

# Adhesion Molecules during Somitogenesis in the Avian Embryo

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**Abstract.** In avian embryos, somites constitute the morphological unit of the metameric pattern. Somites are epithelia formed from a mesenchyme, the segmental plate, and are subsequently reorganized into dermatome, myotome, and sclerotome. In this study, we used somitogenesis as a basis to examine tissue remodeling during early vertebrate morphogenesis. Particular emphasis was put on the distribution and possible complementary roles of adhesion-promoting molecules, neural cell adhesion molecule (N-CAM), N-cadherin, fibronectin, and laminin.

Both segmental plate and somitic cells exhibited in vitro calcium-dependent and calcium-independent systems of cell aggregation that could be inhibited respectively by anti-N-cadherin and anti-N-CAM antibodies. In vivo, the spatio-temporal expression of N-cadherin was closely associated with both the formation and local disruption of the somites. In contrast, changes in the prevalence of N-CAM did not strictly accompany the remodeling of the somitic epithelium into dermamyotome and sclerotome. It was also observed that fibronectin and laminin were reorganized secondarily in the extracellular spaces after CAM-mediated con-

tacts were modulated. In an in vitro culture system of somites, N-cadherin was lost on individual cells released from somite explants and was reexpressed when these cells reached confluence and established intercellular contacts. In an assay of tissue dissociation in vitro, antibodies to N-cadherin or medium devoid of calcium strongly and reversibly dissociated explants of segmental plates and somites. Antibodies to N-CAM exhibited a smaller disrupting effect only on segmental plate explants. In contrast, antibodies to fibronectin and laminin did not perturb the cohesion of cells within the explants.

These results emphasize the possible role of cell surface modulation of CAMs during the formation and remodeling of some transient embryonic epithelia. It is suggested that N-cadherin plays a major role in the control of tissue remodeling, a process in which N-CAM is also involved but to a lesser extent. The substratum adhesion molecules, fibronectin and laminin, do not appear to play a primary role in the regulation of these processes but may participate in cell positioning and in the stabilization of the epithelial structures.

**E**ARLY embryonic morphogenesis proceeds with the formation of a succession of transient tissues that are remodeled into the definitive organs that are established in shape and function. In this remodeling, epithelium-mesenchyme interconversion is one of the most commonly found processes. In many systems, this conversion involves rapid modulations of both cell-cell and cell-substratum adhesion (Wessels, 1977; Hay, 1981; Yamada, 1983; Bernfield et al., 1984; Edelman, 1985; Edelman and Thiery, 1985). Recent progress in defining molecules mediating these adhesion events provides the opportunity to analyze these processes in causal terms.

The formation and subsequent reorganization of the somites in the avian embryo is a striking example of rapid tissue remodeling and constitutes a convenient model system to study some of the roles of adhesion molecules during early morphogenesis. Somites are metameric units found along the

body axis and, in part, they constitute the primordia of the skull and muscles in the trunk (reviewed in Lash, 1985; Belairs et al., 1986). They form from an apparently homogeneous rod of mesenchyme, the segmental plate, located in the caudal region of the embryo. The process of formation of somites is continuous until the total number of segments specific for each species is reached. The sequential formation of somites involves aggregation of cells, epithelialization, and separation from the rest of the segmental plate (Lash, 1985). Within a few hours after their appearance, somites undergo further transformations that lead to the production of a double-layered epithelium, the dermamyotome, and a mesenchyme, the sclerotome; the former differentiates into the dermis and skeletal muscles, whereas the latter gives rise to the cartilage of the vertebrae.

The processes leading to the formation and reorganization of the somites are not yet understood. It has been proposed

that an increase in cell-cell adhesion among segmental plate cells could play a role in somite formation (Bellairs et al., 1978; Cheney and Lash, 1984) and a related idea is that fibronectin may play such a role (Lash et al., 1984). Particularly because of their role in promoting diverse forms of cell adhesion, cell-cell and cell-substratum adhesion molecules might be mutually involved in some aspects of somitogenesis.

Cell-adhesion molecules (CAMs)<sup>1</sup> mediate intercellular adhesion in many tissues, and their distribution and expression are correlated with inductive events, tissue shaping, and tissue remodeling (Edelman, 1985; Edelman and Thiery, 1985; Öbrink, 1986). The major CAMs presently known to play a role during the early events of morphogenesis include N-CAM (neural-CAM) initially isolated from neural tissues (Thiery et al., 1977), L-CAM (liver-CAM; Gallin et al., 1983) present on many epithelial cells and apparently the same as uvomorulin (Hyafil et al., 1980), E-cadherin (Yoshida-Noro et al., 1984), Arc-1 (Imhof et al., 1983), or cell-CAM 120/80 (Damsky et al., 1983), and lastly N-cadherin first described on neural tissues (Hatta et al., 1985; Hatta and Takeichi, 1986) and sharing similarities with the adherens junction-specific A-CAM (adherens junction-CAM; Volk and Geiger, 1984; Geiger et al., 1985; Volk and Geiger, 1986a, b).

Cell-substratum adhesion is mediated by complexes of extracellular molecules, substratum adhesion molecules (SAMs), associated with specific receptors at the cell surface and is also thought to play a key role in a wide variety of morphogenetic events including cell migration, cell aggregation, and tissue remodeling (Ekblom, 1981; Thiery et al., 1985; Duband et al., 1987). Fibronectin (FN) and its 140-kD receptor complex (Hynes and Yamada, 1982; Yamada, 1983; Yamada et al., 1985; Hynes, 1985; Pytela et al., 1985; Horwitz et al., 1985; Chen et al., 1985; Leptin, 1986; Tamkun et al., 1986), and laminin (LN) and its corresponding 67-kD receptor (Timpl et al., 1983; Brown et al., 1983; Liotta et al., 1985; Yamada et al., 1985; Yamada et al., 1987) constitute the two major systems that mediate direct cell-substratum adhesion of both mesenchymal and epithelial cells.

It is important to determine the conjugate and complementary roles of CAMs and SAMs in morphological events during early embryogenesis. In this study, we have examined the distribution of these adhesion molecules during somitogenesis. We then focused on the possible role of these molecules in the maintenance and reorganization of somitic cell clusters using *in vitro* perturbation experiments. Our results demonstrate that N-cadherin and N-CAM are involved in cell adhesion during somitogenesis and suggest that the regulation of N-cadherin expression might play a key role particularly in the formation and disruption of the somitic epithelium.

## Materials and Methods

### Embryos

White Leghorn chick and Japanese quail embryos were used throughout the study. Eggs were incubated at  $38 \pm 1^\circ\text{C}$  in a humidified air chamber, and

1. *Abbreviations used in this paper:* A-CAM, adherens junction CAM; CAM, cell adhesion molecule; FN, fibronectin; HCMF, Hepes-calcium and magnesium-free PBS; L-CAM, liver CAM; LN, laminin; N-CAM, neural CAM; SAM, substratum adhesion molecule.

the ages of the embryos were determined according to the number of somite pairs as well as according to Hamburger and Hamilton stages (1951).

### Antibodies

Rat monoclonal antibodies to N-cadherin (NCD-2) have been described elsewhere (Hatta and Takeichi, 1986). These antibodies were selected on the basis of their inhibitory effect on the calcium-dependent aggregation of neural retina cells (Hatta and Takeichi, 1986). A mouse monoclonal antibody to N-CAM called anti-N-CAM No. 1, which could immunoprecipitate N-CAM molecules and inhibit aggregation of retinal cells, was described elsewhere (Hoffman et al., 1982). It should be stressed that both monoclonal antibodies to N-cadherin and N-CAM did not show any agglutination activity when used as divalent antibodies. Monovalent and divalent rabbit antibodies to L-CAM (Gallin et al., 1983) and to N-CAM (Thiery et al., 1977) were produced as described previously (Brackenbury et al., 1977). Antibodies to laminin were raised in rabbits using essentially the same procedure as Timpl et al. (1979), and goat anti-fibronectin antibodies were kindly provided by Dr. Kenneth M. Yamada (National Cancer Institute, Bethesda, MD). A mouse monoclonal antibody to the adherens junction-specific molecule A-CAM was a generous gift of Dr. Benjamin Geiger (The Weizmann Institute, Rehovot, Israel).

### Embryonic Cell Cultures

Cultures of somites and segmental plates were generated as follows. The trunk regions of embryos incubated for 60 h (i.e., at stage 15 of Hamburger and Hamilton) were excised with a scalpel. Fragments corresponding to the rostral part of the unsegmented region and to the region containing the last four formed somites were incubated for 30–60 min at room temperature with 750 U/ml dispase (Godo Shusei, Tokyo, Japan) in DME. Segmental plates and somites were teased apart with tungsten needles until free of contaminating tissues. After dissociation, tissues were allowed to recover from enzyme treatment by an incubation in DME for 30 min. Tissues were then processed for cell adhesion assays, dissociation experiments, or for immunofluorescence labeling (see below).

### Cell Adhesion Assays

Segmental plates and somites were dissociated by two different treatments to prepare cells that retain either calcium-dependent or calcium-independent adhesion systems (Urushihara et al., 1979; Brackenbury et al., 1981; Aoyama et al., 1985). After treatment with a dispase solution and 30-min recovery in DME (see above), segmental plates and somites were extensively washed in 10 mM Hepes-calcium and magnesium-free PBS, pH 7.4 (HCMF). Tissues were then incubated for 20 min at  $37^\circ\text{C}$  in the presence of either 0.001% trypsin (type XI; Sigma Chemical Co., St. Louis, MO) and 1 mM EDTA in HCMF (light trypsin-EDTA treatment) or 0.01% trypsin and 1 mM  $\text{CaCl}_2$  in HCMF (trypsin-calcium treatment); in the case of the somites, trypsin-calcium treatment was performed with 0.02% trypsin instead of 0.01% trypsin. Explants were collected in a microfuge tube and gently dissociated to single cells with pipetting. After centrifugation at 1,000 rpm for 10 min to remove the bulk of the trypsin, excess soybean trypsin inhibitor (type II-S; Sigma Chemical Co.) was added to the cell suspension, and the cells were washed twice with HCMF. Aggregation of dissociated cells was carried out in 96-well tissue culture seroclusters with U-bottom wells (Costar, Cambridge, MA) previously coated with 0.5% (wt/vol) BSA in HCMF for 5 h at  $37^\circ\text{C}$ . In each well,  $\sim 2-3 \times 10^3$  cells were incubated in 50  $\mu\text{l}$  of HCMF with or without 1 mM  $\text{CaCl}_2$  and with or without 10  $\mu\text{l}$  solution of Fab' fragments of N-CAM antibodies and/or monoclonal antibodies to N-cadherin at final concentrations of 1 mg/ml and 50  $\mu\text{g}/\text{ml}$ , respectively. After a 1-h incubation at  $37^\circ\text{C}$  in a gyratory shaker at 150 rpm, 50  $\mu\text{l}$  of 2% glutaraldehyde solution was added to each well. The wells were observed with a Leitz inverted microscope and total number of particles was counted. The degree of aggregation of cells was measured by the decrease in particle number (% aggregation =  $[1 - \text{number of particles after the aggregation assay}/\text{initial particle number}] \times 100$ ). The inhibition of cell aggregation by antibodies to N-CAM or to N-cadherin was expressed as % inhibition ( $1 - [\% \text{ aggregation with antibodies}/\% \text{ aggregation without antibodies}] \times 100$ ).

### Histological Sections

The distribution of adhesion molecules was studied by immunofluorescent labeling of cryostat sections. After fixation in 3.7% formaldehyde in PBS

for 1–4 h, and extensive washes in PBS, embryos were embedded in a graduated series of sucrose solutions in PBS (12–18% wt/vol) and frozen in Tissue Tek (Miles Laboratories Inc., Naperville, IL) in liquid nitrogen. Sections were cut at 7–12  $\mu\text{m}$  on a cryostat (Bright Instrument Co. Ltd., Huntington, England) and mounted on slides coated with gelatin.

### Immunofluorescent Staining

Immunofluorescent staining of sections was performed essentially as described previously (Duband et al., 1986). Simultaneous staining for N-cadherin, N-CAM, fibronectin, and laminin was performed on successive sections. For staining of cultures, explants of somites were deposited on coverslips previously coated with 20  $\mu\text{g}/\text{ml}$  human plasma FN, and cultured for 24 h or 4 d at 37°C in a humidified 7%  $\text{CO}_2/93\%$  air chamber in the presence of DME supplemented with 10% serum. Cultures were fixed for 1 h in 3.7% formaldehyde in PBS and processed for immunofluorescence as described elsewhere (Duband et al., 1986). Sections and cultures were examined and photographed on a Leitz Orthoplan epifluorescent microscope.

### Dissociation Experiments

For assays of explant dissociation in the presence of molecules interfering with adhesion molecules, tissues from embryos at stage 15 of Hamburger and Hamilton were explanted into 1-cm-diam wells consisting of a section of polyethylene tubing mounted on petri dishes or into Terasaki wells (Falcon Labware, Oxnard, CA). They were then cultured at 37°C in a humidified 7%  $\text{CO}_2/93\%$  air chamber in the presence of antibodies to adhesion molecules at various concentrations in DME supplemented with 10% newborn calf serum, in calcium-free DME (Flow Laboratories, Inc., McLean, VA), or in a 1 mM EDTA solution in DME. Cultures of explants were observed and photographed under a Leitz Diavert inverted microscope. In each case, the effect of the medium were tested on a total of 20 explants in five different experiments.

## Results

### Specificity of Cell Adhesion in Cultured Segmental Plate and Somitic Cells

Segmental plate and somitic cells were first tested for calcium-independent and calcium-dependent cell adhesion systems using a microaggregation assay adapted from previously described procedures (see Materials and Methods).

As shown on Table I, segmental plate and somitic cells possessed both types of cell–cell adhesion systems. (a) In the calcium-dependent system, cells treated with trypsin-calcium aggregated very poorly in calcium-depleted medium but aggregated strongly when calcium was present in the medium; this aggregation could be specifically and strongly inhibited by antibodies to N-cadherin but not by antibodies to N-CAM. These results suggest that the calcium-dependent mechanism of aggregation of segmental plate and somitic cells was mediated by N-cadherin. (b) In relation to the calcium-independent system, cells treated with light trypsin-EDTA could aggregate both in the presence and in the absence of calcium; this aggregation could be partly inhibited by antibodies to N-CAM and only weakly by antibodies to N-cadherin. These results suggest that the calcium-independent mechanism of aggregation of segmental plate and somitic cells could be mediated by N-CAM. No significant differences in rate of aggregation could be detected between segmental plate and somitic cells under both conditions of cell dissociation. In contrast, the sizes of the aggregates varied according to the dissociation treatment. Trypsin-calcium treatment of segmental plate cells usually gave much larger aggregates than light trypsin-EDTA treatments (not shown).

### *In Situ* Distribution of Adhesion Molecules during Formation and Reorganization of Somites

Somitogenesis could be studied either at different levels in the same embryo or at a specific level in different embryos at consecutive stages. We have carried out a detailed study of the distribution of N-cadherin, N-CAM, FN, and LN during somite formation and reorganization using immunofluorescence labeling of sagittal and transverse cryostat sections of embryos at the 25-somite stage (i.e., stage 15 of Hamburger and Hamilton) and of different embryos between 20 and 35-somite stages (i.e., stage 13–18 of Hamburger and Hamilton). Very similar results were obtained in both cases. As previously shown (Thiery et al., 1984), L-CAM was never detected on cells that composed segmental plates, somites, sclerotomes, and dermamyotomes.

**Formation of Somites.** The process of somite formation could be subdivided into four stages according to tissue organization and distribution of adhesion molecules. In the first step, segmental plate cells were organized as a loose mesenchyme with no apparent orientation of cells. These cells were weakly stained for both N-cadherin and N-CAM (Fig. 1, *a* and *b*, stage 1). LN and FN could be detected only minimally (Fig. 1, *c* and *d*, stage 1) and, when present, FN staining was sparse and punctate. The only areas where LN and FN were found were the basal surface of the ectoderm and endoderm (Fig. 1, *c* and *d*). These patterns of distribution of adhesion molecules in the segmental plate differed strikingly from that in the lateral mesoderm. The latter was organized into two epithelial sheets exhibiting high amounts of N-CAM and N-cadherin. These sheets were limited by FN- and LN-rich basement membranes (not shown).

In the second stage, the segmental plate underwent compaction: intercellular spaces were diminished among cells located in the periphery of the plate, which soon became organized as an epithelial-like structure. In contrast, cells located in the internal part of the segmental plate retained a mesenchymal structure. Concomitantly, staining for N-cadherin and N-CAM was significantly increased, particularly among aggregating cells (Fig. 1, *a* and *b*, stage 2). FN was also increased in quantity along the segmental plate but was undetectable in its central portions (Fig. 1 *d*, stage 2), while LN staining was still weak (Fig. 1 *c*, stage 2).

In the third stage, epithelialization was almost complete particularly in the dorsal and ventral aspects of the future somite, and the process of delineation began. N-CAM remained on the whole cell surface of epithelial cells, whereas N-cadherin became predominantly concentrated on their apical surfaces (Fig. 1, *e* and *f*, stage 3). FN was organized as a nearly continuous sheet in the dorsal and ventral side of the nascent somite but could not be detected in the area of separation from the rest of the segmental plate (Fig. 1 *h*, stage 3). LN could now be clearly evidenced as a punctate pattern on the basal surface of epithelial cells (Fig. 1 *g*, stage 3).

In the last stage, the nascent somitic precursor separated from the rest of the segmental plate, resulting in the formation of an additional somite. This consisted of a ball-shaped structure composed of mesenchymal cells (core cells) entirely surrounded by a continuous epithelium. Epithelial cells exhibited staining for N-CAM on their whole membrane and a bright staining for N-cadherin, which was

Table 1. Specificity of Aggregation of Segmental Plate and Somite Cells

Condition of dissociation	Condition of aggregation	Segmental plate cells		Somite cells	
		% Aggregation	% Inhibition	% Aggregation	% Inhibition
Trypsin Ca <sup>++</sup>	-Ca <sup>++</sup> - Ab	7 ± 5	-	21 ± 2	-
	-Ca <sup>++</sup> + anti-N-cadherin	13 ± 8	-	6 ± 5	70 ± 30
	-Ca <sup>++</sup> + anti-N-CAM	20 ± 3	-	16 ± 4	24 ± 12
	-Ca <sup>++</sup> + anti-(N-CAM + N-cadh.)	26 ± 6	-	4 ± 3	80 ± 20
Trypsin Ca <sup>++</sup>	+Ca <sup>++</sup> - Ab	92 ± 2	-	73 ± 7	-
	+Ca <sup>++</sup> + anti-N-cadherin	54 ± 3	44 ± 5	2 ± 2	98 ± 2
	+Ca <sup>++</sup> + anti-N-CAM	84 ± 2	9 ± 2	50 ± 1	32 ± 5
	+Ca <sup>++</sup> + anti-(N-CAM + N-cadh.)	33 ± 6	63 ± 6	2 ± 2	98 ± 2
Light trypsin EDTA	-Ca <sup>++</sup> - Ab	72 ± 1	-	76 ± 2	-
	-Ca <sup>++</sup> + anti-N-cadherin	73 ± 2	2 ± 1	73 ± 4	7 ± 3
	-Ca <sup>++</sup> + anti-N-CAM	46 ± 5	37 ± 7	59 ± 5	23 ± 7
	-Ca <sup>++</sup> + anti-(N-CAM + N-cadh.)	29 ± 2	59 ± 3	51 ± 5	34 ± 7
Light trypsin EDTA	+Ca <sup>++</sup> - Ab	83 ± 1	-	61 ± 4	-
	+Ca <sup>++</sup> + anti-N-cadherin	65 ± 3	22 ± 4	61 ± 3	-
	+Ca <sup>++</sup> + anti-N-CAM	47 ± 2	43 ± 2	47 ± 2	22 ± 10
	+Ca <sup>++</sup> + anti-(N-CAM + N-cadh.)	29 ± 3	65 ± 3	43 ± 5	31 ± 8

Cells were dissociated in the presence of either 0.001% trypsin and 1 mM EDTA (light-trypsin EDTA) or 0.01% trypsin and 1 mM CaCl<sub>2</sub> (trypsin Ca<sup>++</sup>) to prepare cells that retain either calcium-independent or calcium-dependent adhesion systems. Cells were then allowed to reaggregate in wells containing PBS with calcium (+Ca<sup>++</sup>) or without calcium (-Ca<sup>++</sup>) and in the presence of no antibodies (-Ab), of antibodies to N-cadherin (+anti-N-cadherin), antibodies to N-CAM (+anti-N-CAM), or lastly of both antibodies (+anti-[N-CAM + N-cadh.]). The total number of particles was counted in each well. The degree of aggregation of cells was expressed as the percentage of aggregation, i.e., (1 - number of particles after the aggregation assay/initial particle number) × 100. The inhibition of cell aggregation by antibodies was expressed as the percentage of inhibition, i.e., (1 - % aggregation with antibodies/% aggregation without antibodies) × 100. Values represent the mean of 12 wells in four different experiments.

predominantly found on the apical surface. Cells located in the core of the somite were uniformly stained for both N-cadherin and N-CAM; the staining for N-cadherin was weaker on these cells than on epithelial cells (Fig. 1, *e* and *f*, stage 4). FN and LN were now mostly distributed in a continuous basement membrane entirely surrounding the somitic epithelium; they could also be detected as a punctate staining in the core (Fig. 1, *g* and *h*, stage 4). In mature somites (Fig. 1, *e-h*, stage 4), staining for adhesion molecules remained essentially similar.

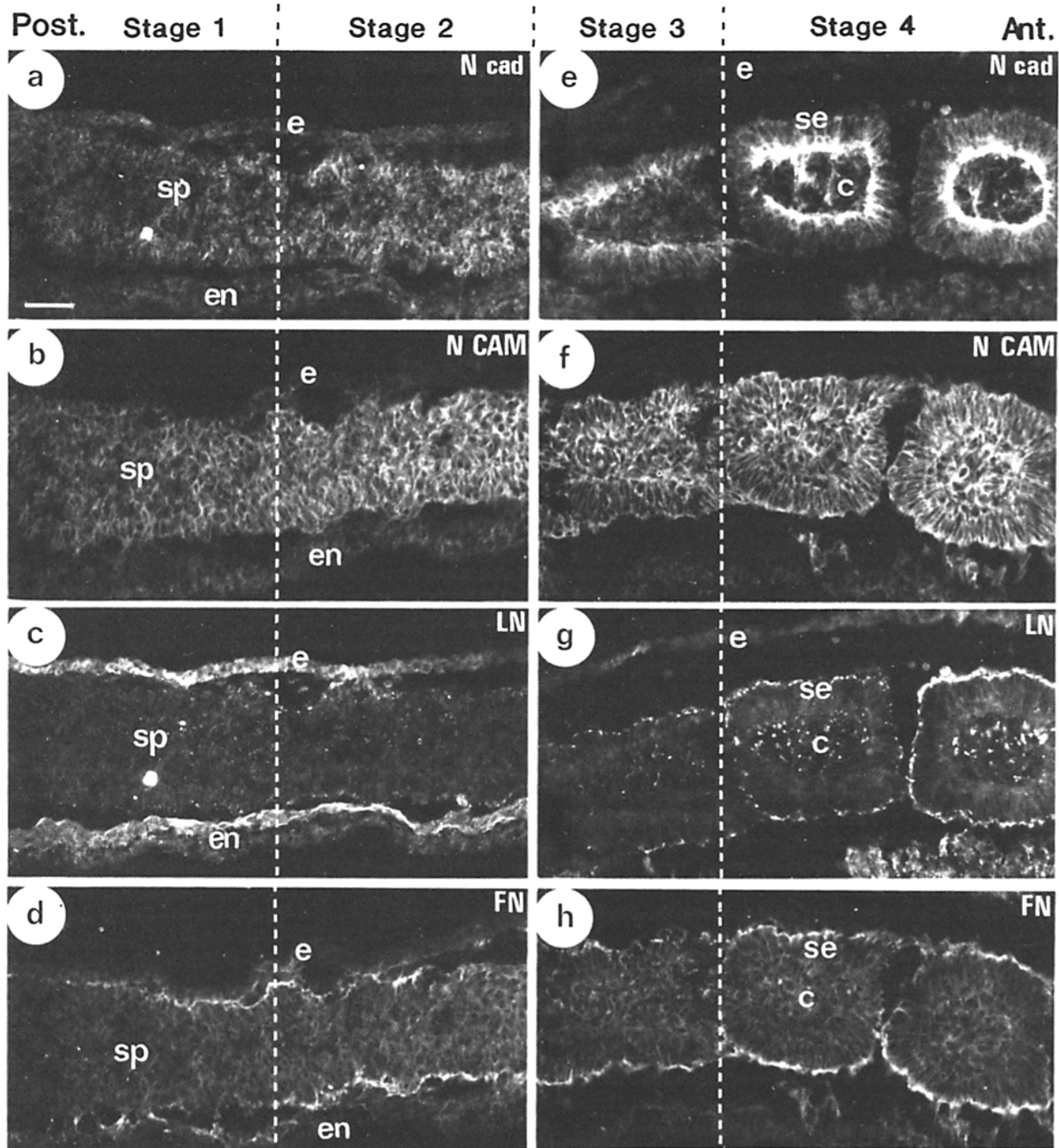
**Reorganization of Somites.** In embryos at stage 13-18 of Hamburger and Hamilton, the disruption of the somitic epithelium could be visualized morphologically at the level of the fifth somite rostral to the last formed somite. However, at the level of the third somite rostral to the last formed somite, i.e., well before any morphological sign of reorganization could be detected, the staining for N-cadherin decreased sharply in the medio-ventral region of the somitic epithelium and among cells in the core (i.e., the presumptive sclerotome), while it remained intense in the dorso-lateral region destined to form the dermamyotome (Fig. 2, *a* and *e*). At the same level, N-CAM, FN, and LN distributions were not significantly altered (Fig. 2, *b-d*). During the time course of somite reorganization, N-CAM remained present in noticeable amounts on both sclerotome and dermamyotome (Fig. 2 *g*); we detected a shift of N-CAM among sclerotomal cells only well after the complete reorganization of the somite (data not shown, but see Thiery et al., 1982). The distribution pattern of FN and LN changed gradually in the medio-ventral region of the somite only after cell dissociation could be detected. Staining for FN and LN first became interrupted in many areas where cells were no longer assem-

bled into an epithelium. Later on, when the somite reorganization into sclerotome and dermamyotome was completed, LN disappeared progressively from the environment of the released sclerotomal cells, while FN was found around the dissociated cells (Fig. 2, *g* and *h*).

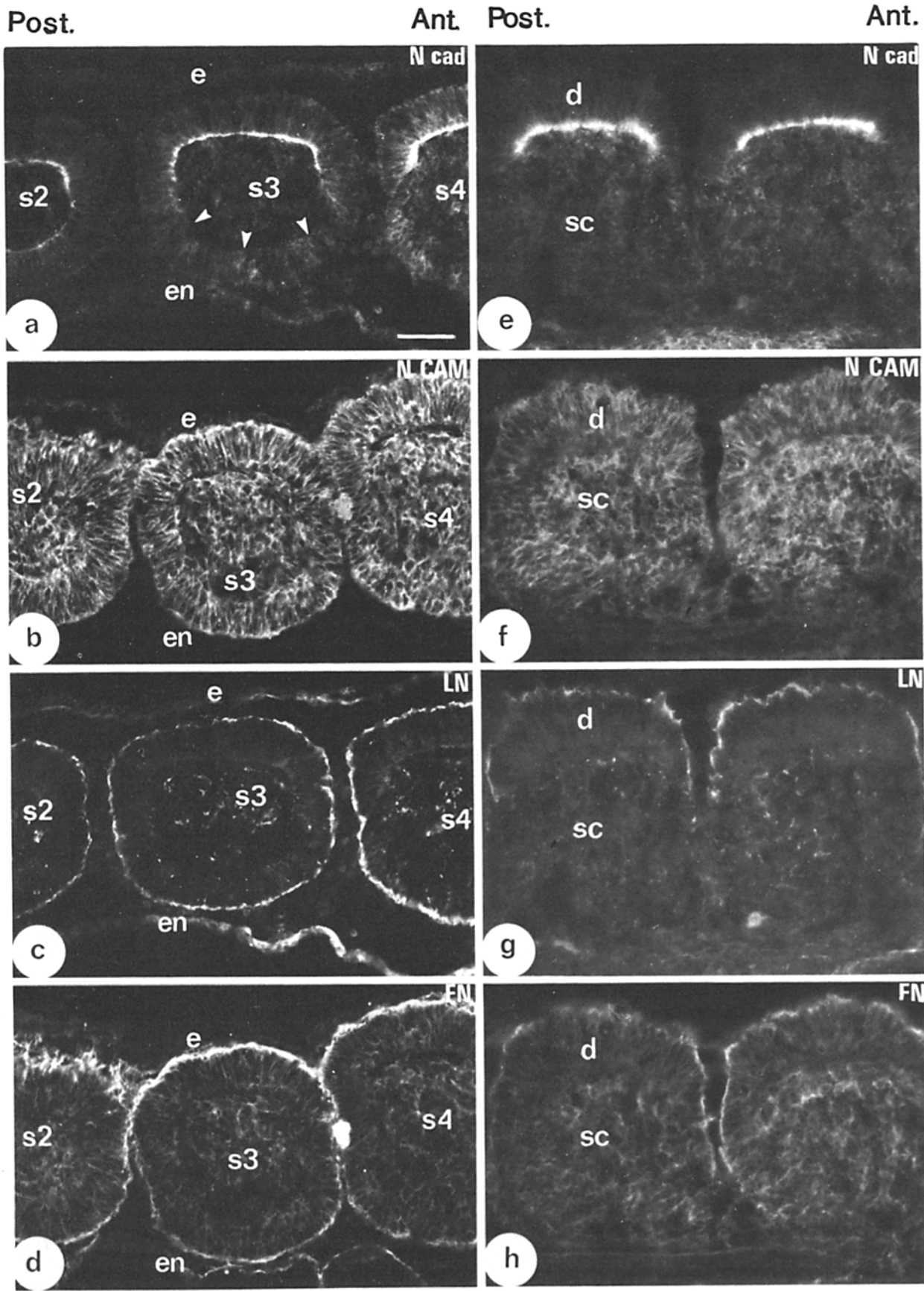
#### Distribution of Adhesion Molecules on Cultured Somitic Cells

Explants of somites cultured in vitro on FN substrata spontaneously dissociated and the released cells spread onto the FN and started migrating. Within 3-4 d of culture, these cells became stationary and established contacts with their neighbors (see also Duband et al., 1986). It was thus of interest to determine whether adhesion molecules show noticeable prevalence regulation during somitic tissue remodeling in vitro in a similar way as previously observed in vivo.

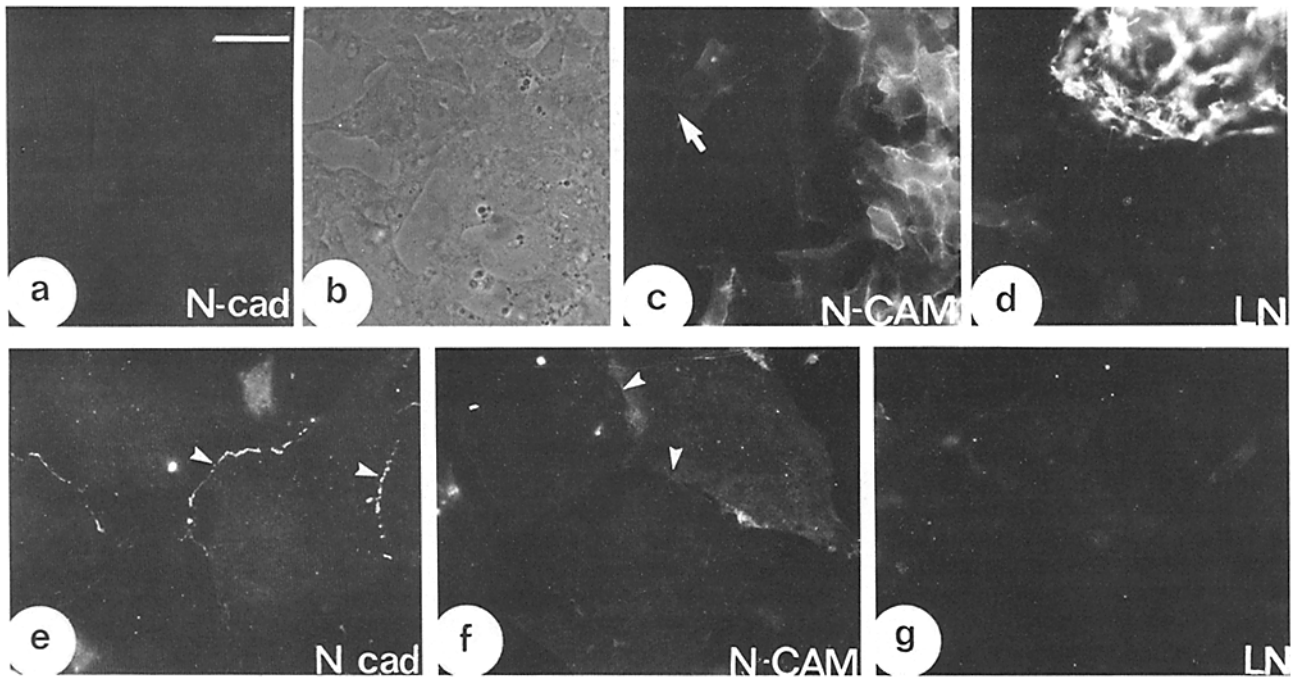
Somitic cells that were separated from their explants and flattened on the substratum were negative for N-cadherin (Fig. 3, *a* and *b*). N-CAM staining gradually decreased from cells located near the explant and became negative on cells in the periphery (Fig. 3 *c*). Closely neighboring cells in the explant showed a strong staining for both cell adhesion molecules (not shown) and for LN (Fig. 3 *d*). When somitic cells became stationary and established contacts after 4 d in culture, N-cadherin but not N-CAM became apparent in the regions of intercellular junctions (Fig. 3, *e* and *f*). LN was undetectable on both migratory and stationary cells (Fig. 3, *d* and *g*). As previously shown (Duband et al., 1986), migratory somitic cells leaving the explant did not express FN in contrast to stationary cells, which deposited a dense FN meshwork.



**Figure 1.** In situ immunofluorescent distribution of adhesion molecules during the formation of the somite in an embryo at stage 15 of Hamburger and Hamilton. (a–d) The segmental plate and (e–f) nascent somites and last somites of the same embryo. a, c and e, g and b, d and f, h show two consecutive sagittal sections double stained for N-cadherin and LN on one side and N-CAM and FN on the other side. Four different stages can be distinguished regarding the distribution of the adhesion molecules and the organization of the tissues; these stages are separated by dotted lines represented on the pictures. While stage 1 is marked by the low level of all adhesion molecules, stage 2 is characterized by the increase of N-cadherin, N-CAM, and FN but not of LN, concomitantly with an increase in cell–cell adhesion. In stage 3, the nascent somite starts separating from the rest of the segmental plate; N-cadherin becomes polarized in the apical surface of the epithelial cells, and FN and LN organize into a basal lamina. The formation of the somite is achieved in stage 4; a continuous basal lamina entirely surrounds the somitic epithelium. *Ant.* and *post.*, cranial and caudal sides of the sections. *c*, core (of the somite); *e*, ectoderm; *en*, endoderm; *se*, somitic epithelium; *sp*, segmental plate. Bar, 50  $\mu$ m.







**Figure 3.** Immunofluorescent detection of adhesion molecules on cultured somitic fibroblasts. Explants of somites were deposited on FN-coated coverslips, grown for 1 d (*a-d*) or 4 d (*e-g*), and stained for N-cadherin (*a, b, and e*), N-CAM (*c and f*), and LN (*d and g*). (*b*) Phase-contrast image of *a*. Cells that emigrated from freshly explanted somites are devoid of N-cadherin (*a and b*) but still express N-CAM (*c*); the staining for N-CAM progressively decreases as cells are distant from the explant (*arrows in c*). LN can only be detected on the explant (*d*). When cells become stationary and establish contacts with their neighbors, N-cadherin (*e*), but neither N-CAM (*f*) nor LN (*g*) appears on the areas of cell contacts (*arrowheads in e and f*). Bar, 10  $\mu$ m.

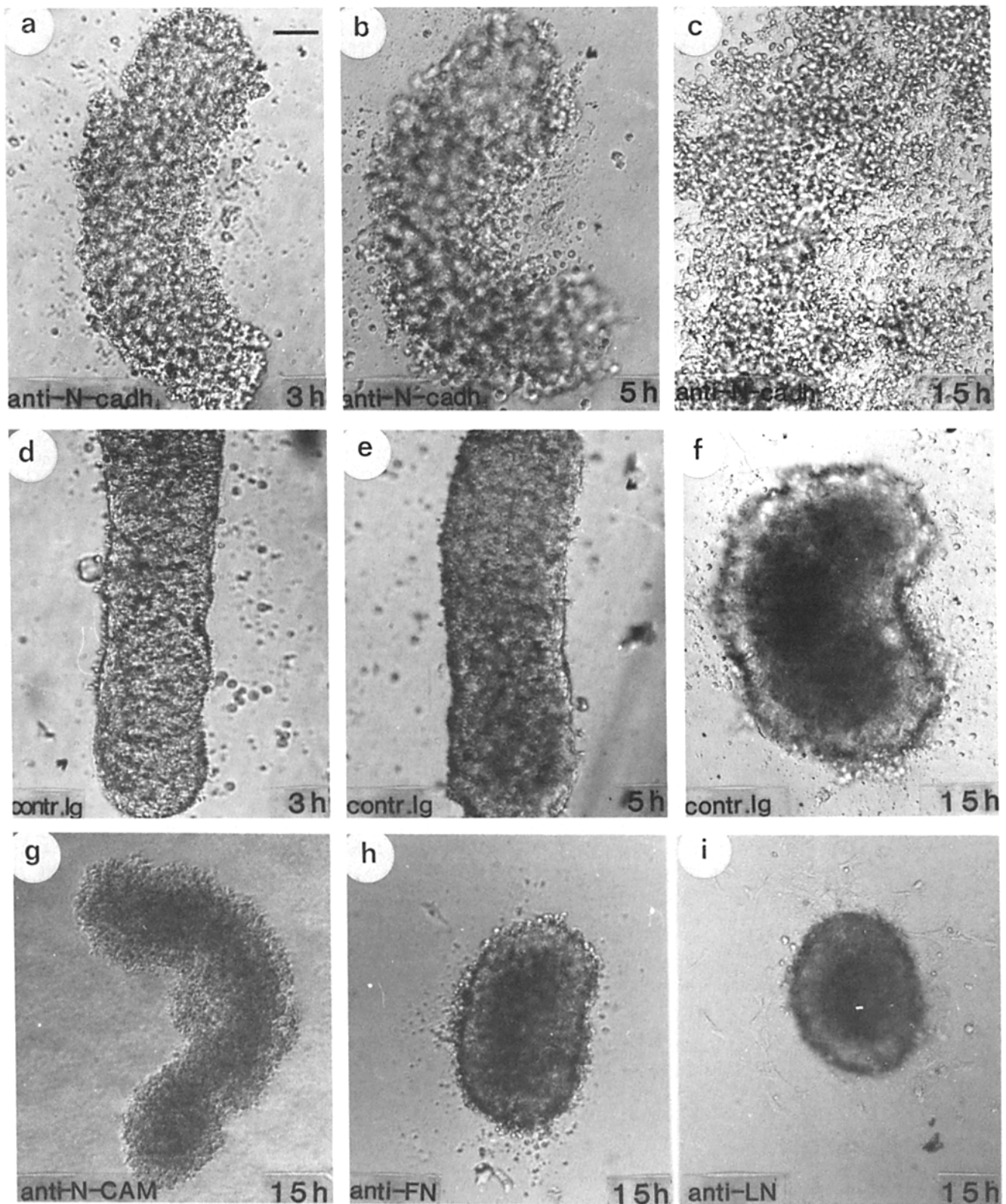
#### **Effects of Antibodies to Adhesion Molecules on Cell-Cell Adhesion in Intact Segmental Plates and Somites In Vitro**

To determine the possible role of adhesion molecules in the compaction of segmental plate cells and in the maintenance of the epithelial structure of somites, we cultured explants of these tissues in the presence of molecules that interfere with the function of adhesion molecules. For this purpose, we used: (*a*) monoclonal antibodies to N-cadherin and to N-CAM; these antibodies were known to inhibit the function of the molecule and could not induce aggregation of dissociated individual cells even when used as divalent forms (Hoffman et al., 1982; Hatta and Takeichi, 1986; see also the first section of the Results); (*b*) monovalent and divalent antibodies to N-CAM and to FN and LN; and (*c*) medium devoid of calcium or containing EDTA.

Incubation of segmental plates with antibodies to N-cadherin caused an extensive alteration in the morphology of the tissues (Fig. 4, *a-c*). Cells detached from the explants within 2–4 h and, after 15 h, segmental plates completely lost their

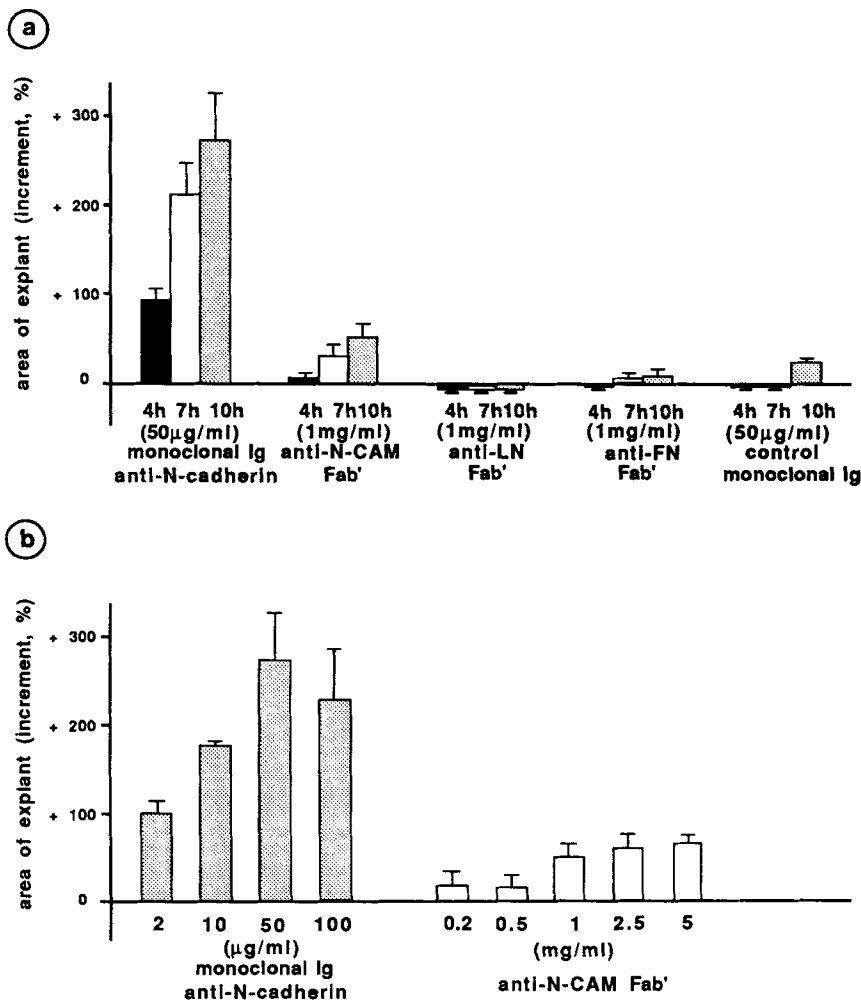
organization (Fig. 4, *a-c*). A very similar but even more rapid effect was obtained in calcium-depleted or EDTA-containing medium in the absence of antibodies (not shown). Both monoclonal and monovalent polyclonal antibodies to N-CAM also caused the dissociation of explants of segmental plates but the effect was slower and weaker than in medium without calcium, or with EDTA, or with antibodies to N-cadherin; only cells located in the periphery of the explant escaped from it, and at no time did we observe complete dissociation of segmental plates (Fig. 4 *g*). In contrast to monovalent antibodies to N-CAM, divalent antibodies to N-CAM did not dissociate explants of the segmental plate (not shown). The combination of N-cadherin and N-CAM antibodies did not significantly increase the dissociation rate of the explants (not shown). Monovalent and divalent antibodies to LN and FN, a combination of these antibodies, and nonimmune monoclonal and polyclonal antibodies were totally devoid of effect on the cohesion of cells within the explants (Fig. 4, *d-f, h and i*). In the presence of these antibodies, the explants acquired the ball shape that is obtained

**Figure 2.** In situ immunofluorescent distribution of adhesion molecules during the reorganization of the somite. (*a-d*) At the level of the second, third, and fourth somite rostral to the last one in an embryo at stage 15 of Hamburger and Hamilton; (*e-f*) at the same level in an embryo at stage 17 of Hamburger and Hamilton. *a, c and e, g and b, d and f, h* show two consecutive sagittal sections double stained for N-cadherin and LN on one side and N-CAM and FN on the other side. The first detectable event in somite reorganization (*a-d*) is the disappearance of N-cadherin from the ventral side of the third somite (*s3*) rostral to the last formed somite (*arrowheads*). Note that in the second one (*s2*), N-cadherin is still present in the ventral side. N-CAM, LN, and FN remain unchanged in their distributions (compare with Fig. 1, *e and f, stage 4*). After the complete disruption of the somite (*e-h*), N-cadherin and LN are absent from the sclerotome, while N-CAM is still detectable on them and FN is now organized as a meshwork typical of mesenchyme. *Ant.* and *post.*, cranial and caudal sides of the sections. *d*, dermamyotome; *e*, ectoderm; *en*, endoderm; *sc*, progenitor cell of the sclerotome; *s2, s3, and s4*, the second, third, and fourth somite rostral to the last formed somite. Bar, 50  $\mu$ m.



**Figure 4.** Effect of antibodies to adhesion molecules on explants of segmental plates. Explants were incubated in the presence of monoclonal antibodies to N-cadherin (a-c), control monoclonal Ig (d-f) both at the concentration of 50  $\mu\text{g/ml}$ , and monovalent antibodies to N-CAM (g), FN (h), and LN (i) at 1 mg/ml. The effects of the antibodies were recorded on the same explant after several periods of time. The dissociating effect of antibodies to N-cadherin can be clearly seen within 3 h (a) and is complete within 15 h (c). In contrast, other antibodies do not alter the cohesion of cells within the explant (d-f, h, and i), with the exception of anti-N-CAM antibodies, which induce the dissociation of cells located only in the periphery of the explant (g). Bar, 50  $\mu\text{m}$ .





**Figure 5.** Quantification of the effect of antibodies to adhesion molecules on explants of segmental plates as a function of time (a) and as a function of the concentration of the antibodies after 10 h of incubation (b). Explants were incubated in the presence of monoclonal antibodies to N-cadherin at concentrations ranging from 2 to 100 µg/ml, control monoclonal Ig at a concentration of 50 µg/ml, monovalent antibodies to N-CAM at concentrations between 0.2 to 5 mg/ml, and monovalent antibodies to FN and LN at 1 mg/ml. The areas of the explants were recorded at various times, and the results are expressed as the increase or decrease of these areas with respect to the initial one. It should be mentioned that the measurement of the area of explants does not take into account the spread cells. Note that the antibodies to N-cadherin are effective at concentrations as low as 2 µg/ml. The values represent the mean of 20 explants in five different experiments.

under normal conditions. As a control of the effect of the antibodies to N-cadherin and N-CAM, we used a monoclonal antibody directed to A-CAM for several reasons. A-CAM shows great similarities with N-cadherin both in its molecular weight and in its *in vivo* distribution on segmental plates and somites (Volk and Geiger, 1986a, b; Duband, J.-L., T. Volberg, J. P. Thiery, and B. Geiger, manuscript in preparation); in addition, the monoclonal antibody to A-CAM does not perturb the function of the molecule (Volk and Geiger, 1986a, b). We never observed, in our assay of tissue dissociation *in vitro*, that this monoclonal antibody affects noticeably the cohesion of cells within the explants of segmental plates (Duband, J.-L., T. Volberg, J. P. Thiery, and B. Geiger, manuscript in preparation).

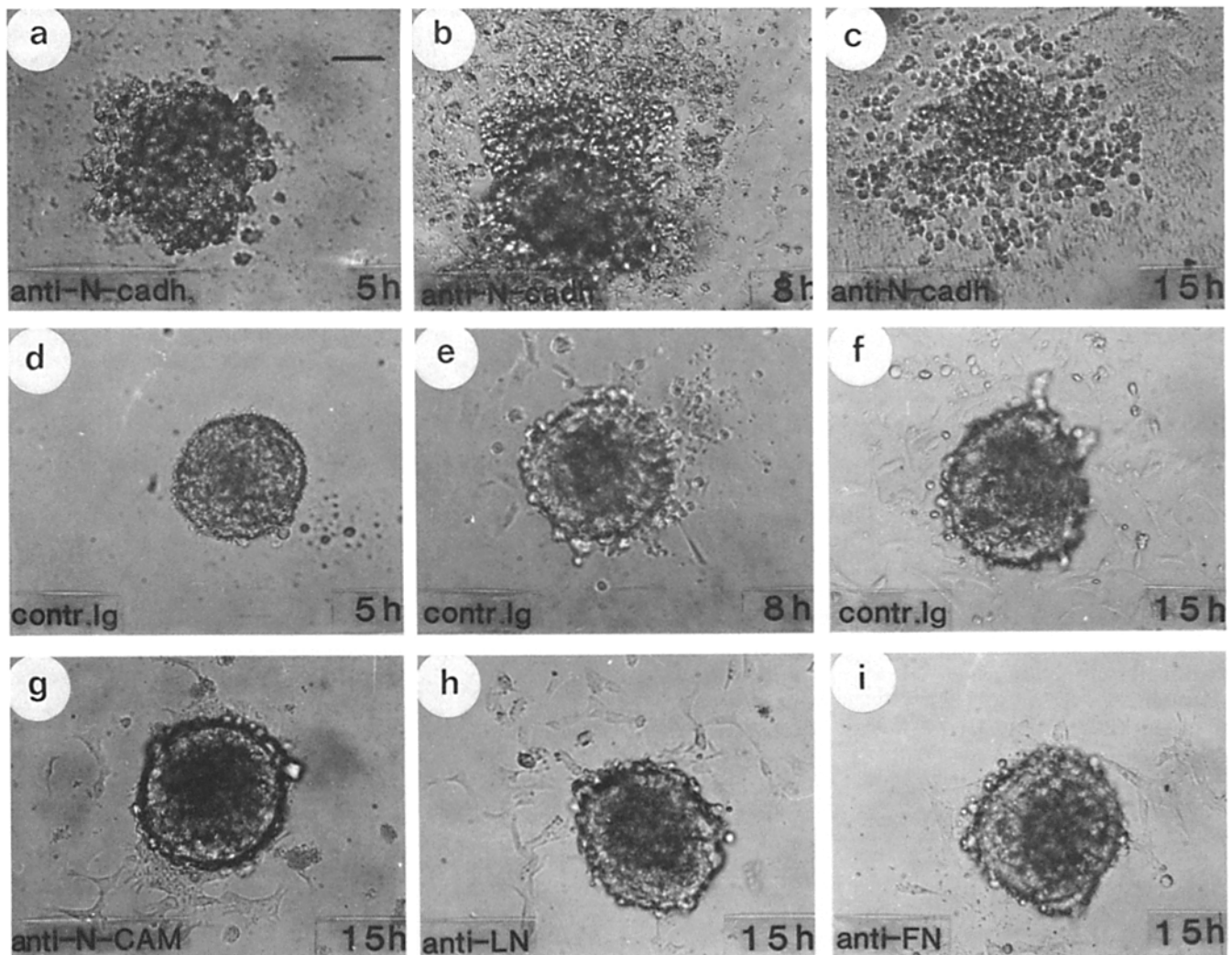
The dissociating effect of the antibodies and of the absence of calcium in the medium was quantified by measuring the total area of the explants. It appeared that the dissociating effect of the antibodies to N-cadherin was effective in both a time- and a dose-dependent manner (Fig. 5). It was noticeable that these antibodies produced a detectable effect on segmental plates even at very low concentrations (2 µg/ml). The effects of the absence of calcium or of the presence of EDTA in the medium were also significant, since within 1 h the areas of the explants were increased by 160% and in 10 h by more than 300% (not shown).

To rule out possible artifacts due to variable penetration of

antibodies to N-cadherin and to N-CAM into the tissue explants, we examined the morphology of the segmental plate explants after incubation with these antibodies. After brief fixation in a 3.7% formaldehyde solution, the cultured explants were sectioned on a cryostat and processed for immunofluorescence. Antibodies to N-cadherin, even at concentrations as low as 2 µg/ml, and monovalent and divalent antibodies to N-CAM at 0.5 mg/ml could be detected inside of the segmental plate (not shown). Explants incubated with either divalent antibodies to N-CAM or control antibodies showed a morphology very similar to that observed *in vivo*. In the presence of monovalent antibodies to N-CAM, only cells located in the periphery of the explants were dissociated, while those present in the core of the segmental plate were still compacted even though antibodies had penetrated deeply inside of the explant. In contrast, cells in explants incubated in the presence of antibodies to N-cadherin were all dissociated and no compact aggregate could be observed in the explants.

Similar results were obtained when explants of somites were used (Fig. 6). However, quantitation of the dissociating effect of the antibodies revealed that the effect of antibodies to N-cadherin was delayed and weaker as compared with segmental plates, and the antibodies to N-CAM, FN, and LN were totally ineffective (Fig. 7).

The dissociating effect of antibodies to N-cadherin and of



**Figure 6.** Effect of antibodies to adhesion molecules on explants of somites. Explants were incubated in the presence of monoclonal antibodies to N-cadherin (a-c), control monoclonal Ig (d-f) both at the concentration of 50  $\mu\text{g/ml}$ , and monovalent antibodies to N-CAM (g), LN (h), and FN (i) at 1 mg/ml. The effects of the antibodies were recorded on the same explant after several periods of time. Antibodies to N-cadherin are strongly effective on somites but their effect can only be seen after 5 h (a-c). Control monoclonal antibodies (d-f) and monovalent antibodies to other adhesion molecules (g-i) are totally ineffective. Bar, 25  $\mu\text{m}$ .

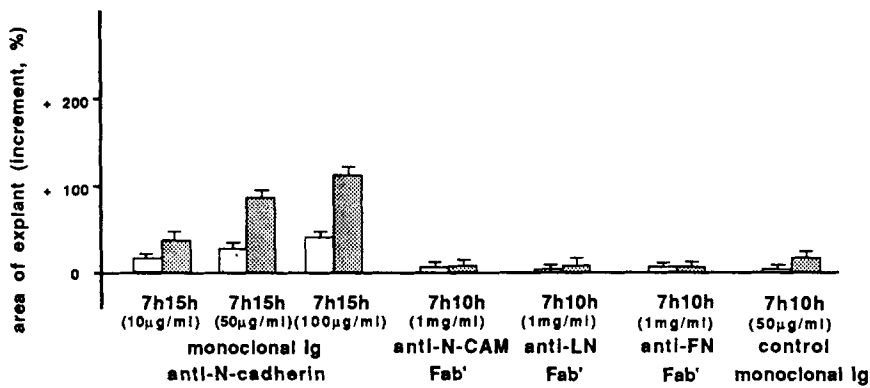
calcium-free medium on segmental plates and somites could not be attributed to a cytotoxic effect of the antibodies, since the removal of the antibodies from the culture medium resulted either in a de novo aggregation of cells into segmental plate- and somite-like structures or at least in the arrest of the dissociation effect of the antibodies (Fig. 8). Nevertheless, cells failed to reassociate after prolonged exposure to the antibodies (not shown).

### Discussion

In this study, we have analyzed the spatio-temporal distributions and possible functional roles of several adhesion molecules (N-cadherin, N-CAM, fibronectin, and laminin) during somitogenesis. We were particularly interested in the correlation of key events with various forms of cell surface modulation. Our major findings are: (a) segmental plate and somitic cells exhibit both calcium-dependent and calcium-independent systems of cell aggregation in vitro; these two

mechanisms are mediated by N-cadherin and N-CAM, respectively; (b) in vivo, the prevalence and cell surface modulations of N-cadherin is strongly correlated with epithelialization and de-epithelialization of the somitic tissue; (c) N-CAM also increases at the surface of segmental plate cells during epithelialization; however, its expression is not as markedly correlated with these events; (d) the cell-substratum adhesion molecule fibronectin, first found in low amounts within the segmental plate, localizes progressively in the basement membrane of the newly formed somites; the basal lamina component laminin is also found to appear late in the process of epithelialization; (e) antibodies to N-cadherin show a noticeable and reversible dissociating effect on explants of segmental plates and somites in contrast to antibodies to N-CAM, which have a weak effect, and to antibodies to FN and LN, which have no effect.

In the vertebrate embryo, the axial mesoderm that is destined to form segmental plates and somites originates during gastrulation and its metameric pattern is laid down very

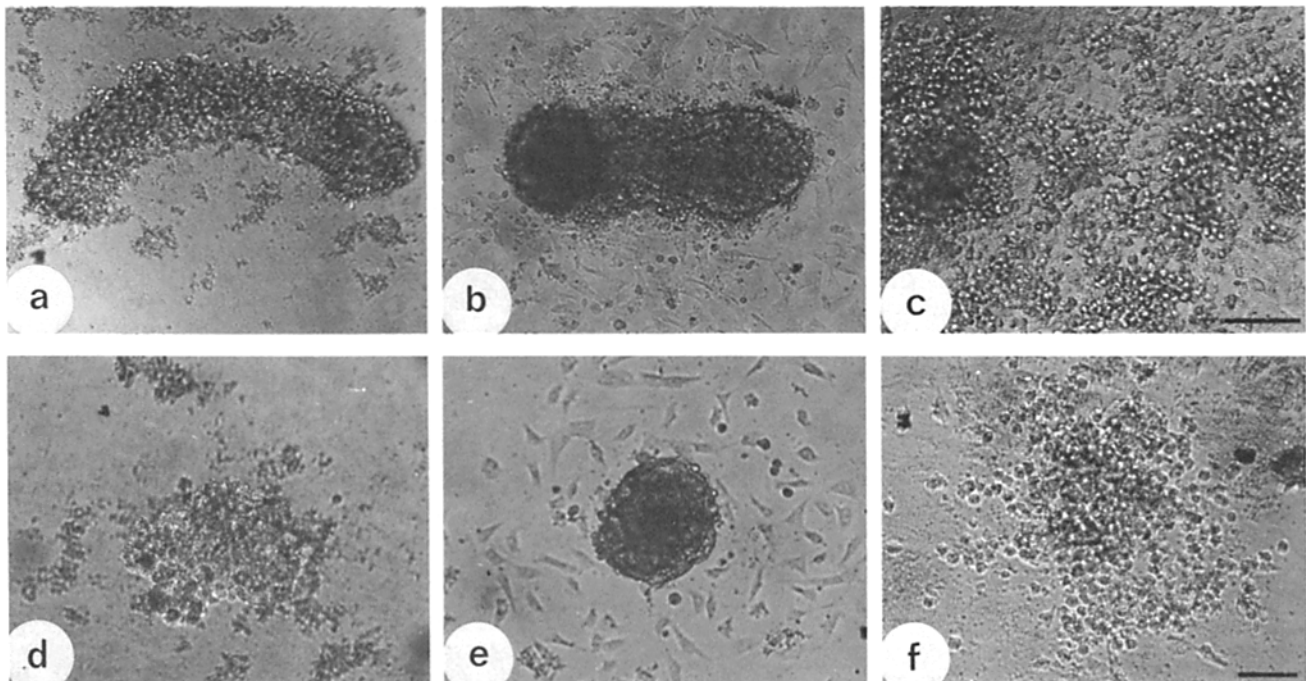


**Figure 7.** Quantification of the effect of antibodies to adhesion molecules on explants of somites as a function of time and of the concentration of the antibodies. Explants were incubated in the presence of monoclonal antibodies to N-cadherin at concentrations between 10 and 100 µg/ml, control monoclonal Ig at a concentration of 50 µg/ml, and monovalent antibodies to N-CAM, LN, and FN at 1 mg/ml. Note that antibodies to N-cadherin do not increase the area of the somitic explants as considerably as on segmental plates; additionally, these antibodies are not active at concentrations below 10 µg/ml.

early, well before it can be visualized (Meier, 1979). The somitic mesoderm derives from epiblast cells located close to Hensen's node and it organizes into segmental plates and subsequently into somites under the action of several inductors including the neural plate, the notochord, and the regressing Hensen's node (Nicolet, 1970; Lipton and Jacobson, 1974). Once the mesoderm of the segmental plates is laid down, somites form gradually and regularly until they reorganize into sclerotome and dermamyotome.

During embryogenesis, adhesion molecules appear as early as before gastrulation, cells frequently carrying several distinct adhesion molecules with different specificities (Edelman, et al., 1983; Crossin et al., 1985; Edelman, 1986). Before gastrulation in the chick, epithelial cells from the epiblast express both L-CAM and N-CAM (Edelman et al.,

1983). During gastrulation, a population of epiblast cells (i.e., those that participate in the conversion into the mesenchyme of the mesoderm) has been shown, so far, to express three CAMs: L-CAM, N-CAM, and N-cadherin (Edelman et al., 1983; Hatta and Takeichi, 1986). In addition, these cells are also associated with LN and FN, possibly through distinct receptors including the 140-kD FN receptor complex (Duband and Thiery, 1982; Mitrani, 1982; Sanders, 1982; Duband et al., 1986; Krotoski et al., 1986). When cells have acquired a mesenchymal state, they readily lose L-CAM, whereas N-CAM diminishes only progressively from their surface and N-cadherin increases in prevalence (Edelman et al., 1983; Hatta and Takeichi, 1986). The expression of N-cadherin decreases only later on the surface of mesodermal cells destined to form the segmental plate. When Hen-



**Figure 8.** Reversibility of the effect of antibodies to N-cadherin. Explants of segmental plates (a-c) and somites (d-f) were incubated in the presence of antibodies to N-cadherin until dissociation could be clearly detected, i.e., 4 and 6 h, respectively (a and d). Antibodies were then removed by extensive washing, and the explants were then incubated in the presence of fresh medium; after 15 h, both segmental plates and somites recovered a compacted shape (b and e). In contrast, explants that remained in the presence of antibodies to N-cadherin were completely dissociated (c and f). Bar in c, 100 µm; a-c are at the same magnification; bar in f, 25 µm; d-f are at the same magnification.

sen's node regresses, allowing mesenchymal cells to assemble into a segmental plate, a subset of adhesion molecules, N-CAM and N-cadherin, is reexpressed progressively on these cells. In contrast to CAMs, the FN-receptor complex as detected by immunofluorescence studies remained on the surface of mesodermal cells throughout these processes (Duband et al., 1986; Krotoski et al., 1986), even though FN was present in low amounts among them (see also Ostrovsky et al., 1983).

As judged by immunofluorescence labeling, the expression of N-cadherin increases rapidly at the onset of epithelialization of the segmental plate; subsequently, N-cadherin shows polarity modulation and localizes predominantly on lateral surfaces facing the lumen of newly formed somites. In contrast, N-CAM and the FN receptors do not redistribute as dramatically on the surface of the somitic epithelial cells (see also Duband et al., 1986). The sclerotome, a new set of mesenchymal cells deriving from part of the somitic epithelium, loses N-cadherin very rapidly, whereas N-CAM and the FN receptor do not diminish as drastically (see also Duband et al., 1986). Recently, it has been shown that a newly discovered SAM, called cytactin (Grumet et al., 1985), is assembled as a basement membrane component of the somites in a cephalocaudal wave and subsequently accumulates very rapidly within the sclerotome at the time of its formation (Crossin et al., 1986). The role of this molecule in regulating structure and movement patterns in and around somites remains to be explored.

These descriptive studies of the prevalence and polarity modulation of CAMs and SAMs suggest that they each play different roles at different stages of development. For instance, N-cadherin is first evidenced at the level of the primitive streak particularly in cells egressing from the epiblastic epithelium (Hatta and Takeichi, 1986), thus becoming associated with migratory cells losing their epithelial structure. Conversely, N-cadherin is expressed during the assembly of segmental plate cells into the somitic epithelium and is lost in cells destined to form the mesenchyme of the sclerotome. Thus, during somitogenesis, the modulation of the expression of N-cadherin is consistent with a role expected for CAMs in mediating cell-cell adhesion. N-CAM expression is increased during the formation of epithelia such as the epiblast and the somite but is not regulated strictly during their reorganization (Edelman et al., 1983). Both epithelial and mesenchymal cells continuously maintain the FN receptor on their surface whether they are in motion or in a stationary state, while FN deposition in the environment of these cells may vary considerably (Duband et al., 1986).

The specificity differences, differences in calcium dependency, and different spatio-temporal distributions of the two CAMs involved in somite cell adhesion also suggest that they have different roles at various stages of somitogenesis. In vitro, dissociated segmental plate and somitic cells all expose functional N-cadherin and N-CAM. However, the effects of antibodies to N-cadherin in the cell dissociation assay on explanted tissues were greater than those of antibodies to N-CAM and, in vivo, N-cadherin disappearance is more closely associated with somite reorganization than N-CAM. Even though combined antibodies to both molecules did not show additive effects, it cannot be excluded that both N-CAM and N-cadherin may act together in crucial stages of somitogenesis. One possible interpretation of our data is that

N-cadherin is strongly associated with tissue remodeling and is critical to the formation of epithelial structures. This may be the case because of its polarity modulation on cells and its possible role in the formation of adherens junctions if it is confirmed that it is closely related to A-CAM (Volk and Geiger, 1986a, b). An alternative view is that both N-cadherin and N-CAM must act synergistically to lead to tissue remodeling. At least for the formation of the somites, such a hypothesis is in accord with a previous report showing that Axolotl mesoderm segmentation is in vivo sensitive to trypsin treatment both in the absence and presence of calcium (Gillespie et al., 1985).

The present data do not allow a sharp choice between the two alternatives mentioned above for a number of reasons: (a) the number of molecules per cell and the relative binding strength of each CAM are unknown; (b) the binding strength and attachment sites of the respective antibodies and Fab fragments are unknown; and (c) the scoring of dissociation in the in vitro assay may not truly reflect more detailed complementary modulation of the two CAMs in vivo, particularly in the case of the somites.

It thus cannot be excluded that other CAMs associated with differentiated junctions may also participate to the processes of cell adhesion occurring during somitogenesis. There is now evidence that desmosomes and intermediate junctions, but not so far tight junctions, are assembled transiently during the formation of epithelia (Aoyama et al., 1985; Geiger, B., unpublished data). In particular, the adhesion molecule A-CAM, which is adherens junction-specific and shows similarities with N-cadherin (Volk and Geiger, 1986a, b), progressively accumulates in developing intermediate junctions during somitogenesis (Duband, J.-L., T. Volberg, J. P. Thiery, and B. Geiger, manuscript in preparation). In addition to A-CAM, desmocollins, the putative adhesion molecules of desmosomes (Cowin et al., 1984) may be involved in the stabilization of the somitic epithelium.

In our study, a direct role has not been found for FN in the compaction and subsequent epithelialization of the segmental plate. However, it has been shown that addition of synthetic peptides containing the major binding site for the FN receptor or addition of cellular but not plasma FN resulted in a premature induction of somites in explanted segmental plates (Lash et al., 1984; Lash and Yamada, 1986). Similar results were also obtained in vivo with the addition of cellular FN but not of plasma FN (Lash et al., 1984). While these results cannot be interpreted unequivocally, particularly because both plasma and cellular FN contain the same cell-binding site, it is still a provocative idea that the major cell-binding domain of FN behaving like a hormone through the FN receptor is a potential inducer of somitogenesis.

Somitogenesis is a multistep process that results from a series of inductive events operating throughout gastrulation (Nicollet, 1970; Lipton and Jacobson, 1974). The regulated expression of CAMs and SAMs is reminiscent of similar situations observed with the ontogeny of neural crest cells (for a review, see Thiery et al., 1985), the development of the kidney (Ekblom, 1981; Thiery et al., 1982, 1984), the patterning of the otic placodes (Richardson et al., 1987), and of the feathers (Chuong and Edelman, 1985a, b; Gallin et al., 1986). Simultaneous and/or reiterative expressions of CAMs and SAMs reflected in cell surface modulation events have

been described during the morphogenesis and differentiation of all of these different structures (Gallin et al., 1986). It is intriguing to postulate that CAM regulation is a major primary event in inductive processes. In the case of the somites, our results indicate that, in association with N-CAM, N-cadherin plays a predominant role possibly in relation to the formation of specific cellular junctions in the mechanisms establishing cell polarity. One of the most challenging tasks for the future will be to identify and trace the biochemical signals responsible for the various modulations seen in structures such as somites.

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