Humoral response to herpes simplex virus is complement-dependent

Xavier J. Da Costa*, Mark A. Brockman*, Elisabeth Alicot†*, Minghe Ma‡*, Michael B. Fischer†*†, Xioaning Zhou†*, David M. Knipe*, and Michael C. Carroll†††

Departments of *Microbiology and Molecular Genetics, †Pathology, and ‡Pediatrics, Harvard Medical School, and ††The Center for Blood Research, 200 Longwood Avenue, Boston, MA 02115

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The complement system represents a cascade of serum proteins, which provide a major effector function in innate immunity. Recent studies have revealed that complement links innate and adaptive immunity via complement receptors CD21/CD35 in that it enhances the B cell memory response to noninfectious protein antigens introduced i.v. To examine the importance of complement for immune responses to virus infection in a peripheral tissue, we compared the B cell memory response of mice deficient in complement C3, C4, or CD21/CD35 with wild-type controls. We found that the deficient mice failed to generate a normal memory response, which is characterized by a reduction in IgG antibody and germinal centers. Thus, complement is important not only in the effector function of innate immunity but also in the stimulation of memory B cell responses to viral-infected cell antigens in both blood and peripheral tissues.

Innate immune mechanisms are the first line of defense encountered by invading infectious agents. Cellular mechanisms include phagocytosis by macrophages and neutrophils and lysis of virus-infected cells by natural killer cells (1, 2). An important component of innate immunity is the complement system, a family of serum proteins that is involved in the protection against pathogens such as enteric bacteria (3, 4). Attachment of complement components to microbial surfaces can lead to direct lysis and/or opsonization by cellular mechanisms. Complement also enhances the neutralization of antibody-bound virus by several mechanisms. These include inhibition of receptor binding, reduction of infectivity by clumping of virions, phagocytosis, and, lastly, direct lysis of enveloped viruses (5).

Bacterial and viral pathogens have evolved innovative mechanisms to evade the host immune response (6) including mechanisms specifically targeting the complement system (5). Among human herpes viruses, whose hallmark is the establishment of a latent infection, several such strategies have been hypothesized (6). Herpes simplex virus (HSV) establishes a latent infection in neuronal tissue, a site that, by itself, is immune-privileged. The lack of expression of detectable viral antigens during latency is also a very effective evasion strategy. However, HSV also undertakes a proactive role in evading the host immune system. Viral-encoded products such as ICP47 (7) have been shown to affect MHC class I antigen presentation in vitro by blocking peptide transport into the endoplasmic reticulum via the TAP transporter (8). Moreover, HSV also expresses at least two surface glycoproteins known to directly engage immune effectors. The viral-encoded gE-gI heterodimer binds the Fc portion of antibody molecules and, thus, prevents virus neutralization (9). Further, reduction in virus neutralization is mediated by a third surface glycoprotein, gC, which directly binds complement component C3b and accelerates the decay of the alternative pathway C3 convertase (10). Mutant viruses lacking gC (gC-null) are dramatically more sensitive to complement lysis in vitro (11) and in vivo (12). The finding that gC-null virus is more virulent in mice deficient in complement C3 supports a role for gC in evasion of innate immunity (12).

The complement system is important not only for innate protection but also for B lymphocyte activation and memory. Guinea pigs (13–15), dogs (16), or humans (17) deficient in complement C3 or mice in which C3 is transiently depleted (18) have reduced responses to thymus-dependent antigens (T-D) injected i.v. These observations have been confirmed and extended recently in mice bearing targeted deficiency in C3 or C4 (19). The enhancing effect of complement is mediated by complement receptors CD21/CD35 because blocking the receptors with either antibody (20) or soluble receptor (21) leads to an impaired humoral response. Mice bearing a targeted deficiency at the C2r locus (Cr2-/-) fail to generate a normal T-D response (22, 23). The defect lies at the B cell level as T cell responses appear to be normal (22, 19). CD21 and CD35 are encoded at a single locus (Cr2) (24, 25) in mice, and the distinct receptors are generated by alternative splicing (26, 27), where CD35 not only includes the CD21-binding site (C3d and iC3b) but also binds C4b and C3b (28). The two receptors are coexpressed on B cells and follicular dendritic cells (FDC), where they are involved in coreceptor signaling and antigen trapping, respectively (29, 30). On B cells, CD21 forms a signaling complex with CD19 and CD81 (Tapa-1) (31, 32). Coligation of the B cell antigen receptor and coreceptor by complexes of C3d-antigen lowers the threshold for B cell activation by 10- to 100-fold (33). These studies have shown that humoral responses to inert antigens in the blood are dependent on complement. However, it was not clear whether humoral responses to antigens in peripheral sites and especially responses to infectious agents also would be affected by complement. Thus, to determine whether complement is important in the host immune response to viral infection at peripheral sites, we compared the immune response of mice deficient in C3, C4, or C2 with control mice after challenge with HSV-1 strain strains HD-2 and KOS1.1. We found that deficiency in either complement C3 or C4 or receptors CD21/CD35 resulted in an impaired memory response to the infectious virus.

Materials and Methods

Mice. Mice deficient in complement component C3 (C3-/-) (34), C4 (C4-/-) (19), or the complement receptors CD21/CD35 (Cr2-/-) (22) were all constructed by gene targeting and were maintained on a mixed C57BL/6 × 129/sv background as described. Studies involving the use of animals were performed in accordance with institutional guidelines. All animals received food and water ad libitum.

Viruses and Infections. Five- to eight-week-old C3-/-, C4-/-, or Cr2-/- or MHC and gender-matched control mice were injected

Abbreviations: FDC, follicular dendritic cells; GC, germinal center(s); HSV, herpes simplex virus; LN, lymph node; pfu, plaque-forming unit; T-D, thymus-dependent; wt, wild type.

To whom reprint requests should be addressed at the I address. E-mail: mcarroll@hcs.bwh.harvard.edu.

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with $2 \times 10^6$ plaque-forming units (pfu) of HSV-1 strain HD-2 or KOS1.1 (35) s.c. Mice received a second and third injection at weeks 3 and 6 with an equivalent dose of virus. Subcutaneous injections were delivered in the right rear flank near the base of the tail.

The ability of HSV to replicate in C3 deficient and control mice was determined by infecting mice ocularily with HSV-1 strain KOS1.1. Each mouse received $2 \times 10^6$ pfu of virus per eye after corneal scarification. Virus shed in tear film was collected by eye swab into modified PBS (36), stored at $-80^\circ$C, and titered by serial dilution plaque assay on Vero cells.

ELISA and T Cell Proliferation Assays. Mice were bled weekly, and $\beta$-galactosidase- and HSV-specific antibody titers were determined by ELISA. Briefly, 2-fold serial dilutions of sera were added to 96-well plates coated with a solution of $\beta$-galactosidase (50 $\mu$g/ml in PBS) (Sigma) or HSV-1 (10 $\mu$g/ml in PBS) (Advanced Biotechnologies, Columbia MD) and serum antibody levels were detected with alkaline phosphatase-conjugated secondary antibody. Plates were read in an ELISA reader at an OD of 405 nM. Antibody titer represents the final dilution, which resulted in above-background optical density. The T cell response was measured as described (19). Briefly, CD3+ T cells were isolated from inguinal lymph nodes (LN) s a week after the third infection with recombinant virus (HD-2). B cells were removed by treating cell suspensions with anti-B220 antisera and rabbit complement. The CD3+ T cells and peritoneal macrophages (serving as antigen-presenting cells) were cultured for 3 days with varying concentrations of antigen (1–50 $\mu$g HSV-1 lysate or $\beta$-galactosidase), and T cell proliferation was determined by uptake of [3H]thymidine.

Germinal Center Response. Inguinal LN were harvested 1 week after the final immunization and quick-frozen in OCT (Miles). Cryosections were prepared and analyzed by immunohistochec- nal staining as described previously (22). Sections were rehydrated and treated with antibodies specific for B220 (PharMin- gen) and with peanut agglutinin, which identifies germinal center B cells.

Results

Mice Deficient in Either C3 or C4 Have Diminished Antibody Responses to HSV and $\beta$-Galactosidase. C3- and C4-deficient mice as well as littermate wild-type (wt) controls were infected with a replication-defective HSV-1 strain, HD2, a mutant virus that expresses the heterologous antigen, $\beta$-galactosidase. Mice received two equivalent doses of virus ($2 \times 10^6$ pfu) s.c. at days 0 and 21 and were bled weekly to determine serum antibody titers. This dose of virus had been shown previously to induce a strong Th1 memory response in BALB/c mice characterized by enhanced production of IgG2a isotype (36). As expected, wt controls expressed specific IgG2a to HSV-virion antigens (Fig. 1A). In contrast, mice deficient in either C3 or C4 had an impaired secondary humoral response to the viral antigens. The levels of HSV-specific antibodies were 5- to 6-fold lower in complement-deficient mice compared with control littermates 1 week after challenge, i.e., $14,208 \pm 3,430$ and $10,528 \pm 3,806$ vs. $63,787 \pm 6,529$, respectively. This effect was much more pronounced after the second infection although some differences in the primary responses between complement-deficient and wt mice were apparent. The finding that C4$^{-/-}$ mice have an impaired response similar to that of C3$^{-/-}$ mice identified the classical pathway as important in humoral response. This was consistent with previous studies involving antigens inoculated i.v.

Gr2$^{-/-}$ Mice Fail to Generate a Memory B Cell Response to HSV. Previous studies had suggested that C3 enhances the humoral response to antigens inoculated i.v. by localizing it to complement receptors CD21/CD35 expressed on splenic B lympho- cytes and FDC (29, 30, 37). To examine the importance of CD21/CD35 in humoral responses to infectious virus administered at peripheral sites, C3$^{-/-}$, C4$^{-/-}$, or wt age- and gender-matched mice were inoculated s.c. or intradermally with $2 \times 10^6$ pfu of recombinant virus strain HD-2 at days 0 and 21. Significantly, the generation of memory responses in C3$^{-/-}$ and C4$^{-/-}$ mice similarly were impaired on secondary immunization at week 3 with either dose (Fig. 1B). As a further measure of the humoral response to live virus, the IgG response to $\beta$-galacto- sidase was measured in C3$^{-/-}$, C4$^{-/-}$, and wt mice (Fig. 1C). Wt mice produced a relatively strong secondary response 1–2 weeks after challenge with recombinant virus. In contrast, both C3$^{-/-}$ and C4$^{-/-}$-deficient strains failed to respond significantly above background.

Strain HD-2, though infectious, is replication-defective. It was possible that viral uptake and spread could overcome the defect in complement. To test the requirement for functional complement to a replication-competent form of the virus, mice deficient in C3 or C4 were injected with $2 \times 10^6$ pfu of wt HSV-1 strain KOS1.1 at days 0 and 21 and the mice were bled weekly. Characterization of the serum antibody response after primary and secondary inoculation with live virus revealed that both C3- and C4-deficient mice had an impaired response compared with wt controls, i.e., mean titers at week 5 were 13,536 $\pm$ 1,434, 10,304 $\pm$ 2,664, and 72,213 $\pm$ 7,158, respectively (Fig. 1D). Thus, the early, classical pathway of complement is important in formation of a B cell memory response to both replication-competent and replication-defective viruses.

Defective Antibody Responses in C3- and C4-Deficient Mice Are Not Due to a Failure to Prime T Cells. The ability of mice to mount a normal humoral response against HSV infection requires a functional T helper response. Thus, the defective humoral response in the complement-deficient mice could have been due to a defect in efficient priming of CD4+ T cells. To address this issue, we examined T cell priming in HD-2 immunized mice by a secondary T cell proliferation assay. LN cells were harvested from infected C3$^{-/-}$, C4$^{-/-}$, and wt mice 7 days after a third injection of virus and enriched for CD4+ and CD8+ cells by

Table 1. Impaired GC response in C3$^{-/-}$ mice infected with HSV-1 strain HD-2

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>No. of LN</th>
<th>No. of follicles</th>
<th>No. of GC</th>
<th>No. of GC per follicle</th>
</tr>
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<tbody>
<tr>
<td>Wt immune</td>
<td>5</td>
<td>3.6 ± 0.5</td>
<td>12.4 ± 3.5</td>
<td>7.2 ± 1.8</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td>C3$^{-/-}$ immune</td>
<td>4</td>
<td>3.5 ± 0.6</td>
<td>9.0 ± 1.8</td>
<td>1.0 ± 0.8*</td>
<td>0.1 ± 0.08*</td>
</tr>
<tr>
<td>Wt nonimmune</td>
<td>3</td>
<td>4.0 ± 0.5</td>
<td>17 ± 6.8</td>
<td>1.3 ± 1.1</td>
<td>0.07 ± 0.07*</td>
</tr>
</tbody>
</table>

Draining LNs (inguinal and popliteal) were harvested from wt and C3$^{-/-}$ mice day 7 after final infection with $2 \times 10^6$ pfu of HD-2. Follicles were enumerated based on staining with B cell-specific antibody (B220). GC were identified by treating sections with PNA, which stains GC B cells.

*Statistical comparison of the number of GC counted for wt immune vs. C3$^{-/-}$ immune; P < 0.0005.
†Statistical comparison of the number of GC per follicle for wt immune vs. C3$^{-/-}$ immune; P < 0.00005.
‡Statistical comparison of the number of GC per follicle for wt immune vs. wt nonimmune; P < 0.001.
§Values represent mean ± SD.
negative selection of B220<sup>−</sup> cells. Analysis of LN cells after 5 days of culture in the presence of HSV lysate at various concentrations (1–50 μg) revealed a similar level of T cell proliferation between the complement-deficient and wt controls (Fig. 2). Similarly, no difference was observed between the three groups at 1 and 50 μg of β-galactosidase. However, at 5 μg of antigen, a significant reduction in T cell uptake of [³H]thymidine was observed in the C3<sup>−/−</sup> and Cr2<sup>−/−</sup> mice (Fig. 2).

C3-Deficient Mice Have Reduced Numbers of Germinal Centers After Virus Infection. A hallmark of the B cell memory response is the formation of germinal centers (GC) within the lymphoid follicles. The GC reaction represents a major site for B cell clonal selection, affinity maturation, and formation of memory cells (38, 39). Previous studies reported that activation of complement C3 (19) and expression of its receptor (CD21/CD35) (22, 23) were critical for GC formation as the number and size of GC were reduced in deficient mice after the second challenge with inert, noninfectious antigens. To dissect further the defect in the humoral response to HSV in C3-deficient mice, inguinal LNs were harvested (7 days after final injection of virus) and quick-frozen in OCT, and cryosections were prepared. Three-color immunohistochemical analysis of cryosections stained with PNA, B220, and CD3 revealed an increase in the number of GC in follicles of infected wt mice as expected (Fig. 3A and C; Table 1). However, an approximate 5-fold reduction in the frequency of GC was observed in C3<sup>−/−</sup> mice, i.e., 0.6 ± 0.06 vs. 0.1 ± 0.08, P < 0.05 × 10⁻³, respectively (Fig. 3 B and D; Table 1). The enhancing effect of C3 in GC formation was most likely mediated via complement receptors CD21/CD35 as found with model antigens. Thus, C3-deficient mice fail to form a normal GC reaction, consistent with an impaired secondary humoral response.

Complement Does Not Appear to Be Essential for Initial HSV Infection. The lack of a humoral response in complement-deficient mice possibly could be because of resistance of these mice to HSV infection. To examine the ability of replication-competent HSV to infect C3<sup>−/−</sup> and control mice, HSV-1 strain KOS.1 wt virus was inoculated after corneal scarification onto mouse eyes. Virus replication was determined by assaying levels of virus shed in tear film collected daily for 1 week postinfection. Both mutant and wt (Fig. 4) shed comparable levels of virus. Thus, C3 deficiency did not impair the ability of the mutant mice to support HSV replication, at least at this site.
Discussion

In this study, we examined the role of complement in the humoral response to antigens introduced at a peripheral site, i.e., antigens expressed by herpes virus after s.c. inoculation. As with inert or noninfectious T-D antigens introduced into the bloodstream, mice deficient in C3 or C4 had a significantly reduced antibody response to viral-encoded antigens compared with similarly infected wt mice. A similar lack of humoral response to HSV-1 infection was observed with mice deficient in CD21/CD35. These mice also failed to develop humoral responses to the heterologous, viral-encoded antigen, β-galactosidase. Examination of CD4+ T cell proliferation identified equivalent responses in mutant and wt mice except at an intermediate dose (5 μg) of β-galactosidase. Thus, as observed previously for antigens introduced in the bloodstream (19, 22), the lack of a humoral response to viral antigens at this dose was not due to an obvious impaired priming of T cells, although it is possible that T cell priming to lower doses of virus could be enhanced in the presence of complement. In this study we used an optimal dose of 2 × 10^6 pfu; however, we have found that complement is also important for memory B cell responses at doses 10- and 100-fold less (results not shown). Although unlikely, one explanation for the lack of a humoral response was resistance to HSV infection by the C3-deficient mice. To address this possibility, we examined acute replication of wt HSV-1 in an ocular infection model. No differences were apparent for viral replication in wts. C3−/− mice throughout the 6-day period in which virus shedding is usually observed. Thus, we consider it very unlikely that the mutant mice are resistant to HSV infection.

Complement enhancement of the antiviral response is mediated by the classical pathway (C4-dependent) and receptors CD21/CD35. Initial recognition of viral antigens most likely is explained by preexisting natural IgM as reported for vesicular stomatitis virus (40, 41), but other recognition proteins such as mannan-binding lectin could be involved. In normal mice, CD21/CD35 are expressed primarily on FDC and B cells, where they function to enhance uptake of antigen and signal transduction via the CD21/CD19/CD81 coreceptor, respectively. The reduction in number of GC observed in C3−/− mice suggests that

![Fig. 2.](image)

![Fig. 3.](image)

![Fig. 4.](image)
at least one defect lies in the absence of signaling via the coreceptor. Studies in chimeric mice constructed such that CD21/CD35 expression is differentially expressed on FDC or B cells identify a critical role for the coreceptor expression (22, 42). The CD21/CD19/CD81 coreceptor not only lowers the threshold of B cell activation in vitro (30) and in vivo (43) but also provides an important survival signal for B cells (43). Based on these earlier studies, it seems probable that the defect in the antiviral response lies both in failure to lower the threshold for initial B cell activation and survival within the GC. Thus, in the absence of CD3 viral complexes or coreceptor expression, viral-specific B cells were not stimulated sufficiently to develop into memory cells. Likewise, mice deficient in CD19 fail to form normal GC or a memory response to inert haptenated T-D antigens (44–46). Interestingly, CD19−/− mice do form GC and make a substantial IgG antibody response to infectious vesicular stomatitis virus (VSV) (47). Although, specific antibody levels do not persist and the memory response is impaired. Thus, despite the presence of GC, B cell memory was not maintained in the infected CD19−/− mice. Interestingly, these same studies identified a role for C3 and CD21/CD35 distinct from that of CD19. CD19−/− mice transiently depleted of C3 or treated with antibody to CD21/CD35 had a more reduced IgM and IgG response to formalin-fixed VSV than did mice deficient in CD19 alone (47). It is most probable that localization of viral antigen to FDC is important for persistence of antibody responses and maintenance of long-term memory as proposed by Klau et al. (37) and supported by recent studies in mice chimeric for CD21/CD35 (48).

Our findings support the “instructional model” of Fearon and Locksley (49), which shows that the complement system directs or instructs the B cell response to pathogens. They also support the general use of complement activation fragment C3d as a natural adjuvant in the development of viral vaccines as proposed by Dempsey et al. (50). In their elegant study, it was demonstrated that attachment of multimers of C3d to hen lysozyme enhanced the humoral response by as much as 10,000-fold. Given the importance of complement in humoral immunity, it would be advantageous for pathogens to evade complement activation. Indeed, HSV expresses a surface protein (gC) that inactivates C3b deposited on the viral surface (10). Significantly, other viruses, bacteria, and yeast express complement inhibitors that block complement activation at the C4b and C3b stages (5, 51). For example, cells infected with vaccinia virus secrete a protein, vaccinia virus complement control protein (VCP), that inhibits complement activation (52). Thus, expression of complement activators by viruses might represent an important protective mechanism to avoid host detection and induction of a vigorous IgG memory response.

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