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Function of C3 in a humoral response: iC3b/C3dg bound to an immune complex generated with natural antibody and a primary antigen promotes antigen uptake and the expression of co-stimulatory molecules by all B cells, but only stimulates immunoglobulin synthesis by antigen-specific B cells

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SUMMARY

Previous studies have shown that an optimal humoral response to a primary protein antigen requires C3 and CR2 (CD21). Sera from non-immunized donors contain natural IgM and IgG antibodies to the primary antigen keyhole limpet haemocyanin (KLH), and these have been previously shown to form immune complexes (IC) that activate the classical pathway of C, fixing iC3b/C3dg onto the KLH antigen. Such KLH IC bind to CR2 on KLH-non-specific B lymphocytes, resulting in antigen processing and MHC class II-dependent presentation to KLH-specific helper T cells. KLH IC also induce B lymphocytes to express the CD80 co-stimulatory molecule via simultaneous CR2 ligation with C3 and FcγRII (CD32) stimulation by IgG natural antibody. The current study demonstrated that KLH IC ligation to either CR2 or FcγRII resulted in activation of a second co-stimulatory molecule, LFA-1 (CD11a, CD18). The possibility of polyclonal B cell stimulation by the presentation of KLH-iC3b/C3dg by antigen-non-specific B cells was excluded by demonstration that *in vitro* cultivation of peripheral blood mononuclear cells (PBMC) with KLH-iC3b/C3dg elicited only anti-KLH, and did not stimulate synthesis of antibodies to hepatitis C virus (HCV) or tetanus toxoid (TT). Of greatest significance, a specific anti-KLH response was only detectable in cultures stimulated with KLH-iC3b/C3dg and not in cultures stimulated with KLH alone or KLH-IgG. Thus, iC3b/C3dg that was bound to a primary protein antigen enhanced recognition and specific immunoglobulin synthesis by antigen-specific B cells, even though the antigen was taken up and processed via CR2 by both antigen-specific and non-specific B cells.

Keywords C3 CR2/CD21 B lymphocytes LFA-1/CD11a CD80

INTRODUCTION

Over the last 23 years, increasing evidence has indicated a role for C3 and CR2 in the immune response. Both C3-depleted mice [1] and individuals with an inherited or acquired deficiency of C3 have diminished immune responses to T cell-dependent primary protein antigens such as keyhole limpet haemocyanin (KLH) or bacteriophage ϕX-174 [2–5]. Identification of the C3 receptors involved in an immune response was facilitated by the development of MoAbs to murine CR1 and CR2 [6, 7], as well as a soluble rCR2/IgG chimaeric molecule that was bivalent in C3d-binding sites [8]. Both primary and secondary responses to T cell-dependent and -independent antigens were suppressed by MoAb that blocked the C3d-binding site of CR2, but not by MoAb that blocked the C3b-binding site of CR1 [6, 7]. The rCR2/IgG chimaera competed with B cells for soluble polymerized C3dg *in vitro*, and in mice suppressed responses to T cell-dependent antigens in the same way as did anti-CR2 [8]. Recently it has also been reported that treatment of mice with anti-CR2 inhibits the development of memory B cells without affecting the priming of helper T (Th) cells [9].

Previous studies from our laboratory suggested that immune responses to primary protein antigens might involve C3-fixation to the antigens following immune complex (IC) formation with natural antibodies and activation of the classical pathway of C. KLH IC formed with normal human serum contained IgM and IgG natural antibody, as well as sufficient iC3b/C3dg for ligation of the antigen to B cells via CR2. Such KLH IC were taken up and processed by all CR2-bearing B cells, regardless of their antigen specificity. Furthermore, KLH IC taken up in this way by non-specific B cells were shown to be presented to KLH-specific, MHC

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class II-compatible Th cells [10]. Induction of the B lymphocyte co-stimulatory molecule CD80 was shown to result from ligation of IC-associated iC3b/C3dg to CR2 followed by FcγRII (CD32) stimulation via IgG natural antibody on the IC [10]. This suggested that antigen-non-specific B cells might be able to function as antigen-presenting cells (APC) for antigen-specific Th cells in the same way as macrophages and dendritic cells.

The current study examined the characteristics of a primary immune response to KLH elicited *in vitro* by cultivation of peripheral blood mononuclear cells (PBMC) with KLH-iC3b/C3dg. Activation of B cell LFA-1 was shown to result from IC binding to either CR2 via iC3b/C3dg or to FcγRII via IgG. Even though all CR2-bearing B cells bound KLH-IC, the processing and presentation of KLH-IC by PBMC only stimulated the production of KLH-specific antibody, and did not result in the production of non-specific antibody.

MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: fetal calf serum (FCS; Hyclone Labs, Logan, UT); RPMI 1640 (Whittaker, Walkersville, MD); KLH (Pacific Biomarine, Venice, CA); avidin-alkaline phosphatase, Ficoll, lipopolysaccharide (LPS), transferrin, Tween 20, *n*-succinimidyl 3-(2-pyridylthio) propionate (SPDP), activated thiol Sepharose-4B (ATS), Sephadex G-25, and *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO); Hypaque M (Winthrop Pharmaceuticals, New York, NY); streptavidin coupled to PE or FITC (Tago, Inc., Burlingame, CA); DTT (Kodak, Rochester, NY); hepatitis C virus (HCV) peptide (Dr J. D. Lambris, University of Pennsylvania, Philadelphia, PA); pokeweed mitogen (PWM; GIBCO-BRL, Grand Island, NY); tetanus toxoid (TT) antigen was from Dr M. Hoover (Coriell Institute for Medical Research, Camden, NJ).

Antibodies

Rabbit (Rab) anti-C3c and C3d were generated [11], the IgG fractions isolated by DEAE anion-exchange chromatography were mixed together in equal proportions, and $F(ab')_2$ fragments were purified following digestion with pepsin [12]. This Rab $F(ab')_2$ anti-C3c/C3d reagent is referred to as '$F(ab')_2$ anti-C3'. Rab anti-CR1 was generated with purified CR1, and the $F(ab')_2$ fragments of IgG were generated [13]. Rab IgG anti-CR2 generated with rCR2 was provided by Dr M. Holers (University of Colorado, Denver, CO). Rabbit anti-KLH were generated with purified KLH and the IgG fraction was isolated [12]. Sheep anti-IgM-FITC was from Sigma. The hybridoma-secreting OKM1 anti-CR3 (CD11b) was obtained from the American Type Culture Collection (ATCC; Rockville, MD), and the IgG2b MoAb was isolated from ascites fluid [14]. Fab AT10 anti-FcγRII MoAb [15], that blocks the binding site of B cell FcγRII, was generously provided by Dr M. J. Glennie (Tenovus Research Laboratory, Southampton, UK). IgG MoAb 24 specific for a Mg^{2+}-dependent activation epitope shared by all three of the β_2-integrin α-chains [16,17] and MoAb 3.9 to CR4 (CD11c) were provided by Dr N. Hogg (Imperial Cancer Research Fund, London, UK). MoAbs 24 and 3.9 were conjugated to biotin for use in immunofluorescence staining in combination with streptavidin-PE or -FITC. Anti-CD19-PE and anti-CD19-FITC were purchased from Becton Dickinson (Mountain View, CA).

PBMC

PBMC, isolated by Ficoll–Hypaque centrifugation from normal citrated blood, were washed five times in RPMI 1640 and maintained on ice until use [18].

Generation of KLH IC

Soluble KLH IC were prepared and analysed by ELISA for bound IgM, IgG, and iC3b/C3dg as before [10]. Briefly, free sulfhydryl groups were introduced into KLH using SPDP, allowing the KLH to be coupled to ATS. The KLH–ATS slurry in veronal-buffered saline containing Ca^{2+} and Mg^{2+} was mixed with fresh human serum and incubated at 37 °C, forming solid-phase immunoglobulin-KLH-iC3b IC via the natural antibody to KLH and C present in normal serum. Soluble immunoglobulin-KLH-iC3b IC were eluted from the ATS matrix with L-cysteine, dialysed, and concentrated. For conversion of complex-bound iC3b into C3dg, ATS-bound IC were incubated secondarily with serum that was both dialysed to lower the ionic strength and supplemented with soluble CR1 to serve as an efficient factor I cofactor.

Assay for induction of the MoAb 24 activation epitope on B cell LFA-1

Samples of 5×10^5 PBMC in 0·1 ml RPMI 1640 were monitored hourly for induction of the MoAb 24 activation epitope on B cells following cultivation with one of the following stimulating agents for 4 h at 37°C: 15 μg/ml KLH, 10 μg/ml KLH IC, 50 μg/ml Rab IgG anti-CR2, or 100 μg/ml Rab $F(ab')_2$ anti-CR1. To assess the potential contribution of FcγRII, IC-associated iC3b/C3dg, or a possible synergism involving both FcγRII and IC-associated iC3b/C3dg in the activation of B cell LFA-1, either the PBMC or IC were incubated for 15 min at 37°C before cultivation with various blocking antibodies: 10 μg/ml Fab anti-FcγRII, 10 μg/ml Rab $F(ab')_2$ anti-C3, or 10 μg/ml of both Fab anti-FcγRII and $F(ab')_2$ anti-C3. Optimal concentrations of stimulators or inhibitors were determined by titrating each agent for the maximum increase or decrease in intensity of B cell staining by MoAb 24.

Following centrifugation of the hourly 0·1 ml cell suspension samples, the cells were stained with anti-CD19-FITC (to identify B cells) and MoAb 24-biotin (to identify activated LFA-1) for 30 min at 4°C. Alternatively, B cells were identified by staining with anti-IgM-FITC, instead of anti-CD19-FITC, and similar results were obtained. The cells were washed by centrifugation through a cushion of 4 ml of 12% bovine serum albumin (BSA)/PBS/azide in 12 × 75 mm glass tubes and double stained by incubation for an additional 30 min at 4°C with streptavidin-PE followed by removal of the unbound stain by centrifugation of the cells through a cushion of 12% BSA/PBS/azide as above. To confirm that MoAb 24 bound primarily to B cell LFA-1 and not to one of the other two β_2-integrins, IC-stimulated PBMC cultures were also examined in parallel for expression of B cell CR3 or CR4 using anti-CD19-PE to identify B cells and biotinylated OKM1 or 3.9 MoAb plus streptavidin-FITC to identify CR3 or CR4, respectively. Immunofluorescence staining of small mononuclear cells was evaluated with a Profile II flow cytometer (Coulter Electronics, Inc., Miami Lakes, FL). The expression of the MoAb 24 activation epitope by $CD19^+$ (or $sIgM^+$) B cells was assessed later by analysing the stored list mode data.

Assay for in vitro *antibody synthesis by PBMC*

PBMC were cultured according to Lane *et al*. [19] using 5×10^6 cells, 12 × 75 mm plastic tubes, and RPMI 1640/10% FCS

medium. Cultures were stimulated by addition of the following antigens or mitogens: 20 μg/ml KLH; 100 ng/ml LPS; 10 μg/ml TT; 20 μg/ml PWM; or 10 μg/ml KLH IC. Appropriate antigen concentrations were determined by titration for maximal stimulation of total immunoglobulin and KLH-specific antibody. The total volume of each tube was brought to 1 ml with medium and 200 μl aliquots were seeded into 96-well flat-bottomed culture plates (Costar, Cambridge, MA). The plates were incubated at 37°C in 5% CO_2 and supernatants were harvested on day 6. To assess the contributions of FcγRII and IC-associated C3 to antibody production, 10 μg/ml Fab anti-FcγRII, 10 μg/ml F(ab′)$_2$ anti-C3 or 10 μg/ml of both Fab anti-FcγRII and F(ab′)$_2$ anti-C3 were incubated with either the PBMC or IC for 30 min at room temperature before cultivation.

Specific and total immunoglobulin in culture supernatants were measured by ELISA [19]. Briefly, flat-bottomed micro-ELISA plates (Dynatech, Alexandria VA) were coated with 10 μg/ml of KLH, HCV, TT, or Rab anti-human polyvalent immunoglobulin (Sigma) in carbonate-bicarbonate buffer pH 9·6, overnight at 4°C. After washing and blocking, 200 μl of culture supernatant were added to each well, and the plates were incubated for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-human immunoglobulin (Sigma) was added and incubated for 2 h at room temperature, followed by colour development with *p*-nitrophenyl phosphate in carbonate-bicarbonate buffer pH 9·6. The plates were evaluated by micro-ELISA reader (Dynatech MR-600) and 410 nm absorbance values were converted to μg/ml immunoglobulin by parallel analysis of a pooled human serum standard (Cappel, West Chester, PA).

RESULTS

CR2- or FcγRII-dependent activation of B lymphocyte LFA-1

B cell antigen presentation requires induced expression of B cell CD80 (B7/BB1) that binds to T cell CD28 and an activation of cell surface LFA-1 molecules that exposes their high-affinity binding sites for T cell intercellular adhesion molecule-1 (ICAM-1) (CD54) [20,21]. The current investigation sought to determine if iC3b/C3dg-bearing KLH IC could induce LFA-1 activation on normal B lymphocytes. PBMC were incubated with KLH IC for up to 4 h and monitored hourly for activation of B cell LFA-1 by two-colour flow cytometry using anti-CD19-FITC (or anti-IgM-FITC) to identify B cells and the reporter MoAb 24 (-biotin/streptavidin-PE) to identify the α-subunit activation epitope corresponding to acquisition of the high-affinity ICAM-1 binding site shared by all of the β_2-integrins [16,17]. Since B cells express little CR3 or CR4, this assay should be specific for activation of LFA-1 unless IC stimulation of B cells induced CR3 or CR4 expression. IC-induction of B cell CR3 and CR4 was excluded when flow cytometry showed $\leqslant$ 5% of CD19$^+$ B cells expressed either CR3 or CR4, and that incubation of PBMC with KLH IC for 4 h did not increase B cell expression of these other β_2-integrins (not shown).

KLH IC bearing iC3b/C3dg activated B cell LFA-1 within 1 h (Fig. 1). IgM/IgG-KLH (generated with heat-inactivated serum) stimulated a lower level of LFA-1 activation, suggesting a role for IC-associated natural IgG antibody and B cell FcγRII. This was confirmed using KLH IC prepared with Rab IgG anti-KLH (Rab IgG-KLH) that promoted LFA-1 activation in the absence of IC-bound IgM or C3. However, an independent ability of CR2 to activate LFA-1 was demonstrated by the finding that Rab anti-CR2, but not Rab anti-CR1, also activated LFA-1. The activation of LFA-1 by iC3b/C3dg-bearing KLH IC was partially blocked by either F(ab′)$_2$ anti-C3 or Fab anti-FcγRII, showing the additive effect of bound C3 and IgG (Table 1). Similarly, treatment of PBMC with anti-FcγRII MoAb blocked the activation of LFA-1 stimulated by IgM/IgG-bearing KLH IC. Near total inhibition of LFA-1 activation by iC3b/C3dg-bearing KLH IC was achieved when both F(ab′)$_2$ anti-C3 and Fab anti-FcγRII were used. However, Fab anti-FcγRII had no effect on LFA-1 activation stimulated by Rab IgG anti-CR2, indicating that anti-CR2 activation of LFA-1 was due to antibody ligation of CR2 and not FcγRII. Thus, CR2 and FcγRII can work independently in the activation of LFA-1.

Humoral immune response of PBMC to KLH IC

Previous investigation had suggested that any CR2$^+$ B cell could process and present a C3-bearing antigen to an antigen-specific Th cell [10]. However, it appeared possible that such processing and presentation by non-specific B cells might result in polyclonal B cell stimulation. To evaluate this possibility, PBMC were incubated with various concentrations of KLH IC and other agonists (i.e. LPS, PWM, TT) and culture supernatants were screened by ELISA for the presence of specific antibody to KLH *versus* antibodies to HCV peptide (a primary antigen), TT (a secondary antigen), and for total immunoglobulin synthesis (Fig. 2). KLH-

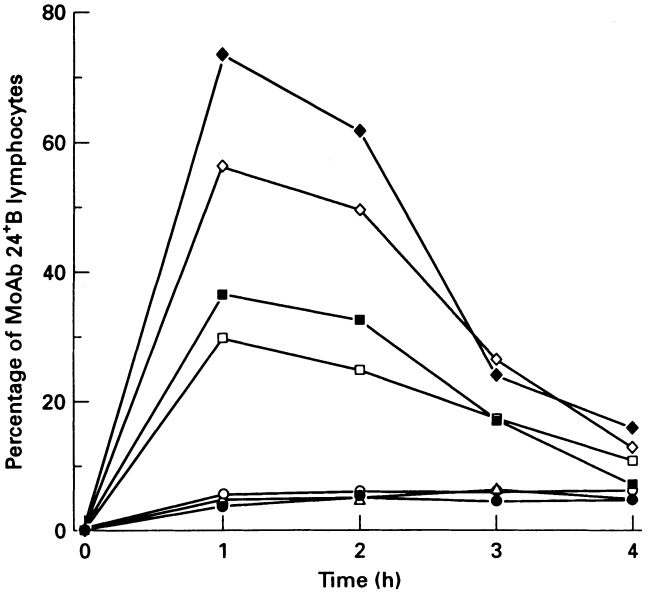

Fig. 1. Two-colour flow cytometry analysis of B lymphocytes for induction of the MoAb 24 activation epitope on LFA-1 following cultivation of peripheral blood mononuclear cells (PBMC) with keyhole limpet haemocyanin (KLH) immune complex (IC). Samples of PBMC cultured in media containing KLH IC were analysed at the indicated time intervals for the proportion of anti-CD19-FITC- or anti-IgM-FITC-stained B cells that were double-stained by MoAb 24-biotin plus streptavidin-PE. Biotinylated MOPC-21 IgG myeloma protein plus streptavidin-PE was employed as a negative control for MoAb 24 staining. The experiment was done twice in which B cells were identified with anti-CD19-FITC and three times in which B cells were identified instead with anti-IgM-FITC with nearly identical results. A typical experiment in which anti-IgM-FITC was used to identify B cells is shown, and results are expressed as the percentage of B lymphocytes that were MoAb 24$^+$. ◆, IgM/IgG-KLH-iC3B/C3dg; ◇, Rab IgG-KLH; ■, IgM/IgG-KLH; □, Rab anti-CR2; ●, KLH; ○, MOPC-21; △, Rab anti-CR1.

Table 1. Blockade of keyhole limpet haemocyanin (KLH) immune complex (IC) mediated activation of LFA-1 on peripheral blood IgM^+ B lymphocytes by anti-C3 and anti-FcγRII

	Percentage of IgM^+/MoAb 24^+ B cells following cultivation with*			
Blocking agent	IgM/IgG-KLH-iC3b/C3dg IC	Rab IgG-KLH IC	IgM/IgG-KLH IC	Rab anti-CR2
None	$73{\cdot}4 \pm 7{\cdot}1$†	$56{\cdot}2 \pm 6{\cdot}7$	$36{\cdot}4 \pm 3{\cdot}9$	$29{\cdot}7 \pm 4{\cdot}6$
$F(ab')_2$ anti-C3	$27{\cdot}5 \pm 4{\cdot}4$	$55{\cdot}3 \pm 9{\cdot}2$	$30{\cdot}2 \pm 7{\cdot}2$	ND
Fab anti-FcγRII	$23{\cdot}9 \pm 2{\cdot}9$	$5{\cdot}8 \pm 2{\cdot}0$	$6{\cdot}4 \pm 1{\cdot}9$	$30{\cdot}3 \pm 7{\cdot}2$
$F(ab')_2$ anti-C3 and Fab anti-FcγRII	$5{\cdot}3 \pm 1{\cdot}9$	$5{\cdot}4 \pm 1{\cdot}7$	$5{\cdot}4 \pm 3{\cdot}2$	ND

* B cells identified with anti-IgM-FITC were evaluated for activation of LFA-1 by double staining with MoAb 24-PE as in Fig. 1, following cultivation of peripheral blood mononuclear cells (PBMC) with KLH IC or Rab IgG anti-CR2 for 1 h at 37°C. To determine the specificity of IC stimulation, either the PBMC or the KLH IC bearing iC3b/C3dg were treated with 10 μg/ml of either AT10 Fab anti-FcγRII or $F(ab')_2$ anti-C3, respectively, for 30 min at 4°C before the 37°C cultivation period.

† Values are mean ± s.e.m. of at least three experiments.

ND, Not done.

iC3b/C3dg IC did not stimulate significant production of non-specific antibody to either HCV peptide or TT, whereas stimulation of PBMC with polyclonal activating agents (LPS, PWM) did stimulate synthesis of antibodies to both HCV peptide and TT (Fig. 2). It was significant that almost no antibody response to KLH was stimulated by cultivation with pure KLH or KLH IC lacking iC3b/C3dg, and that a specific anti-KLH antibody response was only detectable in the cultures stimulated with KLH IC-bearing iC3b/C3dg. This specific response to KLH was inhibited by treatment of the KLH-iC3b/C3dg IC with $F(ab')_2$ anti-C3, but not by treatment of the B cells with MoAb to FcγRII. Further evidence for the KLH specificity of the response to KLH-iC3b/C3dg was the finding that the total immunoglobulin synthesis was within the same μg/ml range as the anti-KLH antibody (Fig. 2). Thus, the iC3b/C3dg-bearing KLH IC were only capable of stimulating KLH-specific B cells to produce antibody, despite their ability to bind via CR2 to the majority of B cells, elicit appropriate co-stimulatory molecule expression on the majority of B cells, and promote the presentation of antigen by non-specific B cells to antigen-specific Th cells [10].

DISCUSSION

This study sought to define further the mechanisms of the C3- and CR2-mediated enhancement of the immune response to primary protein antigens. A major finding was that antigen–iC3b/C3dg complexes elicited only specific antibody in a primary *in vitro* humoral response, despite the demonstration of antigen uptake via CR2 [10] that stimulated most B cells to express co-stimulatory molecules. These findings support the hypothesis that C promotes the humoral immune response largely by enhancing the uptake of antigens by antigen-specific B cells. However, a secondary role for iC3b/C3dg attached to antigen may be to promote the expansion of antigen-specific Th cells through antigen presentation by non-specific B cells that bind and process antigen via CR2 [10]. This pathway requires the presence of low levels of natural antibody to the primary antigen that are sufficient for IC formation and activation of the classical pathway of C. This promotes the attachment of C3 onto the antigen, most of which is broken down into C3dg, promoting antigen uptake by cells bearing CR2. The current investigation showed that KLH-iC3b/C3dg IC stimulated most B cells to express the MoAb 24 activation epitope on LFA-1, and previous work [10] had shown that these same complexes also induced most B cells to express the CD80 co-stimulatory molecule. These data indicate that all of these B cell-activating events are insufficient to elicit immunoglobulin synthesis in the absence of mIg binding to specific antigen.

Although prior experiments had demonstrated antigen presentation to specific Th cells by Epstein–Barr virus (EBV)-transformed B cell lines that had bound KLH-IC via CR2 [10], it was unclear whether such presentation could also occur with normal B lymphocytes. Antigen presentation requires that B cells become activated by antigen to express co-stimulatory molecules that bind to specific counter ligands on Th cells [22]. This study examined LFA-1, a co-stimulatory molecule expressed by APC that must be activated to expose its high-affinity binding site for the Th cell counter ligand ICAM-1 [23]. LFA-1 activation was explored with reporter MoAb 24 that recognizes a cryptic activation epitope shared by all three of the β_2-integrin α-subunits and corresponding to exposure of a common high-affinity ICAM-1-binding state [16,17]. Activation of B cell LFA-1 was shown to require either iC3b/C3dg ligation to CR2 or IgG natural antibody ligation to FcγRII. This finding supports previous reports showing that anti-CR2 [24] or C3d-beads [25] stimulated B cell homotypic aggregation, an event thought to involve both the activation of LFA-1 for binding to ICAM-1 [26], as well as the adhesion of CR2 to CD23 [27]. Furthermore, the signalling of CR2 for such adhesion events is known to be dependent upon a membrane complex consisting of CR2, CD19, and TAPA-1 (CD81), in which TAPA-1 forms a critical part of the signalling complex [28, 29]. The high percentage of B cells responding to KLH IC by activating LFA-1 rules out the possibility that only KLH-specific B cells are responding, and suggests that the entire CR2-bearing B cell subset may be involved in the response. The current data indicate that CR2 and FcγRII can

work additively in the activation of LFA-1. Nevertheless, when IC bore only IgG and no C3, anti-FcγRII alone completely inhibited LFA-1 activation. Likewise, when anti-CR2 was used to ligate CR2 in the absence of FcγRII stimulation, LFA-1 activation occurred but anti-FcγRII was not inhibitory. Although B cell CR1 can also participate in the uptake of iC3b/C3dg-IC, ligation of CR1 with Rab $F(ab')_2$ anti-CR1 failed to activate LFA-1. Although such CR1 ligation would be expected to involve also the CR2 contained in CR1/CR2 membrane complexes [30], such CR2 complexes with CR1 are known to exclude CD19 and TAPA-1 signalling capability [29]. The ability of CR2 and FcγRII to function independently in the activation of LFA-1 is in contrast to the previously demonstrated mechanism for induction of B cell CD80 expression by IC [10]. Although IC binding to B cell CR2 via iC3b/C3dg enhanced the expression of B cell CD80, induction of membrane CD80 required simultaneous stimulation of FcγRII

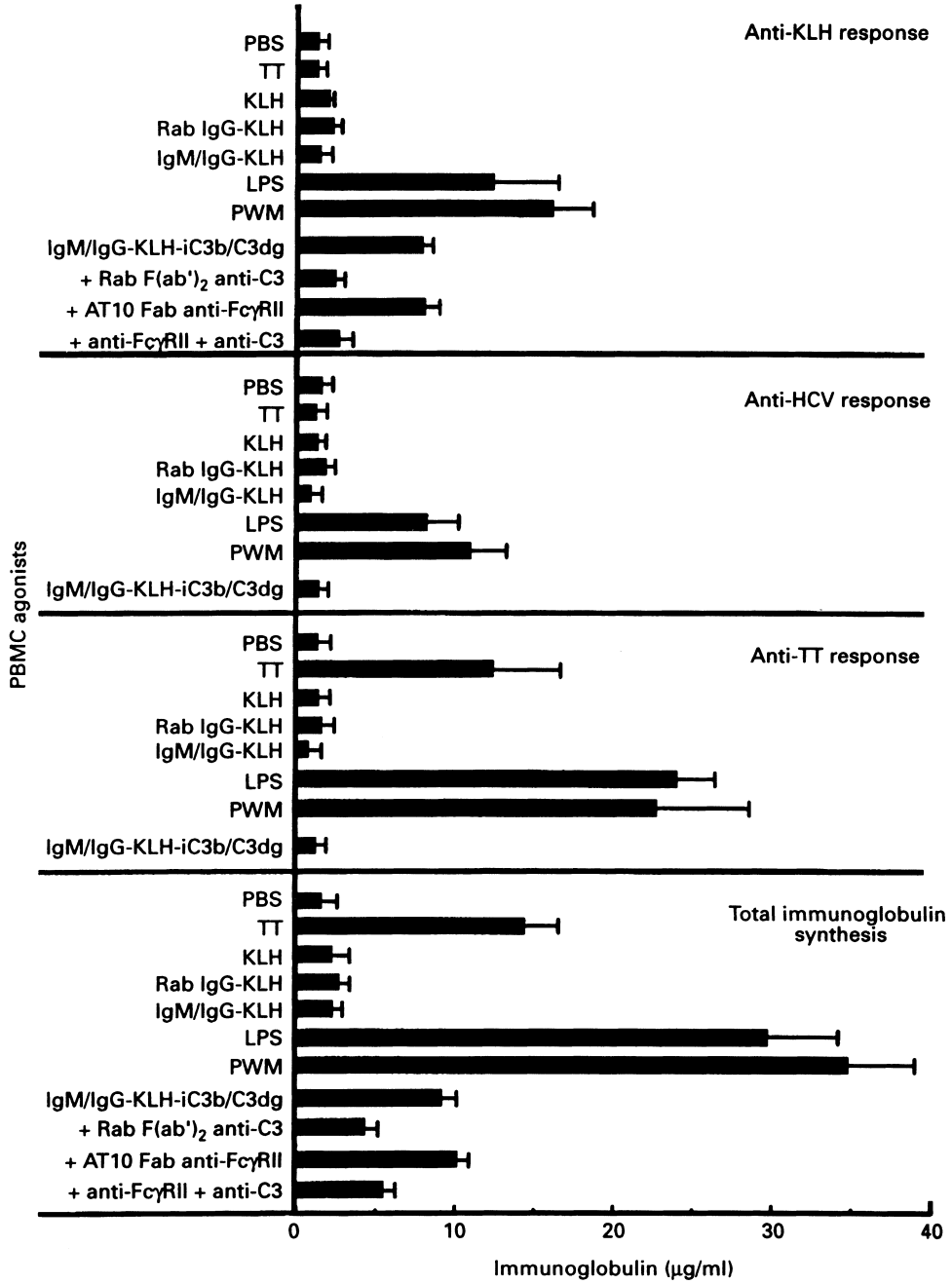

Fig. 2. Assay for an *in vitro* humoral immune response to keyhole limpet haemocyanin (KLH). Peripheral blood mononuclear cells (PBMC) stimulated by cultivation with KLH, KLH immune complex (IC), tetanus toxoid (TT), or the polyclonal B cell activators lipopolysaccharide (LPS) and pokeweed mitogen (PWM) were analysed by ELISA for synthesis of antibodies specific for KLH, hepatitis C virus (HCV) peptide, or TT, and also for total immunoglobulin synthesis. On day 6, supernatants were harvested and tested by ELISA on plates coated with KLH, HCV, TT, or anti-human immunoglobulin. The results were correlated with a standard curve generated with known concentrations of isolated human immunoglobulin to determine the concentration of antibody synthesized in μg/ml. The results represent the mean ± s.e.m. of three or more experiments.

via IC-associated IgG natural antibody, and was not stimulated by Rab anti-CR2 alone.

A major concern was whether the non-specific B cells that processed CR2-bound antigen might be stimulated to secrete immunoglobulin by their interaction with antigen-specific Th cells. It was thus important to show that the KLH IC elicited only a specific antibody response to KLH. It is also significant that IC enhancement of the humoral response required the presence of iC3b/C3dg on the antigen. The KLH-specific response to KLH IC bearing both C3 and IgG was inhibited by Rab $F(ab')_2$ anti-C3 but not by Fab anti-FcγRII. Thus, while stimulation of FcγRII can induce the expression of CD80 and activate LFA-1, antigen uptake mediated only by FcγRII did not stimulate a specific antibody response. B cell FcγRII has long been recognized as an important receptor mediating the down-regulation of immunoglobulin synthesis [31]. These data suggest that simultaneous mIg/CR2 stimulation may override the negative signal generated by IC-associated natural IgG antibody binding to FcγRII.

During a primary immune response, iC3b/C3dg on antigens may promote recognition by CR2-bearing naive B cells that express mIg with too low an affinity to bind the antigen independently of CR2. This might explain the observation that neonates, who have few antigen-specific B cells [32], have a high proportion of $CD5^+$ B cells [33,34], which are associated with the production of low-affinity polyreactive natural antibody [35,36]. According to this model, when neonates were challenged with an antigen that formed IC with circulating natural antibody, the classical pathway of C would be activated, and the resulting antigen-bound iC3b/C3dg would promote antigen uptake by $CR2^+CD5^+$ B cells [37].

CR2 forms part of a B cell transmembrane signalling complex that includes CD19, CD81, and Leu-13 [28,29]. Co-ligation of mIg and the CR2 complex substantially lowers the threshold number of mIg molecules that must be cross-linked for stimulation [38]. Such amplification of small amounts of antigen by CR2 in combination with the ability to interact with low-affinity, polyreactive mIg on $CD5^+$ B cells may be key to an efficient primary response.

As reported previously [10], the possibility also exists for non-specific processing of antigens taken up initially by B cells via CR2 and then presented subsequently to antigen-specific Th cells. Since the current investigation showed that such presentation of antigen by non-specific B cells did not result in non-specific immunoglobulin synthesis, its potential role in the immune response might be the clonal expansion of naive antigen-specific Th cells, thereby increasing the likelihood for an interaction between rarer antigen-specific B cells. Since the amount of antigen required to prime antigen-specific Th cells is known to be much lower than the amount of antigen required to stimulate B cells for a humoral response [39], demonstration of a role for CR2 on non-specific B cells in Th priming would require much lower antigen doses than those used for demonstrating a role of CR2 in humoral responses. This may explain a recently reported failure to demonstrate a necessary role for CR2 in Th cell priming in which the same antigen dose was used in mice for induction of both humoral immunity and Th cell priming [9].

The current findings further support the hypothesis that the immune response to a primary protein antigen may involve natural antibody, the formation of IC that promote activation of the classical pathway of C, covalent attachment of C3dg onto the antigen, and processing of the antigen by any CR2-bearing B cell regardless of mIg specificity [10]. KLH-iC3b/C3dg IC have now also been shown to induce the expression of two B cell co-stimulatory molecules necessary for effective antigen presentation. Nevertheless, antigen-iC3b/C3dg did not induce terminal differentiation of antigen-non-specific B cells, and KLH-iC3b/C3dg elicited only KLH-specific antibodies. While C-mediated antigen recognition is able to enhance responses to antigens present at low levels, it is not essential for an immune response. These results suggest that C provides an important enhancement of antigen recognition that may be essential for responses to some antigens when only a few B cells with low affinity mIg are available [40,41].

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