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ADHESIVE HIERARCHY INVOLVING THE CELL ADHESION MOLECULES L1, CD24, AND α6 INTEGRIN IN MURINE NEUROBLASTOMA N2A CELLS

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The aggregation rate of resuspended neuroblastoma N2A cells depends on the density of the cells in culture prior to their resuspension: isolated, fast growing cells have a weak tendency to aggregate whereas confluent, slowly growing cells reaggregate very strongly. L1 antibody 557 strongly inhibited the slow aggregation of isolated, fast growing cells but not the reaggregation of confluent cells. CD24 (nectadrin) antibodies did not affect the aggregation of isolated or confluent cells but stimulated the aggregation of subconfluent cells. In all stages aggregation was not inhibited when antibody 557 was used together with CD24 antibodies at 37°C in the presence of divalent cations. EA-1 antibody to α6 integrin chain stimulated the aggregation of subconfluent cells but inhibited the reaggregation of confluent cells. Therefore, L1 appears to be an early recognition molecule mediating weak primary adhesion. CD24 appears to participate in activating secondary adhesion mechanisms during primary adhesion, possibly in cooperation with L1, and α6 integrin seems to serve as a secondary, strong adhesion molecule that in early adhesion phases also mediates the activation of itself or of other adhesion mechanisms. These results indicate that neural cells might employ a strategy of adhesion cascade in establishing stable contacts.

The interplay of coexpressed recognition molecules is an important tool in the regulation of correct cell adhesion. In the present work we have examined the differential roles of the cell adhesion molecules L1, CD24, and α6 integrin in mediating N2A cell aggregation. N2A cells resemble cerebellar neurones with respect to their aggregation and expression of the adhesion molecules L1, N-CAM, and CD24 (1-4). L1 is a member of the immunoglobulin superfamily (5) which mediates signal transduction (4), neuronal adhesion (3, 6) and neurite elongation on other neurites (7). CD24, formerly named heat-stable antigen (HSA) and nectadrin, is a small, highly glycosylated surface molecule which mediates B lymphoblast aggregation (8) and cooperates with L1 in mediating strong intracellular Ca^{++} signals in cultured cerebellar neurones and N2A cells (4). The α6 integrins, recognised by antibody EA-1, mediate adhesion between several cell types and between cells and extracellular matrix (9). The results suggest that the investigated

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molecules have sequential roles in mediating N2A cell adhesion that resemble the adhesion cascade (adhesive hierarchy) characteristic of neutrophil-endothelial adhesion (10, 11).

MATERIALS AND METHODS

Antigens and Antibodies

Monoclonal CD24 antibodies 79 and M1/69 (12), monoclonal L1 antibody 557 (13), and monoclonal α6 integrin antibody EA-1 (9) have been described. Antibody 79-15 was obtained from a subclone of the cells producing antibody 79.

Cell Culture and Cell Aggregation

The preparation and culture of neuroblastoma N2A cells has been described (1, 12). Ten million dispersed cells were plated in culture flasks with a floor area of 180 cm^2. At this density the cells cover 1-2% of the floor area after adhering to the substrate. After 24, 48, 72, or 96 hours in culture the cells were suspended in PBS, pH 7.2, containing 2 mg/ml EDTA and dispersed by washing and slow centrifugation (5 min, 100 *g*) as described (3, 12). Aggregation was tested in RPMI-1640 or in absence of divalent cations and in the presence or absence of antibodies and examined by Coulter Counter analysis as described (8, 12).

Statistical Analysis

Statements concerning differences between the quantitative effects of different treatments are based on multiple comparisons among means using the GT2 methods with $\alpha \leq 0.01$ (14).

RESULTS

N2A Cell Aggregation

To examine whether the rate of N2A cell-cell adhesion may be related to the density of the cells in culture, their aggregation was measured after different times in culture. Twenty-four hours after plating the cells at a density of 560 cells/mm^2, the cells were round and 20-50% showed mitotic figures by stereoscopic microscopy. The cells were isolated or present in doublets or triplets as schematically represented in Fig. 1. By Coulter Counter analysis directly following resuspension of the cells, 4±2.3% (mean±SEM) of the cells were present in aggregates measuring more than 30 μm^3 in volume and the mean particle volume (mean volume of all single cells and cell aggregates) was 9±1.4 μm^3. When these cells were dispersed and allowed to aggregate at 37°C their aggregation was slow. Only 9±2.1% of the cells entered aggregates larger than 30 μm^3 within 25 min of aggregation (Fig. 1) and the mean particle volume was then 19±3.0 μm^3. After 60 min of aggregation, 19± 1.8% of the cells were present in aggregates larger than 30 μm^3 (Fig. 1) and the mean particle volume was 20±0.1 μm^3.

After 48-72 hours in culture, the cells have become much more dense. Isolated cells and mitotic figures were still abundant, but most cells were present in isolated small groups of 3-15 cells. Following resuspension and dispersion of these cells, 28±8.2% of the cells entered aggregates larger than 30 μm^3 within 25 min of aggregation (Fig. 1) and the mean particle volume was then 23±2.9 μm^3. Their aggregation at 60 min was not measured.

After 92 hours in culture, the cells were confluent. Many cells had flattened or had sent out neurites, thereby showing morphological signs of differentiation (Fig. 1). Following their dispersion, 56±9.2% of these cells entered aggregates larger than 30 μm^3 within 25 min of aggregation (Fig. 1) and the mean particle volume was 111±116.6 μm^3. After 60 min of aggregation, 95±3.7% of the cells were present in aggregates larger than 30μm^3 (Fig. 1) and the mean particle volume was 420±93.7 μm^3.

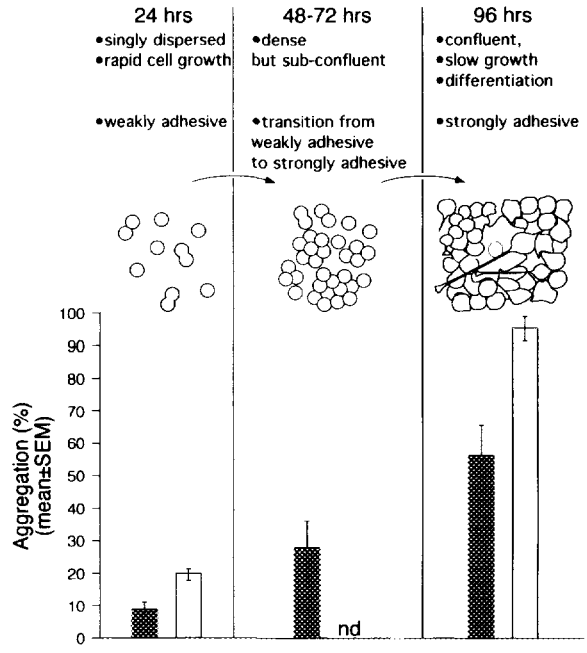

Figure 1. *Schematic representation of N2A cell growth and Coulter Counter analysis of N2A cell aggregation.* Neuroblastoma N2A cells were dispersed and plated in flat culture bottles at a density of 560 cells/mm^2. Following 24, 48, 72, or 96 hours in culture, the cells were examined microscopically, suspended by EDTA, dispersed, and allowed to aggregate in suspension for 25 min (shaded bars) or 60 min (open bars). Aggregation was evaluated by Coulter Counter analysis measuring the partial volume occupied by cells present in aggregates larger than 30 μm^3. Ordinate values denote the difference in the partial volume occupied by such aggregates at the end and start of aggregation; nd – not done; adhesive – denotes common cell-cell contacts in culture and strong aggregation in suspension.

These results suggest that the rate of spontaneous aggregation of dispersed N2A cells may be related to their density or differentiation state in culture prior to their dispersion. The data imply that fast growing, isolated N2A cells have weak adhesion mechanisms whereas stronger adhesion mechanisms are activated as the cells become dense in culture.

To gain more insight into the events underlying the gradual increase in the strength of N2A cell adhesion we have examined the roles of three cell adhesion molecules expressed by these cells, L1, CD24, and α6 integrin, in mediating their aggregation after different times in culture.

Inhibition of N2A Cell Aggregation by Antibodies to L1, CD24, or α6 Integrin

L1 antibody 557 inhibited N2A cell aggregation, as compared to aggregation in the absence of antibodies. In cells having a very low tendency to aggregate inhibition of aggregation by antibody 557 exceeded 100% (Fig. 2Aa). This implies that even the small aggregates present in the cell suspension prior to the onset of aggregation due to the mild dispersion method were dispersed in presence of the antibody. However, whereas the aggregation of slowly aggregating cells was completely inhibited by antibody 557, the aggregation of fast aggregating cells was only slightly perturbed by this antibody (Fig. 2Aa). The ability of antibody 557 to inhibit N2A cell aggregation also depended on the aggregation time. It was strong after 25 min of

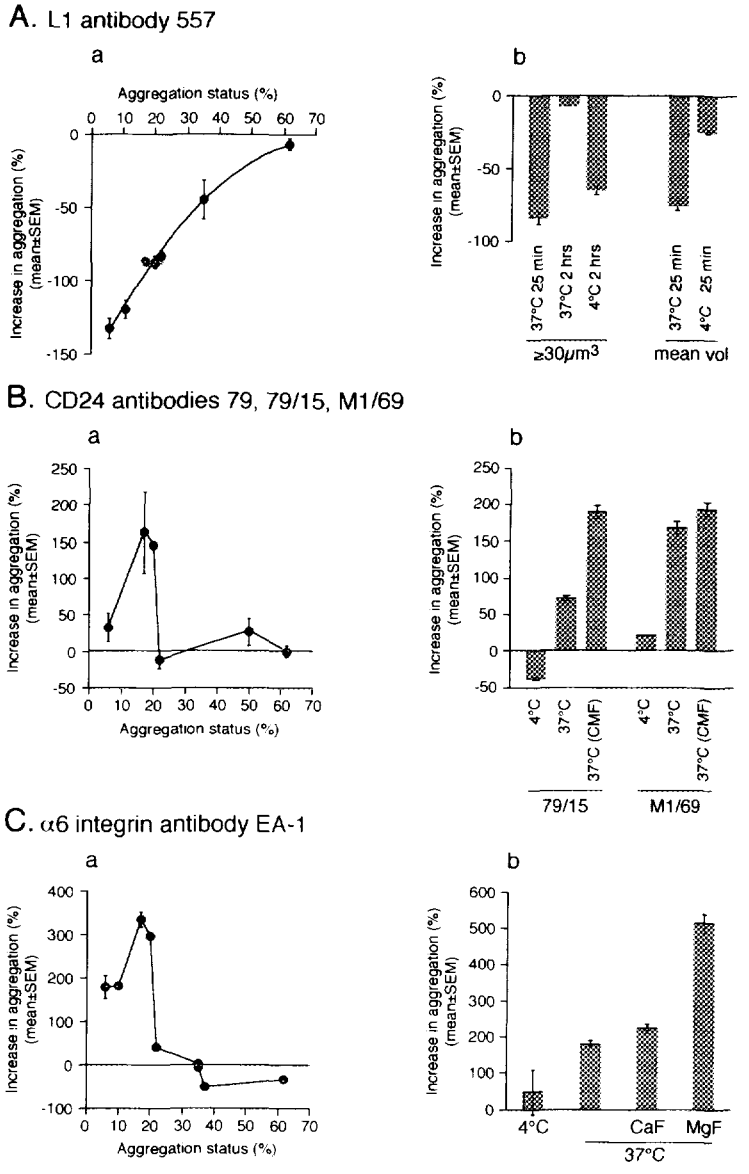

Figure 2. *Coulter Counter analysis of N2A cell aggregation in the presence of antibodies to L1, CD24, or α6 integrin.* N2A cells were plated in culture bottles at a density of 560 cells/mm^2 and were allowed to grow for 24, 48, 72, or 96 hrs (Aa, Ba, Ca) or for 48 hrs only (Ab, Bb, Cb). The cells were then resuspended, dispersed and allowed to aggregate in suspension for 25 min at 37°C in the presence of divalent cations (Aa, Ba, Ca) or under different conditions as denoted under the bars (Ab, Bb, Cb) in the absence or presence of antibodies to L1 (A), CD24 (B), or α6 integrin (C). In Ba, the values for different CD24 antibodies were pooled. Aggregation was evaluated from the increase in the partial volume occupied by aggregates larger than 30 μm^3 (Aa, B, C, and $\geq 30\mu m^3$ in Ab) or from the increase in the mean particle volume (mean vol in Ab). Aggregation status (abscissa values): percent aggregation in the absence of antibodies evaluated as in Fig. 1. Ordinate values: increase in aggregation in the presence of antibodies evaluated relative to the aggregation of cells from the same culture in the absence of antibodies. CaF – Ca^{++}-free; MgF – Mg^{++}-free; CMF – absence of divalent cations. Note that in Aa, Ba, and Ca, the strength of the inhibitory or stimulatory effect of antibodies on aggregation (ordinate value) is related to the aggregation potential of the cells in the absence of antibodies (abscissa value).

aggregation (Fig. 2Ab), weaker after 60 min (not shown), and very weak after 120 min (Fig. 2Ab). As evaluated from the partial volume occupied by large aggregates, antibody 557 had similar inhibitory effects in the presence and absence of divalent cations (not shown; ref. 4) and at different temperatures (Fig. 2Ab). When evaluated by mean particle volume, its effect appeared to be weaker at 4°C than at 37°C (Fig. 2Ab). However, this probably reflected the smaller size aggregates generally have at 4°C (not shown).

CD24 antibodies 79, 79-15, and M1/69 stimulated an increase in the aggregation of slowly aggregating N2A cells at 37°C but did not affect the aggregation of very slowly or fast aggregating cells (Fig. 2Ba). In the presence of divalent cations, but not in their absence, the aggregation-stimulating effect of antibody 79-15 was weaker than the effect of antibody M1/69 (Fig. 2Bb). At 4°C, antibody 79-15 inhibited the aggregation of slowly aggregating N2A cells whereas antibody M1/69 had no effect (Fig. 2Bb).

At 37°C, antibody EA-1 strongly stimulated the aggregation of slowly aggregating N2A cells but inhibited the aggregation of fast aggregating cells by up to 50% (Fig. 2Ca). Its stimulatory influence on the aggregation of slowly aggregating cells was strongest in the absence of Mg^{++} (Fig. 2Cb) and was not observed at 4°C (Fig. 2Cb).

Inhibition of N2A Cell Aggregation by Antibody Combinations

Using cells that had been cultured for 48 hours, the effects of antibody combinations on N2A cell aggregation were also examined. When antibodies to α6 integrin and CD24 were mixed and added together to the cells, their combined effect on aggregation, as evaluated from the increase in the percentage of cells entering aggregates larger than $30\mu m^3$, was similar to the arithmetic sum of their separate effects. However, as evaluated from the increase in the mean particle volume, their combined stimulatory effect at 37°C was 70% stronger than the calculated sum of their separate effects (Fig. 3A), suggesting an increase in the efficacy of adhesion mechanisms. When EA-1 antibody was used in mixture with L1 antibody 557, the cells aggregated at a similar, increased rate as in the presence of antibody EA-1 alone (Fig. 3B).

In the presence of mixed CD24 antibody 79 and L1 antibody 557, aggregation was stronger after 25 min at 37°C than would be expected from the separate effects of these antibodies (Fig. 3C). A similar effect was observed in the presence of mixed CD24 antibody M1/69 and antibody 557 after 60 min but not yet after 25 min of aggregation at 37°C (Fig. 3C). Although much weaker, an increase in aggregation was also noted in the presence of mixed CD24 antibody 79-15 and antibody 557 (Fig. 3C). Interestingly, cell aggregation was stimulated by mixed antibodies 79 and 557 also when the aggregation of cells from the same culture was slightly inhibited by antibody CD24 and strongly inhibited by antibody 557 (not shown). At 4°C (Fig. 3C) as well as in the absence of Mg^{++} at 37°C (not shown), the effect of mixed CD24 and L1 antibodies on aggregation was similar to the arithmetic sum of their separate effects.

DISCUSSION

In the present work we examined the function of L1, CD24, and α6 integrin in mediating N2A cell adhesion with particular reference to the possible sequential roles of these adhesion molecules during cell growth in culture and during the course of cell aggregation in suspension.

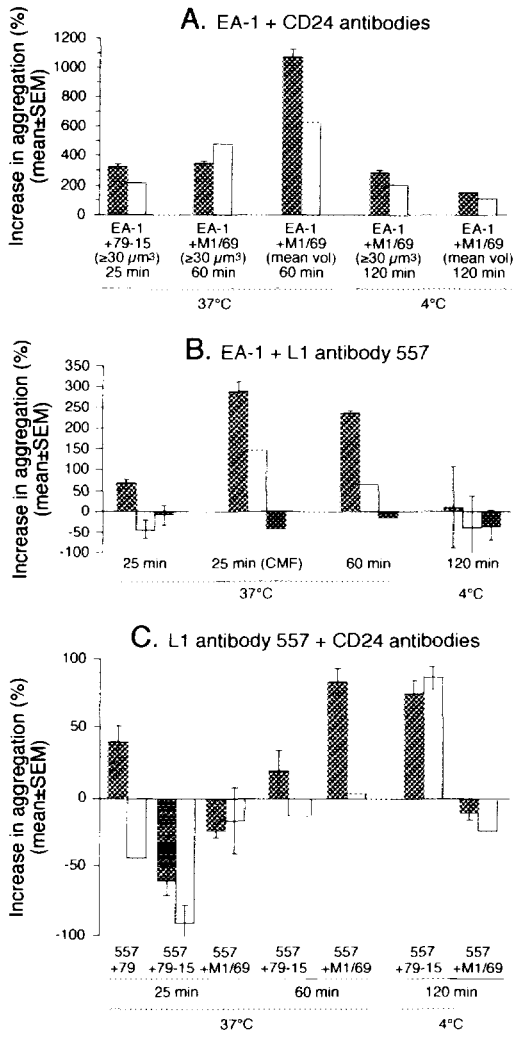

Figure 3. *Coulter Counter analysis of N2A cell aggregation in the presence of mixed antibodies to L1, CD24, and α6 integrin.* N2A cells were plated in culture bottles at a density of 560 cells/mm^2 and were allowed to grow for 48 hrs. The cells were then resuspended, dispersed and allowed to aggregate in suspension at 37°C or 4°C for 25, 60, or 120 min., as indicated under the bars, in the absence or presence of mixed antibodies EA-1 and 79-15 or M1/69 (A), EA-1 and 557 (B), or 557 and 79, 79-15, or M1/69 (C). Aggregation was evaluated from the increase in the partial volume occupied by aggregates larger than 30 μm^3 (B, C, and $\geq 30 \mu m^3$ in A) or from the increase in the mean particle volume (mean vol in A). Increase in aggregation in the presence of antibodies was evaluated relative to the aggregation of cells from the same culture in the absence of antibodies. Striped bars denote the real measured increase in aggregation. Open bars represent the calculated sum of the separate effects of the corresponding antibodies when used on their own. Closed bars in B represent the increase in aggregation in the presence of the mixture of both antibodies as evaluated with respect to the aggregation of cells from the same culture in the presence of antibody EA-1 alone. CMF – absence of divalent cations.

The aggregation of freshly cultured, fast growing, isolated N2A cells was completely inhibited by antibodies to the homophilic adhesion molecule L1. L1 therefore appears to be the major adhesion molecule mediating their aggregation at this stage. In view of the weak aggre-

gation of these cells it is likely that L1 mediates their adhesion with relatively weak binding forces. Studies on the aggregation of L1-expressing lymphatic cells (12, 15) and L1-coated beads (3) have also indicated low avidity for the homophilic binding of L1. However, it has been suggested that in N2A cells the avidity of L1 may be increased through the formation of molecular complexes including L1 and N-CAM (3, 13, 16). At present we do not know whether such complexes exist already in fast growing isolated cells or form only as a result of adhesion-dependent cross linking.

When the cells are kept longer in culture they become dense, increasingly establish mutual contacts, partially differentiate, and acquire the capacity for strong aggregation. The increase in the efficacy of their aggregation at this stage appears to depend on the extent of mutual contacts the cells have experienced prior to the aggregation experiment. In agreement with this assumption we have observed that dense but subconfluent cells regain the properties of fast growing isolated cells when they are passaged and replated at a low density (unpublished observations). Coincidentally with the increase in the ability of the cells to aggregate, the ability of L1 antibodies to inhibit their aggregation decreases. This implies that additional adhesion mechanisms may be activated at this stage.

Taken together, these results suggest that in N2A cells L1 may serve as an early recognition molecule mediating the initial formation of cell-cell contacts following which additional, possibly stronger adhesion molecules become active. This premise is also supported by the observation that the ability of L1 antibodies to inhibit the aggregation of fast growing isolated N2A cells gradually diminishes when the cells are allowed to aggregate for more than 30 min. and is strongly reduced after two hours of aggregation. Since L1 does not only mediate neuronal adhesion but also granule cell migration (17) and neurite extension on other neurites (7) and Schwann cells (18) it is tempting to speculate that L1 may mediate a process of cell body "rolling" on Bergmann glia and growth cone "rolling" on neurites and Schwann cells comparable to the selectin-mediated "rolling" of neutrophils on endothelial cells (10, 11).

Antibodies to CD24 stimulated the aggregation of slowly aggregating N2A cells at 37°C but did not influence their aggregation at 4°C. CD24 also mediates signal transduction when triggered by antibodies (12). Therefore, it is possible that CD24 triggers the activation of resting adhesion mechanisms during the early phases of adhesion. This effect was strongest in the presence of L1 antibodies since cells that did not aggregate in the presence of L1 antibodies aggregated well in the presence of mixed L1 and CD24 antibodies. The stimulation of aggregation by mixed L1 and CD24 antibodies was temperature-sensitive and Mg^{++}-dependent and probably did not result from cross linking or reduction in the binding of L1 antibodies (4). Furthermore, the concomitant triggering of L1 and CD24 stimulates in neurones and N2A cells a very strong intracellular Ca^{++} signal (4). Both this signal and the increase in aggregation in the presence of mixed L1 and CD24 antibodies are similarly sensitive to PMA (4). It is therefore possible that L1 and CD24 cooperate during primary cell recognition in activating via a common signal transduction pathway Mg^{++}-dependent secondary adhesion mechanisms, possibly integrins, that mediate aggregation even in the presence of L1 antibodies.

The α6 integrin(s) recognised by antibody EA-1 did not mediate adhesion in slowly aggregating N2A cells but their triggering resulted in augmented cell aggregation. Conversely, in

fast aggregating cells α6 integrins appeared to mediate aggregation and thus function as active adhesion receptors. At present we do not know whether these α6 integrins undergo self-activation, but they are probably not activated by L1 and CD24 (unpublished observations). Thus, α6 integrins may function in N2A cells similarly to β1 integrins in haematopoietic cells which are activated upon signalling during the three step adhesion cascade (11).

In conclusion, the mechanisms mediating N2A cell adhesion appear to include at least two pathways of adhesive hierarchy. Knowledge of the molecules involved and the underlying regulatory pathways will be crucial to our understanding of the mechanisms by which adhesion molecules mediate the formation of selective and stable neuronal contacts.

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