IN VIVO EFFECTS OF MONOCLONAL ANTIBODIES TO DISTINCT EPITOPES OF Qa-2 ANTIGENS

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A series of new mAbs directed against Qa antigens has recently been described from this laboratory (1). Because Qa antigens are predominantly expressed on mature T cells (2), we have attempted to determine the effects of in vivo treatment with these new mAbs on parameters of T cell immunity. Two antibodies (1-1-2 and 1-9-9) of identical subclass and similar avidity, but directed toward distinct epitopes, were chosen for these studies. Both were found, by immunoprecipitation of ^{125}I-labeled C57BL/10 spleen cell surface antigens, to precipitate 40-kD molecules that could be completely precleared by each other (Park, E., et al., manuscript in preparation), suggesting that both mAbs bind to the same molecules. Very different effects, however, were observed after administration of these antibodies in vivo, 1-1-2 leading to rapid deletion of peripheral T cells, and 1-9-9 showing little immediate effect, and depletion only after several days. Surprisingly, coadministration of both antibodies mimicked the effect of 1-9-9, rather than that of 1-1-2. The present communication details these observations and presents the effects of antibody administration on parameters of T cell immunity. In addition, an attempt is made to examine the mechanism of the differential effects observed in terms of events at the cell surface after antibody administration.

Materials and Methods

Animals. 12–20-wk-old mice of strain C57BL/10Sn (B10) and B10.D2/nSn (B10.D2) were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in a conventional animal facility.

Monoclonal Antibodies. Anti-Qa-2 mAbs 1-1-2 and 1-9-9 were derived from immunization of C3H.KBR (bbk) mice with C3H.SW (bbb) lymphoid cells as previously described (1). These two mAbs are both of the IgG2a subclass and are specific for distinct epitopes of Qa-2. The ascites pools used for these experiments each had titers of ~1:4,000 as measured by complement-mediated cytotoxicity on splenocytes, and of >30,000 as measured by complement-mediated cytotoxicity on thymocytes. Antibodies were purified from ascites fluid using protein A– Sepharose (3) and then fluoresceinated by standard methods (4). Fluoresceinated and biotinylated anti-Thy-1.2 were purchased from Becton Dickinson Immunocytometry System (Mountain View, CA) and Texas red streptavidin (TRA) was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Fluorescein-labeled anti-Ia^b (Y3P) (5) and anti-Fc receptor mAb (2.4G2) (6) were generous gifts from J. Titus (Experimental Immunology Branch, NCI).

Immunofluorescence Staining and Flow Cytometry Analysis. T and B cell depletion in vivo after

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treatment with anti-Qa mAb was determined by staining of PBL with fluoresceinated anti-Thy-1.2 or anti-Ia^b (Y3P) as previously described (7). Blocking of anti-Qa-2 binding was performed by pre-incubating PBL with 1-1-2 or 1-9-9 ascites (diluted 1:10) for 30 min at 4°C followed by incubation with fluorescein-labeled mAbs. Modulation of Qa antigens on T cells was determined by two-color staining with fluoresceinated anti-Qa mAbs (1-1-2 or 1-9-9) (in green) and biotinylated anti-Thy-1.2 followed by TRA (in red). For quantitative comparison of staining intensity, median channel values of T cell fluorescence intensities were converted to linear units using an empirically derived standard calibration curve. Nonspecific staining was assessed with irrelevant mAbs, fluoresceinated or biotinylated anti-Leu-4 (anti-human CD3). Anti-Fc receptor antibody was used at a concentration of 100 μg/ml (10 μl/10^6 cells) to block Fc receptor–mediated antibody binding during the staining procedure. Flow cytometry (FCM) analysis was performed as previously described (8) using a modified B-D Dual Laser FACS II (Becton Dickinson Immunocytometry Systems). One-color fluorescence data were displayed as immunofluorescence profiles in which log fluorescence intensity was plotted on the x-axis and cell number on the y-axis. Two-color immunofluorescence data were displayed as contour diagrams in which log intensities of green (FITC) fluorescence were plotted on the x-axis and log intensities of red (Texas red) fluorescence were plotted on the y-axis.

In Vitro Assays of Cellular Immunity. Proliferative responses of splenocytes to T cell and B cell mitogens, as well as responses to alloantigens, were determined by radiolabeled thymidine incorporation. 5×10^4 spleen cells per well from mice treated 2 d earlier with PBS, or with 1-1-2, 1-9-9 or both mAbs, were incubated in 96-well round-bottomed culture plates for 4 d with either medium (RPMI 1640 plus 0.5% syngeneic mouse serum) alone, 0.5 μg/ml Con-A (Miles Scientific, Naperville, IL), 25 μg/ml LPS (Difco Laboratories, Detroit, MI), or 5×10^4 irradiated (3,000 rad) syngeneic (B10) or allogeneic (B10.D2) spleen cells in 0.2 ml. 8 h before the end of incubation cells were pulsed with [H^3]TdR (1 μCi/well), and thymidine incorporation per well was measured.

Skin Grafts. Unmanipulated B10 (H-2^b) mice and mice treated on day −1 with 1-1-2, 1-9-9 or both mAbs were grafted with tailskin allografts from B10.D2/nSn (B10.D2, H-2^d) donor mice according to the method of Billingham (9). Bandages were removed on day 7 and the grafts were scored daily until rejection (defined as loss of >90% of the graft tissue).

Results

Epitope Specificity of 1-1-2 and 1-9-9 mAb. In previous panel testing of our new series of anti-Qa-2 mAbs, 1-1-2 and 1-9-9 were categorized as belonging to two distinct epitope groups, epitope cluster 1 and epitope cluster 3, respectively (1). Antibody blocking studies confirming this assignment are shown in Fig. 1. As seen in this

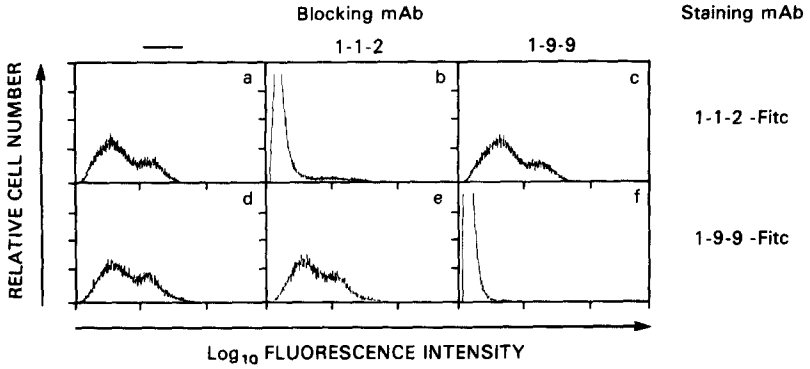

FIGURE 1. 1-1-2 and 1-9-9 anti-Qa-2 mAbs do not block each other's binding. Flow cytometry analysis of B10 spleen cells stained with 1-1-2-FITC (*a*, *b*, and *c*) or 1-9-9 FITC (*e*, *f*, and *g*), before (*a* and *e*) or after preincubation with 1/10 diluted 1-1-2 (*b* and *f*) or 1-9-9 ascites (*c* and *g*).

figure, the binding of fluoresceinated 1-1-2 was inhibited completely by preincubation with unlabeled 1-1-2 mAb (Fig. 1 *b*), but was not diminished at all by unlabeled 1-9-9 mAb (Fig. 1 *c*), as compared with the control (Fig. 1 *a*). Conversely, binding of fluoresceinated 1-9-9 could only be inhibited by the homologous antibody (Fig. 1, *d-f*).

Effects of Treatment with Anti-Qa-2 mAbs on Peripheral T Cells. B10 animals were treated with a single intraperitoneal injection of 0.2 ml of ascites fluid from each of the mAbs. The effects of this treatment on peripheral T cell levels were examined by flow microfluorometry as a function of time, as illustrated in Fig. 2. Treatment with 1-1-2 led to a rapid loss of peripheral T cells, reaching minimal level (10–20% of normal level) by 3 d after injection. In contrast, treatment with 1-9-9 led to only a slight decrease in peripheral T cell levels by day 3, with gradual diminution thereafter, reaching minimal levels only by day 12. Both antibodies depleted CD4 and CD8 T cells to a similar extent (data not shown). Although Qa-2 antigens are expressed on both T cells and B cells (2), the expression is much greater on T cells as demonstrated by the typical bimodal fluorescence histogram (Fig. 1). Only Qa^+ T cells, and not B cells, were depleted by treatment with either antibody (Fig. 3). A similar pattern of T cell depletion was demonstrated in spleen and lymph node but not in thymus. Although these antibodies bound in vivo to the small population of thymocytes expressing Qa antigen (5%), they failed to eliminate these cells from the thymus (data not shown).

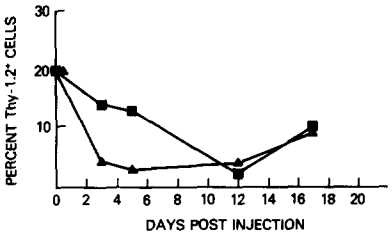

FIGURE 2. Comparison of in vivo PBL depletion by anti-Qa-2 mAbs. B10 mice were injected with 0.2 ml 1-1-2 (▲) or 0.2 ml 1-9-9 (■) mAbs. T cell level was determined at different time points after treatment, by flow cytometry analysis after staining with anti-Thy-1.2-FITC. Data are presented for a representative animal from each treatment group. This pattern was observed on more than five animals in different experiments.

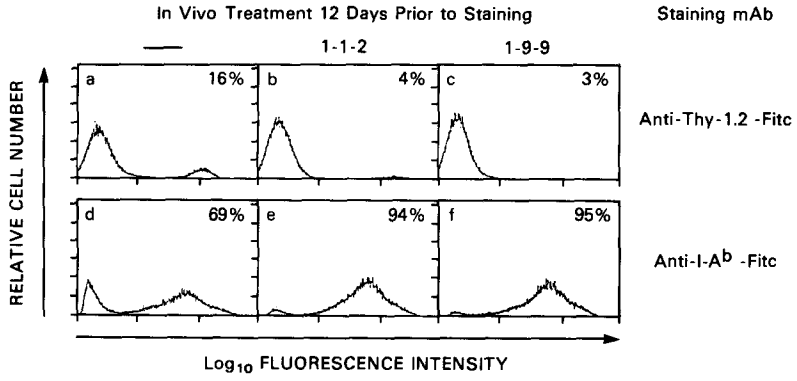

FIGURE 3. Effect of anti-Qa mAbs on T and B cell levels. PBL from B10 mice either unmanipulated (*a*, *d*) or injected 12 d before with 1-1-2 (*b*, *e*) or 1-9-9 (*c*, *f*) anti-Qa mAbs, were stained with anti-Thy-1.2-FITC (*a*, *b*, *c*) or with anti-Ia^b-FITC (*d*, *e*, *f*).

Simultaneous administration of 1-1-2 plus 1-9-9 mAbs produced the surprising finding that peripheral T cells were not depleted at early time points (Fig. 4). At 2 d after treatment, the peripheral T cell levels in animals given both antibodies were similar to those of animals given only 1-9-9, while animals treated with 1-1-2 showed marked depletion. Even at 8 d after treatment, animals treated with both antibodies showed less depletion of peripheral T cells than did animals treated with 1-1-2 alone. These findings were reproduced in three of three separate experiments.

Functional Effects of In Vivo Antibody Treatment. The effects of in vivo administration of 1-1-2 and 1-9-9 on several parameters of cellular immunity are shown in Fig. 5 and Table I. Allogeneic skin grafts placed on B10 animals 1 d after treatment were

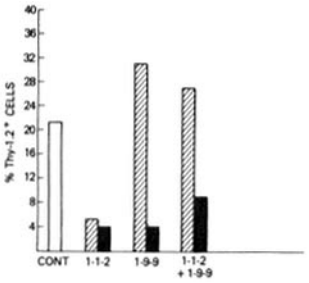

FIGURE 4. Effect of simultaneous administration of 1-1-2 plus 1-9-9 mAbs on time course of T cell depletion. 2 d (▩) and 8 d (■) after treatment with either 1-1-2 or 1-9-9 alone or with both antibodies, PBL were stained with anti-Thy-1.2-FITC and analyzed by flow cytometry.

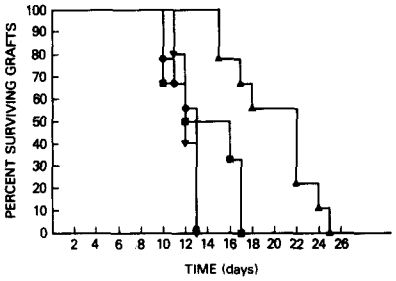

FIGURE 5. B10.D2 skin graft survival on B10 mice treated with anti-Qa-2 mAbs. B10 mice (5–9 mice each group) either unmanipulated (●) or treated with 1-1-2 (▲), 1-9-9 (■) or 1-1-2 plus 1-9-9 (▼), were grafted with B10.D2 allogeneic tail skin one day after treatment. Survival of these grafts was scored daily.

TABLE I

Effect of Anti-Qa-2 mAb Treatment on T and B Cell Functions

Treatment	[^{3}H]Thymidine incorporation*			
	PBS	1-1-2	1-9-9	1-1-2 + 1-1-9
	cpm			
Stimuli				
None	1,680 ± 168	522 ± 51	586 ± 102	710 ± 114
Con A (0.5 μg/ml)	84,785 ± 4,731	28,612 ± 175	43,396 ± 11,505	41,278 ± 5,284
LPS (25 μg/ml)	27,217 ± 1,073	23,835 ± 306	20,072 ± 2,351	17,671 ± 1,458
Syngeneic cells	1,159 ± 624	884 ± 312	1,013 ± 337	887 ± 242
Allogeneic cells	5,743 ± 502	698 ± 21	2,606 ± 1,153	1,880 ± 544

* Spleen cells from B10 mice treated 2 d earlier with PBS or with 1-1-2, 1-9-9, or 1-1-2 plus 1-9-9 anti-Qa-2 mAbs, were stimulated in vitro with T cell mitogen (Con A), B mitogen (LPS), or with irradiated syngeneic (B10) or allogeneic cells (B10.D2). Proliferation was assessed after 4 d by the incorporation of [^{3}H]thymidine added during the final 8 h of culture. Data presented are mean values and standard error of the mean of triplicates.

significantly prolonged by administration of 1-1-2, but not by 1-9-9 alone or the combination of 1-1-2 plus 1-9-9 (Fig. 5). Marked inhibition of T cell stimulation by the mitogen Con A or by an MLR was observed at day 2 after administration of 1-1-2 mAb, but less inhibition was observed after administration of 1-9-9 or a combination of 1-1-2 and 1-9-9 mAbs. By day 13 each antibody, administered individually, inhibited T cell proliferations to the same extent (data not shown). Stimulation with the B cell mitogen LPS, however, appeared to be unaffected (Table I).

Assessment of Antigen Modulation After Antibody Treatment In Vivo. To determine whether or not the observed effects on peripheral T cells might have resulted from differential effects at the cell surface level, a two-color flow cytometry analysis was performed (Fig. 6). For this analysis, spleen cells were removed from animals treated in vivo with monoclonal antibody 24 h before the experiment. Cells were stained with fluoresceinated monoclonal antibodies 1-1-2 or 1-9-9 (green fluorescence) as well as with biotinylated anti-Thy-1.2 mAb followed by TRA (red fluorescence). As seen in Fig. 6, T cells from untreated animals (panels *a* and *d*) showed equivalent staining patterns when either fluoresceinated anti-Qa-2 mAb was used. Cells from animals treated in vivo with 1-1-2 showed marked inhibition of staining with fluoresceinated 1-1-2 (panel *b*), presumably due to blocking with the cold antibody used for treatment. However, when these same cells were examined using fluoresceinated 1-9-9 (panel *e*), a marked diminution in the number of T cells (45%) was observed due to rapid in vivo T cell depletion by 1-1-2 mAb. The staining intensity of 1-9-9 on the remaining cells was moderately changed (21% median relative staining intensity) from that observed for the untreated animals (panel *d*). In vivo treatment with 1-9-9 led to a different result. Staining with fluoresceinated 1-9-9 (panel *f*) was markedly diminished, again presumably due to blocking. However, staining of cells from these animals with fluoresceinated 1-1-2 (panel *c*) showed a smaller decrease (33%) in the number of T cells staining, but a marked decrease in the intensity of T cell staining

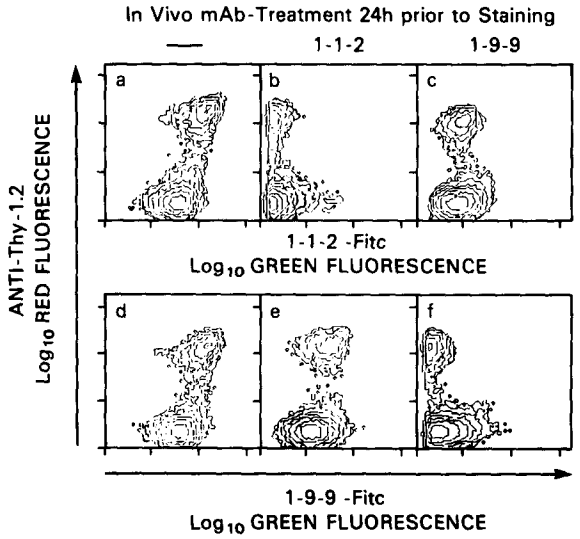

FIGURE 6. Modulation of Qa-2 antigens by anti-Qa-2 mAbs in vivo. PBL from unmanipulated mice (*a*, *d*) or from mice injected 24 h earlier with 1-1-2 (*b*, *e*) or with 1-9-9 (*c*, *f*) were stained with fluoresceinated 1-1-2 (*a*, *b*, *c*) or with fluoresceinated 1-9-9 (*d*, *e*, *f*) (in green) and biotinylated anti-Thy-1.2 plus TRA (in red). The median relative staining intensities of T cells from treated mice compared with T cells from control mice (defined as 100% for each Qa-2 mAb) were: (*c*) 7%, (*e*) 21%, (*b* and *f*) 2%.

(7% median relative staining intensity) after treatment with 1-9-9. Modulation resulting from in vivo treatment with both antibodies was even greater than with 1-9-9 alone, as determined by indirect staining with goat anti-mouse γ-specific antibody (data not shown).

Discussion

In vivo treatment with mAbs has been increasingly important as a therapeutic modality in recent years (10–14). In several cases of treatment with mAbs in primates (15) and mice (16), marked differences have been observed in levels of T cell depletion after treatment with different antibodies. The basis for such differences is not clear, although a role for antibody class (17–19), epitope binding site (20), and/or ability to fix complement (19–20) have been suggested.

In the present studies, the availability of a new panel of anti-Qa-2 mAbs has made it possible to examine the in vivo effects of two mAbs of the same heavy and light chain subclass (IgG2a,k) and similar avidity, but directed against distinct epitopes of the same molecules. Our data indicate that these two antibodies have markedly different in vivo effects. While antibody 1-1-2 led to rapid depletion of peripheral T cells in <3 d, antibody 1-9-9 caused little or no depletion in the first few days, with gradual depletion over the following week. The consequences of these different depletion abilities on prolongation of skin graft survival was exactly as one might have predicted based on their effects on peripheral T cells, with only 1-1-2 leading to significant prolongation. Effects on in vitro parameters of cellular immunity also correlated with the observed depletions, 1-1-2 antibody leading to marked inhibition of Con A responses and MLR responses of spleen cells 2 days following treatment, while either 1-9-9 alone or 1-9-9 plus 1-1-2 had less inhibition capability at this time point. These effects cannot be explained by blocking of T cell functions by anti-Qa mAbs since in vitro addition of the same antibodies had no effects (data not shown). No effect on peripheral B cell levels was observed at any time point and this too correlated with the failure to see an effect on LPS stimulation in vitro and with previous in vitro studies, showing that B cells are less susceptible than T cells to anti-Qa-2 plus complement treatment (2).

Surprisingly, administration of both mAbs simultaneously appeared to protect T cells from rapid depletion. This result was the opposite of what one might have predicted if depletion were merely a function of antibody avidity or of subclass. In the latter case, any of a variety of possible mechanisms for removal of antibody-coated cells might have been responsible for the difference in depletion ability, such as complement fixation or binding of Fc portions to receptors of the reticuloendothelial system. However, neither of these possibilities appeared likely, since both of the antibodies were of the same heavy chain subclass (IgG2a), and no diversity in Fc binding within this subclass has previously been reported.

A more likely explanation for the differential effects of these antibodies, and for the fact that the effect of coadministration mimicked that of administration of the nondepleting antibody alone, is the observed difference in abilities of these two antibodies to lead to modulation of Qa-2 antigens at the cell surface. mAb 1-9-9 depleted peripheral T cells slowly, but led to a marked decrease in the intensity of staining with 1-1-2 within 1 d. Conversely, treatment with 1-1-2 led to rapid depletion of peripheral T cells, but caused less diminution in the intensity of staining with 1-9-9.

Thus, binding of 1-9-9 to the Qa-2 molecules appeared to produce modulation of these antigens from the cell surface by 24 h after antibody administration, while 1-1-2 antibodies led to a lesser change in Qa expression but relatively efficient cell clearance. The ability of an antibody to cause antigen modulation at the cell surface has previously been interpreted as an indication that the antibody can either cause cross-linking (21) or cause an interaction of the antigen with another membrane molecule leading to redistribution (22). In the latter case, modulation has been associated with cell activation (23). Modulation of Qa-2 antigens is particularly surprising in view of the fact that Qa-2 antigens are anchored to the cell membrane not by the ordinary transmembrane and intracytoplasmic domains, but rather by a phosphatidyl inositol (PI) linkage (24). Several other molecules involved in lymphocyte activation have also been found to use such PI linkage (25–27). In this study neither modulation of TCR (as determined by staining with anti-CD3) nor cell activation (as measured by proliferative response to IL-2 and IL-2R expression) was observed as a result of anti-Qa treatment (data not shown). Another possible mechanism for decreased Qa expression could be shedding and secretion of these antigens from the cell surface as had been reported to occur for Qa antigens upon T cell activation (28).

It is tempting to speculate that modulation of Qa antigens may also occur in response to interaction with the natural ligand for this molecule. In this case, the differential effects on Qa expression observed after treatment with these two antibodies may indicate that one of the antibodies (1-9-9) reacts with the very determinant on the Qa-2 antigen that is physiologically relevant for such natural interactions. Since the physiologic function of Qa-2 antigens remains unclear at present, it is difficult to test this hypothesis with respect to any known natural ligand. The fact that the effect of 1-9-9 predominated when both mAbs were administered simultaneously would indicate that the decrease in Qa expression occurs more rapidly than those mechanisms that otherwise might lead to cell clearance.

Summary

The effects of in vivo treatment with anti-Qa-2 mAbs on in vivo and in vitro parameters of T cell immunity have been examined. Two anti-Qa-2 mAbs of the same isotype and with similar avidities but directed against distinct epitopes of the same Qa-2 molecules were studied. mAb 1-1-2 was found to induce rapid T cell depletion, with maximal effect observed within 2–3 d, while administration of mAb 1-9-9 caused little or no depletion in the first few days, and reached maximal effect only by day 8. Surprisingly, administration of both antibodies resulted in a depletion pattern similar to that of the nondepleting antibody 1-9-9. Consistent with these effects on T cell depletion, treatment with 1-1-2 caused significant prolongation of survival of allogeneic skin grafts placed 1 d after antibody administration, while treatment with 1-9-9 or with the combination of both antibodies caused no prolongation. In an attempt to determine the mechanism of this phenomenon, we examined Qa-2 expression on the cell surface by flow microfluorometry after treatment with each of the two mAbs. Our data indicate that mAb 1-9-9 mediates significantly greater modulation of Qa-2 expression from the surface of peripheral T cells within 1 d than does mAb 1-1-2. Apparently, therefore, modulation occurs more rapidly than cell clearance, and the efficiency of T cell depletion and consequent immune suppression is correlated inversely with the ability of each mAb to cause modulation. The

ability of 1-9-9 to cause Qa-2 modulation suggests that it may react with a determinant on this molecule of physiological relevance to the natural ligand interactions of Qa-2 antigens.

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