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In vitro synthesized immunoglobulin A from nu/+ and reconstituted nu/nu mice against a dominant surface antigen of *Giardia lamblia*

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Abstract. Nu/+ mice (ZU.ICR-strain) experimentally infected with *Giardia lamblia* (clone GS/M-83-H7) cleared the infection by day 45 postinfection (p.i.). Athymic nu/nu mice were reconstituted with immune Peyer's patch lymphocytes obtained from self-healed nu/+ littermates and thus acquired the potential to decrease their intestinal parasite mass. Intestinal B-cells from self-healed nu/+ mice as well as from immune-reconstituted athymic nude mice synthesized in vitro parasite-specific immunoglobulin A (IgA). This IgA was subsequently analyzed by immunoblotting, showing a predominant reaction with the major surface antigen (a 72000-Da polypeptide) characterizing the *Giardia* clone in question. The hypothesis on the causative role of intestinal IgA and immune lymphocytes in the control of *G. lamblia* infection thus deserves further attention.

Giardia lamblia is a protozoan parasite that resides in the small intestine of humans and other mammals. Disease manifestations vary from asymptomatic carriage to severe diarrhea and malabsorption. Although the natural history of *Giardia* infections is not well described, it is known that in some persons a self-cure occurs, suggesting the development of immunity or resistance, whereas others develop long-standing chronic infections. Recent studies of *G. lamblia* suggest that the host-parasite interactions are much more complex than previously believed. A number of studies have shown that the surface antigen profiles of *Giardia* are variable (Nash et al. 1983; Nash and Keister 1985; Ungar and Nash 1987) with respect to different isolates and that surface antigenic variation may occur within an isolate (Nash and Aggarwal 1986; Adam et al. 1988; Aggarwal and Nash 1988; Nash et al. 1988; Aggarwal et al. 1989). Although incompletely understood, this phenomenon is likely to play a role in facilitating chronic as well as repeated infections in humans (Gottstein et al. 1991). A complete understanding of the immunopathophysiology of infection and disease requires a model in which both the parasite and the host can be studied.

In model *G. muris* infections it has been shown that parasite-specific intestinal secretory immunoglobulin A (IgA) antibodies may be essential for influencing the course of infection (Anders et al. 1982; Snider and Underdown 1986; Sharma and Mayrhofer 1988; Heyworth et al. 1987, 1988; Heyworth 1989). In contrast, relatively little is known about peripheral and local immunological mechanisms involved in infections with *G. lamblia*. To study these problems we have recently infected neonatal mice (CR:NIH:S) with a cloned human isolate of *G. lamblia* (GS/M-83-H7; Gottstein et al. 1990). Follow-up studies analyzing the major surface antigen of the intestinal trophozoites [defined by a monoclonal antibody (MAb), G10/4] and, in parallel, the cellular and humoral immune responses showed that (a) infections in mice peaked at 2–3 weeks after inoculation and were self-cured by day 42 postinfection (p.i.), (b) the proportion of trophozoites expressing the 72000-Da surface antigen of the initial inoculum had decreased by day 12 and approached zero by day 22 p.i., and (c) the hosts responded with a marked parasite-specific immune response (serum antibodies against the original 72000-Da surface antigen and others) and a lymphoproliferative response to *G. lamblia* antigens of T-lymphocytes [predominantly LY4(CD4)$^+$] isolated from Peyer's patches at 12 days p.i. and later. Further investigations with SCID mice and athymic nude BALB/c and respective immunocompetent control mice have subsequently revealed that a T-cell function determines self-healing, whereas the mechanism involved in inducing "parasite antigen variation" (i.e., expulsion of the initial population of the *Giardia* clone with the antigenic surface variant defined by mAb G10/4, subsequently replaced by new variants) appears to be independent of T-cell interactions (Gottstein and Nash 1991). The present study was designed to investigate in more detail the local immune components that may interact with the intestinal *Giardia* population with respect to its major surface anti-

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gen. We showed that athymic nude mice reconstituted with Peyer's patch lymphocytes from self-healed nu/+ littermates acquired the potential to synthesize intestinal IgA against the major surface *Giardia* antigen and that as a result of reconstitution the intestinal *Giardia* population was markedly reduced as compared with that in nonreconstituted control animals.

Materials and methods

Experimental design

The aim of the present study was to demonstrate the potential of intestinal murine immune cells to synthesize IgA against a single dominant surface antigen of *Giardia lamblia* and to provide some information on the role of parasite-specific immune lymphocytes and respective IgA in controlling infection. The experimental approach included the following:

1. Infection of nu/+ mice with *G. lamblia* to demonstrate an "antigen switch" and a self-healing course of infection. These animals were used to provide intestinal immune lymphocytes for the following experiments.
2. In vitro synthesis by these immune lymphocytes of IgA directed against a dominant surface antigen of the *Giardia* clone in question; antigen specificity was assessed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting.
3. Reconstitution of nu/nu mice littermates with nu/+ immune cells and simultaneous infection of reconstituted mice with *G. lamblia*; demonstration of an "antigen switch," a parasite-reducing course of infection, and the synthesis in vitro of anti-*Giardia* IgA.

Animals

The 3-day-old nu/nu and nu/+ mice and natural mothers used in this study were obtained as litters from the Institut für Labortierkunde, University of Zürich. The specific pathogen-free animals were of the outbred ZU.ICR strain.

Parasite and experimental infections

Five resistant (self-healing course of disease) ZU.ICR nu/+ mice and five susceptible (chronic course of disease) ZU.ICR nu/nu mice (Gottstein and Nash 1991) were infected by intragastric injection of 50000 *G. lamblia* trophozoites (clone GS/M-83-H7) as described previously (Gottstein et al. 1990). Five respective control animals were injected with the same volume of phosphate-buffered saline (PBS) containing 10% TYI-S-33 medium. Animals were killed sequentially at different times (days 31 or 45 p.i., respectively). The kinetics of expression of the major surface antigen was assessed by the use of mAb G10/4 as described elsewhere (Gottstein et al. 1990). The origin, axenization, cultivation, and cloning of the *G. lamblia* GS/M-83-H7 clone (human origin) has been described by Aggarwal et al. (1989). This clone expresses a major 72-kDa antigen on its surface, which is recognized by mAb G10/4. *G. lamblia* was cultivated in TYI-S-33 medium with antibiotics as previously described (Gottstein et al. 1990).

Collection of biological samples

Animals and organs. Lymphocyte donor nu/+ mice (*Giardia*-infected mice with subsequent "self-healing" and noninfected control mice) were killed on day 45 p.i. by CO_2 euthanasia. The small intestines were perfused with 10 ml sterile PBS for removal of the intestinal contents. Peyer's patches were dissected free aseptically and placed in RPMI 1640 medium on ice prior to further processing. Nu/nu mice (*Giardia*-infected mice with subsequent chronic persistence of infection and noninfected control mice) were killed on day 31 p.i. by CO_2 euthanasia. The small intestines were perfused with 10 ml sterile PBS, opened longitudinally with scissors, subsequently rinsed with sterile PBS for removal of the intestinal contents, and placed in TCM (see below) in ice prior to further processing to obtain IgA-synthesizing B-cells.

IgA. Intestinal cell conglomerates from reconstituted nu/nu and nonreconstituted control mice were prepared from intestinal epithelium and lamina propria by slicing off the underlying tissue with a scalpel blade and subsequently macerating it into small pieces. This mucosal material was incubated (4 wells/mouse) in 24-well tissue-culture plates (Nunc, Roslilde, Denmark) in 1 ml TCM (see below) in a humified incubator (5% CO_2 and 95% air at 37° C). After 24 h cultivation the whole cultures were frozen (−20° C) and thawed (water bath, 37° C) three times to disrupt the cells and the whole lysate was subsequently kept frozen at −20° C until testing. For antibody-binding assays (ELISA and immunoblotting), the material was thawed and diluted 1:2 (v/v) with PBS-Tween 20 (see below).

G. lamblia. Trophozoites were recovered by incubation of opened small intestines in ice-cold PBS (resulting in the detachment of trophozoites). The recovered *G. lamblia* trophozoites were semiquantitatively assessed for their number and directly used for immunofluorescent antibody test (IFAT) analysis as described previously (Gottstein et al. 1990).

Host immune-cell suspension. Peyer's patches from self-healed nu/+ mice were rinsed three times with ice-cold RPMI 1640 prior to isolation of single-cell suspensions. Peyer's patches were dissociated into single-cell suspensions by homogenization in Tenbroek's homogenizer. After being washed, the cells were resuspended at appropriate concentrations in tissue-culture medium [TCM:RPMI 1640 containing 10% fetal calf serum (FCS), 12 m*M* HEPES buffer, and 50 μ*M* β-mercaptoethanol and supplemented with L-glutamine (2 m*M*) and penicillin, streptomycin, fungizone (100 units/ml, 100 μg/ml, and 2.5 μg/ml, respectively)] prior to their inoculation into recipient nu/nu mice.

Reconstitution of athymic nu/nu mice

Two athymic nude mice were reconstituted prior to *Giardia* infection with 5×10^6 lymphocytes obtained on day 45 p.i. from the Peyer's patches of (self-healed) nu/+ mice. Cell suspensions were injected intraperitoneally on the same day on which the *G. lamblia* infection was initiated. Nonreconstituted control animals received the same volume of TCM only.

Antibody-detection assays

Immunofluorescent antibody tests. G. lamblia trophozoites recovered from mouse small intestines were washed three times with sterile ice-cold PBS, resuspended in TYI-S-33 medium, and incubated for 1 h at +37° C (gas phase with 7.5% CO_2) in Nunc flat-bottom microplates. Nonadherent cells and culture medium were removed by aspiration and the adherent trophozoites were fixed for 2 min with 2-propanol and air-dried. Fluorescein isothiocyanate (FITC)-labeled mAb G10/4-FITC and irrelevant control mAb 6E7-FITC were diluted 1:40 (v/v) with PBS containing 0.3% Tween 20, 10% TYI-S-33 medium, and 0.5% skim milk (Carnation natural nonfat dry milk) as described previously (Gottstein et al. 1991). Antibody incubation was carried out a +37° C for 1 h. The reaction was read using an inverted Olympus BH-2 immunofluorescence microscope.

Enzyme-linked immunosorbent assay. *G. lamblia* trophozoites were prepared in microplates using the same procedure described for the IFAT. We also used the same buffer solution described above to dilute TCM lysates (1:5, v/v) and conjugate (1:500, v/v) [anti-mouse IgA (α-chain-specific) conjugated to alkaline phosphatase, The Binding Site Ltd.]. Otherwise, we basically followed the procedure described earlier (Gottstein et al. 1984).

Western blotting. Resolution of *G. lamblia* proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and their electrophoretic transfer to Immobilon were performed as previously described (Gottstein et al. 1990) using precast 4%–20% gradient minigels (BioRad Co.). TCM lysates and conjugate [anti-mouse IgA (α-chain-specific) conjugated to alkaline phosphatase; the same as that described for ELISA] were diluted 1:5 and 1:500 (v/v), respectively, in the same buffer used for IFAT.

Antigens

Soluble *G. lamblia* trophozoite antigens for use in SDS-PAGE were obtained by pelleting 7×10^8 *G. lamblia* trophozoites (previously washed with sterile PBS) and resuspending them in 5 ml PBS. Subsequent freezing (lN_2), thawing (water bath, 37° C), and sonication (1 min, 20 W) provided an antigen extract after the removal of cell debris by sedimentation (10 min; 14000 *g*).

Results

Experimental infections and parasites

The course of the *Giardia lamblia* GS/M-83-H7 infection in athymic nude mice (reconstituted and nonreconstituted) and in their nu/+ littermates is shown in Table 1. The proportion of intestinal trophozoites expressing the major surface epitope binding mAb G10/4 was not explicitly listed, as in all experimental steps an antigen switch had occurred (see below). Parallel in vitro control cultures of *Giardia* trophozoites demonstrated the maintenance of the initial antigen variant over the experimental period (45 days).

All nu/+ animals had either cleared their infection by day 45 p.i. or decreased the parasite burden to very few trophozoites that were detectable only after an extensive microscopic search. Nonreconstituted nu/nu mice remained heavily infected on the last respective experimental day (day 31 p.i.), whereas mice reconstituted with lymphocytes from *Giardia*-infected and subsequently self-healed nu/+ mice showed a marked decrease in their parasite burden in that very few trophozoites were detectable only after an extensive microscopic search.

The analysis of *G. lamblia* trophozoites for the presence of the major surface epitope (72000-Da polypeptide) was assessed by IFAT and FITC-labeled mAb G10/4. A complete switch from mAb G10/4-positive to -negative trophozoites was in all experimental steps presented (*Giardia* recovered on day 14 p.i. or later).

IgA synthesis as assessed by ELISA

TCM lysates from lymphocytes cultured in vitro were assessed by ELISA for the presence of parasite-specific IgA; the respective results are shown in Table 1. All experimentally infected nu/+ animals had primed B-cells in that these were subsequently capable of synthesizing parasite-specific IgA in vitro, in contrast to nonreconstituted nu/nu mice. The two mice reconstituted with lymphocytes from *Giardia*-infected and subsequently self-healed nu/+ mice exhibited *Giardia*-specific IgA-binding activity in ELISA. Controls, including noninfected nu/+ and infected but nonreconstituted nu/nu mice, were negative, i.e., they showed no antibody-binding activity in the parasite-specific IgA ELISA.

IgA synthesis as assessed by immunoblotting

Immunoblotting was used to assess the *Giardia* molecules that were responsible for IgA-binding activity in the experiments described above. The migration site of the 72000-Da major surface antigen characterizing our *G. lamblia* clone (GS/M-83-H7) was visualized with an mAb G10/4-alkaline phosphatase conjugate. Under the present experimental conditions, parasite-specific intesti-

Table 1. Analysis of *Giardia lamblia* trophozoites recovered from experimentally infected neonatal mice and demonstration of a respective humoral IgA response by ELISA using in vitro cultivated intestinal lymphocyte lysates

Number of mice	Strain	Experimentally infected/ reconstituted	*Giardia* recovered after necropsy[a]	IgA detected in cultured intestinal lymphocyte lysates by ELISA, mean (range)[b]
5	Nu/nu	No/no	− (5×)	0.04 (0.01–0.05)
5	Nu/nu	Yes/no	+++ (5×)	0.05 (0.01–0.07)
5	Nu/+	No/no	− (5×)	0.04 (0.02–0.05)
3	Nu/+	Yes/no	(+) (3×)	**0.12 (0.11–0.13)**
2	Nu/+	Yes/no	− (2×)	**0.26 (0.12–0.41)**
2	Nu/nu	Yes/yes	(+) (2×)	**0.13 (0.12–0.13)**

[a] *Giardia* recovered on day 31 p.i. All trophozoites had demonstrated a change in their surface antigen variant as shown by a lack of binding capacity with mAb G10/4; the intensity of infection was estimated by microscopic analysis of the fluid obtained after perfusion and of the small intestines and was arbitrarily and semiquantitatively classified as follows: +++, heavy parasite burden; (+), a few trophozoites detectable only in the whole intestine.

[b] Data in boldface indicate *positive* results based upon a threshold of $A_{404\ nm} = 0.10$ as determined by the mean value + 3 SD for the noninfected controls

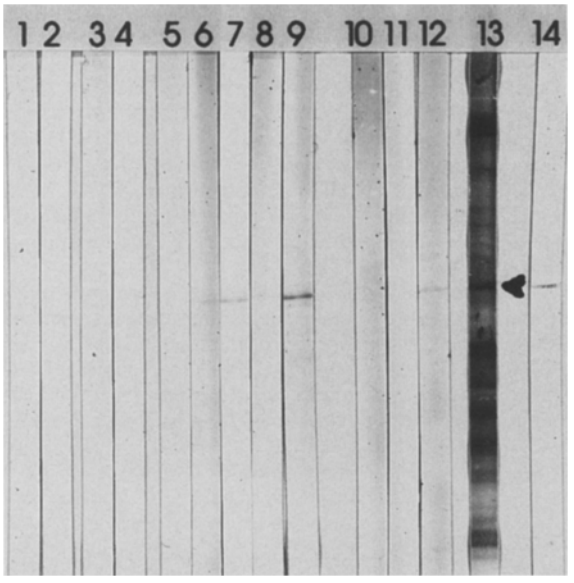

Fig. 1. Immunoblot analysis of parasite-specific in vitro synthesized IgA using antigens from the initial *Giardia lamblia* clone GS/M-83-H7 and lymphocytes from some selected and representative animals. *Lane 1:* Nu/nu, no infection/no reconstitution; *lane 2:* nu/nu, *Giardia*-infected, no reconstitution; *lanes 3, 4:* nu/+, no infection; *lanes 5–9:* nu/+, *Giardia*-infected; *lane 10:* nu/nu, *Giardia*-infected, no reconstituion; *lanes 11, 12:* nu/nu, *Giardia*-infected, reconstituted with immune cells. Identification of the site of the 72000-Da major surface antigen (*arrow*) was done by molecular-weight markers (not shown) and by a reference-strip (*lane 14*) reaction with mAb G10/4. *Lane 13:* Protein stain with India ink

nal IgA bound only to the 72000-Da major surface antigen (Fig. 1), in contrast to total serum immunoglobulins from similar previous experiments (Gottstein et al. 1990), which had recognized other additional bands. The binding activity to the 72000-Da major surface antigen was restricted to experimentally infected nu/+ mice and to one of two nu/nu mice reconstituted with lymphocytes from *Giardia*-infected and subsequently self-healed nu/+ mice.

Discussion

Our previous investigations had demonstrated that a lymphocyte proliferative response to *Giardia*-antigen stimulation was limited to Peyer's patch cells and could not be detected in peripheral lymph nodes or spleen cells. The use of immunodeficient mice in previous studies provided a number of interesting findings. In the nu/nu and nu/+ mouse experiment, it became evident that the course of the *G. lamblia* infection was mainly mediated by functional thymus-dependent T-lymphocytes. These lymphocytes and potentially related synthesized antibodies obviously allowed the corresponding host animals to clear the infection, whereas a basic lack of the respective functional lymphocytes resulted in the chronic persistence of a vast parasite population in the small intestine (Gottstein and Nash 1991). These circumstances indicate the development of protective immunity in (nu/+) mice against the homologous *G. lamblia* clone under investigation and the failure of (nu/nu) mice to develop such immune protection.

The results of the present series of experiments further support the idea that antibody responses, especially with respect to the local synthesis of parasite-specific secretory IgA, may be of causative relevance in vivo. Although nu/nu mice demonstrated the capacity to mount a parasite-specific serum Ig response following experimental infection, they lacked the potential to synthesize intestinal IgA against the 72000-Da major surface antigen characterizing our *Giardia* clone. Nu/nu mice reconstituted with immune Peyer's patch cells obtained from self-healed nu/+ mice acquired the potential to synthesize intestinal IgA against the 72000-Da major surface antigen and simultaneously acquired the potential to decrease their intestinal parasite mass. These results represent the first demonstration of parasite-specific IgA synthesis by immune B-cells from mice experimentally infected with *G. lamblia,* implying their potential relevance for the elimination of *G. lamblia* trophozoites. Of course, we do not exclude the possibility that other types of immune responses not demonstrated in the present work may also be causatively involved in modulating the loss of the initial 72-kDa epitope and the subsequent course of infection. For further experiments, we plan to dissect the nature of the immune reactions listed above by reconstituting SCID mice with subsets of immune cells provided by reconstituted mouse intestines as described in this paper and to prove the in vitro cytotoxicity of anti-72-kDa-IgA as previously shown with mAb G10/4 (Nash and Aggarwal 1986).

The present results and findings emphasize the role of intestinal immune lymphocytes and IgA in recognizing *Giardia* surface antigens with respect to protective mechanisms.

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