Two Levels of Help for B Cell Alloantibody Production

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Summary

We have examined whether T cell stimulation by direct or indirect pathways contributes to alloantibody production by B cells after major histocompatibility complex (MHC)-disparate skin graft rejection in mice. Experiments were performed using normal mice, MHC class II-deficient mice, MHC class II-deficient mice with an intact peripheral CD4+ cell population (due to expression of class II antigens only on thymic epithelium), mice lacking the cytoplasmic tail of their MHC class II antigens, and mice depleted of CD4+ cells by anti-CD4 monoclonal antibody treatment. Depletion of recipient CD4+ cells reduced alloantibody production to barely detectable levels. Absence of donor MHC class II antigens did not affect the production of either immunoglobulin (Ig)M or IgG antibodies directed at class I alloantigens. Absence of recipient MHC class II antigens, however, led to production of only IgM but not IgG antibodies, even if the recipients had an intact CD4+ cell population. Absence of the cytoplasmic tail of the recipient's MHC class II antigens led to the production of slightly reduced amounts of IgG antibody. These findings indicate that (a) CD4+ cells are essential helper cells for B cell alloantibody production; (b) production of IgM alloantibody can occur with help from CD4+ cells, which recognize either donor class II antigens or modified recipient class II antigens; (c) isotype switching from IgM to IgG alloantibody requires help from CD4+ cells activated by antigens presented by recipient MHC class II molecules; and (d) the cytoplasmic domain of the recipient MHC class II molecules may be involved in the mechanism that leads to isotype switching by B cells. Thus, there are two levels of CD4+ mediated help available for B cells responding to alloantigens: one (involving a noncognate interaction) can produce B cell activation, and a second (involving a cognate interaction) is required for differentiation and IgG alloantibody production.

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Figure 1. Two possible schemes by which T cells might be stimulated to provide help for B cell anti-class I alloantibody production. (A) B cells could be stimulated by class I antigens on the surface of donor APC, and CD4+ helper cells could be stimulated directly by the donor class II antigens on the same APC. This would bring the T and B cells into physical contact but would not allow a cognate interaction between the responding T cell receptor with the MHC class II molecule of the B cell. (B) B cells could be stimulated by antigens on donor cells, and CD4+ cells could be stimulated indirectly by foreign peptides presented by the recipient's APC. These CD4+ cells could then provide help for the B cells by a cognate interaction with the B cell's own class II antigens.

in vivo, and they also reveal that CD4+ cells can provide two levels of help for B cells, depending on the nature of the T–B cell interaction.

Materials and Methods

Mice. Male BALB/cByJ (BALB/c) (H-2b) and C57Bl/6J (B6) (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice lacking MHC class II antigens (class II deficient), mice expressing MHC class II antigens only on their thymic epithelium (CD4+ class II deficient), and mice expressing class II antigens with truncated cytoplasmic domains (tailless), were bred in our laboratories.

The genotype and phenotype of mice lacking MHC class II antigens have been previously described (3). For these experiments, mice from the third generation backcross of (B6 × C3H)F1 to B6 mice were used. Prior characterization of the class II-deficient mice has shown that no class II antigen expression can be detected. These mice also show a substantial depletion of CD4+ peripheral T cells, although 3–5% of the peripheral Thy1+ cells are CD4+. Class II-deficient mice have normal levels and development of B cells since T cell-independent B cell responses occur in vivo, and T cell-dependent B cell activation can occur in vitro, despite the absence of MHC class II antigens (4).

Mice expressing MHC class II antigens only on their thymic epithelium were generated by breeding class II-deficient mice with a C57Bl/6-transgenic strain (gift of Dr. D. Lo, Scripps Research Institute, La Jolla, CA), which expresses the I-Aq transgene only on thymic epithelium (5). These mice are MHC class II deficient on all cells other than thymic epithelium, but they do have normal numbers of peripheral CD4+ cells, which proliferate in an allo MLR in vitro (Laufer, T., unpublished observations).

Mice that express a class II Aa molecule with a truncated cytoplasmic domain were generated by mating class II-deficient mice with mice expressing an Aαβ transgene that lacks 13 of its 18 cytoplasmic domain residues. The initial characterization of these mice has been previously reported, demonstrating that they develop normal levels and functions of CD4+ T cells and have normal B cell responses to environmental pathogens (6).

CD4-depleting Therapy. To deplete CD4+ cells in vivo, thymectomized B6 mice were treated with 0.1 ml of GK1.5 ascites on days 6, 3, and 1 before skin grafting. This technique depletes >98% of CD4+ cells for several weeks (7).

Skin Grafting. Trunk skin grafts were placed on mice according to the technique of Billingham and Medawar (8). Mice were anesthetized with chloral hydrate supplemented with ether. After rejecting their grafts, mice were then bled from the retroorbital plexus and subsequently boosted with 10⁶ splenocytes of the same donor type by intraperitoneal injection at weekly intervals for 3 wk. Sera used for the experiments shown were all obtained 3 wk after placing the skin grafts.

Antibody-mediated Complement-dependent Cytotoxicity Assay. These assays were performed in two stages with minor modifications from the technique previously described (9). Sera were heat inactivated at 56°C for 30 min and stored at −20°C until tested. 10⁶ 51Cr-labeled mouse splenocytes of donor type were incubated with serially diluted sera in a final volume of 50 µl in U-bottomed wells for 15 min at 37°C. Cells were washed with medium, pelleted, resuspended, and incubated with rabbit complement (C-6 Diagnostic, Inc., Mequon, WI) at a final dilution of 1:12 for 30 min at 37°C. Cells were washed with medium, pelleted, resuspended, and incubated with rabbit complement (C-6 Diagnostic, Inc., Mequon, WI) at a final dilution of 1:12 for 30 min at 37°C. Cells were washed and eluted with PBS, 1% BSA, and 0.1% sodium azide. 3 × 10⁶ cells were added to each well of a 96-well U-bottomed plate. Mouse FcgIIIR was blocked with 20 µl of 2.4 G2, a rat anti–mouse FcgIIIR mAb, by incubating with the cells at
4°C for 10 min. The cells were then washed twice with 100 µl of FACS® medium. 25 µl of serum from mice that had rejected skin grafts was then added at 1:2 and 1:4 dilutions to the test wells and incubated at 4°C for 30 min. Cells were again washed twice with 100 µl FACS® solution. FITC-conjugated rat anti-mouse antibodies against IgM, IgG1 (Zymed Laboratories, Inc., South San Francisco, CA), IgG2a, or IgG2b (Baxter Healthcare Corp., Mundelein, IL) were then added and incubated with the cells at 4°C for 30 min. Cells were again washed twice with 100 µl FACS® solution, fixed with 2% paraformaldehyde, and analyzed using a FACSscan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Results
To generate alloantibody production, skin grafts from MHC-disparate allogeneic donors were placed on recipient mice. All skin grafts were rejected within 15 d, and neither treatment with anti-CD4 mAb nor the absence of MHC class II antigens on the donor or recipient altered the time to rejection when compared with normal controls. These findings are in keeping with previously reported data (9-11). Sera from recipient mice were tested 3 wk after grafting for the presence of alloantibody, as shown in Fig. 2. Additional sera from later time points were also tested, but the results in all groups were not different in character from those shown.

Normal Mice. Normal recipients of normal MHC-disparate skin grafts produced antidonor cytotoxic alloantibody of IgM isotype and then increasing titers of IgG isotype. The levels at 3 wk are shown in Fig. 2 A. The antibodies in these sera were shown to be directed at class I alloantigens by testing them on targets from MHC-congeneric strains (data not shown).

CD4-depleted Recipients. Recipients treated with thymectomy and anti-CD4 mAb before skin grafting produced very low or undetectable levels of cytotoxic alloantibody, and there was no switch to IgG isotype, as shown in Fig. 2 B. This requirement for CD4+ cells to generate alloantibody production is in agreement with previously reported data (9).

MHC Class II-deficient Mice as Donors. When MHC class II-deficient mice were used as skin graft donors for normal recipients, both IgG and IgM alloantibody production was equivalent to normal controls, as shown in Fig. 2 C. Thus, CD4+ cells stimulated only through the indirect pathway,

![Figure 2. Cytotoxic alloantibody levels in response to skin grafting. Sera were obtained 3 wk after grafting, and alloantibody levels were determined in a 51Cr release antibody-mediated complement-dependent cytotoxicity assay using mouse splenocytes of donor type as targets. IgM combined with IgG levels were measured using untreated sera. IgG levels alone were obtained after treatment of sera with 2-ME. The data presented represent means of lysis by sera from at least four mice in each panel shown. (A) B6 mice (H-2b) received skin grafts from MHC-disparate BALB/c (H-2d) donors. Almost identical results were obtained when BALB/c recipients received B6 skin grafts (data not shown). (B) CD4-depleted B6 mice received skin grafts from BALB/c donors. (C) Normal BALB/c recipients received skin grafts from B6 MHC class II-deficient donors. (D) B6 MHC class II-deficient recipients received skin grafts from BALB/c donors. (E) B6 class II-deficient mice expressing a class II transgene that encodes class II antigens only on their thymic epithelium received grafts from BALB/c donors. (F) B6 class II-deficient mice expressing a class II transgene that encodes class II antigens lacking their cytoplasmic tail received BALB/c skin grafts.](image)
responding to modified self class II antigens, provide help efficiently for alloanibody production.

MHC Class II-deficient Mice as Recipients. MHC class II-deficient mice that received normal skin grafts produced cytotoxic alloanibodies, but in reduced titers when compared with normal controls. The antibody produced was of IgM isotype only, and no IgG was found in these recipients, both in assays for antibody-mediated complement-dependent cytotoxicity as shown in Fig. 2 D and by FITC anti-antibody staining (data not shown).

Although the donor skin grafts in these experiments expressed class II antigens that could stimulate CD4 + cells directly, the recipients had two defects that could account for their reduced antibody production: they lacked class II antigens on their own cells for use in the indirect pathway, and they also had severely reduced numbers of CD4 + cells to provide help for B cells. Therefore, to test whether direct stimulation, in the absence of the indirect pathway, could provide help for B cells, we needed recipients without class II antigen expression but that had normal numbers of CD4 + cells.

MiceExpressing Class II Antigens Only on Thymic Epithelium as Recipients. Class II-deficient mice that express a class II transgene only on thymic epithelium develop normal numbers of mature CD4 + cells but still lack class II antigens on their peripheral APC. When these mice were recipients of normal skin grafts, they produced only moderate titers of total cytotoxic antibody, all of which was again of IgM isotype, as shown in Fig. 2 E. These results were consistent in antibody-mediated complement-dependent assays and in assays using FITC anti-antibody staining (data not shown). Thus, in the presence of direct but in the absence of indirect T cell stimulation, CD4 + cells can provide help for B cells, but only for a limited response.

Mice with Tailless Class II Molecules as Recipients. Finally, recipients lacking the intracytoplasmic domain of their MHC class II antigens were found to produce alloanibody that included both IgM and IgG isotypes. However, in two separate experiments, there was a slight reduction in the IgG component of the total cytotoxic antibody response compared with normal animals (Fig. 2 F).

Discussion

The primary purpose of these experiments was to determine the answer to a long-standing question in transplantation immunology: whether donor class II antigens stimulate helper CD4 + cells for alloanibody production by direct recognition, or whether recipient class II molecules, presenting peptides of donor antigens, stimulate a helper response through what has been termed the indirect pathway. We expected that the indirect pathway would be sufficient, since this is the mechanism for a normal immune response. We also expected, based on previous studies, that both IgM and IgG alloanibody responses after skin transplantation would depend on the presence of CD4 + T cells (9). Finally, we were not surprised that stimulation of CD4 + cells only by the nonphysiologic, direct pathway was unable to stimulate IgG antibody production. These experiments, therefore, settle the issue in transplantation immunology, at least as far as the response to class I antigens on skin grafts. In doing so they also provide further evidence that the so-called indirect pathway in transplantation does play a role in the immune response to foreign tissues and that this pathway is actually critical as far as alloanibody production is concerned.

The result we did not expect (which was, therefore, particularly interesting) was that CD4 + cells stimulated by the direct pathway alone would allow an intermediate B cell response, including IgM but not IgG alloanibody production. This result is of interest beyond the field of transplantation immunology since it indicates that B cells are susceptible to two levels of help for antibody responses. When CD4 + cells are stimulated in a way that links them in physical proximity to B cells, their helper factors can apparently activate the B cells partially, allowing the IgM response. However, apparently only when the CD4 + cells are stimulated in a way that provides both linkage and a cognate interaction through the class II molecules of the B cells does the helper response signal the B cells to switch to IgG production. Only a transplantation experiment, especially one involving the recently available class II knock-out mice, would allow us to see this distinction in vivo. In no other way could one bring B cells, recognizing a foreign protein antigen, into a physical but noncognate linkage with CD4 + helper cells than to stimulate the CD4 + cells with allogeneic class II molecules while eliminating the class II molecules of the recipient’s own APC. Thus, these transplantation experiments provide new insight into the mechanisms of normal B cell physiology.

Our experiments have not yet proven that a physical linkage is required between the CD4 + cells, stimulated by donor class II antigens, and the B cells that respond to donor class I alloantigens. It seems likely that such a linkage is necessary, given the many previous experiments indicating that linkage between T and B cells is a physiologic requirement in vivo (1, 2, 12). However, to demonstrate this requirement in our model we will need to determine whether B cells from mice lacking their own peripheral class II antigen expression can respond to donor alloantigens, such as Thyl, which are not expressed on the same cells that express the class II alloantigens that stimulate CD4 + helper cells.

It is also not clear from our experiments exactly why the cognate interaction between T and B cells, through recognition of the B cells’ class II antigens, should be important in the switch to IgG antibody production. One explanation is that such an interaction is simply the best way to achieve the closest possible physical proximity between helper cells and B cells. The other is that the class II molecules of B cells are involved in the signaling that allows conversion to an IgG response. Previous in vitro studies with transfected cell lines have suggested that the cytoplasmic tail of class II molecules is necessary for the expression of costimulatory molecules (13–16), but subsequent studies with the same transgenic mice used in our experiments, which express
only class II molecules with a truncated intracytoplasmic domain, have indicated that they produce normal IgG responses to nominal antigens, although they appear to have a quantitative defect in their ability to present peptides of foreign proteins (6). In our experiments we found that both IgM and IgG alloantibody responses were fundamentally intact in the mice lacking their class II intracytoplasmic domain, although the IgG response appeared to be weaker in several experiments. Thus, our results are consistent either with the interpretation that slightly diminished presentation of peptides by these truncated class II molecules weakens the physical association of T and B cells, or that the absence of the cytoplasmic portion of the B cells' class II molecules impairs the signaling that leads to IgG conversion.

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