Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system

NICOLE BAUMGARTH*, OMETA C. HERMAN*, GINA C. JAGER*, LORENA BROWN‡, LEONARD A. HERZENBERG*, AND LEONORE A. HERZENBERG*

*Department of Genetics, Stanford University Medical School, Stanford, CA 94305; and ‡Department of Microbiology, University of Melbourne, Melbourne, Victoria 3056, Australia

Contributed by Leonard A. Herzenberg, December 31, 1998

ABSTRACT "Natural" Igs, mainly IgM, comprise part of the innate immune system present in healthy individuals, including antigen-free mice. These Igs are thought to delay pathogenicity of infecting agents until antigen-induced high affinity Igs of all isotypes are produced. Previous studies suggested that the acquired humoral response arises directly from the innate response, i.e., that B cells expressing natural IgM upon antigen encounter, differentiate to give rise both to cells that secrete high amounts of IgM and to cells that undergo affinity maturation and isotype switching. However, by using a murine model of influenza virus infection, we demonstrate here that the B cells that produce natural antiviral IgM neither increase their IgG production nor undergo isotype switching to IgG2a in response to the infection. These cells are distinct from the B cells that produce the antiviral response after encounter with the pathogen. Our data therefore demonstrate that the innate and the acquired humoral immunities to influenza virus are separate effector arms of the immune system and that antigen exposure per se is not sufficient to increase natural antibody production.

Infections with most pathogens, including influenza virus, activate a humoral immune response that is characterized by an early rise of antigen-specific IgM followed by affinity maturation, isotype switching, and the ensuing rise in antigen-specific IgG, IgA, and IgE antibodies. The sera of humans and mice also contain "natural" antibodies, mostly of the IgM isotype, which can bind to a particular antigen or pathogen, even if the host has never been exposed to that antigen (1–4). Although a function for these natural antibodies has not been demonstrated directly, it is generally assumed that these antibodies are part of the innate immune system and delay pathogen replication until the developing acquired humoral and cellular immune responses clear the infection (1–4).

Conflicting data exist in the literature regarding the connection between the natural IgM antibodies found before antigen encounter and the early rise in antigen-specific IgM seen shortly after exposure to antigen (2, 3). Some studies suggest that the production of natural IgM increases after antigen encounter to provide a "platform for antigen specific immune responses" (reviewed in ref. 2). These conclusions are based largely on indirect evidence from studies using hybridoma technology to compare the immune repertoire of antigen-specific B cells before and after immunization. Similarly, others have argued that increases in autoantibody levels, found in various autoimmune diseases, are attributable to increased production, isotype switching, and affinity maturation of natural antibodies after encounter with pathogens that express epitopes cross-reactive to self-antigens (3). Others favor the idea that no connection exists between natural antibodies and the antibodies that are induced by antigen encounter, on experimental (1, 5) or theoretical grounds (6).

Various studies showed that B-1 cells, many of which express CD5, are an important source of "natural" serum IgM, IgA, and IgG and of autoantibodies that collectively comprise the innate humoral immune system in humans and mice (7–11). These self-replenishing B cells differ in their activation requirements from follicular (B-2) B cells: B-1 cells respond to in vitro stimulation with polyclonal activators, including certain influenza virus hemagglutinins, but they do not respond to cross-linking of their surface Ig receptor (12, 13). Moreover, B-1 cells have been shown to recognize preferentially (14), although not exclusively (15, 16), T cell-independent antigens such as polysaccharides and phospholipids. In vivo, B-1 cells are rare in secondary lymphoid organs, including germinal centers (17), but are present in large numbers in coelomic cavities (14).

Recently, studies with T cell-deficient mice demonstrated that T cell-independent IgM, IgG, and IgA production contributes to the humoral immune response induced against a number of viruses (reviewed in ref. 18). The relationship between the T cell-independent antibody response induced by infection with virus and the preexisting natural virus-binding antibodies is not known. The early, largely T cell-independent rise in pathogen-binding IgM could be provided by natural antibody producing cells. Alternatively, the T cell-independent humoral response, like the T cell-dependent response, might be distinct from the innate humoral immunity that exists before antigen encounter.

We present here an in vivo study on influenza virus infection in which we clarify the relationship between the innate and acquired humoral immunity to this virus. Our study clearly demonstrates that these two effector arms of the immune systems differ in cellular origin and in their activation requirements. Therefore, we show that innate and acquired humoral immunities are functionally distinct immune processes.

METHODS

Mice, Influenza Virus, and Antibody Treatment. BALB/c (Igh\(^{h}\)) and C.B-17 (Igh\(^{b}\)) mice were bred and maintained in the Animal Facility at Stanford University. For production of allotype-chimeras, newborn C.B-17 mice received 5 × 10\(^6\) peritoneal cavity lavage cells (PerC) from 2-month-old BALB/c mice and a total of 2 mg of anti-IgM\(^{b}\) (mAb AF6.78), purified from serum-free tissue culture supernatants by DEAE-dextran chromatography, as described (19). Mice were infected intranasally after anesthesia with methoxyflurane (Metofane, Mallinckrodt) with 8 hemagglutinating units of the influenza virus reasortant A/Mem/71 (H3N1) in 50 \(\mu\)l.

Abbreviations: PerC, peritoneal cavity lavage cell; FACS, fluorescence-activated cell sorter;
To whom reprint requests should be addressed: Department of Genetics, Stanford University Medical School, Beckman Center B007, 300 Pasteur Drive, Stanford, CA 94305-5318. e-mail: Baumgarth@Stanford.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
of phosphate-buffered saline. The virus was isolated and stored as described (20).

Nine-Color Fluorescence-Activated Cell Sorter (FACS) Analysis. Single cell suspensions from spleens and PerC were stained on ice for 20 min with cocktails of the following antineurine antibody conjugates in staining medium (bovine, flavin-deficient RPMI 1640 medium supplemented with 4% newborn calf serum, and 0.02% sodium azide): anti-CD21-fluorescein (mAb 7G6) (PharMingen); anti-CD43 phycoerythrin (ST7); anti-CD565 (53–7.3); anti-CD23 Texas red (B3B4); anti-CD11b Texas red; anti-CD11b allophycocyanin (M1/70); anti-IgM Cy7-allophycocyanin (331); anti-IgM Cy7-allophycocyanin (DS-1); anti-IgM6 Cy7-allophycocyanin (AF6.78); anti-IgD Cy7-phycoerythrin (1126); anti-IgD4 Cy7-phycoerythrin (AMS9); anti-IgD3 Cy7-phycoerythrin (AF6.122); anti-B220 Cascade blue (RA3–6B2); anti-IgM6 Cascade blue; anti-CD4 Cascade yellow (GK1.5); anti-CD8 Cascade yellow (53.6.7); and antimacrophage Cascade yellow (F4/80). Cells were washed with staining medium and cells were incubated with streptavidin-Cychrome (PharMingen) as described (21, 22). Propidium iodide was added immediately before analysis at a final concentration of 0.25 μg/ml to discriminate viable cells. Cells were analyzed by using a highly sensitive Becton Dickinson hybrid FACS, described in ref. 23, allowing us to measure simultaneously nine-color parameters plus two scatter signals. Data were analyzed by using the FLUOWIN software (Treestar, San Carlos, CA).

ELISA. The serum concentrations of α- and β-allotype IgM, IgG2a, and IgA were determined by coating 96-well plates (Maxisorp, Nunc) with 10 μg/ml anti-IgM (DS-1), 40 μg/ml anti-IgM6 (AF6.78), 5 μg/ml anti-IgG2aα (20.8.3), 10 μg/ml anti-IgG2aα (3.1), 5 μg/ml anti-IgAα (HY-16), and 10 μg/ml anti-IgAα (HISM-2) in phosphate-buffered saline overnight. Plates were washed and blocked essentially as described (24). Sera were 2-fold serially diluted and incubated for 4 h. Each plate contained as standard the appropriate control myeloma: HPC76 (IgMα), CBPC112 (IgMβ), 27–13 (IgG2aα), CBPCT101 (IgG2aβ), MOPC410d (IgAα), and CBPc-4 (IgAβ). ELISA were developed with biotinylated DS-1; AF6.78; 20.8.3; 5.7 (anti-IgG2aβ), or goat-anti-IgA (Southern Biotechnology Associates) and streptavidin-horseradish peroxidase (Vector Laboratories). o-Phenylenediamine dihydrochloride (Sigma) in citrate buffer was used as substrate. Reaction was stopped with 3 M HCl and read at 495 nm, with 405 nm as reference wavelength on a multiscan 96-well plate-reader. For influenza virus-specific ELISA, the assay was done essentially as described (24), except that allotype-specific antibody-binding was revealed with the biotinylated anti-IgM and IgG2a antibodies listed above and o-phenylenediamine dihydrochloride was used as substrate.

Statistical Analysis. Statistical significance was tested with the nonparametric Wilcoxon/Kruskal–Wallis Rank test. Data were regarded as statistically significant when $P < 0.05$.

RESULTS

Creation of Mice with B-1 and B-2 Cells of Differing Allotypes. To characterize the cellular origins of innate and influenza virus-stimulated antiviral antibodies, we created Igh-a/b allotype-chimeras in which B-1 and B-2 cells produce antibodies marked with distinct Igh allotypes. To achieve this with minimal manipulation of the animals under study, we transferred Igh allotype-congenic peritoneal cavity BALB/c cells (Ighb), a source for B-1 cells, into newborn C.B-17 mice (Ighb) treated with monoclonal anti-IgMβ antibody. This treatment depletes all host (Ighb) B cells for the duration of treatment and allows the Ighβ-expressing B-1 cells to expand. Later, when the anti-IgMβ-treatment is stopped, the Ighβ B-1 cells inhibit the de novo development of most host-derived B-1 but do not inhibit de novo B-2 development (19, 25).

Consistent with previous findings (19), FACS analysis of PerC from chimeric mice 3 months after the end of anti-IgMβ treatment confirms that all of the Igha-expressing cells in PerC are B-1 cells (IgMβa, IgDβa, CD11bβ, CD23-, CD43-), and mainly CD5+, Fig. 1 and data not shown). All B-2 cells (IgMβb, IgDβb, CD11bβ, CD23+, CD43-, and CD5–) and 10–20% of B-1 cells express Ighα. Data for PerC from BALB/c (Ighb) mice, the B-1 donor, and from C.B-17 (Ighb) mice, the treated host, are presented for comparison (Fig. 1).

Neonatal Anti-IgM Treatment Has No Long-Term Effects on Host B Cell Development. Spleens from chimeras contained normal numbers of cells (7.7 × 107 ± 2.5, controls 7.6 × 107 ± 3.1, n = 19) and similar frequencies of follicular (B220+, IgMβb, IgDβb), marginal zone (B220+, IgMβb, IgDβb, CD21hi, CD43–), immature/transitional (B220+, IgMβb, IgDβb, CD21lo, CD43–), and B-1 cells (B220+, IgMβb, IgDβb, CD21lo, CD43–) (Fig. 2 Left) as assessed by nine-color FACS analysis 2–3 months after end of antibody treatment. Bone marrow and lymph node tissues contained very few if any B-1 cells (data not shown). In addition, ~80% of splenic B-1 cells (B220+, CD43+, CD5+) expressed the donor-derived Igha allotype, whereas all B-2 (B220+, CD43–, CD5–) cells were host-derived (Ighb) (Fig. 2 Right). Similar results were obtained when chimeras were rechallenged in the reverse direction, i.e., with Igha hosts and Ighβ PerC (data not shown). Thus, extensive analysis of the lymphoid compartments in the chimeric animals fails to reveal any perturbation in B-2 cell development or localization. B-1 cells, in contrast, localize normally but are derived largely from the PerC donor.

Contribution of B-1 and B-2 Cells to the Serum Ig Pool. Comparison of the Igha and Ighβ serum Ig levels in allotype chimeras with control C.B-17 and BALB/c mice reveals that chimeras have normal concentrations of serum Ig. Approximately 80% of the serum IgM, however, is of the Igha allotype.

![Fig. 1. The peritoneal cavity of allotype chimeras contains B-1 and B-2 cells of differing allotypes. Shown are 5% contour plots of PerC cells from the indicated mouse strains after gating for live, B220+, CD4–, CD8+, F4/80+ cells. Cells were stained either for total or allotype-specific IgM and IgD. Frequencies of B-1 cells (IgMβa, IgDβa) and B-2 cells (IgMβb, IgDβb) were calculated from the indicated gates. These gates were chosen to exclude CD23+ CD43+ CD5+ cells in the B-1 cell gate, and CD11b–, CD5+ cells in the B-2 cells gate (data not shown). In chimeras all Ighβ-expressing cells are B-2 cells, and the majority of Ighα-expressing cells are B-2 cells.](image-url)
and therefore derived from B-1 cells (Fig. 3). Even the remaining 20% of serum Ig derived from the host appear to be largely derived from B-1 cells because, in 15 chimeras analyzed, 20 ± 7% of the B-1 cells in PerC were host-derived (Ighb) and 24 ± 10% of their serum IgM was of the Igh b allotype (see Figs. 1–3). In contrast, most of the serum IgG2a is B-2 cell-derived. Although B-1 cell-derived IgG2a is detectable in chimeric animals, the levels are significantly lower than in Igha controls.

The demonstration that approximately half of the serum IgA in chimeras is B-1 cell-derived (Fig. 3) underscores the importance of B-1 cells as producers of systemic IgA. Our previous studies with irradiation-chimeras indicated that B-1 cells are an important source for locally produced secretory IgA, as approximately half of the IgA-producing plasma cells in the intestinal lamina propria were B-1 cell-derived (26). Thus, although the producers of secretory IgA (dimeric) and serum IgA (monomeric) are presumably different cells, approximately half of the IgA-producing cells in each case are derived from B-1 cells.

**Natural Anti-Influenza Virus-Binding Ig Is Produced by B-1 Cells.** To determine the relationship between the cells that provide innate and acquired humoral immunity, we contrasted the levels of natural and virus-induced serum antibodies in a model of influenza virus infection. Influenza virus is not a natural pathogen for mice. However, influenza virus-binding natural IgM is detected in sera of controls and allotype chimeras at similar levels in all mice before virus exposure (Fig. 4 and 5). Consistent with this, natural polyspecific IgM-producing hybridomas that bind influenza virus hemagglutinin have been obtained from normal mice (27).

Analysis of allotype of the antiviral antibodies present before and after influenza virus infection clearly demonstrates the distinct origins of natural and acquired responses, i.e., the natural antibodies are derived from B-1 cells, whereas the virus-induced antibodies are derived from B-2 cells. The level of B-1 cell-derived (IgMa) natural antiviral IgM in chimeras is similar to that found in control BALB/c mice before infection. The host-derived (IgM<sup>b</sup>) antiviral levels, which are substantially lower than levels in intact C.B-17 controls (P < 0.001) (Fig. 4), mainly reflect residual host B-1 cells because the level of host-derived (IgM<sup>b</sup>) natural antiviral IgM and the frequency of the remaining host-derived B-1 cells are comparable (i.e., in 15 chimeras studied, 18 ± 10% host-derived IgM antiviral...
Control mice (for Igha, BALB/c mice, n = 12; for Ighb, C.B-17 mice, n = 11) were tested before and seven of the chimeras and all controls were also tested at day 7 after influenza virus infection for levels of influenza virus-specific IgMa and IgMb, IgM, and IgG2a. Units anti-influenza Ig were calculated in relation to standard hyperimmune sera. Data are shown as interquartile boxes; disconnected bars above and below the boxes indicate the 90th and 10th percentiles. n.s., not significant. In chimeras, Igha is PerC donor-derived and therefore produced by B-1 cells. IgM levels similar to those found in control Ighb mice (Fig. 4). In contrast, B-1-derived (a-allotype) antiviral IgM levels do not increase over those seen in the absence of infection (Figs. 4 and 5). Furthermore, no B-1-derived IgG2a antiviral antibodies are detectable. Results were similar when chimeras were prepared in the reverse direction, i.e., with Igha hosts and Ighb PerC (data not shown).

The data we presented above leave room for host B-1 cells to participate in the response. However, because host-derived B-1 cells behave similarly to PerC-derived B-1 cells with respect to the natural antiviral Ig production, there is little reason to suspect that they will differ by responding to virus infection with antibody production. Furthermore, the magnitude of the virus-stimulated host-derived response (b-allotype) in chimeras is comparable with that seen in control mice syngeneic to the host (Fig. 4), even though the chimeras have many fewer host-derived B-1 cells, indicating that the magnitude of the antiviral response depends on the frequency of B-2 rather than B-1 cells. Thus, we conclude that, whereas B-1 cells produce the natural antibodies to influenza virus, B-2 cells are responsible for producing the influenza-stimulated (acquired) antibody response.

The striking differences in the responsiveness of the two kinds of B cells are not due to kinetic differences. The host-derived (IgM\textsuperscript{b}) antiviral antibody levels follow the pattern for a typical primary response, which peaks around day 10 after infection (Fig. 5). The levels of B-1-derived antiviral IgM\textsuperscript{b} levels, however, do not change at any point after infection. The nonresponsiveness of B-1 cells to influenza virus infection is rather than B-1 cells. Thus, we conclude that, whereas B-1 cells produce the natural antibodies to influenza virus, B-2 cells are responsible for producing the influenza-stimulated (acquired) antibody response.

Finally, the B-1 nonresponsiveness to the influenza virus is not due to their putative inability to respond to T-dependent antigens. Although B-1 cells are commonly thought to respond only to T-independent antigens, they respond strongly to the phosphorylcholine hapten on keyhole limpet hemocyanin in a T-dependent fashion (15, 16). However, even if B-1 cells only respond to T-independent antigens, they could still respond to influenza virus, because the virus expresses a large number of carbohydrate moieties that can elicit T-independent responses.

**Discussion**

In the study presented here, we investigate the humoral immune response to influenza virus infection in vivo and demonstrate the distinct cellular origins of the innate and
acquired antiviral response. Furthermore, we show that the innate and acquired humoral immune systems respond differently to pathogen encounter, and thus provide direct experimental evidence for the coexistence of two differentially regulated humoral immune effector arms.

Specifically, we show that B-1 cells produce natural antibodies and do not increase antibody production in response to influenza virus infection. B-2 cells, in contrast, contribute little, if any, to the natural antibody pool but respond vigorously to the virus by producing large amounts of pathogen-specific IgM and IgG. These findings are consistent with studies showing that B-1 cells are a source for natural antibodies and autoantibodies (7–11), however, they depart from previous evidence (16), by showing that the cells that make the natural antibodies to influenza virus are not a reservoir for the cells that produce the antibody response to infection. Thus, the innate and acquired humoral effector arms are populated by developmentally distinct B cells with nonoverlapping functions.

These findings, using a viral infection model, contrast sharply with findings from studies in which mice were infected with *Streptococcus pneumoniae*, an opportunistic bacterial pathogen of the respiratory tract. In these latter studies, the B-1 cells that produce the natural antibodies (encoded by variable region heavy chain gene T15) also produce the protective T15 Id+ IgM and IgG3 antibody response to the pathogen (16, 28). The evidence from this streptococcal model, which to our knowledge is the only other relevant in vivo infection study, provides the basis for the common (3), but certainly not universal (1, 5, 6), expectation that cells producing natural antibodies participate significantly in the response to infection. In any event, our findings demonstrate that encounter with distinct pathogens can lead to substantially different response patterns.

The difference in responsiveness of cells producing natural antibodies to influenza virus and *S. pneumoniae* could reflect differences in the binding strength of particular antigens to antibodies expressed by the B-1 cells. However, because B-1 cells do not respond to cross-linking with anti-IgM (13) under conditions where B-2 cells respond strongly, the selective responsiveness of the B-1 cells is more likely due to a requirement for specialized costimulatory (or other types of) signals. Consistent with this, studies in various gene-targeted mice show that B-1 cell homeostasis is affected more strongly by the absence of various costimulatory cell surface molecules than by homeostasis of B-2 cells (29–31).

Collectively, these findings suggest that induction of proliferation and Ig-secretion by cells producing natural antibodies may require qualitatively or quantitatively different signals from those required by cells that only respond to antigenic stimulation. Furthermore, they suggest that influenza virus infection induces the signals necessary to enable antibody responses by these latter cells, whereas infection with streptococci induces signals capable of supporting responses by both types of cells.

Collectively, these findings reveal an unsuspected complexity in the ways in which the innate and acquired arm of the humoral immune system respond to different types of pathogens. It is not clear whether the differential responsiveness of the innate and acquired arms that we have uncovered can be generalized to viral versus bacterial infections. However, based on current evidence, we can expect that certain antigens have the ability to rapidly stimulate increased antibody production by cells producing natural antibodies, whereas other antigens, perhaps many of viral origin, will take longer to stimulate antibody production and will only stimulate antibody production by B-2 cells. But regardless of whether responses to individual pathogens (or antigens) prove to be provided by innate immunity, acquired immunity or both, our findings demonstrate that the immune system itself has two separate effector arms that are independently regulated.

This independence does not rule out key connections between innate and acquired immunity. Because natural antibodies are present at the time of infection, they provide an immediate means for binding antigen, activating complement, and trapping of antigen-antibody complexes on follicular dendritic cells. These functions are crucial prerequisites for the clearance of bacterial, and likely viral, infections. In addition, they are necessary for the optimal induction of humoral immune responses (29, 32–35). Thus, the presence of natural IgM can be expected to significantly affect acquired humoral immune responses, thereby linking innate and acquired humoral immunity even when the producers of the natural and acquired IgM are not identical.

We thank Drs. M. Roederer, F. G. M. Kroese, and J. Wilshire for critical comments on the manuscript and the Stanford FACS Development Group for their efforts in developing and optimizing the nine-color FACS.