humus extract against *T. brucei* infection in mice. Non-specific activation of the host immune system was also investigated to determine the possible mechanism of protection.

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MATERIALS AND METHODS

Humus extract: Light brownish diatomaceous earth (humus) was collected under ground at 5 to 10 m depth in Aino-machi, Nagasaki Prefecture, Kyushu, Japan. Humus extract was prepared from humus using water [8]. To the humus was added 6 volumes of dechlorinated water (v/w); the mixture was agitated every day for 30 days, and then left to stand at 25 to 28°C for 4 months. The resulting supernatant was collected and filtered using a membrane filter (pore size: 25 μm). The resulting humus extract has pH 2.8, and contains various minerals including Al, Ca, Mg, Na and Si. The extract contained 1,500 ppm of sulfate. No culturable bacteria were found in this extract. There were small amounts of protein and carbohydrate (0.7% of the total weight).

Treatment of mice with humus extract: Eight-week-old inbred BALB/c mice of both sexes were divided into groups and administered with 1–6% of humus extract dissolved in dechlorinated tap water *ad libitum* for differing intervals. No unusual drinking behavior was observed in mice administered high concentration of humus extract (up to 10%). Control mice were received water without humus extract.

Challenge with T. brucei: We used the virulent *T. brucei brucei* strain ILtat 1.4 and *T. brucei gambiense* strain Wellcome, donated by Dr. Y. Ohnishi of the Laboratory of

International Prevention of Epidemics, Course of Veterinary Science, Graduate School of Biological and Environmental Sciences, Osaka Prefecture University. The *T. brucei* stock was passed twice in mice by intraperitoneal inoculation of trypanosome-containing peripheral blood prior to the experiment, to maintain the virulence. For the challenge, each mouse was inoculated with 10^1/0.1 m*l* of the bloodstream form of *T. brucei* intraperitoneally. In the first experiment, mice receiving 3% humus extract for 21 days in drinking water were challenged with *T. brucei gambiense*. In the second experiment, challenge tests were performed using *T. brucei brucei* in order to study the relation between the protective effect of humus extract and the duration of administration (0, 5, 10 or 21 days). Challenge tests were also performed (third experiment) using *T. brucei brucei* to study the relation between the protective effect of humus extract and the concentration (1, 2, 3, 4 or 6% administered for 21 days before challenge). Humus extract was administered for 20 consecutive days after trypanosome inoculation. All mice were observed for 20 days to determine their survival. The experiment was performed according to the guidelines of the Osaka Prefecture University Committee in Animal Care and Use.

Spleen cell cytotoxicity test: Spleen cells were separated and used for cytotoxicity assay. The spleens were cut into small pieces in Hanks Balanced Salt Solution (HBSS, pH 7.4; Nissui Pharmaceutical Co., Tokyo, Japan) containing antibiotics. Single cell suspensions were then prepared by passing the cells through stainless steel meshes. After centrifuging at 400 × g for 5 min at 4°C, the resulting cell pellets were mixed in 0.75% ammonium chloride dissolved in 0.15 M Tris-HCl buffer (pH 7.6) in order to lyse the red blood cells. After treatment with the lysis buffer for 60 sec, the cells were washed twice with HBSS. Cell viability, as determined by the trypan blue dye exclusion test, was greater than 90%.

Nonspecific cytotoxic activity of spleen cells against L1210 mouse lymphocytic leukemia line cells was examined using a commercial kit (Cytotox 96 Non-Radioactive Assay, Promega, Madison, Wis., U.S.A.). L1210 target cells were cultivated in RPMI 1640 (pH 7.4; Nissui Pharmaceutical Co.) containing 10% fetal bovine serum and antibiotics at 37°C. They were then suspended in RPMI 1640 at a concentration of 5×10^5 cells/m*l*. Each aliquot of effector cells (0.1 m*l*) and target cells (0.1 m*l*) was placed in 96-well U-bottomed microplates (Iwaki, Tokyo, Japan) at effector cell-to-target cell ratios (E:T) of 20, 10, 5 and 2.5, in quadruplicate. The plates were incubated at 37× for 4 hr. The RPMI 1640 was also added to control target cells for measurement of the release of lactate dehydrogenase (LD). To determine the maximum LD release, control cultures were treated with 10 μl of 10x lysis solution in accordance with the manufacturer's protocol. After centrifuging of the microplates (300 × g for 5 min), 50 μl of supernatant was transferred to 96-well flat-bottomed microplates (Iwaki). At 30 min after addition of 50 μl of substrate solution to each well, the reaction was stopped by adding 50 μl of stop solution. The optical density (OD) of each well was measured using a microplate reader (Model 680, Bio Lad., Tokyo, Japan) at a wavelength of 492 nm. The specific cytotoxicity percentage was then calculated using the following formula:

Specific cytotoxicity percentage =

$$\frac{\text{OD test sample} - \text{OD effector control} - \text{OD target control}}{\text{OD maximum release} - \text{OD target control}} \times 100$$

Cytokine production: Spleen cells suspended in RPMI 1640 were cultivated in 24-well plastic plates (5×10^5 cells/0.5 m*l* in each well, Iwaki) at 37°C for 3 days in the presence of various concentrations of Concanavalin A (Con A; Elastin Products Co., Owensville, Mo, U.S.A.), or without Con A. The concentrations of interferon (IFN)-γ, IL-12 and IL-6 in culture supernatant fluids were measured using a commercial ELISA kits (Biosource Immunoassay, BioSource International, Camarillo, CA, U.S.A.).

RESULTS

Protective effect of humus extract administration against T. brucei challenge: In the first experiment, mice receiving 3% humus extract for 21 days in drinking water were challenged with *T. brucei gambiense*. All control mice died within 7 days of the challenge (see Fig. 1A), whereas 67% of humus-treated mice survived ($P<0.01$ compared to control mice by χ^2 test, n=6 in each group). In the second experiment, challenge tests were performed using *T. brucei brucei* to study the relation between the protective effect of humus extract and the duration of administration (0 to 21 days). Figure 1B shows that administration of humus extract to mice also induced effective protection. Of the 12 untreated mice comprising the control group, all died within 10 days of the challenge. In contrast, the survival rates of mice receiving 3% humus extract for 10 days or 21 days before challenge were respectively 40% ($P<0.05$, n=5) and 52% ($P<0.01$, n=21) at 20 days after challenge. However, none of the mice who received the extract for 5 days before (n=5) or on the same day as the challenge (n=6) survived (not significant). In the third experiment, challenge tests were performed using *T. brucei brucei* to study the relation between the protective effect of humus extract and the concentration (0 to 6%) of the extract. Figure 1C shows that the protective effect depends on the concentration of humus extract in water; the greatest protection was conferred on mice that received 3 % extract for 21 days ($P<0.01$, n=21), and the survival rate of mice receiving 4 % extract was also significantly higher ($P<0.05$, n=11) than in control mice (n=13).

Cytotoxic activity of spleen cells: We measured the nonspecific cytotoxic activities of spleen cells from humus-treated mice against L1210 target cells (Fig. 2). The spleen cells exhibited significantly higher cytotoxicity at E:T ratios of 20 ($P<0.05$) and 10 ($P<0.05$) than untreated controls.

Cytokine production by spleen cells: Spleen cells from

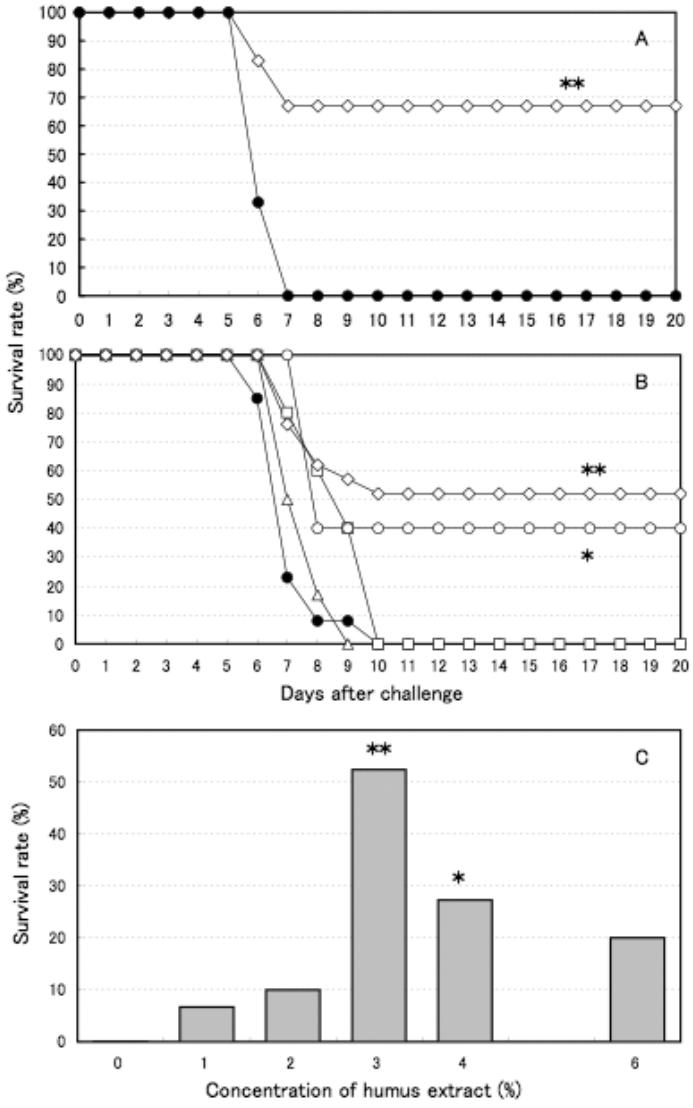

Fig. 1. Survival of mice challenged with two subspecies of *T. brucei.* (A) Humus extract was administered for 21 days orally in drinking water (◇; 3%, n=6) or water containing no extract (●, n=6) prior to challenge with *T. brucei gambiense*. (B) An experiment was repeated in which 3% humus extract was administered for 21 (◇, n=21), 10 (○, n=5), 5 (□, n=5) or 0 (△, n=6) days prior to challenge with *T. brucei brucei*. Control mice (●, n=12) received no humus extract during the experimental period. (C) Survival of mice was also examined by administration of 1% (n=15), 2% (n=10), 3% (n=21), 4% (n=11), 6% (n=15) or without humus extract (n=13) for 21 days prior to challenge with *T. brucei brucei*. In these experiments, all mice were observed for 20 days to determine their survival. The survival rate in these tests was compared statistically (**; $P<0.01$, *; $P<0.05$ estimated by χ^2 test) to that of control mice.

mice that received 3% humus extract for 21 days were stimulated by Con A. Figure 3 shows the concentrations of IFN-γ, IL-12 and IL-6 in the culture supernatant. Production of IFN-γ was dependent on the dose of Con A added to the culture (Fig. 3A). Spleen cells produced significantly higher amount of IFN-γ when the mice were pre-treated with 3% humus extract ($P<0.05$ at Con A concentration of 5 and 2.5 μg/m*l* compared to non-treated mice). IL-12 and IL-6 were also detected in culture fluids after stimulation of the spleen cells with Con A (Figs. 3B and 3C); no significant differ-

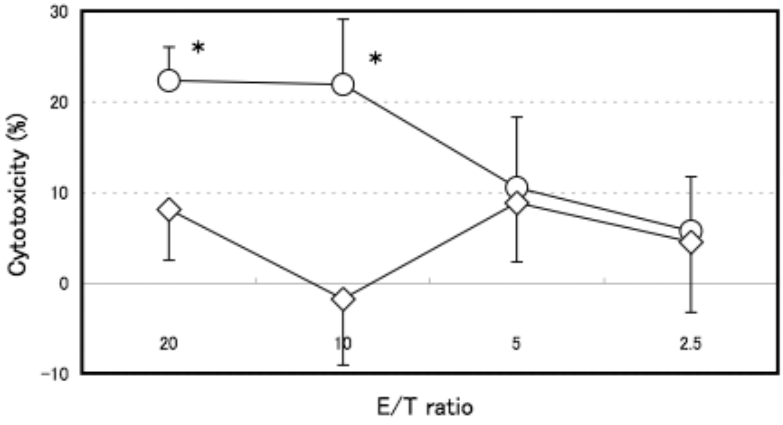

Fig. 2. Non-specific cytotoxic activities of spleen cells from humus-treated mice (3% for 21 days) against L1210 target cells. Tests were repeated four times (mean % ± SE) at E:T ratios of 20, 10, 5 and 2.5. Cytotoxic activity was significantly higher in humus-treated mice than in untreated controls (*; $P<0.05$ by Student's *t*-test).

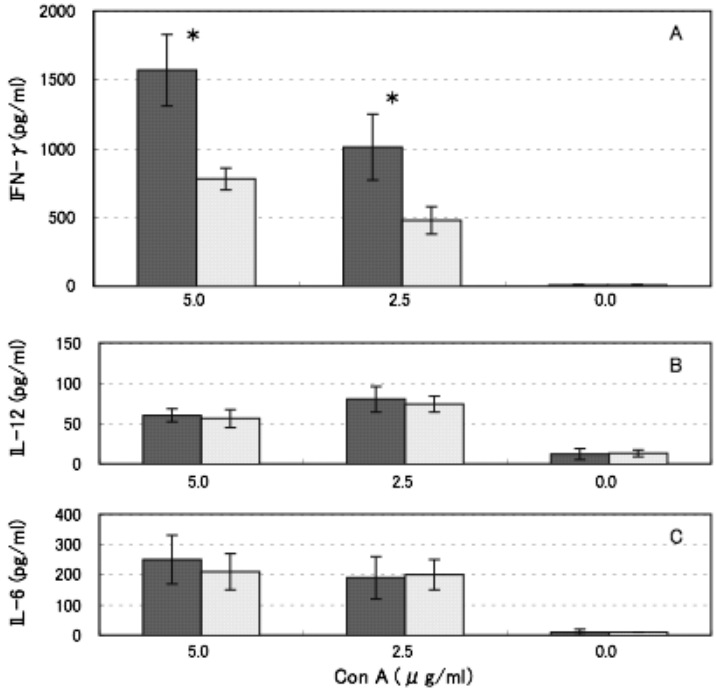

Fig. 3. Cytokine production by spleen cells from mice that received 3% humus extract for 21 days. Cells were stimulated *in vitro* with 5.0 or 2.5 μg/m*l* or without Con A. The concentration of IFN-γ (A), IL-12 (B) and IL-6 (C) in culture supernatant was measured by ELISA, and compared statistically in each group between humus-treated mice (■) and untreated mice (□) (*; $P<0.05$ by Student's *t*-test). Results are shown as mean concentration ± SE from 6 mice.

ences were observed in the concentration of these interleukins between the humus-treated mouse group and the untreated group.

DISCUSSION

Our results clearly show that administration of humus extract protects mice against experimental challenge with both subspecies of *T. brucei*, as shown by reduced mortality

(Fig. 1). After challenge with *T. brucei gambiense* and *T. brucei brucei*, 67% and 52% of mice respectively survived when pre-treated with 3% humus extract for 21 days. The effect of the extract was clear, since untreated control mice all died within 10 days of the challenge. The results shown in Fig. 1C indicate that there exists optimal condition for administration of the extract to mice. Therefore, it is needed to determine optimal concentration and duration of administration of the extract, since we observed similar phenomenon in experimental infections in ulcer disease (caused by *Aeromonas salmonicida*) of fish [9] and anti-tumor effect in mice [8].

The mechanism by which humus extract protects against *T. brucei* infection is not clear, but it is likely that innate immune responses confer protection. To investigate the mechanism of protection, we tested the enhancing effect of humus extract on non-specific cell cytotoxicity against mouse leukemia target cells and cytokine production by spleen cells from mice that received humus extract. Schepetkin *et al.* [17] reported that mumie, which is a semihard black resin formed by long-term humification, enhanced [^{3}H]thymidine uptake by splenic lymphocytes and increased the production of reactive oxygen species and nitric oxide in mouse peritoneal macrophages. Another report found that a peat-based preparation enhanced the proliferative capability of murine thymocytes stimulated by mitogens, and prevented the immuno-suppressive effect of hydrocortisone [13]. These observations indicate that humic substances enhance host immune responses nonspecifically.

Consistent with these reports, the present results demonstrated the induction of cytotoxic activity in spleen cells after administration of humus extract to mice. The cytotoxicity depends on the E:T ratio, and activity was significantly higher than in control mice (Fig. 2). Our preliminary data showed that cytotoxicity was observed in a lymphocyte-rich cell population when spleen cells were separated into an NK cell population and other cells containing lymphocytes and macrophages, using antibody-labeled magnetic beads (unpublished). It is important to clarify the cytotoxic activity of separated cell populations against *Trypanosoma* after treatment of mice with humus extract.

Early incidents in host-parasite interactions are very important in directing the pattern of immune responses. At the time of infection, the host responds with B cell activation, which is important for clearance of trypanosomes from vascular regions. The initial host response toward *T. brucei* is also characterized by the early release of inflammatory mediator associated with a type 1 immune response (namely IFN-γ), which is dependent on activation of the innate immune system [5]. The present results reveal the significant enhancement of Con A-stimulated IFN-γ production in spleen cells of mice that received humus extract (Fig. 3), indicating enhancement of the IFN-γ-dependent immune response. Resistance to *T. brucei* is correlated with the ability of infected animals to produce IFN-γ at an early phase of infection, since Th1 cell responses, such as T lymphocytes or NK cell activation, is associated with IFN-γ production in *Trypanosoma* infection [1, 6, 12].

In contrast to the enhancing effect of Con A on IFN-γ production by spleen cells, no obvious stimulation was observed for IL-12 and IL-6 (Fig. 3). IL-12 is involved in the initiation of immune response and the switching of the Th2 response to Th1 type [10], since IL-12 is a strong stimulator of IFN-γ production during infections. On the other hand, IL-12-independent IFN-γ can be produced, also resulting in resistance against *T. brucei* [1] and *Trypanosoma cruzi* infection [11]. Rapid IFN-γ production might be critical in resistance against *Trypanosoma* infection.

We have previously reported the *in vivo* anti-tumor effect of humus extract on transplantable L1210 leukemia in mice [8], and induction of non-specific resistance against ulcer disease in carp [9]. The detailed mechanisms and substances (major plant species and its degenerates, bacterial components such as cell wall components or lipopolysaccharides etc. existed in humus) involved in anti-tumor and anti-microbial response following humus treatment remain unclear, therefore further studies are required to determine how innate immune responses are induced in these animals by humus extract.

No measurable side effects have been observed with humic substances [15, 21], since these are mostly carboxylic acids and ordinary physiological metabolites. Humic substances and/or humus extract can therefore be used in animal feed as immunopotentiating agents. Analysis for the separation of biologically active components in humus is now in progress.

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