MAJOR ARTICLE

B Cell Memory Is Directed toward Conformational Epitopes of Parvovirus B19 Capsid Proteins and the Unique Region of VP1

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Background. Loss of antibody reactivity against linear epitopes of parvovirus B19 (B19) capsid proteins VP1 and VP2 occurs after infection; however, it is unclear whether B cell memory is established against linear epitopes.

Methods. B cell enzyme-linked immunospot assay was used to evaluate B19-specific B cell memory in volunteer donors ($n = 22$).

Results. B cell memory is maintained against conformational epitopes of VP2 and is absent against linear epitopes of VP2. Individuals seronegative for IgG against the unique region of VP1 have detectable B cell memory, with the potential to mount a humoral response on reexposure to B19. Conversely, in mice immunized with VP2, long-lasting IgG against linear epitopes of VP2 and a strong B cell–memory response are observed.

Conclusions. B cell memory is established and maintained against conformational epitopes of VP2 and against linear epitopes of VP1 but not against linear epitopes of VP2. These findings further our understanding of the immune response to B19 and suggest that analysis of B19-specific B cell memory merits consideration for future B19-vaccine studies.

Parvovirus B19 (B19) is a small, nonenveloped erythrovirus that usually causes a mild, self-limiting disease (called "erythema infectiosum") in children. However, immunocompromised patients (such as patients with HIV or patients with cancer who are undergoing chemotherapy), pregnant women, and, increasingly, transplant recipients are at significant risk for B19 infection [1, 2]. Many such individuals are unable to produce neutralizing antibodies to clear the virus, and this can lead to a persistent infection resulting in anemia [1]. It is significant that many blood-product companies have begun to implement screening protocols to minimize the B19 viral load in such blood-derived products as packed red blood cells, albumin, and intravenous immunoglobulin [3].

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B19 encodes 3 main proteins: a nonstructural protein, NS1 (77 kDa), and 2 structural proteins, VP1 (83 kDa) and VP2 (58 kDa) [4]. The latter 2 proteins, which compose the viral capsid (5% VP1; 95% VP2), are colinear, except that VP1 contains a 227-residue amino-terminal extension (hereafter, "unique region"), compared with VP2 [5]. A transient, high viremia is observed in individuals after infection with B19, followed by the development, within a number of days, of B19-specific IgM [6]; within a number of weeks, the presence of B19-specific IgG directed against linear and conformational epitopes of viral capsid proteins VP1 and VP2, in addition to the presence of IgG directed against NS1, is evident [3, 7, 8]. B19 infection leads to an intriguing immunological phenomenon—a specific loss of IgG, initially reactive against linear epitopes of VP1 and VP2, occurs after infection [8, 9]. In fact, specific IgG against linear epitopes of VP2 disappears within 6–12 months after infection [8]. More dramatically, there is a near-total diminution of NS1-specific IgG in previously infected individuals [10]. It is, therefore, firmly established that detection of B19-specific IgG against conformational epitopes is currently the most reliable method for the detection of previous infection with, and probable immunity to, B19. However,

the reason for the postinfection loss of IgG reactivity against linear epitopes of B19 capsid proteins has not yet been elucidated, and few mechanisms that would explain this loss have been suggested [8].

Recently, considerable effort has been directed toward the elucidation of the nature of the T cell response to B19 and toward the definition of T cell epitopes, yet the nature of B cell memory directed against B19 remains unclear [11, 12]. Memory B cells make a significant contribution to protective immunity and are characterized in terms of (1) a rapid proliferative response, accompanied by cellular differentiation after antigen reexposure, to produce affinity-matured, antibody-secreting plasma cells; (2) a lower activation threshold, compared with that of naive B cells, in response to cytokine and antigen; and (3) an absence of spontaneous immunoglobulin secretion. Recently, the identification of antigen-specific memory B cells against vaccine antigens (diphtheria and tetanus), whereby isolated peripheral-blood mononuclear cells (PBMCs) were nonspecifically stimulated by bacterial antigens and interleukin-2 (IL-2), followed by the specific detection of antigen-specific memory B cells, has been described [13]. This approach may also prove to be useful in the elucidation of virus-specific B cell–mediated immunity.

Although B19 linear epitope–specific IgG is absent in previously infected individuals, it is currently unclear whether B cell–memory response is ever established against linear or conformational epitopes of B19 proteins in infected individuals. Furthermore, in the absence of reliable cell-culture techniques for the detection of B19, no method is currently available for the confirmation of either past exposure to the virus or serology results for plasma cell–derived B19-specific IgG. Here we describe the investigation of B cell memory against B19, an investigation using a B cell enzyme-linked immunospot (ELISpot) assay to enumerate circulating B cells that are specific to either conformational or linearized B19 capsid proteins. This technique could also be used to define the immune status achievable with candidate B19 vaccines and to aid in the design of immunization protocols that adequately induce mature immune responses and so ensure long-lasting protection. Data are presented that further our knowledge of B cell memory directed against specific viral antigens; the use of a B19-specific B cell ELISpot assay offers the possibility that past exposure to, and infection with, B19 can be confirmed.

SUBJECTS, MATERIALS, AND METHODS

Study population. Heparinized venous-blood samples (10 mL; $n = 22$) were drawn from a cohort of healthy adult volunteers (mean age, 26 years; age range, 21–41 years) and were processed $\leq$2 h after collection. Prior to the collection of samples, consent was obtained from all volunteers, and approval was obtained from the ethics committee of the National University of Ireland, Maynooth. The human-experimentation guidelines of the authors' institution were followed in the conduct of this research, as were the animal-experimentation guidelines of the government of Ireland's Department of Health and Children.

Antigens. B19 recombinant proteins VP1, VP2, and NS1 were produced in, and purified from, *Spodoptera frugiperda* (Sf9) insect cells infected with recombinant baculovirus encoding either VP1, VP2, or NS1 open-reading frames, as described elsewhere [10, 14–16].

Antibody assays. Plasma obtained from blood samples was used to evaluate B19-specific IgG reactivity to the viral capsid proteins. B19-specific IgG against native VP2 (VP2-N) was determined by a commercial EIA (Biotrin International) and was quantified using the World Health Organization B19-specific IgG international-standard preparation (code 93/724; National Institute for Biological Standards and Control). IgG reactivity against denatured VP1 (VP1-D) and denatured VP2 (VP2-D) was determined by EIA formats developed in-house, as described elsewhere [16]. In brief, microtiter plates (Nalge Nunc) were coated overnight at 4°C with either VP1-D or VP2-D. The microtiter plates were then washed and blocked before being incubated with plasma samples that had been diluted 1:100 in PBS containing 0.05% (v/v) Tween-20 (PBST); the plates were then incubated with horseradish peroxidase (HRP)–conjugated anti–human IgG (Dako) for 30 min. After a wash step (4 washes with PBST), tetramethylbenzidine substrate was added to the wells, which were then incubated for 10 min. The enzymatic color reaction was terminated by adding 1 N H_2SO_4. The absorbance was read at 450 nm and at 630 nm. Immunoassay cutoffs were defined as the absorbance plus 2 SDs greater than the mean absorbance, as determined from a panel of B19-specific IgG–negative specimens. Results are expressed as index values (IV), calculated as specimen absorbance divided by cutoff absorbance (IV <1, B19-specific IgG negative; IV >1.0, B19-specific IgG positive).

Avidity assays. The avidity of B19-specific IgG antibody against VP1 and VP2 was determined in the same manner as was used in the antibody assays described above, but with a modification of the wash step. Microtiter plates were coated as described above and were then incubated with plasma samples in duplicate on 2 separate plates. After being incubated, the plates were washed differently to determine avidity; one plate was washed with 4 M of urea and PBST for 5 min, whereas the other plate was washed with only PBST for 5 min. Both plates were then washed twice with only PBST for 5 min. Antigen-bound IgG was detected as described above, by use of HRP conjugate. The avidity index (AI) was calculated as the ratio of the absorbance obtained for the plate washed with urea and PBST and that obtained for the plate washed with only PBST;

AI is expressed as a percentage. High and low AIs are indicative of past and recent infection, respectively.

Isolation of PBMCs and stimulation of cells. PBMCs were isolated by density-gradient centrifugation, as described elsewhere [17]. PBMCs (5×10^6 cells/mL) were cultured for 5 days in complete RPMI 1640, in the presence of heat-killed *Staphylococcus aureus* Cowan strain I cells (SACs) (Calbiochem) diluted 1:5000 and of 10 ng of IL-2/mL (Serotec). SACs and IL-2 jointly function to induce generalized antibody production in resting memory B cells [13].

B cell–memory ELISpot assay. After culture, cells were washed and quantified by ELISpot assay using a modification of a protocol described elsewhere [13]. Nitrocellulose-lined 96-well plates (Millipore) were coated with either VP2-N (10 μg/mL), VP2-D (10 μg/mL), VP1-D (10 μg/mL), or rabbit anti–human IgG (10 μg/mL; Dako) in 50 mmol of sodium carbonate buffer/L (pH 9.6) overnight at 4°C. Rabbit anti–human IgG facilitated detection of the total number of IgG-secreting B cells for each donor, to ensure that memory B cell activation had occurred. Plates were washed with PBS and then were blocked with 20% (v/v) fetal calf serum in RPMI 1640. After stimulation, cells (at concentrations of 1×10^6, 1×10^5, and 1×10^4 cells/well) were added to the plates, incubated in complete RPMI 1640 for 18 h at 37°C, and then removed by washing with PBS. Secreted IgG was detected using biotinylated rabbit anti–human IgG (1 μg/mL) and subsequent use of streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich) at a dilution of 1:1000 (1% [w/v] bovine serum albumin in PBS). Spots were developed by the addition of 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride substrate (Sigma-Aldrich) to the wells and were counted after 15 min by light microscopy. The numbers of spots in triplicate wells were averaged, and resultant data were reported as the mean number of spot-forming cells (SFCs) per 1 million starting cells (SCs).

Murine serology and B cell–memory response. BALB/c mice were immunized intraperitoneally with either 50 μg of VP1-D ($n = 3$) or 50 μg of VP2-N ($n = 3$) in Freund's complete adjuvant (Sigma-Aldrich). The murine IgG response was analyzed at weeks 6 and 32 after immunization, by the antibody assays described above for the human antibody response, except that an anti–mouse IgG–HRP conjugate (Bio-Rad Laboratories) was used. Murine B cell ELISpot assays were also performed on isolated spleen cells taken from individual mice, according to the protocol described above, except that (1) goat anti–mouse IgG (Sigma-Aldrich) was used to capture total B cell–derived IgG and (2) biotinylated rabbit anti-mouse IgG (1 μg/mL) was used to detect secreted IgG.

Statistical analysis. Frequencies of the level of SFCs obtained from the ELISpot assays were subjected to comparative analysis by Student's *t* test; comparison of antibody data with ELISpot results was assessed by Pearson's correlation; titers of mouse antibody were compared by a repeated-measure analysis of variance. The statistical-software package GraphPad InStat was used for all analyses.

RESULTS

The present study was designed to investigate whether B cell memory is established against B19. It can be seen from figure 1 that all individuals who were seropositive for B19-specific IgG ($n = 17$; table 1) exhibit strong evidence of B cell memory, whereas individuals who were seronegative for B19-specific IgG ($n = 5$; table 1) do not appear to harbor any significant level of B19-specific memory B cells. In fact, the difference between the level of SFCs in seropositive individuals and that in seronegative individuals was statistically significant for both VP2-N and VP1-D ($P = .003$ and $P = .009$, respectively); a total of 15 seropositive individuals exhibited B19-specific memory (mean ± SD, 87 ± 154 SFCs/1 million SCs; range, 7.8–606 SFCs/1 million SCs) against VP2-N, and 12 seropositive individuals exhibited B19-specific memory against VP1-D (mean ± SD, 69 ± 110 SFCs/1 million SCs; range, 4.4–406 SFCs/1 million SCs) (figure 1). There was no statistically significant difference between the number of memory B cells directed against VP1-D and that directed against VP2-N ($P = .2$); however, the greatest number of SFCs were specific to VP2-N and were evident in 2 individuals (S008, mean ± SD, 222 ± 16.4 SFCs/1 million SCs; S014, mean ± SD, 606 ± 69 SFCs/1 million SCs). B cell memory could not be detected in the absence of IL-2 and SAC stimulation prior to ELISpot analysis (data not shown); neither was there evidence of memory directed against NS1 in 5 individuals (S008, S009, S010, S011, and S012) seropositive for B19-specific VP2 IgG, all of whom exhibited B cell memory against either VP1-D or VP2-N.

When the IgG avidity of the 5 specimens (S003, S008, S010, S013, and S014) positive, by EIA, for B19-specific IgG against VP1-D were analyzed, a statistically significant positive correlation ($r = 0.96$) was found between the level of SFCs and avidity. Such a correlation was not observed for VP2-N–specific avidity ($r = 0.27$); neither was there a correlation between the level of SFCs and the endpoint titer of B19-specific IgG (data not shown).

Although 3 individuals (S003, S008, and S012) had circulating VP2-D IgG (detectable by EIA in all 3 [table 1] and by Western blot in S003 only), there was no evidence of any significant B cell memory against linear epitopes of VP2 (figure 1). Furthermore, the level of memory B cells observed in this cohort was not significantly different from that observed in seronegative individuals ($P > .999$). Induction of memory against epitopes of the B19 capsid proteins is, therefore, confined to 2 regions—linear epitopes in the unique region of VP1 and conformational epitopes present on VP2-N. VP1 cannot

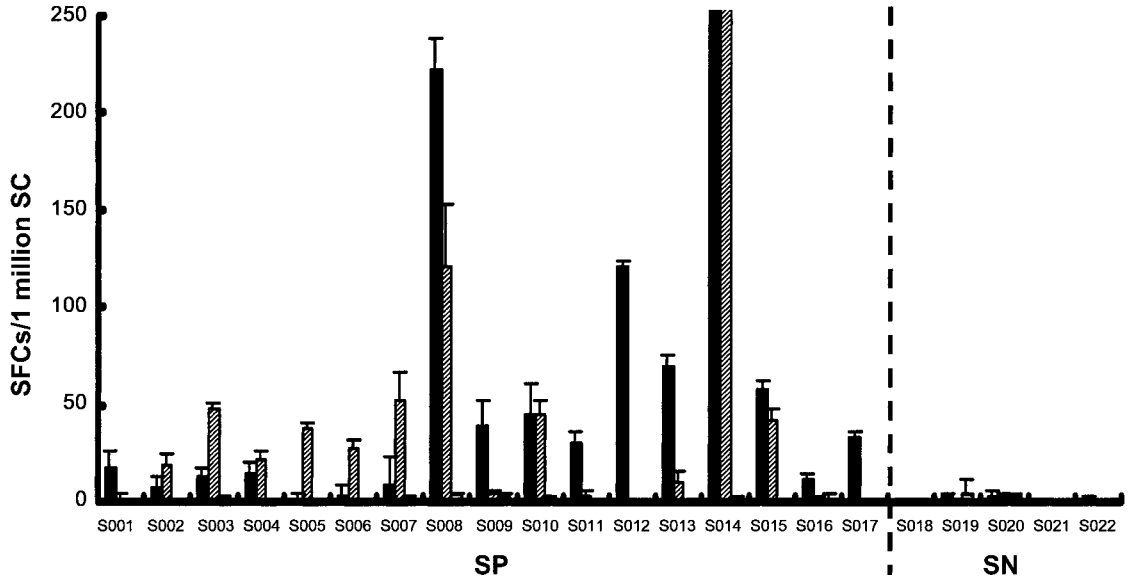

Figure 1. Human parvovirus B19 (B19)–specific B cell memory in blood samples obtained from seropositive (SP) and seronegative (SN) volunteer donors. Results of B cell memory are expressed as B19-specific spot-forming cells (SFCs)/1 million starting cells (SCs) and are the mean ± SD of triplicate wells. ELISpot microplates were coated with either native VP2 *(black bars),* denatured VP1 *(diagonally shaded bars),* or denatured VP2 *(horizontally shaded bars).*

be purified in a conformationally intact form, and so no information with respect to the conformational B cell epitopes of this protein is available at present.

To test the hypothesis that there is a species-specific difference between the antigenicity of linear and conformational epitopes of the B19 capsid proteins and to explore the appropriateness of animal-model systems in B19 immunogenicity studies, a murine model was employed. The murine B19-specific IgG response against either VP1 ($n = 3$) or VP2 ($n = 3$) was analyzed at weeks 6 and 32 after immunization. Equally strong responses were observed against both linear (endpoint titer, $\log_{10}$ 4.0) and conformational (endpoint titer, $\log_{10}$ 4.6) epitopes of VP2 at week 6 (figure 2*A*); the antibody response against linear epitopes showed no evidence of waning, because analysis of VP2-specific IgG reactivity at week 32 revealed no statistically significant difference ($P = .1$) between conformational epitopes and linear epitopes (VP2-N endpoint titer, $\log_{10}$ 4.9; VP2-D endpoint titer, $\log_{10}$ 4.2). In addition, there was equally strong immunoreactivity against VP1-D at week 6 (endpoint titer, $\log_{10}$ 3.9) and at week 32 (endpoint titer, $\log_{10}$ 4.1) (figure 2*A*).

Similar results were obtained in murine B cell ELISpot analysis. However, unlike the human response to B19 infection, where there was no evidence of memory against linear epitopes of VP2, in the murine response there was strong B cell memory against both the conformational and linear epitopes of VP2 (mean ± SD, 103 ± 55 and 129 ± 11 SFCs/1 million SCs, respectively) at week 32 after immunization (figure 2*B*). B cell memory against VP1-D was also strongly induced (mean ± SD, 104 ± 6 SFCs/1 million SCs) and was not significantly different ($P = .8$) from the response induced against VP2-N. These data indicate a strong, long-lasting, intrinsic antigenicity of the B19 capsid proteins that is directed against both the conformational and linear epitopes.

DISCUSSION

The present study is the first to demonstrate B cell memory against B19 VP2 and against the unique region of VP1. We have shown that B cell memory is maintained against the unique region of VP1, even in the absence of any detectable antibody against the unique region. In addition, we have shown a statistically significant positive correlation ($r = 0.96$) between the level of antibody directed against the unique region of VP1 and the level of SFCs. Finally, whereas B cell memory is not maintained against linear epitopes of VP2, we have shown that, in immunized mice, B cell memory does persist against linear epitopes of VP2. This result suggests that interaction between VP2 itself and the human immune system (or, conceivably, between other B19 proteins [e.g., NS1] and the human immune system) during infection may be responsible for the absence of B cell memory against linear epitopes of VP2.

IgG reactivity against conformational epitopes of VP2 is now accepted as the most reliable indicator of past infection with B19 [9, 18, 19]. In the present study, 88% (15/17) of individuals demonstrating IgG reactivity against VP2-N exhibited B cell memory against VP2-N, whereas only 71% (12/17) showed evidence of B cell memory against the unique region of VP1. The absence of detectable B cell memory against linear epitopes of VP2 was quite marked, especially since 18% (3/17) of se-

Table 1. Summary of donor serology: IgG response, in plasma obtained from adult volunteers, against the parvovirus B19 (B19) capsid proteins VP1 and VP2, in native and denatured forms.

Donor (n = 22)	Titer of B19-specific IgG against VP2-N, IU/mL	Index value[a]	
		VP2-D	VP1-D
B19-specific IgG seropositive			
S001	90	0.5	0.5
S002	49	0.5	0.6
S003	122	2.3	1.2
S004	120	0.5	0.6
S005	118	0.5	0.3
S006	21	0.6	0.5
S007	18	0.4	0.4
S008	147	1.8	2.0
S009	27	0.4	0.4
S010	105	1.0	1.2
S011	43	0.6	0.6
S012	53	1.2	0.5
S013	68	0.6	0.8
S014	140	0.6	1.2
S015	160	0.2	0.8
S016	152	0.5	0.7
S017	38	0.2	0.3
B19-specific IgG seronegative			
S018	1	0.4	0.4
S019	1	0.5	0.7
S020	3	0.4	0.3
S021	2	0.4	0.3
S022	1	0.1	0.4

NOTE. VP1-D, denatured VP1; VP2-D, denatured VP-2; VP2-N, native VP-2.

[a] Index values are calculated as specimen absorbance divided by cutoff absorbance.

ropositive individuals exhibited IgG reactivity against linear epitopes of VP2. To our knowledge, the present study provides the first confirmation that B cell memory is never established against linear epitopes of VP2 and adds further credence to the concept that long-lived plasma cells and memory B cells are 2 separate forms of independently controlled immunological memory [20, 21]. A recent report of the enumeration, in the adenoids of children, of IgG memory B cells against 4 pneumococcal antigens has also shown a broad antigen-specific response, which suggests that certain antigens are more likely than others to elicit the establishment of B cell memory [22]. It is also likely that the B19-specific B cell ELISpot assay could find application in the confirmation of past infection with B19.

It is well established that the humoral immune response to B19 evolves gradually, from a population of antibodies with reactivity against both linear and conformational epitopes to one with predominantly conformational-epitope specificity [8, 9, 23, 24]. In the present study, although only 5 individuals exhibited, by either microplate immunoassay or Western blot, IgG reactivity against VP1-D, 12 individuals displayed a positive B cell–memory response against the unique region of VP1. It has been well documented that the unique region of VP1 exhibits characteristics associated with immunodominance, including the stimulation of ex vivo T cell proliferation and of cytokine secretion [16]. In addition, this region appears to elicit neutralizing-antibody responses more effectively than do other linear epitopes of B19 [3]—specifically, more effectively than the VP1 region corresponding to aa 60–100 [25]. Our analysis further reveals a statistically significant correlation between antibody avidity against the unique region of VP1 and the level of SFCs specific for VP1-D, indicating that the strength of the antibody response against the unique region of VP1 may be related to the induction of B cell memory. Conceivably, the level of reexposure to B19—or, perhaps, B19 persistence—could account for the different B cell responses to B19 infection in previously infected individuals.

Even though 3 of the individuals tested (S003, S008, and S012) displayed IgG reactivity against linear epitopes of VP2 and had high avidity (AIs, 80.7, 93.2, and 40.2, respectively),

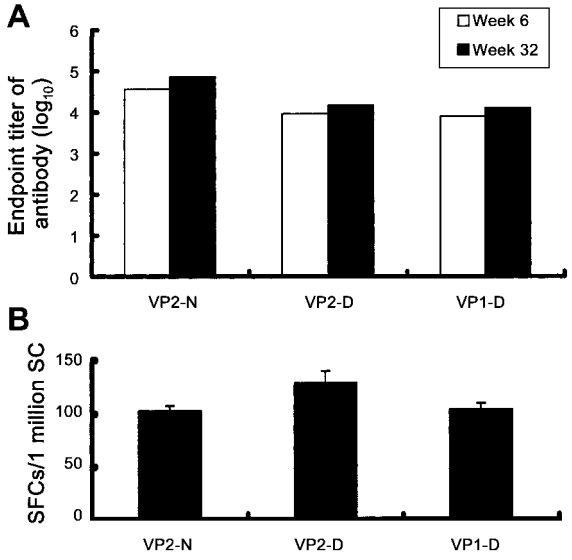

Figure 2. *A,* Murine IgG response to parvovirus B19 after a single intraperitoneal immunization with B19 VP1 and VP2 antigens. At weeks 6 and 32 after immunization, thoracic-blood samples were taken from mice and levels of IgG specific for native VP2 (VP2-N), denatured VP2 (VP2-D), and denatured (VP1-D) in serum were determined by ELISA. Results are expressed as the mean log_{10} endpoint titers of antibody, calculated by regression of the straight part of the curve of $OD_{450/630\ nm}$ versus serum dilution, to a cutoff of 2 SDs above background control values obtained from serum in naive mice. *B,* B19–specific B cell memory in immunized mice at week 32 after immunization. Results of B cell memory are expressed as B19-specific spot-forming cells (SFCs)/1 million starting cells (SCs) and are the mean ± SD of triplicate wells.

there was no evidence, by ELISpot assay, of memory against these VP2-specific epitopes. This phenomenon suggests that the observed IgG reactivity is a product of long-lived plasma cells that are established early in the response and is not a product of memory B cells, which are mostly established after undergoing selection in the germinal center [26]. The induction of memory after infection may favor conformational epitopes over linear epitopes because of simple stoichiometry [27]. Linear epitopes are less likely to be multivalent, thereby reducing B cell receptor cross-linking; this reduction, in turn, would lead to the imposition of a selective disadvantage in the competitive environment of the germinal center [28]. This phenomenon is exemplified in equine infectious anemia virus infection—although the initial antibody response is predominantly directed against linear determinants of the envelope protein, within 1–2 months after infection the IgG reactivity gradually progresses to a more conformationally dependent antibody population [29]. In addition, conformational epitopes are likely to exist on intact capsids, which, when internalized, are processed by specific B cells, resulting in the presentation of a broader range of peptides to specific T cells [16]. Therefore, even though the T cells recognize only linear peptides, assistance would be preferentially directed toward B cells that are specific for conformational or discontinuous epitopes. This situation is thought to occur in immunity against polioviruses, where B cell memory against conformational epitopes is central to protection but requires support by T cells that recognize peptides from either internal or external capsid proteins [30, 31]. On the other hand, there may be other explanations for this phenomenon; for example, it is known that strong immune-activation signals can induce lymphocyte apoptosis [32]. Linear epitopes of VP2 can elicit a strong initial IgG response, and it is conceivable that such stimulation could induce B cell apoptosis; however, this scenario seems unlikely, given that no loss of memory is observed in immunized mice. Alternatively, the lack of establishment of B cell memory against linear epitopes of VP2 could be due to the absence of a "maintenance" signal, essential to the maintenance of mature B cells, from a functional B cell receptor [33], although it is difficult to construct a scenario, beyond those described above, in which this would occur selectively.

Despite the absence of memory against linear epitopes of VP2 in any of the human specimens tested, in the murine model there was a strong B cell–memory response induced against VP2-D and VP1-D. The maintenance of high titers of antibody at week 32 after immunization (VP2-D endpoint titer, $\log_{10}$ 4.2; VP1-D endpoint titer, $\log_{10}$ 4.1) (figure 2*A*), in parallel with a high level of SFCs at week 32 for VP2-D (mean ± SD, 129 ± 11 SFCs/1 million SCs) (figure 2*B*), demonstrates that both VP1 and VP2 possess linear epitopes that are intrinsically immunogenic and that facilitate the establishment of B cell memory. It is interesting to note that the level of SFCs observed in the present study after mice were immunized with B19 antigens is comparable to that observed (50–100 SFCs/1 million SCs) when mice were naturally infected with lymphocyte choriomeningitis virus [34]. Although this analogous B cell–memory response in infection and in immunization is significant, it does not help explain why specific epitope reactivity is lost after natural infection in humans. Furthermore, these results illustrate the difference between the immunity generated by infection in the natural host, and the difficulty of using a murine model to assess immunogenicity. The different responses observed in the human and murine models may be partly explained by the fact that Freund's adjuvant is powerful and, therefore, may have induced stronger immunoreactivity to both linear and conformational epitopes than would be seen in natural infection. In addition, the route of delivery was different in each model: in the murine model, delivery was intraperitoneal (and, therefore, systemic), whereas the delivery was most likely mucosal in natural infection. Replicating virus may be responsible for inducing in humans host-specific cellular factors that cause specific B cell deletion and, therefore, antibody loss against linear regions of VP2. Because NS1 is a cytotoxic protein [35] that is capable of inducing apoptotic effects in the host [36], the possibility that NS1 might play a role in the loss of epitope specificity cannot be ruled out.

Initial B19-vaccine studies that have been performed in mice, guinea pigs, and rabbits [37] have focused on the level of circulating B19 antibody and its associated ability to neutralize the virus. Although a correlation has been observed between the development of antibody against the unique region of VP1 and neutralization of the virus [38], the differing natures of the human and murine immune responses to B19 antigens would suggest that caution be taken in the extrapolation and application of the results of nonprimate vaccine studies to humans. More recently, the in vitro efficacy of a candidate vaccine for B19 (MEDI-491; Medimmune) has been demonstrated; in a double-blind, phase 1 trial, 24 volunteers all produced neutralizing antibody after 3 immunizations [39]. Despite the development of a candidate B19 vaccine [37, 39], there has been no analysis of the potential role that memory B cells may play in either the efficacy of B19-subunit vaccines against infection or in the persistence of immunological protection. The B19-specific B cell ELISpot assay presented here would be an ideal ex vivo diagnostic tool for the evaluation of whether candidate B19-subunit vaccines have the capacity to establish B cell memory. Such an approach would be particularly important for understanding the interplay between individual titers of B19-specific IgG and B19-specific memory B cells, both to establish the relative importance of each parameter in combating B19 infection in immunized individuals and to define correlates of protection. It would also have important implications in the design of booster strategies, as has been the case in vaccine

evaluation for tetanus toxoid and diphtheria, where it has been concluded that the level of antigen-specific SFCs is an indirect measure of immunological memory [13].

To summarize, analysis of the induction of B cell memory after B19 infection shows that, although B cell memory is strongly maintained against conformational epitopes of the major capsid protein VP2, it is not maintained against its linear epitopes. Furthermore, although previously infected individuals may no longer have circulating IgG against the unique region of VP1, memory B cells persist and have the potential to mount a humoral response on reexposure to B19. Additional studies of selected patient cohorts (e.g., persistently infected individuals or individuals with a B19 infection of defined duration) should further elucidate the mechanism and extent of the absence of B cell memory against linear epitopes of B19 capsid proteins. The findings of the present study have implications for both the confirmation of B19 infection and the development of a vaccine to protect against B19 infection.

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