Antibody-Mediated Immune Control of a Retrovirus Does Not Require the Microbiota

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Commensal microbes are often required to control viral infection by facilitating host immune defenses. However, we found that this does not hold true for retroviral infection. We report that retrovirus-resistant mice control the pathogen with virus-neutralizing antibodies independently of commensal microbiota. This is in contrast to orthomyxoviruses and arenaviruses, where resistance is ablated in animals depleted of microbiota. Clearly, when it comes to antiviral immunity, the role of the microbiota cannot be generalized.

It is well established that the host's microbiota influences immunological defense against numerous bacterial pathogens (1, 2). The microbiota can also influence protective antiviral immunity, as in the case of systemic dissemination of arenaviruses and orthomyxoviruses (3, 4). However, an intact microbiota can also promote viral propagation and transmission, as in the case of orally transmitted picornaviruses and reoviruses (5).

Previously, we showed that when genetically susceptible mice were rederived as germfree (GF) and injected intraperitoneally (i.p.) with an orally transmitted retrovirus, the animals became infected but failed to transmit infectious virus to their offspring (6). Therefore, similar to that of picornaviruses and reoviruses, transmission of a milk-borne retrovirus in susceptible animals depends entirely on the host's microbiota. Unlike retrovirus-susceptible mice, retrovirus-resistant mice do not pass infectious virus even in the presence of microbiota; these animals generate antiviral immune responses capable of eliminating the virus (7, 8). In this current work, we set out to determine if production of protective retrovirus-specific immune responses in retrovirus-resistant mice requires the microbiota.

GF mice exhibit normal production of antigen-specific antibodies (Abs) in response to immunization. There have been conflicting reports regarding the ability of GF animals to mount an efficient humoral response after immunization with innocuous antigen (9–11). Therefore, we needed to ensure that mice from various genetic backgrounds, including retrovirus-resistant strains, did not exhibit a defective immune response upon immunization. Accordingly, we immunized GF and specific-pathogen-free (SPF) C57BL/6J, C3H/HeN, and BALB/cJ mice with ovalbumin (OVA) using the protocol described in reference 9.

GF C57BL/6J mice were obtained from Eugene Chang (The University of Chicago). BALB/cJ and C3H/HeN mice were rederived as GF at Taconic Farms (Germantown, NY) and maintained at The University of Chicago gnotobiotic facility. SPF C57BL/6J and BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), whereas C3H/HeN mice have been maintained in our colony for the past 10 years. All studies were conducted with approval by the Institutional Animal Care and Use Committee, and all animals were housed in accordance with *The Guide for Care and Use of Laboratory Animals* (National Research Council, 8th edition, 2011) and AAALAC International.

To confirm the sterility of the GF isolators, DNA was extracted

from freshly frozen cecal contents or fecal pellets and amplified with a set of primers that hybridize to all bacterial 16S rRNA gene sequences (12). Tests were conducted weekly using fecal samples from individual cages. In addition, microbiological cultures were set up with GF fecal pellets.

For immunization, a suspension of OVA, fraction VI (Sigma), and complete Freund's adjuvant (CFA) was prepared by combining equal volumes of OVA solubilized in phosphate-buffered saline (PBS) and CFA. Eight-week-old mice were immunized as described by Lamoussé-Smith et al. (9). Primary OVA-specific IgG and IgM responses were tested via an enzyme-linked immunosorbent assay (ELISA) 10 days after immunization. OVA fraction VI (5 µg/ml) was bound to plastic in borate-buffered saline (pH 8.0) overnight. Nonspecific binding was blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C followed by incubation with mouse sera at 4°C for 1 h. Goat anti-mouse IgGs or IgM coupled to horseradish peroxidase (HRP) was used to develop the ELISA. For all experimental samples, the values of optical density at 450 nm (OD₄₅₀) obtained from the incubation with preimmune sera alone were subtracted. In each ELISA, the serum samples were run in duplicate.

We found that mice from all strains produced OVA-specific IgG Abs and that this production was independent of the environment in which they were reared (Fig. 1). Specifically, GF mice from all three strains produced levels of antigen-specific Abs within the same range as those produced by their microbially replete counterparts, suggesting that the results are broadly generalizable. Notably, the same result was obtained when either the diet or the duration of sterilization was altered (data not shown).

Humoral response to a retrovirus does not require the microbiota. Murine leukemia virus (MuLV) is a gammaretrovirus

Received 27 January 2014 Accepted 12 March 2014

Published ahead of print 19 March 2014

Editor: S. R. Ross

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doi:10.1128/JVI.00251-14

FIG 1 Immunization with an antigen induces the Ab response, which does not require the microbiota. OVA-immunized GF and SPF animals from 3 different strains were bled 10 days postimmunization to screen for OVA-specific IgG or IgM Abs. Graphs show OD₄₅₀ values at each serial dilution of sera. Results for three mice per group are displayed. Average OD₄₅₀ values are demonstrated for 10⁻³ and 10⁻² dilutions for IgG and IgM, respectively. Error bars represent standard deviation (SD).

that is transmitted as an exogenous or an endogenous virus (13). Exogenous MuLV is passed through the blood and the milk of infected animals and primarily infects cells of lymphoid origin (14, 15). Susceptible mice develop severe splenomegaly and subsequently succumb to leukemia (15). Unlike mice from susceptible strains, MuLV-infected I/LnJ mice eliminate the infectious pathogen and resist leukemia (7, 16). In these animals, retrovirus neutralization is predominantly mediated by the humoral response, as sera of MuLV-infected I/LnJ mice completely neutralize the virus *in vitro* and *in vivo* by interfering with virus entry (16, 17). A single recessive locus, virus infectivity controller 1 (*vic1*) mapped to chromosome 17, controls Ab-mediated retrovirus resistance in I/LnJ mice and confers resistance to mice from susceptible backgrounds congenic for the *vic1* I/LnJ locus (6, 16). To determine whether the microbiota is required for the *vic1*-mediated antiretrovirus protective responses, we rederived BALB/cJ mice congenic for the *vic1* I/LnJ locus (BALB/cJ^{vic11/LnJ}) as GF and compared their ability to generate antiviral protective responses with that of SPF BALB/cJ^{vic11/LnJ} mice.

For this work, the BALB/cJ^{vic11/LnJ} congenic line, which contains the *vic1* region of the I/LnJ chromosome between markers *D17Mit143* and *D17Mit34*, was produced by 10 repetitive backcrosses of I/LnJ to BALB/cJ mice. BALB/cJ^{vic11/LnJ} mice were rederived as GF at Taconic Farms along with BALB/cJ mice (virus-susceptible control).

GF and SPF BALB/cJ^{vic11/LnJ} and BALB/cJ mice were challenged by an i.p. injection of Rauscher-like MuLV (RL-MuLV) (10³ PFU) (18). RL-MuLV is a mixture of an ecotropic and mink cell focus-forming MuLVs capable of causing splenomegaly, which progresses to erythroleukemia in BALB/cJ mice (18). Infected mice were screened for antiviral Abs 9 weeks after infection. MuLV-specific ELISA was performed as described previously (16) with the exception that 1% bovine serum albumin (BSA) was used for blocking. IgG2a-specific Abs (I/LnJ mice produced a predomi-

nantly IgG2a-specific antiviral response [7]) coupled to HRP were used at the second step. For all experimental samples, the OD₄₅₀ values obtained from the incubation with secondary Abs alone were subtracted.

Both GF and SPF BALB/cJ^{vic11/LnJ} mice but not BALB/cJ mice produced antiviral Abs (Fig. 2), indicating that only the I/LnJ *vic1* locus determines the virus-specific humoral response. In addition, the levels of virus-specific Ab did not differ between the GF- and SPF-resistant mice (Fig. 2).

To test whether anti-MuLV Abs produced by GF-resistant mice were capable of neutralizing the virus, we measured splenomegaly and examined the viral titers in the spleens of virus-injected animals (described in reference 16). In agreement with previous reports (18–21), the virus replicated efficiently in GF and SPF BALB/cJ mice, as they developed splenomegaly and carried high viral titers in the spleen (Table 1). Unlike BALB/cJ mice, both virus-resistant GF and SPF BALB/cJ^{vic11/LnJ} mice neutralized the virus, as they did not develop splenomegaly and had no detectable virus in the spleen (Table 1). Thus, Ab-mediated retrovirus-specific responses are equally neutralizing in GF and SPF mice.

C57BL/6J mice carry a resistance locus (*recovery from Friend virus no. 3* [*rfv3*]) mapped to chromosome 15 (22). Mice that harbor the *rfv3* resistance allele mount an Ab-mediated antiviral immune response (8, 23). To ensure that the ability to produce a microbiota-independent antiviral immune response is not a unique property of the *vic1*-mediated mechanism, we compared antiviral Ab responses in C57BL/6J mice with and without microbiota. Accordingly, SPF and GF C57BL/6J mice were i.p. injected with 10³ PFU of MuLV. Infected mice were screened for antiviral Abs 3 weeks after infection via the MuLV-specific ELISA as described above with the exception that total IgG-specific HRP-coupled Abs were used at the second step. In addition, virus titers in the spleen were assessed via the XC-plaque assay (16). The results

FIG 2 Vic1-mediated production of virus-neutralizing Abs is microbiota independent. (A) RL-MuLV-injected virus-resistant BALB/c^{vic11/LnJ} (R) and virus-susceptible BALB/cJ (S) GF and SPF mice were bled 9 weeks postinfection, and sera collected from all groups of mice were tested for reactivity to RL-MuLV virion proteins in an ELISA. A total of 13 SPF BALB/c^{vic11/LnJ}, 13 GF BALB/c^{vic11/LnJ}, 11 SPF BALB/cJ, and 7 GF BALB/cJ mice were used in three independent experiments. The average OD values for all mice within a group are shown. Error bars represent the standard deviation (SD). (B) Western blot analysis of MuLV virion proteins with sera of MuLV-infected R and S mice. Anti-mouse total IgG-specific Abs coupled to HRP were used as secondary Abs. While all sera were analyzed via Western blotting, only some samples are shown. 2°, secondary Abs alone. gp70Env and p30Gag, processed virion proteins products of *env* and *gag*, respectively. p65Gag, partly processed Gag. Lanes 1 to 16 represent individual mice. p30, a-p30CAGag monoclonal Ab.

obtained with C57BL/6J mice mirrored those obtained with the BALB/c^{vic11/LnJ} mice; both the GF and SPF C57BL/6J mice produced a virus-specific humoral response that effectively eliminated the virus (Fig. 3 and Table 1). It should be noted that the humoral response and viral burden were examined at single time points during the course of infection (3 months and 3 weeks for *vic1* and *rfv3* mice, respectively), at which time no differences were observed between SPF and GF mice. However, it is possible that

the commensal flora may influence the kinetics of the antiretroviral humoral response during the initial stages of infection.

Nevertheless, our data clearly demonstrate that an antiretrovirus Ab-mediated protective response can be generated independently of the microbiota. Furthermore, this response appears to be the dominant factor controlling infection, as all infected GF mice cleared the virus. Conversely, high susceptibility of GF mice to influenza in the presence of antiviral Abs (3) suggests that the protective function of

TABLE 1 Comparison of the susceptibilities of GF and SPF mice to MuLV infection^a

Mouse line	Housing condition	Infection status	Mean spleen wt (g) ± SD (no. of mice)	Mean no. of PFU/10 ⁶ spleen cells ± SD (no. of mice)	Anti-MuLV Abs
BALB/cJ	SPF	Infected	0.31 ± 0.14 (3)	6,167 ± 1,266 (3)	No
BALB/cJ	GF	Infected	0.21 ± 0.05 (3)	8,400 ± 1,907 (3)	No
BALB/cJ ^{vic11/LnJ}	SPF	Infected	0.10 ± 0.01 (6)	0 (6)	Yes
BALB/cJ ^{vic11/LnJ}	GF	Infected	0.10 ± 0.04 (5)	0 (5)	Yes
C57BL/6J	SPF	Infected	0.07 ± 0.01 (4)	0 (4)	Yes
C57BL/6J	GF	Infected	0.07 ± 0.01 (3)	0 (3)	Yes
BALB/cJ	SPF	Uninfected	0.09 ± 0.01 (3)	0 (3)	No
BALB/cJ	GF	Uninfected	0.10 ± 0.04 (4)	0 (4)	No
C57BL/6J	SPF	Uninfected	0.07 ± 0.01 (4)	0 (4)	No
C57BL/6J	GF	Uninfected	0.07 ± 0.004 (3)	0 (3)	No

^a Spleens from randomly selected BALB/cJ and BALB/cJ^{vic11/LnJ} mice and from all C57BL/6J GF and SPF mice were subjected to the XC plaque assay to determine the virus load. Numbers of mice tested are indicated in parentheses. Yes, presence of antiviral Abs determined by ELISA or Western blotting as shown in Fig. 2 and 3; no, absence of antiviral Abs.

FIG 3 Rfv3-mediated control of MuLV does not require commensal bacteria. RL-MuLV-injected GF and SPF C57BL/6J mice were bled 3 weeks postinfection, and sera collected from all groups of mice were tested for reactivity to RL-MuLV virion proteins in an ELISA. A total of 4 SPF and 3 GF C57BL/6J mice were used. The average OD values for all mice within a group are shown. Error bars represent the standard deviation (SD).

the microbiota in the case of influenza is unrelated to Abs. Such protection could be due to the reduction in tissue damage caused by this cytopathic virus (24).

ACKNOWLEDGMENTS

These studies were supported by AI090084 (to T.G.), by AI082418 and Juvenile Diabetes Research Foundation grant 17-2011-519 (to A.C.), by T32 AI 007090 (to J.W.), and by P30 DK42086 (to the Digestive Disease Research Core Center).

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