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RAPID COMMUNICATION

Contribution of the Innate Immune System to Autoimmune Diabetes: A Role for the CR1/CR2 Complement Receptors[1]

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B lymphocytes are required for diabetogenesis in nonobese diabetic (NOD) mice. The complement component of the innate immune system regulates B cell activation and tolerance through complement receptors CR1/CR2. Thus, it is important to assess the contribution of complement receptors to autoimmune diabetes in NOD mice. Examination of the lymphoid compartments of NOD mice revealed striking expansion of a splenic B cell subset with high cell surface expression of CR1/CR2. This subset of B cells exhibited an enhanced C3 binding ability. Importantly, long-term *in vivo* blockade of C3 binding to CR1/CR2 prevented the emergence of the CR1/CR2^{hi} B cells and afforded resistance to autoimmune diabetes in NOD mice. These findings implicate complement as an important regulatory element in controlling the T cell-mediated attack on islet β cells of NOD mice.

INTRODUCTION

Autoimmune diabetes is the end result of specific T cell-mediated destruction of islet β cells. Recently, B lymphocytes have been demonstrated to be critical participants in driving the evolution of T cell-mediated diabetes in NOD[2] mice (1–4). Mechanistically, B cells may act as antigen-presenting cells which mediate the activation of potentially diabetogenic T cells (4–7). Indeed, islet-specific expression of a transgenic costimulatory molecule, B7, or inflammatory cytokines such as IL-10 can obviate the need for antigen presentation by B cells in NOD mice (4, 8). Thus, in considering the APC role of B cells in NOD diabetogenesis, it is essential to examine the routes of Ag uptake through which islet autoantigens might gain access to the antigen processing and presentation machinery of these cells. An important non-Ig-mediated pathway of antigen uptake by B cells is the complement system, which can serve to efficiently direct antigens through the receptors CR1 (CD35) and CR2 (CD21) (9, 10). In fact, these receptors serve as the crucial link between the complement components of the innate immune system and adaptive immune responses (9, 11–13). By binding to C3-conjugated antigens, CR1 and CR2 not only enhance antigen uptake and presentation by B cells but also lower the threshold for B cell activation (14, 15). C3- and C4-deficient mice, as well as CR1/CR2-deficient mice, are compromised in their ability to mount an efficient immune response to T-dependent antigens (16, 17). Additionally, C3 deposition on antigen-presenting cells has been shown to lower the activation threshold of antigen-specific T cells (18). Therefore, in delineating the parameters which allow B lymphocytes to contribute to the T cell-mediated autoimmune attack on islet β cells, the influence of the innate immune system via complement receptors, CR1/CR2, must be explored. The present study characterizes the unique expansion of a CR1/CR2^{hi} B cell subset with an enhanced C3 binding ability in NOD mice. Furthermore, by demonstrating a protective effect of blocking C3 binding to CR1/CR2 *in vivo*, this study implicates a role for the complement cascade in NOD diabetogenesis.

MATERIALS AND METHODS

Mice

NOD/LtJ, C57BL/6, and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free bar-

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[2] Abbreviations used: NOD, nonobese diabetic; MFI, mean flouresence index.

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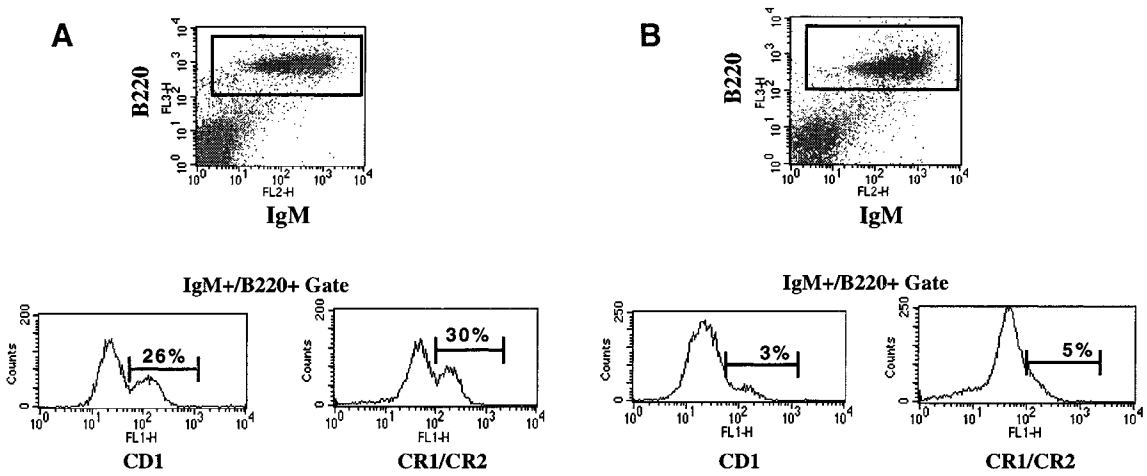

FIG. 1. Flow cytometric analysis of splenocytes from 10-week-old female NOD mice (A) and age- and sex-matched BALB/c mice (B). Splenic lymphocytes were stained with anti-IgM and anti-B220 to identify B cells and counterstained with either anti-CR1/CR2 or anti-CD1d to assess the expression of these latter markers by $IgM^+/B220^+$ gated lymphocytes. The presented data are representative of 20–30 individual mice per strain. This figure documents the expansion of $CR1/CR2^{hi}$ and $CD1^{hi}$ splenic B cells in NOD mice compared to nonautoimmune BALB/c mice.

rier conditions. Female NOD mice in our colony exhibited a spontaneous diabetes incidence in the range of 70–80%, developing between 15–25 weeks of age with peak incidence at 18–20 weeks. It is well known that cyclophosphamide treatment of NOD mice >10 weeks of age induces the accelerated onset of diabetes within 2 weeks after treatment in 100% of treated mice (19). In the present work, a cohort of 10 female NOD mice were treated with twice-weekly injections (200 μg) of purified anti-CR1/CR2 (7G6) mAb starting at birth until 12 weeks of age, at which time they were treated with cyclophosphamide to induce diabetes. An additional cohort of 10 control female NOD mice was treated with isotype control IgG and subsequently with cyclophosphamide at 12 weeks of age.

Induction of Diabetes by Cyclophosphamide

Mice were treated intraperitoneally with 200 mg/kg of cyclophosphamide (Sigma Chemical Co., St. Louis, MO) dissolved in PBS. Two weeks following the initial treatment, the remaining nondiabetic mice were treated a second time. Mice were followed every other day for the development of diabetes defined as three consecutive nonfasting daily blood glucose measurements of >250 mg/dl. Blood glucose measurements were made using CHEMSTRIP bG (Boehringer Mannheim, Indianapolis, IN).

Flow Cytometry

Cells (1×10^6) were surface stained according to a previously described protocol (20). The following antibodies were used: RA3-6B2-biotin (anti-B220), 7G6-FITC (anti-CD21/35), 7E9-FITC (anti-CD21/35), 1B1 FITC (anti-CD1) (PharMingen, San Diego, CA), polyclonal anti-IgM-PE (Southern Biotechnologies, Birmingham, AL), and streptavidin-Red670 (GIBCO BRL, Gaitherburg, MD). C3-FITC was generated by FITC-conjugating affinity-purified human C3. All samples were analyzed on FACScan (Becton Dickinson, Mountain View, CA) using Cellquest software. Twenty thousand events were collected within a live lymphoid gate set based on forward and side scatter.

RESULTS AND DISCUSSION

NOD Mice Harbor an Expanded Subset of Splenic B Cells with a $CR1/CR2^{hi}$ Phenotype

Analysis of splenic B cells from adult (7–20 week old) NOD mice consistently revealed the presence of a greatly expanded pool of splenic B cells characterized by a $CR1/CR2^{hi}$ surface phenotype (Fig. 1). This population made up to $23 \pm 6.5\%$ ($n = 30$) of splenic B cells of both male and female adult NOD mice. In contrast, the splenic B cell compartment of BALB/c and C57BL/6 mice contained a far smaller proportion of such B cells ($4 \pm 2.2\%$; $n = 21$). Additionally, NOD B cells with the $CR1/CR2^{hi}$ phenotype were also IgM^{hi} and $CD1^{hi}$ (Figs. 1 and 2). $CR1/CR2^{hi}$ B cells in NOD mice underwent a sequential expansion beginning at 3 weeks and reaching a peak by 6–7 weeks of age. Interestingly, the emergence and expansion of these B cells were coincident, with the initiation and progression of insulitis in NOD mice starting at 4–5 weeks of age.

In addition to being $CR1/CR2^{hi}$, $CD1^{hi}$, and IgM^{hi}, these B cells revealed a $CD23^{(-)}$ and IgD low to high cell surface expression (data not shown). B cells with this phenotype are thought to be antigen experienced and reside in the splenic marginal zones (21–23). Thus,

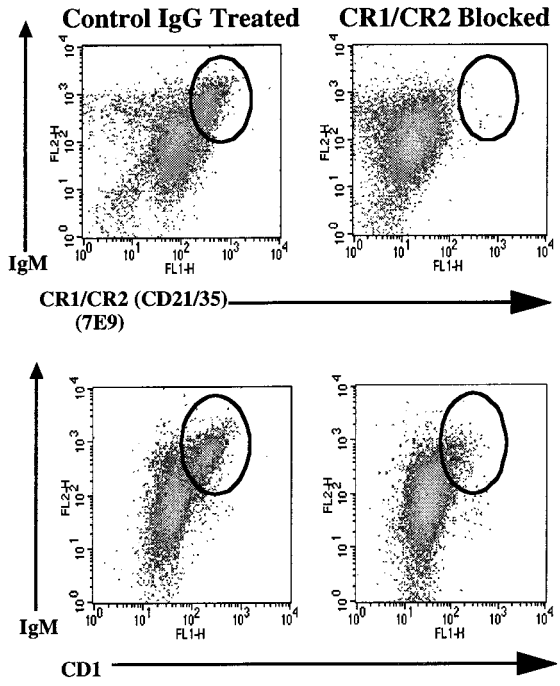

FIG. 2. Flow cytometric analysis of IgM vs CR1/CR2 and IgM vs CD1 expression by $B220^{+}$ gated splenic B cells. Splenocytes were isolated from female NOD mice which had been treated since birth for 11 weeks with anti-CR1/CR2 (7G6) or an isotype control IgG antibody. This figure demonstrates that long-term *in vivo* blockade of CR1/CR2 in NOD mice, using 7G6, does not deplete B lymphocytes and clearly prevents the emergence of the $CR1/CR2^{hi}$, $CD1^{hi}$, IgM^{hi} splenic B cell subset. The circles in each panel demarcate the expected position of $CR1/CR2^{hi}$, $CD1^{hi}$, IgM^{hi} splenic B cells in the dot plots.

it is likely that this pool of $CR1/CR2^{hi}$ NOD B cells may have been activated as a result of an encounter with antigen. In support of this possibility, we found that this uniquely expanded population of B cells displays a forward-scatter profile indicative of enlarged size, a characteristic of antigen-mediated activation.

The Emergence of $CR1/CR2^{hi}$ B Cells Is a Complement Receptor-Driven Process

Since $CR1/CR2^{hi}$ B cells may be activated as a result of antigenic experience we sought to determine whether their emergence is dependent upon CR1/CR2 binding to the C3 ligand. To answer this question, CR1/CR2 binding to C3 was blocked, *in vivo*, using the well-characterized blocking mAb, 7G6 (24). This antibody does not deplete B cells and, in fact, abrogates immune responses to antigens and, in particular, T cell-dependent antigens (14, 24). In agreement with these published reports, the B cell compartment of NOD mice treated over the course of 10–12 weeks with 7G6 was found to contain a normal absolute number of $B220^{(+)}$ lymphocytes. As shown in Fig. 2, the expression of CR1/CR2 in 7G6-treated NOD mice was assessed using the mAb, 7E9, with specificity for a CR1/CR2 epitope distinct from that recognized by 7G6. Utilizing 7E9 mAb, we found that B cells from 7G6-treated mice exhibited down-modulated cell surface expression of CR1/CR2 when compared to control B cells.

Interestingly, long-term treatment of NOD mice with 7G6 prevented the emergence of the IgM^{hi}, $CR1/CR2^{hi}$, $CD1^{hi}$, and $CD23^{(-)}$ B cells in NOD mice, suggesting that the expansion of this subset of cells in NOD mice is dependent upon a complement receptor-mediated process. Based on this conclusion, we reasoned that the appearance of the $CR1/CR2^{hi}$ B cells might be driven by Ag–C3 ligands. Determination of the validity of this hypothesis awaits the development of $C3^{-/-}$ NOD mice, currently underway.

$CR1/CR2^{hi}$ B Cells Have a Markedly Augmented Capacity for Binding C3

The elevated expression of CR1/CR2 endows these cells with an augmented ability to bind C3 ligands. As shown in Fig. 3, the $CR1/CR2^{hi}$ subset of NOD B cells exhibited approximately fivefold greater C3 binding than that of mature resting splenic B cells ($CR1/CR2^{intermediate}$). It has been shown that increased CR1/CR2 expression by B cells endows them with an enhanced ability for C3–Ag conjugate uptake and presentation to T cells (25). In addition, C3 deposition on B cells via CR1/CR2 has been shown to enhance the proliferation of antigen-specific T cells (18). Therefore, we reasoned that the $CR1/CR2^{hi}$ B cells, by virtue of their enhanced capacity for binding and uptake of C3-opsonized autoantigens, might contribute to the activation of diabetogenic T cells in NOD mice. To test this hypothesis we sought to block C3 binding to CR1/CR2 in NOD mice.

In Vivo Blockade of C3 Binding to CR1/CR2 Renders NOD Mice Resistant to Diabetes

The coincident kinetics of the emergence of $CR1/CR2^{hi}$ B cells with the initiation of insulitis in NOD mice suggested the intriguing possibility that the C3 complement component, via CR1/CR2 on B cells, might play an important role in diabetogenesis. To test this possibility the interaction of the C3 ligand with CR1/CR2 was blocked *in vivo,* using 7G6. Figure 3 shows that B cells from 7G6-treated NOD mice exhibited a five- to sixfold reduction in C3 binding compared to control IgG-treated mice. This finding is in accordance with previous reports demonstrating inhibition of C3 binding to CR1/CR2 by 7G6 (14). Furthermore, as mentioned above, long-term treatment of NOD mice with 7G6 prevented the emergence of the expanded population of $CR1/CR2^{hi}$ splenic B cells. With the assurance that *in vivo* 7G6 treatment of NOD mice does not deplete B cells and leads to effective blockade of C3 binding to CR1/CR2 *in vivo,* the contribution of C3

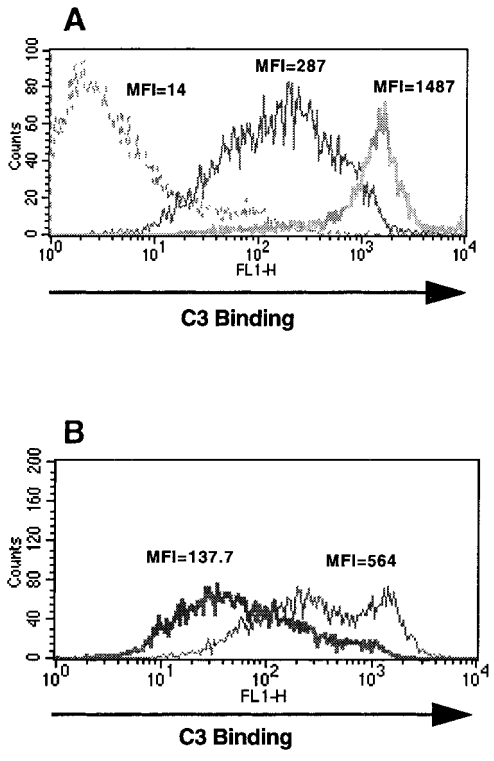

FIG. 3. Determination of the relative C3 binding ability of splenic B lymphocytes using flow cytometry. (A) C3 binding to the CR1/CR2^{hi}, B220$^{(+)}$ splenic B cell subset (gray line) is compared with C3 binding to the CR1/CR2^{intermediate} splenic B cell subset (black line). The dotted line is indicative of the C3 binding ability of B220$^{(-)}$ splenocytes in NOD mice. The presented data are representative of three separate experiments. This panel demonstrates the five- to sixfold-enhanced ability of the CR1/CR2^{hi} B cell subset for binding C3 when compared to the CR1/CR2^{intermediate} subset. (B) Determination of the C3 binding ability of B220$^{(+)}$ splenic B cells from long-term *in vivo* anti-CR1/CR2(7G6) (bold line) and isotype control IgG-treated female NOD mice (lightface line). This panel demonstrates decreased C3 binding by NOD B lymphocytes upon long-term *in vivo* treatment with 7G6. MFIs in this figure indicate mean flourescence index as a measure of C3 binding.

binding to CR1/CR2 to NOD diabetogenesis was assessed.

Two separate cohorts of female NOD mice were treated with either 7G6 or an isotype-matched control Ig for up to 12 weeks of age, at which time they were treated with cyclophosphamide to rapidly induce diabetes. It is well established that, as early as 10–12 weeks of age, 100% of unmanipulated female NOD mice develop diabetes vigorously following treatment with cyclophosphamide (26). The induction of diabetes in cyclophosphamide-treated NOD mice is known to be dependent on the severity of islet inflammation. Interestingly, 7G6-treated NOD mice were markedly resistant to cyclophosphamide-induced diabetes compared to their control IgG-treated counterparts (Fig. 4). In fact, histologic examination of pancreata from representative 7G6-treated NOD mice at 12 weeks of age demonstrated the presence of periinsulitis in <50% of islets (data not shown). This was in contrast to the invasive insulitis present in pancreata from control IgG-treated NOD mice. Thus, the resistance of 7G6-treated NOD mice to cyclophosphamide-induced diabetes suggests that the nature of islet cell inflammation seen in these mice is benign compared to that seen in the control group, a finding confirmed by the histologic data described above.

The diabetes-resistant phenotype of 7G6-treated NOD mice correlates with the five- to sixfold reduced C3 binding capacity of B cells in 7G6-treated mice (Fig. 3) and parallels the absence of CR1/CR2^{hi} B cells from these mice (Fig. 2). These findings suggest a pivotal link between the innate immune system, via complement, and B lymphocytes in mediating the T cell attack on islet β cells. Future analysis of the contribution of complement to the dysregulated state of T and B cell tolerance to marker islet autoantigens, such as GAD65, will provide insight into the interplay of innate and adaptive immunity in NOD diabetogenesis.

In summary, this work characterizes the presence of an unusually expanded B cell population with CR1/CR2^{hi} cell surface expression and a markedly enhanced C3 binding capacity. Most notably, C3 binding to CR1/CR2 is implicated in the rapid progression of the anti-islet T cell response after the initial targetting of β cells has spontaneously occurred in NOD mice.

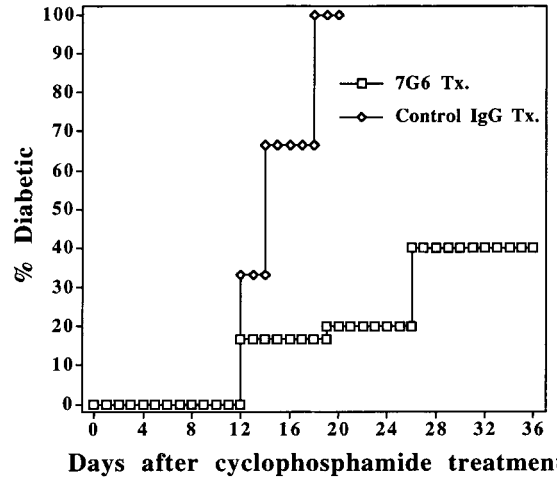

FIG. 4. Diabetes incidence in a cohort of 7G6-treated female NOD mice (n = 10) is compared to that in isotype control IgG-treated (n = 10) female NOD mice. The experimental cohort of female NOD mice was treated with 7G6 starting at birth up to 12 weeks of age (squares). The control group was treated with an isotype control IgG starting at birth up to 12 weeks of age (diamonds). At 12 weeks of age NOD mice from both groups were treated with a first injection of cyclophosphamide. At 14 weeks of age all nondiabetic mice remaining in each cohort were treated with a second injection of cyclophosphamide. This figure documents the marked resistance of 7G6-treated female NOD mice to the development of cyclophosphamide-induced diabetes.

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