Impaired intestinal tolerance in the absence of a functional complement system

Pirkka T. Pekkarinen, MD,[a] Kirsi Vaali, PhD,[a,b,c] Hanna Jarva, MD, PhD,[a,d] Eliisa Kekäläinen, MD, PhD,[a] Ilvo Hetemäki, MB,[a] Sami Junnikkala, PhD,[a,e] Merja Helminen, MD, PhD,[f] Outi Vaarala, MD, PhD,[b] Seppo Meri, MD, PhD,[a,d] and T. Petteri Arstila, MD, PhD[a] *Helsinki and Tampere, Finland, and Bergen, Norway*

Background: Cells of the innate immune system regulate both adaptive immune responses and the maintenance of tolerance, especially in the gut. However, relatively little is known about the effects of complement on lymphocyte homeostasis.
Objective: This study explored complement C3 deficiency in mice and human subjects for its effect on intestinal tolerance.
Methods: C3-deficient mice and control C57BL/6 mice were fed ovalbumin (OVA) by means of gavage, and subsequent response to immunization with OVA in Freund's adjuvant was monitored. Serum antibodies against commensal microbes were measured, and the activation status of peripheral blood lymphocytes bearing mucosal homing markers was determined from 2 rare cases of C3-deficient patients.
Results: We show in C3-deficient mice and human patients that intestinal tolerance fails in the absence of functional complement. In contrast to wild-type control animals, in which oral tolerance was induced, intragastric administration of OVA did not result in a significantly decreased response to subsequent subcutaneous OVA challenge in C3-deficient mice. In the jejunum of C3-deficient mice the cytokine ratio between IL-10 and IFN-γ or IL-17 levels was decreased, indicating a shift in favor of proinflammatory cytokines. In 2 C3-deficient children the frequency of gut-homing T cells expressing activation markers was increased, and the patients had increased serum IgG levels against gut commensal microbes. The data also suggest that the impaired oral tolerance was at least partly caused by the absence of signaling through C3-binding complement regulators in T cells.
Conclusions: Taken together, our results identify complement as an important and nonredundant regulator of intestinal tolerance. (J Allergy Clin Immunol 2013;131:1167-75.)

Key words: *Mucosal immunity, peripheral tolerance, complement C3, T lymphocytes, humoral immunity*

Reflecting the importance of first-line defense, intestinal mucosa–associated lymphoid tissues represent the largest lymphocyte accumulation in the body, accounting for an estimated 70% of all lymphoid cells.[1] At the same time, the gut is a particularly challenging environment for the immunologic decision process. In no other organ does the immune system come regularly into such close contact with microorganisms and foreign antigens, a few of which are pathogenic, although most are not. Thus the local mechanisms have to choose between initiating an immune response, ignoring the foreign antigen, or actively inducing tolerance to it.

The pathogens using mucosal surfaces as a route of entry possess virulence factors that allow them to penetrate the epithelium and to avoid destruction by phagocytes, complement, and other innate mechanisms.[2,3] Proliferation of the pathogens leads to increased signaling through pattern-recognition receptors and, ultimately, to dendritic cell activation and induction of an adaptive immune response in the gut-associated lymphoid tissues.[4,5] The activated effector cells express mucosal homing receptors, which allow them to migrate to the gut epithelium, with subsequent clearing of infection.[5]

However, the default mode of response in the gut favors tolerance.[4,6,7] The interaction of the immune system and normal microbial flora is characterized by limited local IgA responses and systemic ignorance.[3] Commensal microbes lack the virulence factors necessary for penetration of the epithelium and are rapidly cleared by innate immune cells if they reach the lamina propria. Therefore antigens derived from commensal organisms are scarce outside the gut lumen, and the activation of both innate and adaptive immunity is limited.[8,9]

Another potential outcome of intestinal antigen exposure is systemic unresponsiveness, which is often termed oral tolerance. Dendritic cells presenting antigens in the absence of proinflammatory signals induce regulatory T cells in the mesenteric lymph nodes, which then mediate systemic antigen-specific tolerance.[7] In addition to forkhead box P3 (FOXP3)–positive regulatory T cells, especially the IL-10–producing T_R1 cells, have been implicated in the maintenance of intestinal homeostasis.[10]

From [a]the Department of Bacteriology and Immunology, Haartman Institute, and [c]the Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki; [b]the Department of Vaccination and Immune Protection, Immune Response Unit, National Institute for Health and Welfare, Helsinki; [c]the Institute of Medicine, University of Bergen; [d]HUSLAB and Helsinki University Central Hospital, Helsinki; and [f]the Paediatric Research Centre, Tampere University and University Hospital, Tampere.

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Corresponding author: Pirkka T. Pekkarinen, MD, Haartman Institute, Department of Bacteriology and Immunology, PB 21, 00014 University of Helsinki, Finland. E-mail: pirkka.pekkarinen@helsinki.fi.

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Abbreviations used
$C3_{ctrl}$: C3 knockout mice, saline feeding, ovalbumin immunized
$C3_{OVA}$: C3 knockout mice, ovalbumin feeding, ovalbumin immunized
FOXP3: Forkhead box P3
HRP: Horseradish peroxidase
KO: Knockout
OVA: Ovalbumin
PE: Phycoerythrin
TCR: T-cell receptor
WT: Wild-type
WT_{ctrl}: Wild-type mice, saline feeding, ovalbumin immunized
WT_{OVA}: Wild-type mice, ovalbumin feeding, ovalbumin immunized

Although ultimately mediated by adaptive immunity, the decision between a response and tolerance depends on the particular combination of microbe-associated molecular signals sensed by innate immunity.[9] Thus the function of innate immune cells and cellular receptors has been studied in increasing detail and elucidation. In contrast, very little is known about the role of complement in gut-associated adaptive immune responses. Yet complement is a potent modulator of the adaptive immune system on several levels.[11] Complement-mediated opsonization enhances phagocytosis and helps follicular dendritic cells to bind antigens to their surface, thus affecting antigen presentation to lymphocytes.[12-14] The soluble complement cleavage products C3a and C5a participate in regulating cellular traffic.[15] More directly, complement C3d receptor CR2 on B cells decreases their activation threshold and enhances proliferation and survival in the germinal centers.[16,17] T-cell function is also modulated by complement. T-cell responses against viruses are impaired and rejection of allografts is delayed in C3-deficient mice.[18,19] This is at least partly due to disrupted T_H1 differentiation in the C3-deficient animals, as reported in several studies.[20-22]

Here we have examined the role of complement in the establishment of oral tolerance. Our data, obtained from both C3-deficient mice and 2 rare cases of total C3 deficiency in human subjects, identify complement as an important and nonredundant regulator of oral tolerance.

METHODS

Study subjects

PBMCs were isolated from patients 1 and 2 when they were 8.5 and 7 years old, respectively, and sera at the age of 2.5 years. Control samples were collected from children (n = 19; age, 3 days to 3 years) and adults (n = 3; age, 21-38 years). PBMCs were isolated with Ficoll-Paque PLUS (GE Healthcare, Waukesha, Wis) density gradient centrifugation. All subjects were free of acute infections at the time of sampling. Written informed consent was obtained from the study subjects or their parents. The study plan was accepted by the ethics committee of the Joint Municipal Authority of the Pirkanmaa Hospital District and by the ethics committee for pediatrics at the Helsinki University Central Hospital.

Mice and immunizations

The mice used were C3 knockout (KO) mice on the C57BL/6 background, with sex- and age-matched wild-type (WT) C57BL/6 mice used as control animals (kindly provided by Professor Marcela Pekna, Gothenburg University, Gothenburg, Sweden). The mice were given an intragastric dose of 1 mg of ovalbumin (OVA) in 100 μL of saline or saline only twice a week for 4 weeks. After gavage, the mice were immunized subcutaneously in the neck once with 50 μg of OVA (grade V; Sigma-Aldrich, St Louis, Mo) in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich) and reimmunized twice with 50 μg of OVA in Freund's incomplete adjuvant (Difco Laboratories) at intervals of 2 weeks. Animals were killed 1 to 2 weeks after the last subcutaneous boost. The experiment was repeated 3 times independently, each time with 6 mice in each group. The study was approved by the Laboratory Animal Board of the Southern Finland Regional State Administrative Agency.

Western blotting and complement measurements

Serum samples were run in a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked with 5% milk in PBS/0.05% Tween 20. Polyclonal rabbit anti-human C3c antibody (1:10,000; Dako, Glostrup, Denmark) was added and incubated at +4°C overnight. The membrane was washed with PBS/0.05% Tween, incubated with horseradish peroxidase (HRP)–conjugated goat anti-rabbit antibody (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, Pa) for 1 hour at room temperature, and again washed. Goat anti-mouse C3 antibody (1:2000; Bethyl Laboratories, Montgomery, Tex) and HRP donkey anti-goat (1:5000; Jackson ImmunoResearch Laboratories) were used to measure mouse C3 levels in undiluted cell-culture supernatants. The bound antibodies were detected by means of electrochemiluminescence.

Complement total hemolytic activity of the classical pathway (CH100Cl) was measured according to standard diagnostic procedures with the enzyme immunoassay, and the serum concentration of C3 was measured with immunoturbidimetry.

ELISA

Mouse OVA-specific immunoglobulin levels were measured as described previously and are presented as OD at 405 nm.[21] Human immunoglobulin levels and vaccine responses were measured by using standard diagnostic ELISA procedures (HUSLAB and National Institute for Health and Welfare, Helsinki, Finland).

Heat-inactivated serum samples from human subjects were used to determine antibodies against commensal microbes. Strains of commensal organisms were isolated from clinical samples or healthy volunteers and grown in suitable broth until the mid-log phase. The microbes were washed twice with PBS, and the concentration was adjusted to 0.6 OD at 600 nm. MaxiSorp microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 μL per well of the microbe suspension and allowed to dry overnight at +37°C. The wells were washed with PBS/0.05% Tween, and serum samples were incubated in the wells for 1 hour at room temperature. Bound IgG and IgA were detected by using HRP-conjugated anti-human IgG or IgA (1:5000, Jackson ImmunoResearch Laboratories), followed by OPD substrate solution (Dako). The amount of bound antibody was measured as OD at 492 nm.

Mouse IL-10 levels were measured from cell-culture supernatants with a commercial ELISA reagent set (Mouse IL-10 ELISA Ready-SET-Go; eBioscience, San Diego, Calif), according to the manufacturer's instructions. The supernatants were stored at −70°C until used undiluted for ELISA. Plates were read at 450 nm, and absorbance values of 560 nm and background OD from blank wells without added samples were subtracted.

Cell stimulation assays

Mouse spleens were homogenized mechanically, and erythrocytes were lysed with RBC lysing buffer (Sigma-Aldrich). Splenocytes were cultured at 2×10^5 cells per well on 96-well plates, as described previously.[21,23] The cells were stimulated in triplicate with OVA (10 μg/mL) for 5 days and then pulsed for 6 hours with tritiated thymidine (3.7×10^4 Bq per well; GE Healthcare, Buckinghamshire, United Kingdom). The cells were harvested with a Skatron harvester (Newington, NH) and analyzed with a Microbeta liquid scintillation counter (Wallac, Turku, Finland) by using OptiScint HiSafe scintillation fluid (PerkinElmer, Waltham, Mass). The data are presented as the stimulation index, which was calculated by dividing the average cpm value measured in stimulated wells by the average cpm in control wells.

For Crry/CD3 stimulation, splenocytes from WT C57BL/6 mice (2 individual experiments, n = 4 in each) were stimulated with plate-bound anti-mouse CD3 (clone 145-2C11, 0.3 μg/mL) and Crry (clone 1F2, 2 μg/mL)