A Role for Complement in Feedback Enhancement of Antibody Responses by IgG3

Teresita Díaz de Ståhl,[1] Jörgen Dahlström,[1] Michael C. Carroll,[2] and Birgitta Heyman[1]

[1]*Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden*
[2]*Center for Blood Research, Harvard Medical School, Boston, MA 02115*

Abstract

IgG1, IgG2a, and IgG2b, passively administered with soluble Ags, enhance specific Ab responses. The effect of IgG3 in this type of feedback regulation has not been studied previously. We immunized mice with trinitrophenyl (TNP)-coupled carrier proteins (bovine serum albumin [BSA] or ovalbumin [OVA]) alone or complexed to monoclonal TNP-specific IgG3. The carrier-specific Ab responses were enhanced by several hundred-fold by IgG3. Enhancement was significantly impaired in mice depleted of complement factor C3 and in mice lacking complement receptors 1 and 2 ($Cr2^{-/-}$). In contrast, mice lacking the common Fc-receptor gamma chain ($FcR\gamma^{-/-}$), resulting in reduced expression of FcγRI and lack of FcγRIII, and mice lacking FcγRIIB ($Fc\gamma RIIB^{-/-}$), responded equally well to immunization with IgG3-complexed Ag as wild-type controls. These findings demonstrate that IgG3 can induce feedback enhancement and that IgG3, in analogy with IgM, uses the complement system for this function.

Key words: rodents • Fc receptors • cellular activation • transgenic/knockout

Introduction

IgG3 is the predominant IgG subclass in responses against T cell–independent type 2 antigens in mice whereas it constitutes a very low fraction of IgG responses to T cell–dependent protein Ags (1, 2). A similar situation is seen with human IgG2 which is considered to be the equivalent of murine IgG3 (3, 4). These isotypes have been postulated to play an important biological role in the defense against bacterial infections. In support of this, IgG3-deficient mice were shown to be more susceptible to pneumococcal sepsis than wild-type animals (5). IgG3 Abs are efficient activators of C, and although they were first believed to act only via the alternative pathway, it was recently shown that monoclonal anti-erythrocyte IgG3 can activate the classical pathway (6). Some biological functions of IgG3 are dependent on C activation, such as the protective effect against *Candida albicans* infections and the pathogenicity in hemolytic anemia (6, 7). Another possible effector pathway of IgG3 is via Fc-receptors (FcRs).* Early studies demonstrated that macrophages could phagocytose IgG3-coated particles and suggested the existence of a specific FcR for IgG3 (8). This receptor has been elusive, and for many years IgG3 was not believed to bind to any of the known murine Fc-receptors for IgG (FcγRI, FcγRIIB, or FcγRIII; reference 9). However, recent data suggest that FcγRI is the IgG3-binding receptor (10, 11).

Apart from mediating various effector functions, Abs have the ability to feedback regulate the production of themselves (for a review, see reference 12). Passively administered, or actively produced, specific Ab can enhance or suppress Ab responses to the Ag they bind to. Regulation is potent, frequently resulting in 99% suppression or several 100-fold enhancement, and only responses to determinants within the immune complex are affected. When TNP-specific IgG1, IgG2a, IgG2b, or IgE are administered together with soluble hapten-carriers, e.g. OVA-TNP, BSA-TNP, or KLH-TNP, the anti-carrier responses are enhanced (13–15). Enhancement by IgG2a and IgG1 takes place in the absence of C activation (16) and in $Cr2^{-/-}$ mice lacking CR1 (CD35) and CR2 (CD21; reference 17), but is severely impaired in $FcR\gamma^{-/-}$ mice (14). These mice lack the common signaling γ-chain used by both FcγRI and FcγRIII and do not express FcγRIII (18), although 1/5 of FcγRI was recently shown to remain (11). The data therefore suggest an important role for FcγRs in IgG1- and IgG2a-mediated enhancement. IgE-mediated enhancement is exclusively dependent on the low affinity

Address correspondence to Birgitta Heyman, Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden. Phone: 46-18-6113868; Fax: 46-18-55-89-31; E-mail: Birgitta.Heyman@genpat.uu.se

**Abbreviations used in this paper:* BCR, B cell receptor; CVF, cobra venom factor; FcR, Fc-receptor.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2003/05/1183/8 $8.00
Volume 197, Number 9, May 5, 2003 1183–1190
http://www.jem.org/cgi/doi/10.1084/jem.20022232

receptor for IgE, CD23 (15, 19, 20). Also, IgM can enhance Ab responses and this effect is dependent on C activation. Mutant monoclonal IgM that has lost its ability to activate C cannot enhance, normal IgM cannot enhance in C-depleted mice or in $Cr2^{-/-}$ mice lacking CD35 and CD35 (17, 21), and monomeric IgM, which cannot activate C, does not enhance (22). Murine CD21 and CD35 are alternatively spliced from the same Cr2 gene and knockout mice therefore lack both receptors (23, 24). CD21 associates with the signaling molecule CD19 in the B cell membrane and cocrosslinking of the B cell receptor (BCR) and CD21/CD19 lowers the threshold for B cell activation (25). $Cr2^{-/-}$ mice have poor Ab responses to suboptimal doses of various antigens (for a review, see references 23 and 24) and it was recently shown that IgG3 was the most severely affected isotype (26). These mice were more susceptible to *Streptococcus pneumoniae* infection than wild-type controls (26), a finding agreeing well with a role for IgG3 (5) and C3 (27) in defense against bacterial infections.

Whether IgG3, in addition to the isotypes discussed above, can act to enhance humoral responses has not been examined. We here report that TNP-specific IgG3 mAbs, administered in complex with BSA-TNP or OVA-TNP, dramatically increase the carrier-specific responses, sometimes >1,000-fold. IgG3 enhances Ab responses equally well in $FcR\gamma^{-/-}$ and $Fc\gamma RIIB^{-/-}$ as in wild-type control mice. In contrast, the capacity of IgG3 to enhance Ab production was markedly reduced in C3-depleted as well as in $Cr2^{-/-}$ mice, suggesting that IgG3, in analogy with IgM, enhances the Ab response via the C system.

Materials and Methods

Antigens. BSA (fraction V, A-3059), OVA (grade V, A-5553), and TNP (picrylsulfonic acid/hydrate) were from Sigma-Aldrich and KLH from Calbiochem. TNP was conjugated to BSA or OVA as described (28). The number of TNP residues/Ag molecule was 12 for BSA and 3 for OVA determined according to (29). SRBC from the National Veterinary Institute (Uppsala, Sweden), were conjugated to TNP (SRBC-TNP) as described (30).

Antibodies. 5-mo-old BALB/c mice (Bommice) were immunized in the tail vein with 4×10^8 SRBC-TNP in 0.1 ml PBS. 3 d before the fusion, mice were boosted intravenously with 4×10^8 SRBC-TNP and 10 μg LPS (Sigma-Aldrich) intraperitoneally. Splenocytes were mixed with the fusion partner Sp 2/0 in a ratio of 5:1 in PBS containing 50% polyethylene glycol 4000 (Merck), at 37°C for 1 min. The fusion mixture was slowly diluted with serum-free DMEM, spun down, and incubated for 15 min at 37°C. The cells were then washed and diluted in DMEM containing 10% heat-inactivated FCS, seeded out in 96-well microtiter plates (Nunc), and cultured overnight at 37°C together with 3×10^6 rat thymocytes per ml. Next day, HAT (Hypoxanthine-Aminopterin-Thymidine) medium (Sigma-Aldrich) was added and the cells were cultured in HAT until growing clones were established. Hybridomas were screened for production of IgG3 anti-TNP by ELISA. Positive hybridomas were expanded and cloned by limiting dilution and clones IM-F10 and IM-H11 were selected for further studies.

IgG3 anti-TNP (IM-F10 and IM-H11), IgG2a anti-TNP (C4007B4, 7B4; reference 13), and IgE anti-TNP (IgEL-A5, mouse IgE/κ; reference 31) were cultured in DMEM (4.5 g/liter glucose) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamin, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and 5% heat-inactivated FCS. IgG mAbs were purified on a protein A Sepharose column (Amersham Biosciences) and IgE on a Sepharose column coupled with monoclonal rat anti–mouse κ 187.1.10 as described (17). Abs were concentrated by Microsep® 10K concentrator (Pall Filtration Corp.) and the storage buffer was changed to PBS using a PD10 column (Amersham Biosciences). Protein concentrations were determined by absorbance at 280 nm, assuming that an absorbance of 1.5 equals 1 mg/ml of Ab. The subclasses of each IgG preparation were confirmed by subclass-specific ELISA. mAbs were sterile filtered and IgG2a and IgE stored at –20°C whereas IgG3 was used immediately or stored in liquid nitrogen.

Mice. BALB/c, CBA/J, and DBA/1 were from Bommice. H-2^b mice have an I-A^b-linked low responsiveness to IgE/BSA-TNP and IgG/BSA-TNP complexes (28, 32) and the knockout mice used in the present study were backcrossed to responder strains. $FcR\gamma^{-/-}$ mice (18) were backcrossed/intercrossed to DBA/1 (H-2^q) for five generations and offspring from homozygous mutant $FcR\gamma^{-/-}$/ H-2^q and wild-type $FcR\gamma^{+/+}$/H-2^q animals were used in experiments. $Fc\gamma RIIB^{-/-}$ mice (33) were backcrossed to CBA/J mice (H-2^k). The fifth generation was intercrossed and offspring from homozygous mutant $Fc\gamma RIIB^{-/-}$/ H-2^k and wild-type $Fc\gamma RIIB^{+/+}$/H-2^k mice were used in experiments. $Cr2^{-/-}$ mice (34), first backcrossed to H-2^k mice (17), were further backcrossed to DBA/1. Mice from the second generation were intercrossed and offspring from homozygous mutant $Cr2^{-/-}$/H-2^q and wild-type $Cr2^{+/+}$/H-2^q animals, identified by PCR analysis (described below), were used in experiments. Mice were bred and maintained at the Department of Genetics and Pathology, the Department of Animal Development and Genetics or at the Biomedical Center, Uppsala University.

PCR Reactions. The FcRγ genotype was analyzed according to (18), FcγRIIB as described (35), and H-2^k, H-2^b, and the Cr2 genotype according to (17). H-2^q was analyzed in a PCR reaction using two primers: αK1:2 (5′ TAT CAG TCT CCT GGA GAC ATT G 3′) and q2 (5′ GTC AAA GCT TGT CAA TTG GC 3′) resulting in a 128 bp band. Gene amplification was done in 20 μl 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTPs, 0.22 μM of primers, and 2.5 U AmpliTaq DNA polymerase (PerkinElmer) for 35 cycles (30 s, 94°C; 40 s, 54°C; 50 s, 72°C).

Immunizations. Mice were immunized in the tail vein with Ag alone or Ab/Ag complexes in 0.2 ml PBS. Complexes were formed by incubating TNP-conjugated Ag with TNP-specific mAb for 1 h at 37°C, immediately before the immunization. Non-crossreacting Ag (unconjugated OVA, BSA, or KLH) was included in the Ag mixture as a specificity control.

Cobra Venom Factor Treatment. Mice were injected intraperitoneally with four doses of 0.1 ml of 100 U/ml cobra venom factor (CVF) from *Naja haje* (Cordis Laboratories Inc.) during 24 h. Control groups received PBS in the same regimen. All mice were bled individually from the tail 7 d before the first and 2 h after the last CVF injection. C3 levels in sera were assayed by radial immunodiffusion using a polyclonal goat anti-mouse C3 antiserum (Cooper Biomedical, Inc.). Titers are expressed as the reciprocal of the highest dilution giving a precipitation line (e. g. dilution 1:27 = 27).

ELISA. Blood was collected from tail veins and sera were tested using IgG anti-BSA-, IgG anti-KLH, or IgG anti-OVA-

specific ELISAs (36). Statistical differences were determined by Student's *t* test. P values are presented as: not significant, NS; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***. Stimulation indices (SI) were calculated as the geometrical mean of the experimental group divided by the geometrical mean of the control group.

Hemolysis. Hemolytic titers are defined as the highest antibody dilution able to lyse a 0.25% suspension of SRBC-TNP_5 (prepared as described [30] but with a fivefold lower concentration of picryl sulfonic acid) in the presence of a 1:160 dilution of guinea pig serum in veronal-buffered saline (VBS). Alternative pathway activation was assayed in VBS with Mg-EGTA (37).

Results

Monoclonal TNP-specific IgG3 Enhances Ab Responses to BSA-TNP and OVA-TNP. To assess the ability of IgG3 to augment Ab production, mAbs IM-F10 and IM-H11 were administered to CBA/J mice together with BSA-TNP and OVA. Both mAbs were able to enhance the IgG anti-BSA responses (51- and 13-fold respectively) compared with control groups immunized with Ag alone (Table I, experiments 1 and 2). The response to OVA, included as a specificity control, was not affected by IgG3. Ab responses to BSA in two other strains of mice, BALB/c and DBA/1, were also efficiently enhanced by IgG3 (1344- and 316-fold, respectively; Table I, experiments 3 and 4). IM-F10 administered to BALB/c mice together with another Ag, OVA-TNP, induced an 81-fold enhancement of the OVA-specific response (unpublished data). Thus, two out of two TNP-specific IgG3 mAbs were able to specifically enhance Ab responses to BSA-TNP or OVA-TNP in three different mouse strains.

Impaired Ab Responses to IgG3/Ag Complexes in C3-depleted and $Cr2^{-/-}$ Mice. The hemolytic titer of purified IgG3 (1.8 mg/ml) in the presence of Ca^{2+} and Mg^{2+} (both required for C activation via the classical pathway) was 1:256 whereas the titer in the absence of Ca^{2+} (allowing only alternative pathway activation) was reduced to <1:4. This established that IM-F10 is an efficient activator of the classical pathway, confirming previous data (6).

We next examined whether IgG3-mediated enhancement was C dependent. Mice where the MHC-II I-A region is of the b haplotype are low-responders to IgE/Ag (28), IgG2a/Ag (32), and IgG3/Ag (unpublished data). Available C3 and C4-deficient mice are on C57BL/6 and/or 129/Sv (H-2^b) backgrounds. Since the C4 locus is located within the MHC region, backcrossing to a responder MHC is impossible. The C3 locus is located outside, but close to, the MHC region, and backcrossing to a responder MHC is feasible, albeit complicated. An alternative approach is to deplete mice of C3 using CVF which acts by

Table I. *Enhancement of Ab Responses by Monoclonal IgG3 Ab*

			IgG anti-BSA			IgG anti-OVA		
Exp	Strain	Immunization	Log_{10} ng/ml ± SD (geom. mean)[a]	SI[b]	P[c]	Log_{10} ng/ml ± SD (geom. mean)[a]	SI[b]	P[c]
1	CBA/J	BSA-TNP + OVA	2.25 ± 0.35 (178)	1	c	1.64 ± 0.08 (44)	1	c
		BSA-TNP + OVA + 50 μg IgG3 (IM-F10)	3.96 ± 0.26 (9,076)	51	<0.001	1.54 ± 0.11 (35)	0.8	NS
2	CBA/J	BSA-TNP + OVA	2.20 ± 0.15 (160)	1	c	2.92 ± 0.19 (830)	1	c
		BSA-TNP + OVA + 50 μg IgG3 (IM-H11)	3.30 ± 0.52 (2,010)	13	<0.005	2.81 ± 0.11 (650)	0.8	NS
3	BALB/c	BSA-TNP + OVA	2.46 ± 0.40 (290)	1	c	1.98 ± 0.14 (97)	1	c
		BSA-TNP + OVA + 50 μg IgG3 (IM-F10)	5.59 ± 0.15 (389,814)	1,344	<0.001	1.74 ± 0.21 (54)	0.6	NS
4	DBA/1	BSA-TNP + OVA	2.41 ± 0.05 (259)	1	c	2.21 ± 0.12 (162)	1	c
		BSA-TNP + OVA + 50 μg IgG3 (IM-F10)	4.91 ± 0.43 (81,835)	316	<0.001	2.38 ± 0.07 (240)	1.5	NS

Groups of five mice were immunized intravenously with 20 μg BSA-TNP + 20 μg OVA, alone or together with 50 μg TNP-specific mAb IgG3, IM-F10 or IM-H11. 14 d after immunization, mice were bled and sera tested in ELISA.

[a]Mean of log_{10} ng/ml for IgG anti-BSA or IgG anti-OVA. Figures within parentheses represent geometrical mean (anti-log).

[b]SI, stimulation index, geometrical mean of the experimental group divided by the geometrical mean of the control group.

[c]P value vs. control value (c) as determined by Student's *t* test; NS, not significant ($P > 0.05$).

replacing C3b in the formation of alternative pathway C3 convertase resulting in CVFBb instead of C3bBb complexes. Whereas C3bBb is relatively labile, CVFBb has a half-life of several hours, resulting in transient C3-depletion (production is not affected; reference 38). Normal CBA/J mice were treated with CVF or sham-treated with PBS. C3 levels in CVF-treated animals were <5% of the titers in sham-treated mice (C3 titers ± SEM: 2.3 ± 0.25 vs. 49 ± 9.5, respectively). Both groups were immunized with BSA-TNP alone or in complex with 50 μg IgG3 (IM-F10) and tested for BSA-specific IgG. C3-depletion resulted in a significant reduction of responses to IgG3/BSA-TNP complexes (Fig. 1, A and B). In contrast, C3-depletion had no significant effect on responses to IgE/BSA-TNP, included as a control (Fig. 1, C and D). IgE-mediated enhancement is exclusively dependent on the low affinity receptor for IgE, CD23 (15, 19, 20) and therefore operates independently of C. Although IgG3-mediated enhancement was significantly impaired in CVF-treated mice, it was not completely absent (Fig. 1 B). One reason for this could be the residual levels of C3.

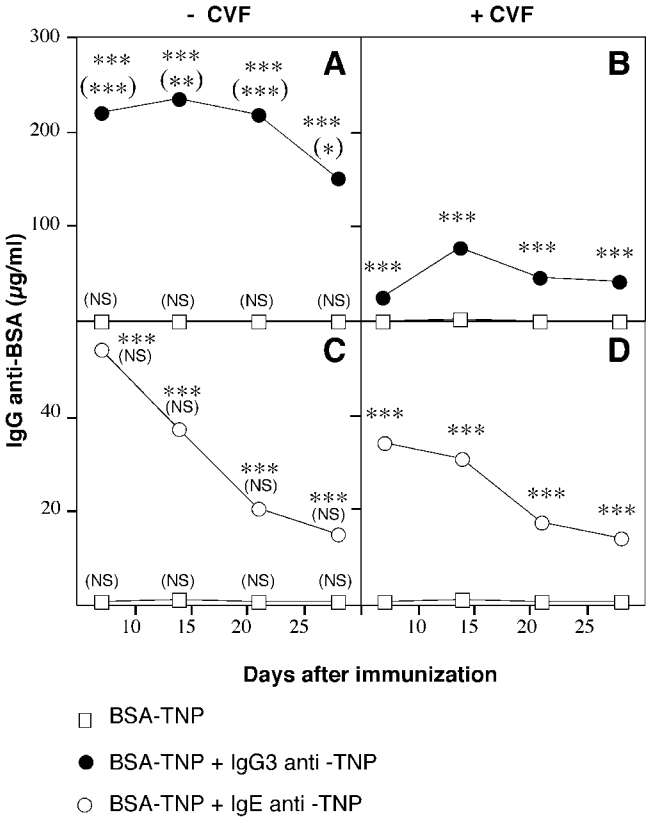

Figure 1. IgG3-, but not IgE-, mediated enhancement is impaired in CVF-treated mice. Groups of five FcRγ$^{+/+}$ mice, treated with CVF or sham-treated with PBS, were immunized intravenously with 20 μg BSA-TNP and 20 μg OVA alone or in combination with 50 μg TNP-specific IgG3 (A and B) or IgE (C and D). On the indicated days, mice were bled and the IgG anti-BSA and anti-OVA titers were measured by ELISA. OVA-specific responses were not enhanced by IgG3 or IgE (unpublished data). Asterisks indicate the statistical differences between experimental and control groups within the same strain; asterisks within parentheses indicate the statistical differences between corresponding not-C3-depleted and C3-depleted groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

$Cr2^{-/-}$ mice respond normally to complexes consisting of IgE/Ag and IgG2a/Ag whereas responses to IgM/Ag are very low (17). To further test the C dependence of IgG3-mediated enhancement, $Cr2^{-/-}$ and wild-type mice were immunized with OVA-TNP alone or in complex with 50 μg IM-F10 (Fig. 2). IgG3-mediated enhancement was severely impaired in $Cr2^{-/-}$ animals at all times tested. To verify that the impaired responses to IgG3/OVA-TNP were not just an effect of a general low-responsiveness, $Cr2^{-/-}$ mice were immunized also with IgG2a/Ag (Fig. 3). Whereas IgG3-mediated enhancement decreased markedly (SI = 16 in wild-type mice and 5.6 in $Cr2^{-/-}$ mice), IgG2a-mediated enhancement was stronger in $Cr2^{-/-}$ mice (SI = 17 in wild-type mice and 32 in $Cr2^{-/-}$), thus confirming earlier studies (17). The results show that IgG3, but not IgG2a, loses most of its enhancing capacity in mice lacking CD21/CD35. Unlike another $Cr2^{-/-}$ line, which expresses a hypomorphic variant of CD21/CD35 (39), the $Cr2^{-/-}$ line used here completely lacks expression of CD21/CD31 (40). Therefore, the residual enhancement seen in $Cr2^{-/-}$ mice (Fig. 2 B) cannot result from enhancement via a hypomorphic receptor.

Normal Ab Responses to IgG3/Ag Complexes in FcRγ$^{-/-}$ Mice. Since a small enhancement by IgG3 could be detected both in C3-depleted and $Cr2^{-/-}$ mice (Figs. 1–3), we wanted to test whether FcγRs may also be involved. FcRγ$^{+/+}$ and FcRγ$^{-/-}$ mice were immunized with OVA-TNP alone or in complex with IgG3 (IM-10). IgG3 enhanced the OVA-specific Ab response equally efficiently in

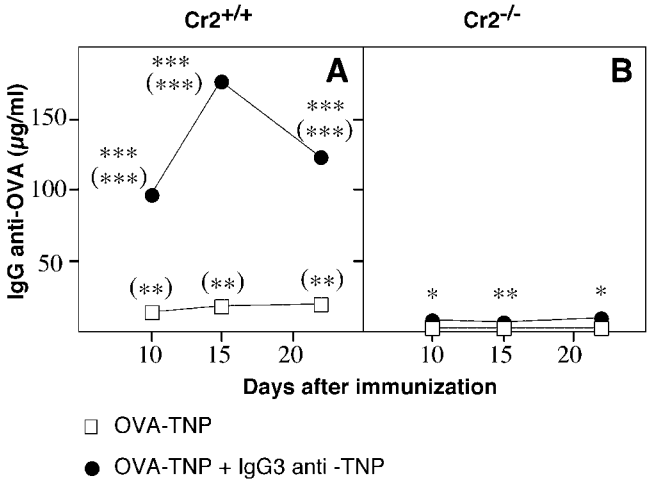

Figure 2. IgG3-mediated enhancement is impaired in $Cr2^{-/-}$ mice. Groups of five $Cr2^{+/+}$ (A) and $Cr2^{-/-}$ (B) mice were immunized intravenously with 20 μg OVA-TNP and 20 μg KLH alone or in combination with 50 μg TNP-specific IgG3. On the indicated days, mice were bled and the IgG anti-OVA and anti-KLH titers were measured by ELISA. KLH-specific responses were not enhanced by IgG3 (unpublished data). Asterisks indicate the statistical differences between experimental and control groups within the same strain; asterisks within parentheses indicate the statistical differences between the corresponding $Cr2^{+/+}$ and $Cr2^{-/-}$ groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

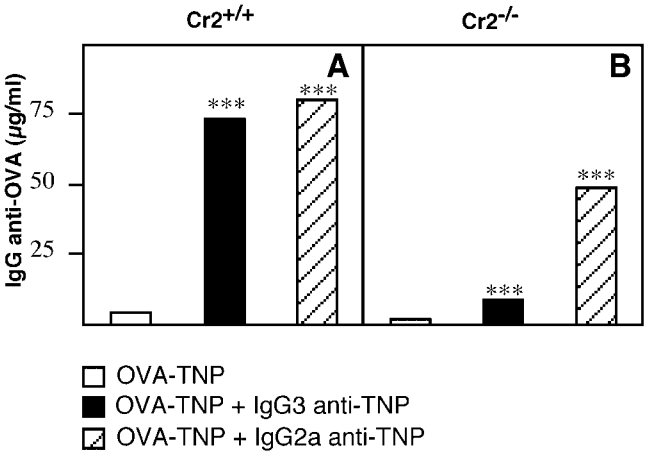

Figure 3. IgG2a-mediated enhancement is normal in $Cr2^{-/-}$ mice. Groups of five $Cr2^{+/+}$ (A) and $Cr2^{-/-}$ (B) mice were immunized intravenously with 20 μg OVA-TNP and 20 μg KLH alone or in combination with 50 μg TNP-specific IgG3 or IgG2a. Mice were bled 14 d after immunization and the IgG anti-OVA and anti-KLH titers were measured by ELISA. KLH-specific responses were not enhanced by IgG3. Asterisks indicate the statistical differences between experimental and control groups. ***, $P < 0.001$.

both strains (Fig. 4). These experiments were repeated using BSA-TNP as the Ag and yielded similar results (unpublished data). Thus, IgG3 enhances well in mice lacking expression of FcγRIII and with reduced expression of FcγRI.

Normal Ab Responses to IgG3/Ag Complexes in FcγRIIB$^{-/-}$ Mice. Enhancement by TNP-specific IgG1, IgG2a, and IgG2b mAbs administered with BSA-TNP increases dramatically in FcγRIIB$^{-/-}$ mice compared with wild-type controls (14). IgG3 is the only IgG subclass reported not to bind to FcγRIIB and it was of interest to test whether IgG3-mediated enhancement was indeed independent on this receptor. Wild-type and FcγRIIB$^{-/-}$ mice were immunized with BSA-TNP alone or in complex with 50 μg IgG3 (IM-F10) and their sera tested for BSA-specific IgG. The Ab responses were enhanced both in FcγRII$^{+/+}$ (11–16-fold of controls) and FcγRIIB$^{-/-}$ mice (16–25-fold of controls; Fig. 5). Although IgG3 induced a twofold higher Ab response in FcγRIIB$^{-/-}$ compared with wild-type mice, this difference was not significant and was far less pronounced than the 20–100-fold increased enhancement previously reported with IgG1-, IgG2a-, and IgG2b-complexes (14). The response to uncomplexed Ag is similar in FcγRIIB$^{-/-}$ and wild-type mice (Fig. 5). In light of the fact that immunization with proteins without adjuvant induces very little production of IgG, which is a prerequisite for ligation of FcγRIIB and initiation of its negative regulation, this is not surprising. Thus, IgG3-mediated enhancement takes place in the absence of FcγRIIB and this receptor does not exert a significant inhibitory influence on responses to IgG3-complexed Ags.

Discussion

Here we provide the first evidence that IgG3 can positively feedback-regulate Ab production. The augmentation is impressive, frequently yielding several-hundred-fold increases in Ab titers, and is severely impaired in mice lacking CD21/CD35 or in mice with reduced C3 levels. It is well

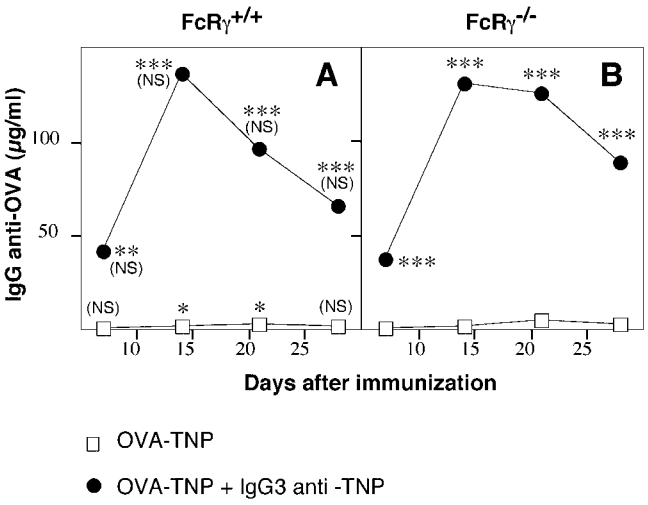

Figure 4. IgG3-mediated enhancement is normal in FcRγ$^{-/-}$ mice. Groups of four FcRγ$^{+/+}$ and FcRγ$^{-/-}$ mice were immunized intravenously with 20 μg OVA-TNP and 20 μg KLH alone or in combination with 50 μg TNP-specific IgG3. On the indicated days mice were bled and the IgG anti-OVA and anti-KLH titers were measured by ELISA. Anti-KLH responses were not enhanced by IgG3 (unpublished data). Asterisks indicate the statistical differences between experimental and control groups within the same strain; asterisks within parentheses indicate the statistical differences between the corresponding FcRγ$^{+/+}$ and FcRγ$^{-/-}$ groups. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

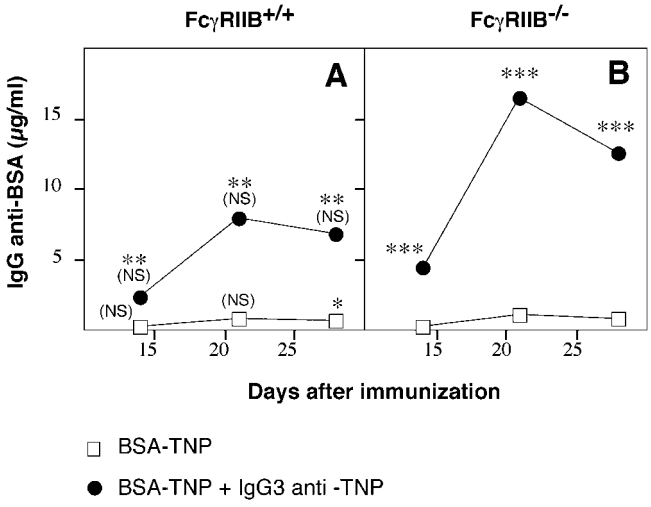

Figure 5. IgG3-mediated enhancement is normal in FcγRIIB$^{-/-}$ mice. Groups of five FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ mice were immunized intravenously with 20 μg BSA-TNP and 20 μg OVA alone or in combination with 50 μg TNP-specific IgG3. On the indicated days mice were bled and the IgG anti-BSA and anti-OVA titers were measured by ELISA. OVA-specific responses were not enhanced (unpublished data). Asterisks indicate the statistical differences between experimental and control groups within the same strain; asterisks within parentheses indicate the statistical differences between the corresponding FcγRIIB$^{+/+}$ and FcγRIIB$^{-/-}$ groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

known that C plays an important role for primary as well as secondary Ab responses, and animals lacking C1q, C2, C4, C3, as well as CD21/CD35 have severely impaired Ab responses (for reviews, see references 23 and 24). In contrast, factor B–deficient mice have normal Ab responses (41; unpublished data) suggesting that the alternative pathway is not required for up-regulation of Ab responses and that classical pathway activation (via immune complexes) plays a major role. One explanation for the role of C in Ab responses is that immune complexes, containing C factors, cocrosslink BCR and the CD21/CD19-receptor complex on the B cell surface and thereby lowers the threshold for B cell activation, as shown to take place in vitro (25). A mutually not exclusive hypothesis is that Ab/Ag/C complexes are captured by follicular dendritic cells in the spleen and lymph nodes, increasing the effective concentration of Ag (22, 42, 43). Our present data suggest that not only IgM (17, 21, 22), but also IgG3, uses the C system to initiate feedback "help" for early Ab production. Since classical pathway C activation is important for normal primary Ab responses in animals immunized with Ag alone, an interesting question is how specific Abs, able to recognize Ag and form immune complexes, can be available in naive mice. Mice lacking secretory IgM have impaired primary Ab responses to noncomplexed Ags (44–46) and a normal response could be reconstituted by transfer of IgM from naive mice. These animals may have several abnormalities in their immune system, secondary to the lack of secretory IgM, and were also reported to have developmental defects in their B cell compartment (45, 47). The underlying reason for the impaired IgG responses observed (44–46) is not known. However, one intriguing explanation would be that "natural" IgM, present without prior (deliberate) immunizations, initiates positive feedback of early responses. Should the enhancing effect of natural IgM be caused by its ability to activate C, which has not been formally proven, it is possible that recognition of Ag by natural IgM is the first step in the C-dependent chain of events leading to efficient early Ab responses to suboptimal doses of Ag. This would explain how classical C activation can play a role already for primary Ab responses. Our present data raises the question whether "natural" IgG3 has a similar role as IgM in enhancing early Ab responses.

IgM has only been reported to enhance responses to large Ags such as erythrocytes (21, 48), malaria parasites (49), and KLH (22), whereas responses to small Ags, e.g. BSA-TNP, have not been reported to be enhanced (unpublished data). An explanation for this would be that IgM must bind to an Ag large enough to permit binding of several of its arms in order to achieve the conformation change necessary to bind C1q. IgG3, bound to the surface of e g group A streptococci, is able to facilitate the binding of other IgG3 molecules to the same Ag (50, 51). This so called cooperative binding is probably mediated by Fc–Fc interactions and increases the functional affinity of IgG3. The interaction between IgG3 molecules is also evidenced by their tendency to self-aggregate (52) and to act as cryoglobulins, i.e. to precipitate in the cold (53). Cooperative binding of IgG3 would increase the chances that two or more IgG3 molecules end up close together (and thereby can bind C1q) also on Ags which are too small for IgM to bind with all five arms. This may explain why IgG3, but not IgM, can utilize C to enhance responses to Ags like BSA and OVA. The probability that two IgG2a or IgG2b molecules, subclasses which do not show cooperative binding, bind to the same Ag close enough to be able to fix C1q is smaller and could be the reason that these subclasses preferentially utilize FcγRs for feedback enhancement.

Unlike enhancement mediated by IgG1 and IgG2a, IgG3-mediated enhancement was unperturbed in FcRγ$^{-/-}$ mice. Although FcγRI in these animals has lost its signaling function, 20% of the receptor is still expressed (11). As FcγRI was also reported to bind IgG3 (10, 11), we cannot at this point exclude that FcγRI, or another yet unidentified IgG3-binding receptor, acts redundantly to C in IgG3-mediated enhancement. This would explain the small residual enhancement seen in C-depleted and Cr2$^{-/-}$ mice. Direct experiments to prove this point will have to await the availability of FcγRI$^{-/-}$ mice on a responder background, as H-2^{b} mice, commonly used to produce knockout mice, are nonresponders to soluble IgE- and IgG-immune complexes (28, 32).

Mice lacking FcγRIIB have augmented humoral, anaphylactic, and inflammatory immune responses (for a review, see reference 54). This receptor contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM), which upon cocrosslinking to receptors containing immunoreceptor tyrosine-based activation motifs (ITAMs), such as the BCR, FcγRI, FcγRIII, and FcεRI, inhibits activation of the ITAM receptor. Ab production after immunization with IgG1/Ag, IgG2a/Ag, or IgG2b/Ag complexes is considerably higher in FcγRIIB$^{-/-}$ mice than in wild-type controls, implying that FcγRIIB negatively regulates the response to Ag complexed to these isotypes (14). IgG3 does not bind FcγRIIB and we here show that enhancement by IgG3 is similar in FcγRIIB$^{-/-}$ and wild-type mice. Therefore, IgG3/Ag complexes have the for IgG isotypes unique possibility to enhance Ab responses without inducing simultaneous negative feedback via cocrosslinking of BCR and FcγRIIB. This feature may be useful to quickly generate a potent primary Ab response to e g bacterial Ags. IgG3 is an isotype frequently involved in autoimmunity (55–58). In addition to its ability to initiate inflammation via C-activation, the ability of IgG3 to enhance autoantibody production without negative feedback via FcγRIIB may play a role in its pathogenicity.

We thank Ms. I. Brogren and Ms. T. Wahlström for skillful technical assistance, Dr. M. Wabl for IgE, and Dr. P. Coulie for IgG2a hybridomas, Dr. H. Molina for Cr2$^{-/-}$, Dr. J.V. Ravetch for FcRγ$^{-/-}$ and FcγRIIB$^{-/-}$, and Dr. S. Kleinau for FcRγ$^{+/+}$ and FcRγ$^{-/-}$ (H2^{q}) founder animals.

This work was supported by Agnes and Mac Rudberg's Foundation; Ellen, Walter and Lennart Hesselman's Foundation; Hans von Kantzow's Foundation; King Gustaf V:s 80 Years Foundation; Lilly and Ragnar Åkerhams's Foundation; The Swedish Foundation for Health Care Science and Allergy Research; The Swedish Medical

Research Council; The Swedish Association against Rheumatism; and Ollie and Elof Ericsson's Foundation.

Submitted: 30 December 2002
Revised: 2 April 2003
Accepted: 2 April 2003

References

1. Perlmutter, R.M., D. Hansburg, D.E. Briles, R.A. Nicolotti, and J.M. Davie. 1978. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* 121:566–572.
2. Rubinstein, L.J., and K.E. Stein. 1988. Murine immune response to the *N. meningitidis* group c capsular polysaccharide: ontogeny. *J. Immunol.* 141:4352–4356.
3. Shackelford, P.G., D.M. Granoff, S.J. Nelson, M.G. Scott, D.S. Smith, and M.H. Nahm. 1987. Subclass distribution of human antibodies to *H. influenzae* type b capsular polysaccharide. *J. Immunol.* 138:587–592.
4. Stein, K.E. 1992. Thymus-independent and thymus-dependent responses to polysaccharide antigens. *J. Infect. Dis.* 165 (Suppl. 1):S49–S52.
5. McLay, J., E. Leonard, S. Peterson, D. Shapiro, N.S. Greenspan, and J.R. Schreiber. 2002. γ3 gene-disrupted mice selectively deficient in the dominant IgG subclass made to bacterial polysaccharides. II. Increased susceptibility to fatal pneumococcal sepsis due to absence of anti-polysaccharide IgG3 is corrected by induction of anti-polysaccharide IgG1. *J. Immunol.* 168:3437–3443.
6. da Silveira, S.A., S. Kikuchi, L. Fossati-Jimack, T. Moll, T. Saito, J.S. Verbeek, M. Botto, M.J. Walport, M. Carroll, and S. Izui. 2002. Complement activation selectively potentiates the pathogenicity of the IgG2b and IgG3 isotypes of a high affinitiy anti-erythrocyte autoantibody. *J. Exp. Med.* 195: 665–672.
7. Han, Y., T.R. Kozel, M.X. Zhan, R.S. MacGill, M.C. Carroll, and J.E. Cutler. 2001. Complement is essential for protection by an IgM and an IgG3 monoclonal antibody against experimental, hematogenously disseminated candidiasis. *J. Immunol.* 167:1550–1557.
8. Diamond, B., and D.E. Yelton. 1981. A new Fc receptor on mouse macrophages binding IgG3. *J. Exp. Med.* 153:514–519.
9. Hulett, M.D., and P.M. Hogarth. 1994. Molecular basis of Fc receptor function. *Adv. Immunol.* 57:1–127.
10. Gavin, A.L., N. Barnes, H.M. Dijstelbloem, and P.M. Hogarth. 1998. Identification of the mouse IgG3 receptor: Implications for antibody effector function at the interface between innate and adaptive immunity. *J. Immunol.* 160:20–23.
11. Barnes, N., A.L. Gavin, P.S. Tan, P. Mottram, F. Koentgen, and P.M. Hogarth. 2002. FcγRI-deficient mice show multiple alterations to inflammatory and immune responses. *Immunity.* 16:379–389.
12. Heyman, B. 2000. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu. Rev. Immunol.* 18:709–737.
13. Coulie, P., and J. Van Snick. 1985. Enhancement of IgG anti-carrier responses by IgG2-anti-hapten antibodies in mice. *Eur. J. Immunol.* 15:793–798.
14. Wernersson, S., M. Karlsson, J. Dahlström, R. Mattsson, J.S. Verbeek, and B. Heyman. 1999. IgG-mediated enhancement of Ab responses is low in FcRγ chain deficient mice and increased in FcγRII deficient mice. *J. Immunol.* 163:618–622.
15. Gustavsson, S., S. Wernersson, and B. Heyman. 2000. Restoration of the antibody response to IgE/antigen complexes in CD23-deficient mice by $CD23^+$ spleen or bone marrow cells. *J. Immunol.* 164:3990–3995.
16. Wiersma, E.J., M. Nose, and B. Heyman. 1990. Evidence of IgG-mediated enhancement of the antibody response in mice without classical pathway complement activation. *Eur. J. Immunol.* 20:2585–2589.
17. Applequist, S.E., J. Dahlström, N. Jiang, H. Molina, and B. Heyman. 2000. Antibody production in mice deficient for complement receptors 1 and 2 can be induced by IgG/Ag and IgE/Ag, but not IgM/Ag complexes. *J. Immunol.* 165: 2398–2403.
18. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J.V. Ravetch. 1994. FcRγ chain deletion results in pleiotrophic effector cell defects. *Cell.* 76:519–529.
19. Heyman, B., T. Liu, and S. Gustavsson. 1993. In vivo enhancement of the specific antibody response via the low affinity receptor for IgE. *Eur. J. Immunol.* 23:1739–1742.
20. Fujiwara, H., H. Kikutani, S. Suematsu, T. Naka, K. Yoshida, K. Yoshida, T. Tanaka, M. Suemura, N. Matsumoto, S. Kojima, et al. 1994. The absence of IgE antibody-mediated augmentation of immune responses in CD23-deficient mice. *Proc. Natl. Acad. Sci. USA.* 91:6835–6839.
21. Heyman, B., L. Pilström, and M.J. Shulman. 1988. Complement activation is required for IgM-mediated enhancement of the antibody response. *J. Exp. Med.* 167:1999–2004.
22. Youd, M.E., A.R. Ferguson, and R.B. Corley. 2002. Synergistic roles of IgM and complement in antigen trapping and follicular localization. *Eur. J. Immunol.* 32:2328–2337.
23. Carroll, M.C. 1998. The role of complement and complement receptors in induction and regulation of immunity. *Annu. Rev. Immunol.* 16:545–568.
24. Fearon, D.T., and M.C. Carroll. 2000. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu. Rev. Immunol.* 18:393–422.
25. Carter, R.H., and D.T. Fearon. 1992. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science.* 256:105–107.
26. Haas, K.M., M. Hasegawa, D.A. Steeber, J.C. Poe, M.D. Zabel, C.B. Bock, D.R. Karp, D.E. Briles, J.H. Weis, and T.F. Tedder. 2002. Complement receptors CD21/CD35 link innate and protective immunity during *Streptococcus pneumoniae* infection by regulating IgG3 antibody responses. *Immunity.* 17:713–723.
27. Botto, M., and M.J. Walport. 1993. Hereditary deficiency of C3 in animals and humans. *Int. Rev. Immunol.* 10:37–50.
28. Gustavsson, S., S. Hjulström-Chomez, B.-M. Lidström, N. Ahlborg, R. Andersson, and B. Heyman. 1998. Impaired antibody responses in $H\text{-}2A^b$ mice. *J. Immunol.* 161:1765–1771.
29. Good, A.H., L. Wofsy, C. Henry, and J. Kimura. 1980. Preparation of hapten-modified protein antigens. *In* Selected Methods in Cellular Immunology. B.B. Mishell and S.M. Shiigi, editors. W.H. Freeman and Co, San Francisco, CA. 343–350.
30. Karlsson, M.C.I., S. Wernersson, T. Diaz de Ståhl, S. Gustavsson, and B. Heyman. 1999. Efficient IgG-mediated suppression of primary antibody responses in Fcγ receptor-deficient mice. *Proc. Natl. Acad. Sci. USA.* 96:2244–2249.
31. Rudolph, A.K., P.D. Burrows, and M.R. Wabl. 1981. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Ig^a or Ig^b heavy chain haplotype. *Eur. J. Immunol.* 11:527–529.

32. Gustavsson, S., S. Chomez, and B. Heyman. 1999. Low responsiveness to immunization with immunoglobulin E/antigen and immunoglobulin G/antigen complexes in H-2A^b mice. *Scand. J. Immunol.* 50:45–51.
33. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J.V. Ravetch. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature.* 379:346–349.
34. Molina, H., V.M. Holers, B. Li, Y.-F. Fang, S. Mariathasan, J. Goellner, J. Strauss-Schoenberger, R.W. Karr, and D.D. Chaplin. 1996. Markedly impaired humoral immune responses in mice deficient in complement receptors 1 and 2. *Proc. Natl. Acad. Sci. USA.* 93:3357–3361.
35. Kleinau, S., P. Martinsson, and B. Heyman. 2000. Induction and suppression of collagen-induced arthritis is dependent on disctinct Fcγ receptors. *J. Exp. Med.* 191:1611–1616.
36. Diaz de Ståhl, T., and B. Heyman. 2001. IgG2a-mediated enhancement of antibody responses is dependent of FcRγ$^+$ bone marrow-derived cells. *Scand. J. Immunol.* 54:1–7.
37. Dodds, A.W., and R.B. Sim. 1997. Complement. A practical approach. A Practical Approach Series. D. Rickwood and B.D. Hames, eds. Oxford University Press Inc., New York. 22–29.
38. Zabern, I.v., B. Hinsch, H. Przyklenk, G. Schmidt, and W. Vogt. 1980. Comparison of Naja n. naja and Naja h. haje cobra-venom factors: correlation between binding affinity for the fifth component of complement and mediation of its cleavage. *Immunobiology.* 157:499–514.
39. Hasegawa, M., M. Fujimoto, J.C. Poe, D.A. Steeber, and T.F. Tedder. 2001. CD19 can regulate B lymphocyte signal transduction independent of complement activation. *J. Immunol.* 167:3190–3200.
40. Wu, X., N. Jiang, C. Deppong, J. Singh, G. Dolecki, D. Mao, L. Morel, and H. Molina. 2002. A role for the Cr2 gene in modifying autoantibody production in systemic lupus erythematosus. *J. Immunol.* 169:1587–1592.
41. Matsumoto, M., W. Fukuda, A. Circolo, J. Goellner, J. Strauss-Schoenberger, X. Wang, S. Fujita, T. Hidvegi, D.D. Chaplin, and H.R. Colten. 1997. Abrogation of the alternative complement pathway by targeted deletion of murine factor B. *Proc. Natl. Acad. Sci. USA.* 94:8720–8725.
42. Fang, Y., C. Xu, Y.-X. Fu, V.M. Holers, and H. Molina. 1998. Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *J. Immunol.* 160:5273–5279.
43. Barrington, R.A., O. Pozdnyakova, M.R. Zafari, C.D. Benjamin, and M.C. Carroll. 2002. B lymphocyte memory: role of stromal cell complement and FcγRIIB receptors. *J. Exp. Med.* 196:1189-1200.
44. Ehrenstein, M.R., T.L. O'Keefe, S.L. Davies, and M.S. Neuberger. 1998. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc. Natl. Acad. Sci. USA.* 95:10089–10093.
45. Boes, M., C. Esau, M.B. Fischer, T. Schmidt, M. Carroll, and J. Chen. 1998. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J. Immunol.* 160:4776–4787.
46. Baumgarth, N., O.C. Herman, G.C. Jager, L.E. Brown, L.A. Herzenberg, and J. Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J. Exp. Med.* 192:271–280.
47. Baker, N., and M.R. Ehrenstein. 2002. Selection of B lymphocyte subsets is regulated by natural IgM. *J. Immunol.* 169: 6686–6690.
48. Henry, C., and N. Jerne. 1968. Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J. Exp. Med.* 128:133–152.
49. Harte, P.G., A. Cooke, and J.H.L. Playfair. 1983. Specific monoclonal IgM is a potent adjuvant in murine malaria vaccination. *Nature.* 302:256–258.
50. Cooper, L.J., J.C. Schimenti, D.D. Glass, and N.S. Greenspan. 1991. H chain C domains influence the strength of binding of IgG for streptococcal group A carbohydrate. *J. Immunol.* 146:2659–2663.
51. Greenspan, N.S., and L.J.N. Cooper. 1992. Intermolecular cooperativity: a clue to why mice have IgG3? *Immunol. Today.* 13:164–168.
52. Grey, H.M., J.W. Hirst, and M. Cohn. 1971. A new mouse immunoglobulin: IgG3. *J. Exp. Med.* 133:289–304.
53. Abdelmoula, M., F. Spertini, T. Shibata, Y. Gyotoku, S. Luzuy, P.H. Lambert, and S. Izui. 1989. IgG3 is the major source of cryoglobulins in mice. *J. Immunol.* 143:526–532.
54. Ravetch, J.V., and S. Bolland. 2001. IgG Fc receptors. *Annu. Rev. Immunol.* 19:275–290.
55. Takahashi, S., M. Nose, J. Sasaki, T. Yamamoto, and M. Kyogoku. 1991. IgG3 production in MRL/lpr mice is responsible for development of lupus nephritis. *J. Immunol.* 147:515–519.
56. Fulpius, T., F. Spertini, L. Reininger, and S. Izui. 1993. Immunoglobulin heavy chain constant region determines the pathogenicity and the antigen-binding activity of rheumatoid factor. *Proc. Natl. Acad. Sci. USA.* 90:2345–2349.
57. Swanson, P.C., C. Ackroyd, and G.D. Glick. 1996. Ligand recognition by anti-DNA autoantibodies. Affinity, specificity, and mode of binding. *Biochemistry.* 35:1624–1633.
58. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity.* 11:141–151.