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Antigen density on target cells determines the immunosuppressive potential of rat IgG_{2b} monoclonal antibodies*

The current studies were designed to determine the relevance of T cell antigen density, besides antibody isotype, with regard to the success of antibody serotherapy. We compared the immunosuppressive effects of two rat IgG_{2b} monoclonal anti-Thy-1 antibodies, RmT1 and 30-H12, with distinct binding sites in a graft-*vs.*-host disease (GVHD) model of fully H-2 and I-A region-mismatched bone marrow transplantation, making use of the difference in Thy-1.2 antigen density between homozygous (BALB/c) and heterozygous (BALB/c × AKR/J)F_1 GVHD-promoting donor cells. Antibodies RmT1 (directed against a monomorphic determinant on mouse Thy-1) and 30-H12 (reactive with the Thy-1.2 allele-specific determinant) did not differ in their anti-GVHD activity with regard to Thy-1.2 homozygous grafts. However, in the region of a critical number of binding sites a small difference in the amounts of the two antibodies bound (about 8×10^3 IgG molecules/cell) obviously accounts for a great difference in anti-GVHD activity. This is shown in a two haplotype host-graft disparity between C57BL/6 recipients treated with either RmT1 or 30-H12 before challenging them with (BALB/c × AKR/J)F_1 grafts, where the Thy-1.2 antigen concentration is approximately 50% compared to the density on BALB/c lymphocytes. Here, mAb 30-H12 loses its remarkable *in vivo* immunosuppressive quality, whereas RmT1 treatment protects mice against lethal GVHD. Binding sites were quantitated using a computerized approach for the analysis of data from ligand binding experiments of the respective mAb, RmT1 and 30-H12, coated to LN cells of BALB/c and F_1 hybrid origin. Furthermore, the *in vivo* immunosuppressive activity of rat IgG_{2b} antibodies directed against Thy-1 was found to correlate with their ability to generate stable antibody-C1q complexes on the cell surface of immunocompetent T cells.

1 Introduction

In the murine model there is evidence that monoclonal serotherapy prevents marrow rejection by T cell depletion of recipients and assists tolerance induction, even with sub-lethal irradiation [1, 2]. Here, engraftment was readily achieved by a more specific and less toxic pretransplant conditioning regimen. Parallel with these efforts, prevention of acute and chronic GVHD was observed in graft-*vs.*-host disease (GVHD) models after treatment of recipient mice with a single injection of mouse or rat anti-Thy-1 mAb of selected isotype [3, 4]. In these experiments, long-term survival of donor-type chimeras was afforded across MHC barriers. These results may suggest useful approaches to the problems of graft failure and prevention of acute and chronic GVHD in human marrow transplant recipients. However, these encouraging findings are empirical and it is, therefore, not possible to predict whether the immunosuppressive potency of appropriate mAb in animal models is directly applicable to the human situation. It has, therefore, become important to establish which factor(s) other than antibody isotype may have any relevance in respect of mAb serotherapy. In a previous communication [4] we described the immunosuppressive activity of rabbit anti-thymocyte globulin to be far superior to that from a rat IgG_{2b} monoclonal anti-Thy-1 antibody in terms of survival time of skin allografts. From these observations we concluded that unabsorbed polyclonal antiserum is more effective due to its ability to recognize multiple epitopes and, thus, be a more effective eliminator of antibody-coated cells. In order to examine this phenomenon we have compared the anti-GVHD activity of two rat IgG_{2b} anti-Thy-1 mAb with distinct antigen-binding sites using mouse models of fully mismatched BM transplantation (BMT). We show here that in the region of a critical number of binding sites a small difference in the amounts of the two antibodies bound on GVHD-promoting donor cells substantially influences the extent of immunosuppression. We also demonstrate that with the deposition of sufficient C1q molecules on the cell surface, conditions are optimal for immunosuppression.

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Abbreviations: **BMT:** Bone marrow transplantation **GVHD:** GVH disease

2 Materials and methods

2.1 Animals

Inbred mice of the BALB/c, CBA/J and C57BL/6 strains homozygous for the Thy-1.2 alloantigen were raised and maintained in our breeding facilities. (AKR/J ×

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BALB/c)F_1 mice were bred from stock in our own laboratory. The genotype of this F_1 hybrid determines coexpression of the Thy-1.1 and Thy-1.2 alloantigen. Both male and female animals between 2 and 3 months of age were used in these studies.

2.2 mAb

Two rat IgG_{2b} mAb against Thy-1 were selected for this study. The serologic specificity and molecular characteristic of mAb RmT1 has been described previously [4]. The second rat IgG_{2b} mAb directed against the Thy-1.2 allelic product was produced by cells of hybrid 30-H12 [5] obtained from American Type Culture Collection (Rockville, MD). Ascites fluid was used as source of antibody in all *in vivo* experiments. Purified mAb were obtained from culture SN by affinity chromatography on columns of Sepharose-4B coupled with a mouse anti-rat IgG_{2b} mAb [6].

2.3 Cell suspensions

Single-cell suspensions were isolated from LN obtained from donor mice as described [4]. LN cells were adjusted to the appropriate cell density in ice-cold, isotonic Veronal-buffered saline (VBS; 335 mOsm/kg; composed of 0.17 M NaCl and 0.005 M sodium barbital, pH 7.35, with 0.3 mM $CaCl_2$ and 2 mM $MgCl_2$) containing 20% heat-inactivated mouse serum and 0.1% sodium azide. Viability, as judged by trypan blue exclusion, was always >95%.

2.4 Radiolabeling

Purified mAb (RmT1 and 30-H12) were radiolabeled as described previously [7]. This method of linkage gave specific activities of about 560 Ci/mmol = 20.72 TBq for [^{3}H]RmT1 and of about 520 Ci/mmol for [^{3}H]30-H12. Isolated C1q was labeled with tritium as previously reported [4]. The resultant specific activity was about 480 Ci/mmol.

2.5 Saturation binding assay

Binding of radiolabeled mAb RmT1 or mAb 30-H12 to LN cells (10^6 cells/determination) was performed in Eppendorf (Hamburg, FRG) test tubes (1.5 ml) in a final volume of 250 µl. The tubes were incubated for 90 min on ice with intermittent agitation. During this incubation time the total amout (T) of ligand added to each tube was defined by measuring an aliquot (50 µl) of these cell suspensions. The separation process was initiated by adding 1.0 ml of ice-cold VBS buffer/2% inactivated mouse serum to each tube, followed by centrifuging briefly (3 min, 800 × g). Subsequently the SN containing the hot ligand in the free form was removed and the amount of cell-bound hot ligand (B) determined by counting in a liquid scintillation spectrometer (Canberra Packard GmbH, Frankfurt, FRG).

2.6 Analysis of binding data

The computer program system LIGAND of Munson and Rodbard [8] was used for the analysis of saturation binding data, model fitting, parameter estimation and graphical output. Based on the theory of Feldman [9] this computerized analysis method makes it possible to fit the most appropriate physico-chemical binding model to the experimental data. The parameters avidity, K, and binding capacity R of the specified model were estimated by means of a weighted nonlinear least squares algorithm. The goodness of fit of the model was analyzed with the LIGAND program using the Runs test' of Bennett and Franklin [10]. Nonspecific binding represented < 1% of the total [^{3}H]mAb bound and was handled as a computer-generated parameter. LIGAND was run on an IBM XT personal computer.

2.7 FCM analysis

A FACScan fluorescence-activated cell analyzer (Becton Dickinson GmbH, Heidelberg, FRG) was used to determine percentages of specifically stained cells. Aliquots of 10^6 LN cells were stained with both mAb directed against Thy-1 and then with an appropriate dilution of fluorescinated mouse anti-rat IgG (Dianova GmbH, Hamburg, FRG) that had been optimized in previous experiments.

2.8 Assay of C1q uptake

Interaction of tritium-labeled C1q with mAb-coated single-cell suspensions of LN cells was quantified by using an RIA. The assay was carried out in 1.5-ml Eppendorf test tubes in a total volume of 320 µl. Target cells (2.5×10^6 cells/determination) in 200 µl were incubated for 90 min at 4 °C with saturating concentrations of the individual mAb (100 µl) and a constant concentration of labeled human C1q (20 µl) in known excess. To reproduce the *in vivo* temperature, C1q uptake studies were also performed at 37 °C. Nonsaturable binding was measured by adding a large excess of unlabeled ligand, thus displacing virtually all the specifically bound C1q from the cell-bound mAb. The remainder of the procedure was a described above.

2.9 X-irradiation

Prospective graft recipients were X-irradiated using a ^{137}Cs small animal irradiator (Gammacell, HWM-D-2000, Ottawa, Canada) with a source-to-skin-distance of 35 cm at a computed delivery rate of 1.04 Gy/min for 8.2 min. A lethal dose of total body irradiation of 850 Gy/animal was delivered during this exposure time.

2.10 Treatment of recipient mice

Groups of 12 C57BL/6 mice were given a single injection of mAb RmT1 or mAb 30-H12 in a final volume of 0.25 ml i.p. (equivalent to about 1 mg of active antibody) 4 h post irradiation and about 20 h prior to transfusion of a mixture of BM and spleen cells. Control animals received 0.25 ml sterile saline but were equivalent in all other respects to the antibody-treated groups. Irradiated but not marrow-reconstituted mice all died within 2 weeks after irradiation.

2.11 BMT

BM cells were flushed from donor femoral and tibial bones and washed with isotonic HBSS (adjusted to 335 mOsm/kg with tenfold concentrated HBSS; Gibco, Karlsruhe, FRG). Cells were counted with trypan blue and adjusted to a concentration of 2×10^7 viable cells. In all experiments, 5×10^7 spleen cells from the same donor were added to the inoculum. The cell suspension was then administered in a final volume of 0.25 ml into the tail vein of recipient mice irradiated 24 h before. The animals were weighed twice a week and examined every day for other signs of GVHD.

2.12 Test for chimerism

To monitor the chimeric state of BM reconstituted mice, randomly selected 100-day survivors (4 per group) were simultaneously grafted with donor-type (BALB/c) or (BALB/c × AKR/J)F_1 and third-party (CBA/J) tail skin grafts, both placed on the lateral thoracic wall. Dressings were removed on day 12, and grafts were inspected every second day for signs of rejection.

3 Results

3.1 Comparison of the anti-GVHD activity of anti-Thy-1 and anti-Thy-1.2 antibodies

To assess precisely whether the anti-GVHD activity of potentially immunosuppressive mAb is sensitive to the number of antibody molecules bound to donor lymphocytes and, thus, to the number of antigen copies on immunocompetent T cells, fully H-2 and I-A region-mismatched BMT were carried out using donors which are genotypically partially different for the Thy-1 locus. Recipients in each case were C57BL/6 mice treated with mAb carrying distinct antigen-binding sites but sharing the constant region structure of the rat IgG_{2b} subclass. BM grafts were obtained from homozygous (Thy-1.2) BALB/c mice expressing a single allelic product or from (BALB/c × AKR/J)F_1 mice whose genotype determines the presence of both Thy-1 alloantigens on their T cells. To simulate the blood-contaminated BM in human BMT more closely, donor spleen cells were added to marrow grafts, thereby heavily favoring the development of lethal GVHD. Transplantation of fully allogeneic BM plus spleen cells from BALB/c or F_1 hybrid mice into lethally irradiated C57BL/6 recipients causes an acute 100% mortality of GVHD within 2 weeks, as demonstrated in Fig. 1A and B (controls). *In vivo* immunosuppression of GVHD from homozygous or heterozygous grafts could be readily achieved by treating recipients with a single injection of RmT1 (anti-monomorphic) with resulting long-term survival of chimeras (Fig. 1A and B). However, the Thy-1.2 allele-specific 30-H12 antibody suppressed disease activity only when Thy-1.2 homozygous BM combined with spleen cells both obtained from BALB/c mice were transplanted (Fig. 1A). In this group of grafted animals a protection from GVHD was nearly complete (> 90% of mice were alive with body weight curves similar to the syngeneic control group) and a donor-type chimerism was observed. In contrast, similar recipients transplanted with heterozygous F_1 donor cells did not survive until day 21 (Fig. 1B). We did not determine whether the 1 mg dose of each mAb given i.p. was actually saturating the Thy-1 epitopes *in vivo*. However, our unpublished data showed that for different maximally histoincompatible strain combinations (including BALB/c into C57BL/6) an average of 250 μg of each of the mAb was an effective and thus GVHD-preventing dose. The present results are thus probably an indication that the reduced Thy-1.2 determinant density on GVHD-promoting T cells from F_1 hybrids is the primary cause of acute GVHD in C57BL/6 mice given mAb 30-H12 prior to transplantation of (BALB/c × AKR/J)F_1 grafts. We therefore aimed to quantitate the number of antibody molecules of both specificities attached to their corresponding antigen on homozygous and heterozygous T cells.

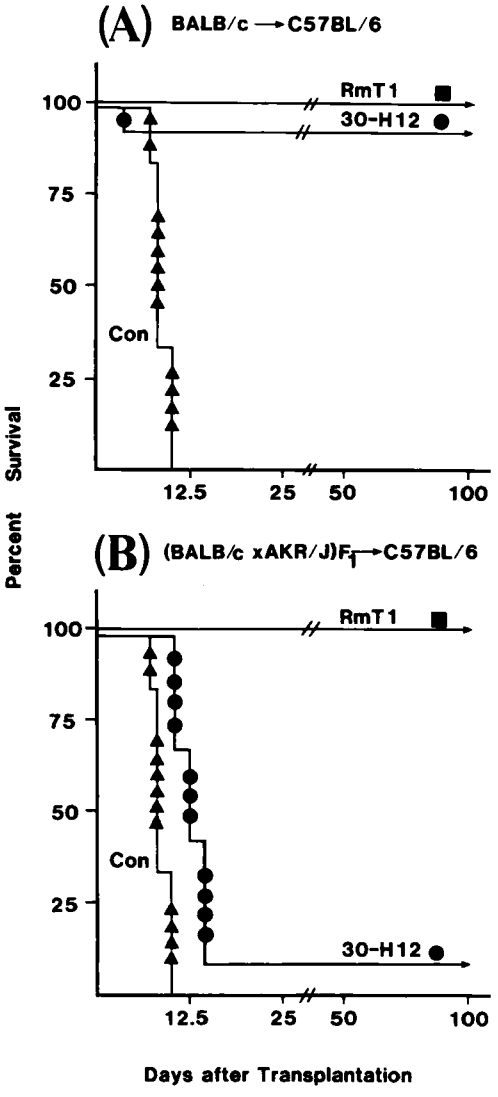

Figure 1. Suppression of GVHD with rat IgG_{2b} mAb detecting a common (RmT1) or the Thy-1.2 allele-specific determinant (30-H12) on Thy-1. Survival time is shown for groups of 12-week-old C57BL/6 mice (12 per group) preirradiated with 8.5 Gy and given a single i.p. injection of individual mAb (equivalent to about 1 mg of active antibody) prior to transfer of fully incompatible BM (2×10^7) and spleen cells (5×10^7) from homozygous BALB/c (A) or from heterozygous (BALB/c × AKR/J)F_1 donor mice (B). Controls received no antibody.

3.2 Calculation of the number of antibody molecules bound to individual T cells

To estimate the average number of antibody molecules bound per cell it was necessary to know two values, the binding capacity of the individual labeled mAb, and the percentage of Thy-1$^+$ cells. The latter was established for LN cells of both donor strains by using the FACS. The distribution of RmT1 bound at saturation to homozygous BALB/c cells was indistinguishable from that of antibody 30-H12, with about 57%–58% of the cells stained (Table 1). Qualitatively similar results were obtained with (BALB/c × AKR/J)F_1 LN cells (Table 1). However, the average fluorescence intensity of the stained cells was less for both mAb in the heterozygous populations (data not shown).

The binding capacity and functional affinity was estimated by using a computerized approach for the analysis of data from ligand binding experiments of the respective mAb RmT1 and 30-H12 coated to LN cells of BALB/c and F_1 hybrid origin. (see Sect. 2.6 for further details). In all experiments the mathematical model for a single ligand binding to one class of specific sites and to an additional class of nonsaturable, nonspecific sites was used as a basis for calculation of binding parameters. Table 1 shows the numerical output and Fig. 2 the graphical presentation of this analysis. On the basis of the computer-generated estimate of the binding capacity together with the percentage of Thy-1$^+$ cells measured on the cytofluorograph, the number of binding sites of each antibody was calculated. Table 1 shows that the two anti-Thy-1 antibodies, when assayed on LN cells of BALB/c mice, gave similar numbers of binding sites/cell (RmT1 5.9 × 10^4; 30-H12 6.5 × 10^4). The average reduction in 30-H12 antibody molecules bound to the F_1 hybrid lymphocyte is approximately 50% (3.25 × 10^4) and the corresponding value for RmT1 is 4.05 × 10^4 binding sites/cell (Table 1). A comparison of the latter two estimates clearly shows that the Thy-1.2 site is more abundant on F_1 lymphocytes. This is not surprising because we know from saturation binding experiments that the level of expression of Thy-1.1 and Thy-1.2 determinants on this target is about half that of the respective parental strain (data not shown). For the RmT1 antibody we found only 1.4 × 10^4 binding sites/cell when measured on LN cells of the AKR/J parental strain carrying the Thy-1.1 alloantigen (data not shown). The 30-H12 antibody gave identical functional affinity constants in the two situations, the value being 1.6 × 10^9 M^{-1} (Table 1). Also shown in Table 1 are the estimates for RmT1, namely 6.1 × 10^9 M^{-1} (homozygous, Thy-1.2) and 5.6 × 10^9 M^{-1} (heterozygous, Thy-1.1 × Thy-1.2). This difference in the avidity constants was not surprising because this mAb reacts with the two Thy-1 gene products with a differential affinity (manuscript in preparation).

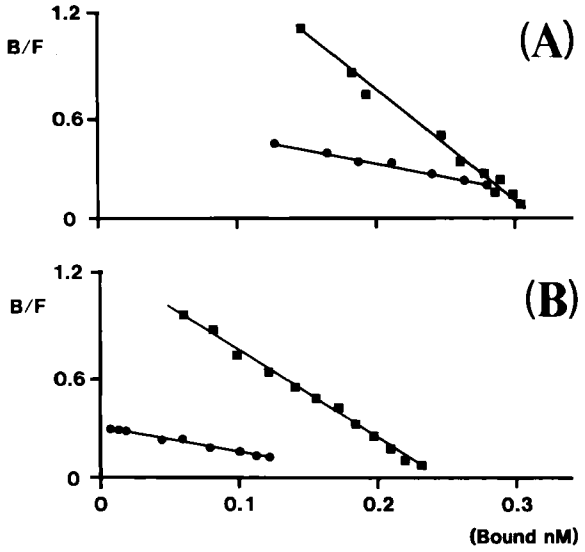

Figure 2. Scatchard analysis for binding of [^{3}H]RmT1 (■—■) or [^{3}H]30-H12 (●—●) to homozygous BALB/c (A) or heterozygous (BALB/c × AKR/J)F_1 LN cells (B). For each target cell equilibrium binding data from two experiments performed in duplicate were analyzed, making use of a computerized approach as described in Sect. 2.6. In each case binding of both mAb is characterized by a linear Scatchard plot. The curves correspond to parameters given in Table 1. B/F = bound/free.

3.3 C1q uptake studies

Both homozygous and heterozygous lymphocytes were coated with saturating concentrations of RmT1 or 30-H12 antibody and then incubated with a known excess of tritium-labeled human C1q in order to quantify the subsequent uptake. The use of the readily available purified human C1q instead of its murine counterpart is justified by the fact that fresh or EDTA-treated mouse serum, when tested in an RIA, totally inhibited subsequent uptake of biologically active purified human C1q (data not shown). As shown in Fig. 3A, the plateau level of C1q binding on RmT1- or 30-H12-coated homozygous T cells was almost identical, which was expected because in this strain the level of expression of monomorphic determinants is concordant with that of the Thy-1.2 allelic product. Further analysis

Table 1. Data on binding parameters, distribution of Thy-1 determinants and the calculated number of bound RmT1 and 30-H12 antibody molecules on LN cells obtained from homozygous BALB/c or heterozygous (BALB/c × AKR/J)F_1 mice

	BALB/c		(BALB/c × AKR/J)F_1	
	RmT1	30-H12	RmT1	30-H12
Equilibrium constant, K × 10^{-9} (M^{-1})	6.1	1.6	5.6	1.6
Binding capacity, R (nM)/5 × 10^5 cells	0.35	0.40	0.25	0.20
Proportion of Thy-1$^+$ cells (%)	58	57	57	58
Antibody molecules bound/cell	59 000	65 000	40 500	32 500

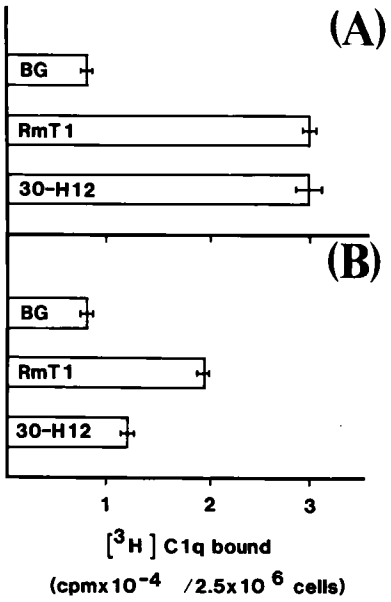

Figure 3. [^{3}H]C1q binding to homozygous BALB/c (A) or heterozygous (BALB/c × AKR/J)F_1 LN cells (B) precoated with anti-Thy-1 mAb (RmT1 or 30-H12). Nonsaturable binding (BG) was estimated by the addition of a 100-fold excess of unlabeled ligand. Data are depicted as cpm ^{3}H/2.5 × 10^6 cells and represent the mean ± SD (bars) of six separate values.

with the (BALB/c × AKR/J)F_1 strain showed that C1q uptake on 30-H12-coated LN cells was drastically reduced, resulting in a marginal reaction (Fig. 3B). Quantitatively less impressive but qualitatively similar results were obtained in a related study in which RmT1-coated F_1 LN lymphocytes were assayed. In this experiment, C1q uptake gave about half that of the RmT1-coated homozygous lymphocytes. The uptake studies were performed at 4 °C and 37 °C to reproduce the *in vivo* temperature. In both cases similar results were obtained (data not shown), suggesting that an altered fluidity of the plasma membrane at physiologic temperature does not raise the probability of creating more frequently juxtaposed antibody pairs to be cross-linked by C1q.

4 Discussion

Serotherapy with anti-Thy-1 mAb of selected isotype has been found to provide an effective means of regulating transplantation immunity in the murine skin and marrow graft recipient [3, 4, 11, 12]. The results described here confirm these findings. However, they also indicate that the outcome of marrow transplantation in a genetic disparity differing at class I and class II major histocompatibility loci depends to a large extent on the Thy-1 antigen density on GVHD-promoting cells.

It is clear that a monomorphic and, for example, the Thy-1.2 polymorphic site of the Thy-1 molecule are topographically, but not genetically separated structures of the molecule when a strain is homozygous for the Thy-1.2 allele. We therefore anticipated that RmT1 (anti-monomorphic) and 30-H12 (anti-polymorphic) would not differ in their anti-GVHD activity with regard to the BALB/c into C57BL/6 combination. Our results show that treatments with either RmT1 or 30-H12 are indeed both equally effective in affording long-term survival of chimeras. In contrast, the same amount of 30-H12 given to recipients receiving (BALB/c × AKR/J)F_1 grafts carrying the Thy-1.1 and Thy-1.2 alloantigen hardly delayed GVHD mortality. However, RmT1 treatment in the similar host-graft combination completely protected mice against lethal GVHD and promoted lymphohematopoietic chimerism.

Interpretation of the above results assumes that elimination of 30-H12-coated F_1 lymphocytes was incomplete due to the reduced number of binding sites, allowing expansion of the remaining cells upon antigenic challenge. It is unlikely that other factors might have influenced the outcome of marrow/spleen cell transplantation in the (BALB/c × AKR/J)F_1 into C57BL/6 combination for three reasons: (a) factors such as histocompatibility differences, variations in T cell numbers, differences in suspectibility to irradiation, environmental conditions and the general state of health of animals have been ruled out due to the experimental set-up differing only in the conditioning of recipients with either RmT1 or 30-H12; (b) the observation that both antibodies were potently immunosuppressive in the BALB/c into C57BL/6 combination indicates that binding to distinct epitopes on Thy-1 does not influence the effectiveness of immunosuppressive serotherapy; (c) and most important, our recent work has shown that the inclusion of a mouse IgG_{2a} anti-Thy-1.1 antibody into 30-H12 serotherapy totally prevented alloreactivity in C57BL/6 recipients grafted with F_1 marrow and spleen cells (Kummer, U. et al., manuscript in preparation). This pair of mAb possesses a degree of immunosuppressive activity that could not be accounted for by the anti-GVHD activity of either alone. Thus, the reduced impact of 30-H12 monotherapy on GVHD cannot be explained on the grounds that (BALB/c × AKR/J)F_1 cells are themselves more potent in eliciting GVHD, and that the 30-H12 mAb is simply less effective than the RmT1 antibody. We are, therefore, confident that the striking difference between RmT1 and 30-H12 in protecting C57BL/6 recipients against GVHD-causing F_1 donor cells is obviously related to the average reduction in binding sites for 30-H12 (compare the data for (BALB/c × AKR/J)F_1 lymphocytes where we found approximately 4.05 × 10^4 RmT1 molecules/cell at saturation and 3.25 × 10^4 binding sites with 30-H12).

Binding sites are not necessarily the same as antigenic determinants. Although this parameter has not been determined directly, it is justified to assume that the reduction in bound 30-H12 on F_1 hybrid lymphocytes is concordant with the reduced Thy-1.2 antigen density for the following reason: the 30-H12 antibdoy gave identical functional affinity constants for the Thy-1.2 molecule on BALB/c and (BALB/c × AKR/J)F_1 target cells. This suggests that there is no substantial change in valency of binding of 30-H12 antibody to the different targets, thus eliminating one complicating factor in analyzing antigenic determinants on the cell surface. We therefore conclude that (a) the outcome of the serological assays reflects the *in vivo* situation of antibody binding to their corresponding targets and (b) the average reduction in 30-H12 binding sites is equivalent to the Thy-1.2 antigen concentration on the F_1 hybrid lymphocyte, which is approximately 50% compared to the density on BALB/c LN cells.

It comes as a surprise that the small difference in the amounts of the two antibodies bound (about 8×10^3 binding sites/T cell) may influence the extent of immunosuppression and account for the great difference in anti-GVHD effect. In the region of a critical number of binding sites on GVHD-promoting cells the basis of this quantitative factor is most likely the inability of the Fc portion of the 30-H12 antibody to interact with that effector mechanism in the host which is most significant *in vivo* for therapeutic efficacy. The nature of this interaction is not yet precisely understood. However, we have recently shown that the *in vivo* immunosuppressive activity of monoclonal and polyclonal anti-Thy-1 antibodies correlates with their ability to generate stable antibody-C1q complexes on the cell surface of immunocompetent T cells [4]. As a result of binding of C1q it appears that conditions are optimal for the subsequent elimination process. At present we favor the view that bound C1q is capable of directing an opsonization mechanism by using the host's reticuloendothelial system (RES). Evidence supporting this possible mechanism of cell elimination includes the above results, which demonstrate that below a certain threshold of determinant density on GVHD-promoting cells the potential protective mAb 30-H12 loses its ability to efficiently bind the C1q subunit and in parallel its remarkable *in vivo* immunosuppressive quality as shown in a two-haplotype host-graft disparity with (BALB/c × AKR/J)F_1 as BM and spleen cell donor. Because of the requirement for a firm and thus at least two-headed binding, the number of 30-H12 binding sites on an immunocompetent F_1 hybrid T cell (about 3.25×10^4 antibody molecules/cell) is such that at any one time too few mAb will be appropriately juxtaposed to create sufficient stable C1q-binding sites (Fig. 3B). Interestingly, the small increase in binding sites obtained with RmT1 on F_1 lymphocytes (about 4.05×10^4 antibody molecules/cell) raises the probability of creating antibody pairs suitable for a stable, two-headed C1q-binding as shown in Fig. 3A. The plausibility of these results is enhanced by the findings of Borsos and Rapp [13], who recognized that the amount of bound C1q increases exponentially as the antibody density increases linearly. The explanations for participation of C1q in the clearance process are speculative. However, we feel that the correlation between *in vitro* C1q uptake and *in vivo* immunosuppression can hardly be a coincidence. The data presented here probably constitute the strongest experimental support for the C1q concept yet available.

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