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Immunology

Overexpressed Ly-6A.2 mediates cell–cell adhesion by binding a ligand expressed on lymphoid cells

(Ly-6 protein/thymus)

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ABSTRACT **The Ly-6 locus encodes several cell surface proteins whose functions are unknown. Although it is hypothesized that these proteins may be receptors, there is no direct evidence that they bind a ligand. Herein we present evidence that Ly-6A.2, a Ly-6 protein expressed on T lymphocytes, binds a ligand expressed on normal thymocytes and splenic B and T cells. We find that transgenic thymocytes that overexpress Ly-6A.2 spontaneously aggregate in culture. This homotypic adhesion requires the overexpression of Ly-6A.2 because it is not observed in cultures of nontransgenic thymocytes. The aggregation of Ly-6A.2 transgenic thymocytes is inhibited by phosphatidylinositol-specific phospholipase C (which removes Ly-6A.2 and other glycosylphosphatidylinositol-anchored proteins from the membrane). Some anti-Ly-6A.2 monoclonal antibodies, including nonactivating ones and Fab′ fragments, inhibit this aggregation. In contrast, other anti-Ly-6A.2 monoclonal antibodies increase the aggregation of transgenic but not nontransgenic thymocytes. To further examine whether Ly-6A.2 mediates adhesion (versus inducing another adhesion pathway) reaggregation assays were performed with paraformaldehyde-fixed Tg^+ thymocytes. Paraformaldehyde-fixed Tg^+ thymocytes reaggregate in culture and this aggregation is also blocked by phosphatidylinositol-specific phospholipase C and anti-Ly-6A.2 monoclonal antibodies. These results indicate that the homotypic adhesion of cultured Ly-6A.2 transgenic thymocytes is directly mediated by Ly-6A.2 and, more importantly, strongly suggests that Ly-6A.2 binds a ligand that is expressed on thymocytes. Tg^+ thymocytes also bind to nontransgenic thymocytes, B cells, and T cells, indicating that normal cells naturally express the Ly-6A.2 ligand.**

The Ly-6 locus is a multigene family on chromosome 15 of mice that encodes several proteins that are differentially expressed on hematopoietic stem cells, lymphocytes, monocytes, and granulocytes (1–9). The functions of these genes are largely unknown. All of the known Ly-6 molecules are expressed on the cell surface; however, it is not known whether they bind an extracellular ligand. Recently, Johnson *et al.* (10) have reported indirect evidence that one Ly-6 molecule, Ly-6C, might interact with a molecule expressed on fibroblasts. To further understand the function of Ly-6 proteins it is important to determine whether they interact with an external ligand.

Ly-6A.2 is a member of this gene family that is expressed on pluripotent hematopoietic stem cells (SCA-1), on some developing thymocytes, and on the majority of mature T lymphocytes (8, 11–13). The expression of Ly-6A.2 is markedly increased upon T-cell activation or after stimulation with cytokines (14, 15). Although the precise function of Ly-6A.2 is not resolved, some data have suggested that it is involved in T-cell activation (16, 17).

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We have recently overexpressed Ly-6A.2 in transgenic mice under the control of the human CD2 enhancer (28). The overexpression of Ly-6A.2 in the thymus of these animals leads to a block in T-cell development. These results imply that this molecule is active during thymic development and might interact with an intrathymic ligand. In this report we present evidence that such a ligand is expressed on the surface of lymphoid cells and can mediate homotypic adhesion.

MATERIALS AND METHODS

Mice. A CD2-Ly-6A.2 transgene was introduced in (C57B/6 × C3H)F_1 mice and then backcrossed to BALB/c for three or four generations (28). Mice that were 4–8 weeks old were used in all experiments. Age and sex matched nontransgenic littermates were used as controls.

Antibodies. Anti-Ly-6A.2 monoclonal antibodies (mAbs) used in this study were 3A7, 3E7, and 8G12 (18–20). Anti-Ly-6A/E (D7) (21) was generously made available by Ethan Shevach and anti-H-2 class I (M1/42) (22) and anti-H-$2D^{d,q}$ (28-14-8s) (23) were obtained from American Type Culture Collection. Culture supernatants containing mAbs were prepared as described (24). Antibodies were purified from culture supernatant (D7, 28-14-8s) or ascites fluid (3A7) on a protein A- or protein G-Sepharose column. Fab′ or 3A7 was prepared as described (24, 25). Other reagents used for immunofluorescence and flow cytometric analysis were phycoerythrin-conjugated anti-Ly-6A/E (D7, Pharmingen), anti-Thy-1-biotin (Becton Dickinson) and anti-CD45R (anti-B220, GIBCO/BRL).

Cell Purification. Spleen cells from nontransgenic mice were treated with Tris NH_4Cl and 40×10^6 cells were incubated in rabbit anti-mouse immunoglobulin (RAMG)-coated plates for 45 min on ice. The splenic B cells and T cells were purified after two rounds of positive selection or negative selection on RAMG-coated plates, respectively.

Cell Culture and Aggregation Assays. Thymocytes (1×10^6) were cultured in flat-bottom 96-well plates for 18–24 hr at 37°C in a final volume of 200 μl of culture medium (19). The precise culture conditions are indicated in the respective experimental procedures. In some experiments aggregated thymocytes obtained from these cultures were disrupted into single cell suspension by pipetting and further cultured either live or after paraformaldehyde fixation (see below) for 18–24 hr at 37°C in V-bottom microtiter wells.

Thymocytes were fixed with 1% paraformaldehyde for 10 min at room temperature. Fixation was stopped by adding 10 ml of medium (RPMI 1640 medium, glutamine, and antibiotics) (Irvine Scientific) with 5% fetal calf serum. Cells were further washed three times and resuspended in culture medium (19).

Abbreviations: Tg, transgenic; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; mAb, monoclonal antibody.

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For quantitation of cells in aggregates, cultures of either liver or paraformaldehyde-fixed thymocytes in V-bottom microtiter wells were used. The cells were gently pipetted with wide-bore pipette tips and counted in a hemocytometer. The percentage of cells forming aggregates was calculated by counting the free (single) cells and applying the following formula: % aggregation = (1 − number of free cells/number of total cells) × 100. The experiments were carried out in duplicates and all data are representative of at least three independent assays. Brackets show the SD in the illustrated representative experiment.

Separation of Aggregates and Immunofluorescence. Transgenic (Tg^+) thymocytes (2.5×10^6) were mixed with an equal number of Tg^- thymocytes, purified splenic B cells (purified by positively panning on RAMG-coated plates), T cells (purified by negatively panning on RAMG-coated plates), or red blood cells and cultured in 10-ml round-bottom tubes in 1 ml of culture medium under sterile conditions. Parallel cultures using only 2.5×10^6 Tg^- or Tg^+ were also set up. After 18 hr of incubation at 37°C, the cells in each tube were gently resuspended and 5 ml of warm wash medium was added. The cells were allowed to sediment for 15 min at 37°C and then the upper 5.5 ml of the medium was removed. The resuspension, sedimentation, and aspiration steps were repeated four times. After the last wash the sedimented aggregates were disrupted into a single cell suspension by pipetting vigorously. The cell suspensions were then stained by immunofluorescence and analyzed by flow cytometry as described (19).

RESULTS

Homotypic Aggregation of Ly-6A.2 Transgenic Thymocytes. When Ly-6A.2 transgenic (Tg^+) thymocytes were cultured at 37°C, we observed the formation of large aggregates of cells (Fig. 1*c*). The clusters of Tg^+ thymocytes could be disrupted by vigorous pipetting and the cells would reaggregate if placed back in culture. Under these conditions, 45–60% of the thymocytes underwent aggregation (Fig. 1*e*). In contrast, control (Tg^-) thymocytes remained monodispersed (Fig. 1*a*) even when these cells were cultured for >7 days (data not shown). In quantitative assays, only 4–6% of Tg^- thymocytes were clustered (Fig. 1*e*). Moreover, the Tg^- thymocytes that were enriched in $CD4^-CD8^-$ cells also did not show any increased aggregation (data not shown). These observations imply that overexpression of Ly-6A.2 stimulates homotypic aggregation of thymocytes.

Homotypic Aggregation of Ly-6A.2 Transgenic Thymocytes Is Sensitive to PI-PLC. The Ly-6A.2 molecule is linked to the plasma membrane by a glycosylphosphoinositol (GPI) anchor (26). To investigate the role of GPI-anchored proteins in the homotypic aggregation of Tg^+ thymocytes, these proteins were removed with the enzyme PI-PLC. Incubation of thymocytes in the presence of PI-PLC inhibited their aggregation by 80–90% (Fig. 1 *d* and *e*). These results indicated that GPI-anchored molecules were involved in the homotypic adhesion of Tg^+ thymocytes. PI-PLC also blocked the reaggregation of previously cultured thymocytes and could dissociate preformed cell aggregates (data not shown). These latter observations provided the first indication that GPI-anchored proteins might directly mediate adhesion as opposed to indirectly stimulating some other adhesion pathway.

Anti-Ly-6A.2 Antibodies Inhibit or Augment the Homotypic Aggregation of Ly-6A.2 Transgenic Thymocytes. PI-PLC removes other GPI-anchored proteins in addition to Ly-6A.2. To directly examine the role of Ly-6A.2 in the homotypic aggregation of Ly-6A.2 transgenic thymocytes, these cells were cultured in the presence of anti-Ly-6A.2 antibodies. The anti-Ly-6A.2 mAbs 3A7 (Fig. 2 *b* and *e*) and D7 (data not shown) inhibited the aggregation of Tg^+ thymocytes by 80–90%. Several other anti-Ly-6A.2 mAbs did not affect cell clustering (data not shown). Presumably these other mAbs are against functionally distinct epitopes. The inhibition of aggregation appeared to be specific since control isotype-matched mAbs and mAbs to other T-cell surface proteins (e.g., anti-HSA, anti-LFA1, anti-class I, anti-CD3, and anti-CD4) either did not affect or in some cases modestly increased cell clustering of both Tg^- and Tg^+ thymocytes (Fig. 2*e* and data not shown).

mAb crosslinking of Ly-6A.2 molecules can stimulate T lymphocytes (18). Several lines of evidence suggested that inhibition of thymocyte aggregation by the anti-Ly-6A.2 mAbs was not a secondary event arising from the stimulation of cells by the antibody reagents. Only some anti-Ly-6A.2 mAbs were stimulatory (18–20), yet both stimulatory (e.g., 3A7) and nonstimulatory mAbs (e.g., 1E7 and 3E7) (data not shown) inhibited aggregation. The aggregation of Tg^+ thymocytes could also be inhibited by Fab′ fragments of the 3A7 mAb (Fig. 2*f*). In contrast, the stimulation of T cells by 3A7 or other

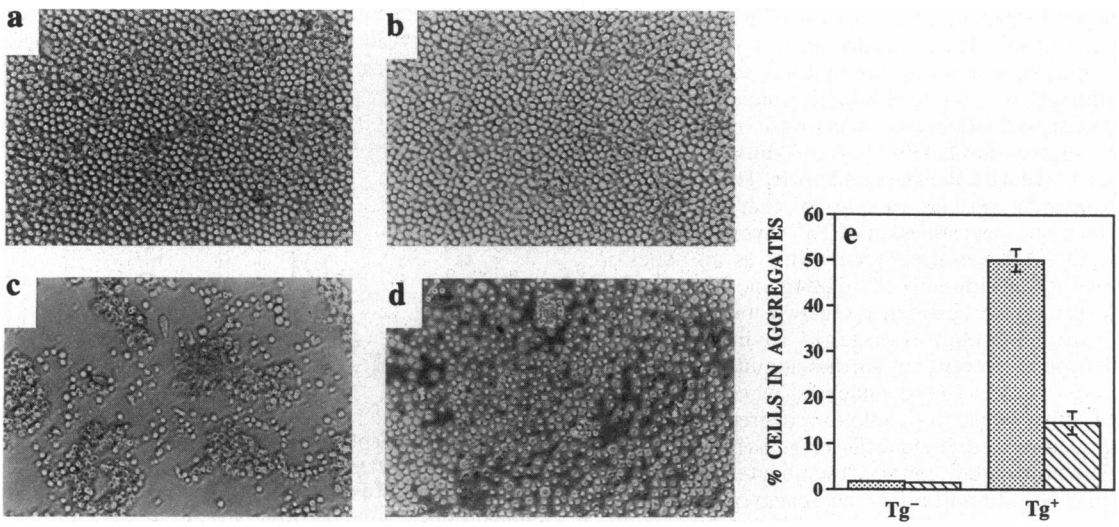

FIG. 1. Homotypic aggregation of thymocytes and effect of phosphatidylinositol-specific phospholipase C (PI-PLC). Tg^- (*a* and *b*) and Tg^+ (*c* and *d*) thymocytes were incubated alone (*a* and *c*) or in presence of PI-PLC (*b* and *d*) at a concentration of 1 unit/ml for 18 hr. Cell cultures were photographed using a phase-contrast microscope. (×200.) (*e*) Tg^- and Tg^+ thymocytes were incubated with either medium alone (dotted) or PI-PLC (hatched) as described above, and aggregates were quantified (see text).

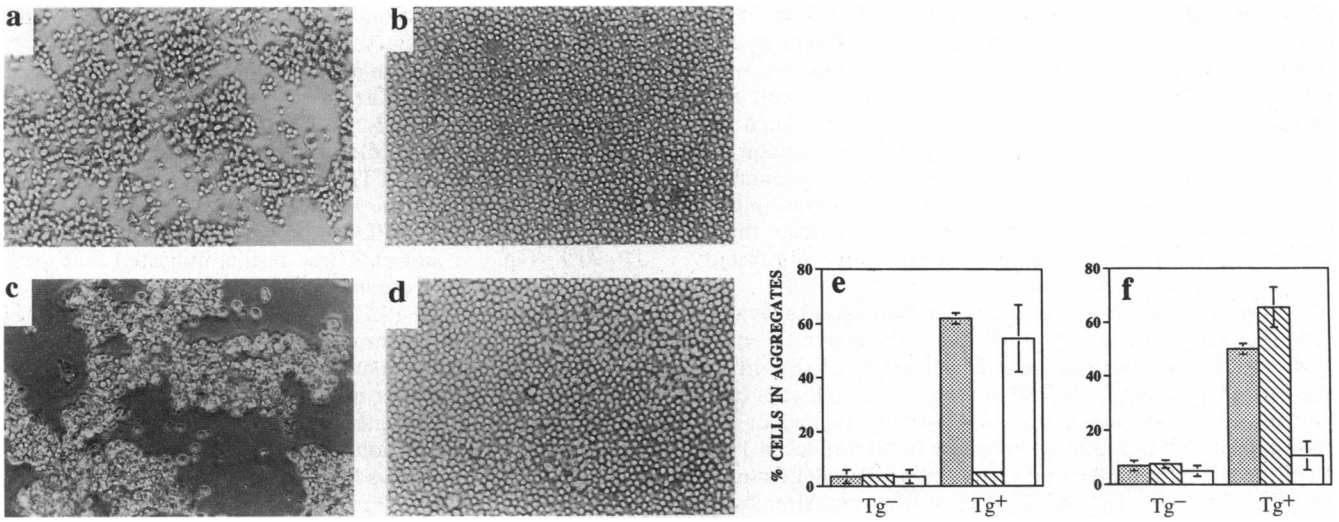

FIG. 2. Effect of anti-Ly-6A.2 mAbs on homotypic aggregation of Tg^+ thymocytes. Tg^+ thymocytes were cultured in the presence of medium (*a*), 3A7 (1:4 SN) (*b*), 8G12 (1:4 SN) (*c*), and 3A7 Fab′ (5 μg/ml) (*d*) and photographed as described in the legend to Fig. 1. (*e*) Tg^- and Tg^+ thymocytes were incubated with medium alone (dotted), 3A7 (1:4 SN) (hatched), or 28.14.8s (1:4 SN) (white) and aggregates were quantified as described in the legend to Fig. 1. (*f*) Thymocytes from Tg^- and Tg^+ mice were incubated with either media alone (dotted), 8G12 (1 μg/ml) (hatched), or Fab′ of 3A7 at 5 μg/ml (white).

anti-Ly-6A.2 mAbs requires intact, bivalent antibodies (25). The anti-Ly-6A.2 mAbs that inhibited thymocyte aggregation also blocked the reaggregation of previously cultured Tg^+ thymocytes (Fig. 2 *e* and *f*). These observations again suggested that Ly-6A.2 might be directly involved in the intercellular adhesion; this point is addressed further below.

We also observed that some anti-Ly-6A.2 mAbs (e.g., 8G12 and 2D7) enhanced the aggregation of Tg^+ thymocytes, resulting in larger aggregates (Fig. 2 *c* and *f*). These mAbs could similarly enhance the reaggregation of cultured Tg^+ thymocytes. In contrast, 8G12 had no effect on fresh or cultured control Tg^- thymocytes. The antibody-stimulated aggregation of Tg^+ thymocytes was partially inhibited by PI-PLC. Both activating (8G12) and nonactivating (2D7) anti-Ly-6A.2 mAbs could cause this effect, suggesting that stimulation was not required for this phenomenon. It is possible that this enhanced cell clustering arises from agglutination of the cells by the mAb. However, other mAbs that bound the T cell in similar amounts, including other anti-Ly-6A.2 mAbs, did not cause thymocyte agglutination (data not shown). Furthermore, the aggregation of thymocytes in the presence of anti-Ly-6A.2 was inhibited at 4°C (data not shown). These results suggest that the mAb binding of a particular epitope on Ly-6A.2 might promote adhesion, although we cannot absolutely rule out that antibody-mediated agglutination was occurring.

Homotypic Aggregation Is Observed in Transgenic Thymocytes That Are Fixed with Paraformaldehyde. The experimental results described thus far established that Ly-6A.2 was involved in the homotypic adhesion of Tg^+ thymocytes. It was possible that Ly-6A.2 was itself functioning as an adhesion receptor or that it was indirectly stimulating another adhesion pathway. To distinguish between these two mechanisms we treated cultured Ly-6A.2 transgenic thymocytes with paraformaldehyde to prevent any further stimulation through Ly-6A.2. Fixed Ly-6A.2 Tg^+ thymocytes, when cultured in V-bottom microtiter wells, were able to reaggregate, albeit to a lesser degree than untreated cells (Fig. 3*a*). In contrast, paraformaldehyde-treated control thymocytes aggregated minimally (Fig. 3*a*). Importantly, the reaggregation of Ly-6A.2-Tg^+ thymocytes was inhibited by anti-Ly-6A.2 antibodies (Fig. 3*a*) and PI-PLC (Fig. 3*b*). Furthermore, the 8G12 anti-Ly-6A.2 mAb enhanced the aggregation of the Tg^+ but not Tg^- thymocytes (Fig. 3*b*). Control antibodies (e.g., anti-major histocompatibility complex class I) had no effect on the aggregation of these cells (Fig. 3*a*). These experiments strongly suggest that Ly-6A.2 is directly responsible for the aggregation of Tg^+ thymocytes rather than stimulating another adhesion receptor.

Binding of Tg^+ Thymocytes to Lymphocytes from Tg^- Mice. We next sought to determine whether Tg^+ thymocytes would bind normal (nontransgenic) lymphocytes. For this purpose equal numbers of Tg^+ and Tg^- thymocytes (Fig. 4) or purified splenic B cells or purified splenic T cells (Table 1) were mixed in cultures. The cell aggregates that formed were separated by $1 \times g$ sedimentation and then stained for Ly-6A.2-expression. The aggregates were heterotypic containing both Ly-6A.2 bright (transgenic) and Ly-6A.2 weak (nontransgenic) cells (Fig. 4*d* and Table 1). Aggregates from cultures on Tg^+ thymocytes without Tg^- cells were 100% Ly-6A.2 bright (Fig. 4*c*). Since Tg^- lymphocytes do not aggregate when cultured alone, essentially no cells were recovered from the sedimentation of cultures of these cells. This latter finding verified that our fractionation procedure isolated aggregated and not free lymphocytes. Furthermore, red blood cells (Table 1) and a B-cell line, A20 (data not shown), did not coaggregate with Tg^+ thymocytes. This observation indicates that the hetero-

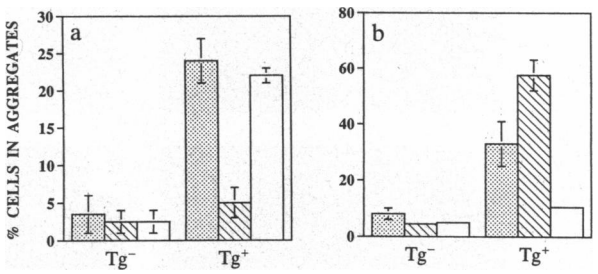

FIG. 3. Effect of paraformaldehyde fixation on homotypic reaggregation of thymocytes. (*a*) Tg^- and Tg^+ thymocytes were incubated for 24 hr at 37°C and dissociated into single cell suspension. The cells were fixed with paraformaldehyde and incubated for 24 hr at 37°C in V-bottom microtiter wells in the presence of medium alone (dotted), 3A7 at 1 μg/ml (hatched), and 28.14.8s at 1 μg/ml (white). (*b*) Similar to *a*, except incubations were in the presence of medium alone (dotted), 8G12 at 1 μg/ml (hatched), and PI-PLC at 1 unit/ml (white). Aggregates were then quantitated (see text).

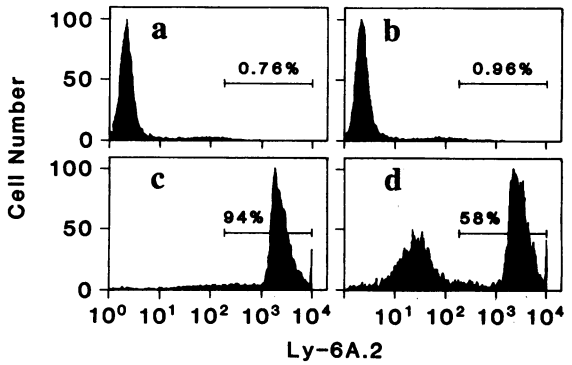

FIG. 4. Binding of Tg^+ thymocytes to Tg^- thymocytes. Tg^+ and Tg^- thymocytes were cocultured and aggregates were separated (see text). Tg^- thymocytes that were cultured and not subjected to purification were stained with either goat anti-rat IgG conjugated to fluorescein isothiocyanate (FITC) (Kirkegaard & Perry Laboratories) only (*a*) or D7 (anti-Ly-6A/E) (*b*). A single cell suspension of aggregates of only Tg^+ thymocytes (*c*) or a mixture of Tg^- and Tg^+ thymocytes (*d*) was stained with D7 and goat anti-rat IgG-FITC. Since Tg^- cells do not aggregate, no cells were recovered in the sedimenting fractions of Tg^- cells cultured alone. Staining of aggregated cells from Tg^+ only or cocultured Tg^- and Tg^+ thymocytes with goat anti-rat IgG-FITC alone was the same as shown in *a*. The cells (5000) were analyzed by cytofluorometry.

typic adhesion between Tg^- lymphocytes and Tg^+ thymocytes is not due to a nonspecific trapping of Tg^- cells. The cultured thymocytes from Tg^- mice showed dim-negative expression of Ly-6A.2 (Fig. 4*b*). Pretreatment of Tg^- thymocytes with blocking anti-Ly-6A.2 antibodies did not affect its ability to coaggregate with Tg^+ thymocytes (data not shown). Moreover, aggregation of Tg^+ thymocytes with Pronase-treated Tg^- thymocytes was reduced by 43–69% as compared to the untreated cells (data not shown). The above results indicate that the molecule on the Tg^- thymocytes that mediates cell–cell adhesion is a protein other than Ly-6A.2 that is expressed on the cell surface.

DISCUSSION

The overexpression of Ly-6A.2 molecule on thymocytes induces homotypic adhesion. Several lines of evidence demonstrate that this requires Ly-6A.2 molecules. (*i*) The aggregation is only seen with Tg^+ and not with Tg^- thymocytes. (*ii*) The adhesion of Tg^+ cells is inhibited by PI-PLC and therefore requires a GPI-anchored protein (presumably Ly-6A.2 and/or its ligand). (*iii*) The adhesion is blocked by some anti-Ly-6A.2 antibodies as well as Fab' fragments. (*iv*) Other anti-Ly-6A.2 mAbs stimulate temperature-dependent homotypic aggregation. Our data further indicate that Ly-6A.2 is directly participating in adhesion because fixed Tg^+ thymocytes show similar clustering, which is also blocked by some anti-Ly-6A.2 antibodies or PI-PLC and augmented by other anti-Ly-6A.2 mAbs. To mediate homotypic aggregation, Ly-6A.2 must be interacting with a molecule on thymocytes.

Table 1. Binding of Tg^+ thymocytes to other cell types

Genotype	Cell type	Source	% cells in aggregates*	$n^\dagger$
Tg^-	T cell	Spleen	35.5 ± 4.4	4
Tg^-	B cell	Spleen	28.4 ± 7.2	5
Tg^-	RBC	Blood	4 ± 1	2

The Tg^+ thymocytes were cocultured with Tg-purified splenic B, T, and red blood cells (RBC) and the aggregates were separated. The aggregates with B cells were stained with anti-CD45R-red613 and anti-Ly-6A/E-phycoerythrin in a two-color immunofluorescence. Similarly, aggregates with T cells were stained with anti-Thy-1-biotin followed by strepavidin-red613 in conjunction with anti-Ly-6A/E-phycoerythrin RBCs in the aggregates were detected by the absence of staining in a two-color analysis.
*Mean ± SEM.
†Total number of experiments.

A major conclusion of this study is that Ly-6A.2 binds an extracellular ligand. This provides direct evidence that a Ly-6 molecule is a receptor. Earlier studies had suggested this possibility because an anti-Ly-6.C mAb blocked the stimulation of a T-cell clone by fibroblasts but not other antigen-presenting cells (10). In future studies it should be possible to use the assays described in this report to determine the identity of the Ly-6A.2 ligand.

Lymphoid cells from control animals coaggregate with Tg^+ thymocytes, and this heterotypic aggregation is not due to cell trapping. This finding indicates that the Ly-6A.2 ligand is expressed on normal thymocytes and at least some splenic T and B cells. Tg^- thymocytes do not spontaneously aggregate in culture even when enriched for $CD4^-CD8^-$ cells (which account for 20–30% of Tg^- thymocytes). The aggregation of Tg^+ thymocytes is observed with normal splenic B (Ly-6A.2 negative) as well as Tg^- thymocytes that are pretreated with anti-Ly-6A.2 antibody. These experiments indicate that Ly-6A.2 does not mediate adhesion through a like–like interaction. The sensitivity of the ligand to Pronase digestion indicates that it is a cell surface protein.

The augmentation of adhesion that is induced by some anti-Ly-6A.2 mAbs suggests that different affinity states of Ly-6A.2 might exist. Similar effects have been observed when mAbs bind to other adhesion receptors (27). However, we cannot rule out that these particular mAbs are aggregating cells due to agglutination.

Although we present evidence that Ly-6A.2 can mediate intercellular adhesion, it is unclear whether this molecule plays a significant role in adhesion under physiological conditions. Homotypic aggregation of thymocytes requires high levels of Ly-6A.2 expression, much higher levels than are present on resting T lymphocytes. However, Ly-6A.2 expression is markedly up-regulated to levels approaching those observed in our Tg^+ animals upon T-cell activation or stimulation with cytokines and in this setting might contribute to cell–cell interactions (refs. 14 and 15; unpublished data). Even if Ly-6A.2 does not contribute to adhesion, it would be expected to engage in ligand during cell–cell interactions and might subserve other functions (e.g., contributing to T-cell stimulation; reviewed in refs. 2 and 3). In support of this concept it is of interest that the overexpression of Ly-6A.2 in the thymus, wherein the Ly-6A.2 ligand is expressed, leads to a block in T-cell development (28).

Ly-6A.2 is a member of a large multigene family on chromosome 15. Various members of this family are expressed on different cells of the hematopoietic lineage. Since these genes are highly homologous to one another it is likely that they will also interact with extracellular ligands. We anticipate that the further characterization of Ly-6 ligands should give insight into the function of this poorly understood gene family.

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