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Mitogenicity of anti-Thy-1 monoclonal antibodies attributable to an Fc-dependent mechanism*

We analyzed the mechanism by which certain anti-Thy-1 monoclonal antibodies (mAb) activate T cells directly without additional stimuli. Using a panel of rat anti-Thy-1 antibodies which included more than 30 IgG2c mAb, we found that only the IgG2c isotype was able to induce a strong proliferative response in both resting T cells and a T cell lymphoma, suggesting that this form of T cell activation is isotype restricted and might be a consequence of a unique physico-chemical property of the IgG2c heavy chain. Results from surface distribution studies of Thy-1 molecules, following specific interactions with anti-Thy-1 antibodies of different isotypes, again showed that only IgG2c mAb formed Thy-1 aggregates of high valence on the surface of a T cell lymphoma, and such clustering always evoked a biological response. This led us to propose that IgG2c mAb have the inherent tendency to self-associate, probably through homophilic Fc-Fc contacts, and that this feature renders anti-Thy-1 mAb mitogenic. To prove this, we set up cross-inhibition studies with randomly selected mitogenic (IgG2c) and non-mitogenic (IgG2b) anti-Thy-1 mAb. The results clearly demonstrated that IgG2c antibodies enhance their own binding, analogous to the new form of antibody binding that was recently demonstrated between murine IgG3 mAb and a multivalent antigen. Confirmation of this was also provided by IgG2c-derived $F(ab')_2$ fragments, which were unable to cause proliferation. Furthermore, masking the Fc part of cell-bound IgG2c mAb with a monomeric and thus non-aggregating IgG-binding protein A-derived fragment cancelled their mitogenic ability. Finally, induction of T cell proliferation appeared to be independent of cross-linking via FcγR. The results support a model in which noncovalent intermolecular homophilic contacts of the Fc regions of the IgG2c isotype bring about effective aggregation of Thy-1 molecules, thereby stimulating the mitotic apparatus of the cell.

1 Introduction

Antigen-specific T cell activation involves the interaction of the TcR/CD3 complex with foreign antigen peptides presented in the context of MHC molecules [1]. In addition to the specific immune response, this effect can be mimicked by antibodies directed against the TcR/CD3 complex and several other cell surface antigens expressed on T cells [2–7]. Among these, the glycosylphosphatidyl-inositol (GPI)-linked murine T cell differentiation antigen Thy-1 [8–10], which exists in two allotypic forms [11], has been shown to be a member of the family of signal transduction molecules.

Anti-Thy-1 mAb are capable of increasing the intracellular Ca^{2+} concentration and inducing IL-2 receptor expression, IL-2 secretion, and proliferation of resting peripheral T cells and T cell hybridomas [12–17]. In addition, some anti-Thy-1 mAb can stimulate the secretion of IFN-γ by cytolytic T cell clones [14]. The extracellular conditions necessary for activation by these mAb can be divided into two categories: mAb belonging to the first group require additional stimuli such as FcγR-bearing accessory cells (AC), cross-linking second antibodies or the presence of the comitogen PMA in order to induce stimulation [16]. The second group comprises mAb which *directly* activate T cells without the help of co-stimulatory agents and FcγR-providing AC [13–15]. However, the mechanism by which mAb directly deliver a mitotic signal has remained an enigma.

Mitogenic anti-Thy-1 mAb appear to adequately represent the physiological situation by mimicking the interaction of the "natural ligand". It was therefore important for us to investigate in detail the requirements necessary for this type of mAb to have a mitogenic effect. The results presented here support a model in which, because of a unique physico-chemical property of their heavy chains, rat IgG2c anti-Thy-1 mAb bring about effective oligomerization of Thy-1 molecules, thereby stimulating the mitotic apparatus of the cell.

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2 Materials and methods

2.1 Hybridomas, mAb and cell lines

Hybridomas secreting anti-Thy-1 mAb were selected from a fusion between the rat myeloma Y3/Ag1.2.3 [18] and Lou/c rat spleen cells immunized to BALB/c thymocytes. The mAb isotype was determined by ELISA using murine mAb specific for different rat isotypes (American Type

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Culture Collection, ATCC, Rockville, MA). The rat anti-Thy-1 mAb RmT1 (IgG2b) and RmT2 (IgG2c) have been described previously [19]. Anti-DNP mAb of different isotypes, which served as control in the proliferation assays, were generously provided by Dr. H. Bazin (University of Louvain, Belgium). Purified BD30-H12 was purchased from Becton Dickinson, Heidelberg. The murine T cell line LBRM-33 4A2 (ATCC) was used for the induction of IL-2 production by anti-Thy-1 mAb. An IL-2-dependent CTLL was provided by Dr. K. Stuenkel (Bayer, Wuppertal).

mAb were either purified from culture supernatants on a protein A-Sepharose column (rat IgG2c and mouse IgG3) or a protein G-Sepharose column (rat IgG2b).

2.2 Culture medium

All cultures were carried out in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 U/ml, L-glutamine (2 mM), sodium pyruvate (1 mM) and 10% heat-inactivated fetal calf serum (FCS). In all functional assays, 2-ME was added to get a final concentration of 5×10^{-5} M. All reagents were purchased from Gibco, Eggenstein.

2.3 Competition ELISA

Serial dilutions of rat anti-Thy-1 mAb (50 µl/well) were incubated with 3×10^5 C57BL/6 Thy-1.2 thymocytes/well in dried milk-blocked microtiter plates at indicated temperatures. After 90 min, cells were incubated with biotinylated RmT1 (10 µl; half saturation) on ice without a wash-centrifugation step. At the end of the second incubation (usually 30 min), the plates were washed twice with PBS containing 1% dried milk and 0.1% sodium azide, then a 1 : 500 dilution of avidin-conjugated horseradish peroxidase (Camon, Wiesbaden) was added. Following this incubation (45 min), the plates were washed again and the assay was developed with H_2O_2 and o-phenylenediamine (OPD; Sigma, Deisenhofen). Absorbance was measured by a Dynatech ELISA reader at 405 nm. Results are expressed as percent binding relative to the saturation binding level (1.8 U) for the biotin-labeled RmT1, which was determined in the absence of a competitor. Background absorbance, assayed with an excess of unlabeled RmT1, ranged between 0.3 to 0.4 U, which is equivalent to about 20 to 30% binding of the indicator mAb RmT1. Data are expressed as the average of duplicates. RmT1 was biotinylated as described previously [20] using a 100-fold molar excess of biotinyl-ε-amino caproic acid N-hydroxy-succinimide ester to IgG.

2.4 Proliferative assay

Single-cell suspensions of lymph node cells were prepared from peripheral and mesenteric lymph nodes of Thy-1.2 C57BL/6 mice. Cells (4×10^5/well) were cultured in complete medium in 96-well round-bottom microtiter plates in the presence of purified anti-Thy-1 antibody preparations, each at a concentration of 75 µg/well in a total volume of 200 µl. Proliferation, measured as DNA synthesis, was determined by adding 1 µCi of [^{3}H] dThd (Amersham Buchler, Braunschweig) to each well for the last 18 h of culture. The plates were harvested with a semiautomatic device (PHD cell harvester, Dunn, Asbach). When indicated, 20 µl/well of a protein A-derived IgG binding fragment (Sigma) was used at a fivefold molar excess compared with the added mAb.

2.5 Assay of IL-2 production

LBRM-33 cells (2×10^5/well) were co-cultured with the various antibody preparations (100 µl/well) in a total volume of 200 µl. After 24 h, 100 µl supernatant was removed from each well and assayed for IL-2 activity. The amount of secreted IL-2 was assessed by standard microassay [21] based on the IL-2-dependent proliferation of CTLL cells. Cultures of CTLL cells were pulsed after 24 h with 1 µCi/well [^{3}H] dThd and harvested 18 h later. Stimuli used in positive controls included PHA (Welcome, Burgwedel) at a final concentration of 2.5 µg/ml in the case of LBRM-33 cells and rIL-2 at 20 U/ml in the case of CTLL cells.

2.6 Immunofluorescence labeling and detection

For antibody-induced distribution of Thy-1 molecules, LBRM-33 cells were incubated overnight at 37 °C in flat-bottom microtiter plates with various rat anti-Thy-1 mAb of the IgG2b or IgG2c isotypes, each at 10 µg/well in a final volume of 200 µl. To remove unbound antibody, samples containing 2×10^5 cells were washed once with warm (37 °C) PBS. This procedure prevented any further cold-induced antibody aggregation. Cells were then fixed with 1% (w/v) paraformaldehyde, immediately treated with FITC-conjugated $F(ab')_2$ fragments of rat anti-mouse IgG (Dianova, Hamburg), washed again and finally plated on coverslips. Microscopy and photography were performed according to a method described by Kupfer et al. [22].

2.7 Preparation of $F(ab')_2$ fragments

According to Rousseaux et al. [23], $F(ab')_2$ fragments were produced by digestion of rat IgG2c mAb with pepsin (Boehringer, Mannheim) in 0.1 M sodium acetate buffer, pH 4.5, for 1 h at 37 °C at an enzyme to antibody ratio of 1% w/w. Bivalent binding fragments were separated from undigested IgG and large Fc fragments on a protein A-Sepharose column. Dialysis was then performed to remove smaller, more thoroughly digested Fc fragments.

3 Results

3.1 Monoclonal antibodies to mouse Thy-1

Initially, antibody activities in culture supernatants were tested for their ability to block the binding of a well-characterized biotinylated rat IgG2b anti-Thy-1 indicator antibody with specificity for Thy-1 (RmT1). The series of mAb which showed specific and effective blocking were then analyzed on allotype-specific cell lines and a Thy-1-negative mouse lymphoma line (S49; ATCC). The binding of the selected mAb exhibited the classic reaction pattern of antibodies detecting a monomorphic Thy-1 determinant.

3.2 Anti-Thy-1-induced activation is isotype-restricted

The observation that mitogenic rat anti-Thy-1 mAb exclusively belong to the IgG2c isotype [13–15] suggested that this form of anti-Thy-1-induced activation demonstrates isotype specificity. To prove this, we compared the secretion of IL-2 by the murine Thy-1.2-bearing T cell line LBRM-33 in response to a panel of anti-Thy-1 mAb belonging to different rat IgG isotypes. The results depicted in Fig. 1 show that all 8 IgG2c mAb induced a marked secretion of the lymphokine in the absence of a co-stimulatory agent, while only marginal IL-2 release, if any, was promoted by mAb belonging to other IgG subclasses and the IgM class. Similar results were observed with another panel of more than 50 anti-Thy-1 mAb, including 30 more IgG2c antibodies (data not shown).

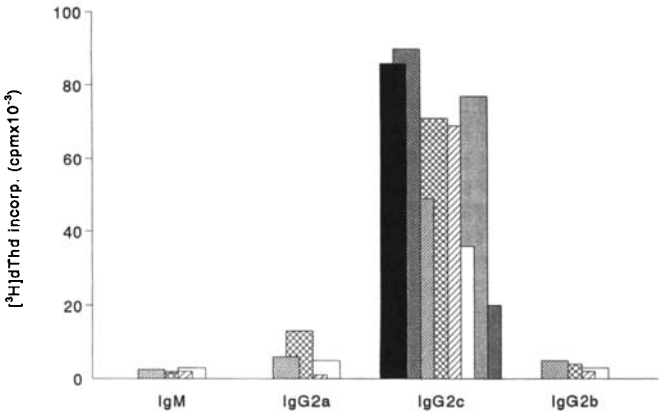

Figure 1. IL-2 production induced by rat anti-Thy-1 mAb of different isotypes. LBRM-33 cells (2×10^5) were cultured with supernatants of originally obtained hybridomas in a final volume of 200 µl. Supernatants (100 µl; cell free) were removed after 24 h, and IL-2 activity was tested in a standard proliferation assay using IL-2-dependent CTLL indicator cells. The results represent [³H]dThd uptake of CTLL cells in cpm shown for the mean of quadruplicate cultures. IL-2 activity in supernatants of unstimulated LBRM-33 cultures was less than 5% and was assayed using irrelevant mAb of each IgG subclass and IgM.

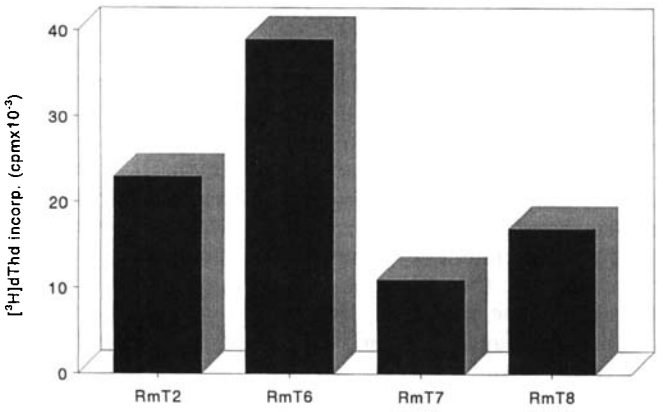

Figure 2. Thy-1.2 expressing lymphocytes of C57BL/6 mice were cultured for 72 h with rat IgG2c anti-Thy-1 mAb RmT2, RmT6, RmT7 and RmT8, each at a concentration of 75 µg/well in a final volume of 200 µl. [³H]dThd was added to the cultures 18 h before harvesting. Data represent mean cpm of quadruplicate cultures and standard errors were less than 15% of the mean. Control experiments with irrelevant rat IgG2c mAb (75 µg/well) showed background values of about 1200 cpm comparable with the medium control.

As shown below (Fig. 4), IgG2c anti-Thy-1 mAb bind less effectively to their corresponding antigen at higher temperatures. This property could explain why the amounts of some IgG2c mAb used as culture supernatant in the functional assay were too low to induce optimal IL-2 responses. Indeed, when we treated target cells with a higher concentration (10 µg/2 × 10^5 LBRM-33 cells) of these "poor stimulators" responses were equal to those obtained with optimal concentrations of PHA (data not shown).

Another series of functional experiments was set up with resting peripheral T cells obtained from lymph nodes of C57BL/6 mice. Fig. 2 shows a representative example of mitogenic IgG2c anti-Thy-1 mAb. Taken together, these findings strongly suggested that T cell activation via anti-Thy-1 antibodies is isotype restricted.

3.3 Surface distribution of Thy-1 after interaction with specific mAb

To visualize the cell surface distribution of Thy-1 molecules following a specific Ag-Ab interaction, LBRM-33 cells were pulsed overnight with either non-mitogenic or mitogenic anti-Thy-1 mAb. A representative example is shown in Fig. 3. Cells coated with RmT1 (IgG2b) displayed a uniformly dispersed immunolabeling. However, a clustering of Thy-1 was observed when LBRM-33 cells were treated with the mitogenic antibody RmT6 (IgG2c). RmT6-induced clustering of Thy-1 molecules paralleled the secretion of IL-2, as determined by the biological assay (data not shown). Conversely, the uniformly distributed RmT1 did not induce any detectable IL-2 (data not shown).

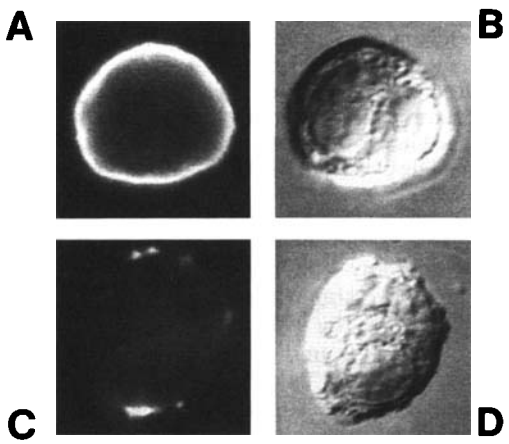

Figure 3. Representative example for the distribution of Thy-1 molecules on LBRM-33 cells that were pulsed overnight with (A) the non-mitogenic RmT1 (IgG2b) and (C) the mitogenic RmT6 (IgG2c). The right panel (B and D) is the Nomarski image of the respective antibody-coated cells.

3.4 Mitogenic anti-Thy-1 mAb have the inherent tendency to self-associate

The above results suggested that the ability of the IgG2c isotype to cause proliferation might be a consequence of a unique physico-chemical property of its heavy chain. To test this idea, we set up cross-inhibition experiments with

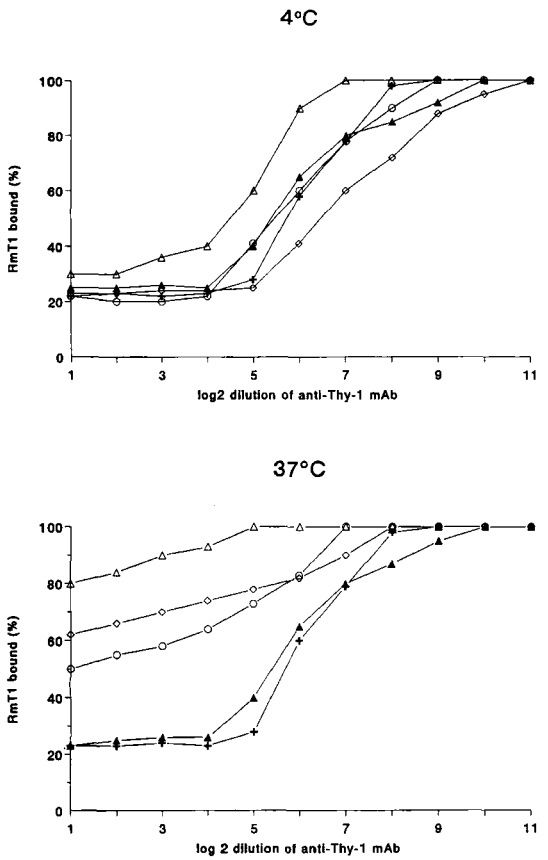

Figure 4. Temperature-sensitive enhancement of IgG2c binding to Thy-1. The competition ELISA was carried out by incubating C57BL/6 Thy-1.2-bearing thymocytes (50 µl) subsequently with serial dilutions (50 µl) of IgG2c (RmT2◇; RmT6△; RmT8○) and IgG2b anti-Thy-1 antibodies (RmT1▲; BD30-H12+) at 4°C or 37°C for 90 min and biotinylated RmT1 (20 µl) at 4°C for an additional 30 min. Results are expressed as percent binding relative to the saturation binding level (1.8 U) for the indicator antibody RmT1 which was determined in the absence of any competitor. Background absorbance assayed with an excess of unlabeled RmT1 ranged between 0.3 to 0.4 U which is equivalent to about 20 to 30% binding of labeled RmT1. Data are expressed as the average of duplicates.

randomly selected anti-Thy-1 mAb of IgG2b and IgG2c origin and Thy-1.2-expressing thymocytes of C57BL/6 origin. Fig. 4 shows that the selection of IgG2c mAb (RmT2, RmT6 and RmT8) bound more efficiently to their antigen at 4°C than at 37°C. This "bonus effect" on the binding strength of IgG2c-Thy-1 interactions is analogous to the previously reported cooperative binding between murine IgG3 and a multivalent antigen, which is proposed to result from the tendency of the heavy chains to self-associate. In contrast, this temperature-sensitive cooperative binding phenomenon was not seen with anti-Thy-1 mAb belonging to the IgG2b isotype (RmT1 and BD30-H12; Fig. 4).

3.5 Mitogenicity of IgG2c anti-Thy-1 mAb requires an intact and unmodified Fc region

If the cooperative binding and the activating property of IgG2c anti-Thy-1 mAb are consequences of the same basic mechanism, then it should be demonstrable that (i) the Fc region of the antibody is mandatory for its mitogenicity and (ii) an interruption of Fc-Fc contacts should abolish effective aggregation of Thy-1 molecules and thus the activating property of this antibody isotype. We therefore examined the capability of an $F(ab')_2$ fragment derived from the mitogenic mAb RmT6 to cause proliferation. Unlike the intact RmT6, Fc-free fragments which still retained their binding to Thy-1 (data not shown) were unable to induce a T cell response (Fig. 5). This basic pattern has been observed even when substantially greater quantities (100 or 150 µg/well) of fragments were used (data not shown). To check the second point, we masked the cell-bound RmT6 mAb with a monovalent and thus non-aggregating IgG binding protein A-derived fragment at the beginning of a proliferation assay. Again, Fig. 5 clearly shows that this treatment totally prevented triggering of a proliferative signal by the mitogenic RmT6. These experiments also counter the argument that the fragmentation process changes the degree of interdomain flexibility of $F(ab')_2$ molecules and therefore is responsible for the subsequent failure of the fragment to stimulate the mitotic apparatus of the cell. Thus, the results provide strong evidence that the Fc region is the causative part of the molecule that confers on the IgG2c anti-Thy-1 mAb the ability to induce directly the full spectrum of T cell activation.

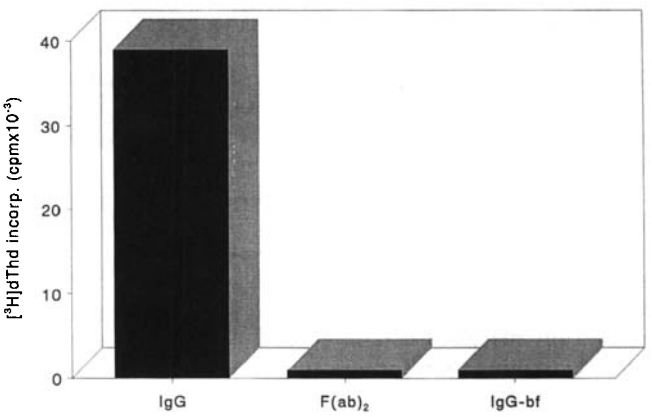

Figure 5. Effect of Fc-free or Fc-masked IgG2c anti-Thy-1 mAb RmT6 on the proliferative response of resting peripheral T cells. Single-cell suspensions of Thy-1.2-expressing lymphocytes obtained from Thy-1.2 C57BL/6 mice were cultured for 72 h with intact RmT6, the $F(ab')_2$ fragments of RmT6, or RmT6 in the presence of a protein A-derived IgG binding fragment (IgG-bf; 5-fold molar concentration of the ligand compared to the concentration of RmT6), each at a concentration of 75 µg/well. The proliferation assay was the same as described in the legend to Fig. 2. [³H]dThd incorporation in the presence of the fragmented or masked RmT6 was comparable with the medium control (about 1200 cpm). Data represent mean cpm of quadruplicate cultures and standard errors were less than 15% of the mean. Similar results were obtained in two independent experiments.

4 Discussion

The goal of the present study was to discover why certain anti-Thy-1 mAb can induce activation of resting peripheral T cells directly [13–15] while other anti-Thy-1 mAb must rely on co-stimulatory agents such as secondary cross-linking antibodies and the presence of the PKC activator

PMA. Receptor dimerization or oligomerization is a universal phenomenon among growth factor receptors [24, 25], and other molecules involved in signal transduction [26] which also appears to apply to Thy-1. The latter is based on the studies of Kroczek et al. [16], who found that a critical signal required in their system was triggered by the interaction of an mAb bound to Thy-1 with a second cross-linking antibody. They concluded that the requirement of a second antibody for activation of resting peripheral T cells via Thy-1 is dependent on the formation of large aggregates on the cell surface, since many of the anti-Thy-1 mAb used were IgM and would be expected to bind several Thy-1 molecules simultaneously. The authors therefore speculated on the role of self-aggregation of directly mitogenic antibodies in mediation of this effect, although no definitive evidence was presented. Among all rat anti-Thy-1 mAb tested so far, only the IgG2c isotype possessed directly activating properties [13–15]. Using a panel of more than 50 anti-Thy-1 antibodies of different isotypes, our laboratory readily replicated those experiments. Fig. 1 shows such an experiment with cells from a T cell lymphoma, in which the induction of IL-2 secretion has been determined. Since the activation requirements may differ between malignant and normal T cells, the latter were similarly examined. Again, activation of resting peripheral T cells was only observed in response to cocultivation with IgG2c anti-Thy-1 antibodies (Fig. 2). These findings suggested that this form of anti-Thy-1-induced activation is isotype restricted and might be due to a unique physico-chemical property of the IgG2c heavy chain.

The first distinct evidence of the ability of specific mAb to induce Thy-1 oligomerization came from our surface distribution experiments (Sect. 3.3 and Fig. 3). The studies revealed that only IgG2c anti-Thy-1 antibodies formed large aggregates on the cell surface following interaction with their corresponding antigen. Most importantly, such clustering was sufficient to evoke a biological response. These findings led us to propose that an inherent property which would render an anti-Thy-1 antibody mitogenic is its tendency to self-associate. The correctness of this prediction has been confirmed in cross-inhibition studies with randomly selected mitogenic (IgG2c) and non-mitogenic (IgG2b) anti-Thy-1 antibodies. In this series of experiments we observed that, in fact, IgG2c antibodies enhance their own binding, and the binding strength decreases substantially with increasing temperature. This temperature-sensitive enhancement is analogous to the new form of antibody binding recently discovered by Greenspan et al. [27, 28] in the murine system. The authors showed that the interaction of murine IgG3 mAb with a multivalent antigen is characterized by a positive cooperative activity and concluded that the enhancement results from self-associating properties of the Fc regions of this antibody isotype, which create Ag-Ab interactions of high valency. An important aspect, in perfect agreement with experimental data on the cooperative binding of both rat IgG2c and mouse IgG3 is the striking sequence homology between the heavy chain constant region of these IgG isotypes at the nucleotide and amino acid levels [29].

A prediction of our hypothesis was that mitogenic antibodies require the intact Fc region to initiate the proliferative response. To address this question, cells were pulsed with an Fc-free fragment derived from a mitogenic antibody which still retained its ability to bind to Thy-1 on the cell surface. A variation on the theme of how important intermolecular Fc-Fc contacts are for this form of an antibody-driven proliferation is provided by the experiment performed with a recombinant IgG-binding fragment, obtained from protein A by genetic engineering, which was used to mask the Fc region. Unlike intact antibody, neither the Fc-free fragment nor the Fc-manipulated potentially mitogenic antibody was able to trigger a proliferative response.

Finally, it might be argued that this clustering phenomenon is mediated simply via FcγR. However, a variety of considerations and experimental results argue against the involvement of FcγR. First, as shown above, clustering of IgG2c-coated Thy-1 molecules was effective with cells of the murine T cell lymphoma LBRM-33 in the absence of FcγR-bearing AC. Because FcγRII is the only FcγR found on T cells [30, 31], it is also the only candidate that would be expected to mediate this effect. However, this low-affinity receptor is specific for membrane (m)IgG1, mIgG2a and mIgG2b, but not for mIgG3. The striking sequence homology between the heavy chain constant region of rat IgG2c and mouse IgG3 suggests that rat IgG2c is the homologue of mIgG3. Therefore, we suppose that a putative FcγRII on LBRM-33 cells binds neither mIgG3 nor rat IgG2c and so it cannot be responsible for the IgG2c-induced aggregation phenomenon. Second, our studies on the mitogenic effect of a murine IgG2a anti-Thy-1 mAb along with resting T cells or LBRM-33 cells did not result in significant proliferation above background levels of both target cells, although MΦ were present from the beginning of the proliferation assays (data not shown). It is known that MΦ express FcγRI, which binds monomeric IgG2a with high affinity [6, 7]. Third, Gunter et al. [13] showed that a vigorous proliferative response was induced by a mitogenic anti-Thy-1 mAb (G7, rat IgG2c), although the T cell population was cleared of contaminating FcγR-providing AC by passage over nylon wool columns prior to cultivation. Thus, the alternative explanation that FcγR is essential to this form of antibody-induced activation cannot be ruled out, but it seems extremely unlikely.

How can we explain that manifestation of mitogenic activity requires a concentration of IgG2c antibodies that is two orders of magnitude higher than is necessary to fully saturate the Thy-1 binding sites? The clue may again come from the antibody isotype. It is apparent from the cross-inhibition studies (Fig. 4) that IgG2c anti-Thy-1 antibodies are of low intrinsic affinity, making dissociation of the ligand a favorable event. Given the fact that any Fc-Fc association requires prior binding to antigen [27], it appears likely that in this situation antibody excess would guarantee a maximum number of simultaneously bound ligands, which in turn would raise the probability of a clustering event to a virtual certainty.

Generic properties that may favor or, to the contrary, prevent the clustering of a cell surface antigen are (i) its density and (ii) its translational mobility. The average number of Thy-1 sites on resting peripheral T cells was recently investigated in our laboratory [32]. From this study, it was apparent tht the level of expression is about 1×10^5–1.2×10^5 Thy-1 molecules/cell. More recent studies revealed that the Thy-1 sites on LBRM-33 cell are even more abundant, the average value being more than 10^6

Thy-1 antigens/cell. Based on these studies, we are confident that LBRM-33 cells, in any case, but also resting peripheral T cells of C57BL/6 origin provide the necessary density of Thy-1 antigens to endow the cellular-based Ag-Ab system with multivalence. GPI-linked membrane proteins like Thy-1 are known to have a translational diffusion constant that may be one order of magnitude higher than the one calculated for conventional transmembrane proteins [33]. In this context, another GPI-linked membrane protein, CAMPATH-1, which is widely distributed on most human lymphocytes, was recently reported to be yet another component which plays a role in T cell activation [34]. In that study, it was shown that among different anti-CAMPATH-1 antibodies, only the rat IgG2c isotype induced a proliferative response in human lymphocytes. One possible explanation for this was the ability of mitogenic antibodies to cross-react with other cell surface molecules such as CD2 or CD3. Although we cannot present evidence to the contrary, we are confident that these findings, particularly of the isotype-restricted activation phenomenon, strongly support our concept. Although certain attributes of the membrane environment appear to favor rather than to impede the clustering even of transmembrane proteins, it will have to be investigated whether this phenomenon is unique to the connection of a GPI-linked cell surface target being attached to a certain isotype.

The findings presented here support a model in which intermolecular homophilic Fc-Fc contacts create Ag-Ab interactions with high valency, which in turn bring about effective aggregation of Thy-1 molecules on the cell surface, ultimately triggering the activation process. This unique effector function offers new strategies for using these antibody isotypes in manipulation of selected GPI-linked cell surface structures, thereby defining their physiological function.

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5 References

1 Weiss, A., Imboden, J., Hardy, K., Manger, B., Terhorst, C. and Stobo, J., *Annu. Rev. Immunol.* 1986. *4:* 593.
2 Meuer, S. C., Hussey, R. E., Fabbi, M., Fox, D., Acuto, O., Fitzgerald, K. A., Hodgdon, J. C., Protentis, J. P., Schlossman, S. F. and Reinherz, E. L., *Cell* 1984. *36:* 897.
3 Malek, T. R., Ortega, G., Chan, C., Kroczek, R. A. and Shevach, E. M., *J. Exp. Med.* 1986. *164:* 709.
4 Davis, L. S., Patel, S. S., Atkinson, J. P. and Lipsky, P. E., *J. Immunol.* 1988. *141:* 2246.
5 Thompson, L. F., Ruedi, J. M., Glass, A., Low, M. G. and Lucas, A. H., *J. Immunol.* 1989. *143:* 1815.
6 Carrel, S., Moretta, A., Pantaleo, G., Tambussi, G., Isler, P., Perussia, B. and Cerottini, J. C., *Eur. J. Immunol.* 1988. *18:* 333.
7 Thompson, C. B., Lindsten, T., Ledbetter, J. A., Kunkel, S. L., Young, H. A., Emerson, S. G., Leiden, J. M. and June, C. H., *Proc. Natl. Acad. Sci. USA* 1989. *86:* 1333.
8 Low, M. G. and Kincade, P. W., *Nature* 1985. *318:* 62.
9 Tse, A. O. D., Barclay, A. N., Watts, A. and Williams, A. F., *Science* 1985. *230:* 1003.
10 Homans, S. W., Ferguson, M. A., Dwek, R. A., Rademacher, T. W., Anand, R. and Williams, A. F., *Nature* 1988. *333:* 269.
11 Reif, A. E. and Allen, J. M., *Nature* 1966. *209:* 523.
12 Lancki, D. W., Ma, D. I., Havran, W. L. and Fitch, F. Q., *Immunol. Rev.* 1984. *80:* 67.
13 Gunter, K. C., Malek, T. R. and Shevach, E. M., *J. Exp. Med.* 1984. *159:* 716.
14 MacDonald, H. R., Bron, C., Rousseaux, M., Horvath, C. and Cerottini, J. C., *Eur. J. Immunol.* 1985. *15:* 495.
15 Pont, S., Regnier-Vigouroux, A., Naquet, P., Blac, D., Pierres, M. and Marchetto, S., *Eur. J. Immunol.* 1985. *15:* 1222.
16 Kroczek, R. A., Gunter, K. C., Seligmann, B. and Shevach, E. M., *J. Immunol.* 1986. *136:* 4379.
17 Kroczek, R. A., Gunter, K. C., Germain, R. N. and Shevach, E. M., *Nature* 1986. *322:* 181.
18 Galfre, G., Milstein, C. and Wright, B., *Nature* 1979. *277:* 131.
19 Kummer, U., Thierfelder, S., Hoffmann-Fezer, G. and Schuh, R., *J. Immunol.* 1987. *138:* 4069.
20 Stähle, C., Miggiano, V., Stocker, J., Staehelin, T. H., Häring, P. and Takacs, B., *Methods Enzymol.* 1983. *92:* 242.
21 Gillis, S., Ferm, M. M., Ou, W. and Smith, K. A., *J. Immunol.* 1978. *120:* 2027.
22 Kupfer, A., Singer, S. J., Janeway, Jr., C. A. and Swain, S. L., *Proc. Natl. Acad. Sci. USA* 1987. *84:* 5888.
23 Rousseaux, J., Rousseaux-Prevost, R. and Bazin, H., *Methods Enzymol.* 1986. *121:* 663.
24 Ullrich, A. and Schlesinger, J., *Cell* 1990. *61:* 203.
25 Robinson, P. J., *Immunol. Today* 1991. *12:* 35.
26 Altman, A., Cogshall, M. and Mustelin, T., *Adv. Immunol.* 1990. *48:* 227.
27 Greenspan, N. S., Monafo, W. J. and Davie, J. M., *J. Immunol.* 1987. *138:* 285.
28 Greenspan, N. S., Dacek, D. A. and Cooper, L. J., *FASEB J.* 1989. *3:* 2203.
29 Brüggemann, M., Delmastro Galfre, P., Waldmann, H. and Calabi, F., *Eur. J. Immunol.* 1988. *18:* 317.
30 Fridman, W. H., Bonnerot, Ch., Daeron, M., Amigorena, S., Teillaud, J.-L. and Sautes, C., *Immunol. Rev.* 1992. *125:* 49.
31 Revetch, J. V. and Kinet, J.-P., *Annu. Rev. Immunol.* 1991. *9:* 457.
32 Kummer, U., Thierfelder, S. and Mysliwietz, J., *Eur. J. Immunol.* 1990. *20:* 107.
33 Ishihara, A., Hou, Y. and Jacobson, K., *Proc. Natl. Acad. Sci. USA* 1987. *84:* 1290.
34 Hale, G., Xia, M. Q., Tighe, H. P., Dyer, M. J. and Waldmann, H., *Tissue Antigens* 1990. *35:* 118.