Teratocarcinoma Stem Cell Adhesion:

The Role of Divalent Cations and a Cell Surface Lectin

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ABSTRACT   We describe two additive systems of intercellular adhesion in teratocarcinoma stem cells (Nulli cell line). One component is divalent cation-dependent (Ca^{++} or Mg^{++}) and the other involves a cell surface fucan/mannan-specific lectin, previously identified on stem cells by an erythrocyte rosetting assay. The existence of these two systems is inferred from the observation that reaggregation of stem cells was partially inhibited by the removal of divalent cations or by the presence of lectin inhibitors such as fucoidan, but reaggregation was completely blocked when the two conditions were combined. Our results are related to recent work describing a calcium-dependent system of intercellular adhesion in teratocarcinoma stem cells.

Intercellular adhesion is fundamental to many diverse biological processes and therefore elucidating its molecular basis is of great interest. One approach to studying the mechanism of cell-cell adhesion in several systems (1–4) has recently focused on the observation that two systems appear to contribute to intercellular adhesion, one calcium-dependent and the other calcium-independent. The calcium-dependent system is well defined and in some cases, specific molecules have been implicated, whereas the calcium-independent system remains poorly defined.

A different approach to studying the mechanism of cell-cell adhesion has focused on the role of carbohydrate-binding proteins ([CBP], lectins; [5, 6]). We have previously identified a CBP on the surface of teratocarcinoma stem cells. Its existence was initially inferred from the observation that stem cells can form rosettes with certain erythrocyte types and that this rosetting is inhibited by specific carbohydrates (7). More recently, a soluble hemagglutinin from stem cells has also been described with an erythrocyte and carbohydrate specificity almost identical to that of the rosette-mediating factor, suggesting that the hemagglutinin and the rosette-mediating factor are identical (8). This CBP recognizes fucans as well as certain mannans, although in both assays fucans are the more potent inhibitors by 2–3 orders of magnitude.

We have suggested a role for the lectin in intercellular adhesion, since mannans can partially interfere with the reaggregation of stem cells in rotary cultures. This suggestion is supported by the observation described here that fucans are also inhibitors of stem cell reaggregation and, as was observed for rosetting and hemagglutination, that the fucans are more effective than the mannans. It is also noteworthy that fucan-specific lectins may be involved in mediating a number of diverse cell-cell adhesion events including fertilization in *Fucus* (9), sea urchins (10), and guinea pig (11), as well as lymphocyte attachment to endothelial cells during lymphocyte recirculation (12). In this paper we present evidence that suggests that the teratocarcinoma stem cell lectin is involved in a divalent cation-independent system of cell-cell adhesion.

MATERIALS AND METHODS

Materials: *Arbacia* fucan was a generous gift of Dr. W. J. Lennarz (Johns Hopkins University School of Medicine, Baltimore, MD). Fucoidan was obtained from ICN K&K Laboratories, Inc. (Plainview, NY). Heparin (H3125), chondroitin sulfate (C3254), dextran sulfate (D7515), dermatan sulfate (C4259), hyaluronic acid (H1751), fetuin (F2379), ovalbumin (A2512), glycogen (G9251), bovine serum albumin (A4378) (BSA), DNase (D0876), trypsin (T8253), and egg white trypsin inhibitor (T2011) were obtained from Sigma Chemical Co. (St. Louis, MO). Fucoidan was desulfated and hydrolyzed as previously described (8).

Cell Harvesting and Reaggregation: The Nulli SCC-1 line of mouse teratocarcinoma stem cells was cultured as described previously (13). Stem cells were harvested as a single-cell suspension following 10-min incubation at 37°C in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). Viability as evaluated by trypan-blue exclusion was >90%. Cells were washed in CMF-PBS and allowed to reaggregate in rotary culture for 30 min at 37°C as previously described (7). Briefly, cells were added to 40 mM Tricine-buffered Eagle's minimum essential medium (MEM) (with or without $CaCl_2$ and $MgSO_4$) that contained 1 mg/ml BSA and 20 μg/ml DNase. EDTA, EGTA, and the various glycoconjugates were added at concentrations stated in the text. 0.5 ml of these cell suspensions ($1–2 \times 10^6$ cells/ml) was then dispensed into individual Linbro wells (Linbro Chemical Co., Hamden, CT) in duplicate, reaggregated by rotary gyration, and counted by Coulter counter (Coulter Electronics, Inc., Hialeah, FL), as previously described (7). Percent aggregation was calculated as the percent reduction in total particle number from time zero. All data expressed in terms of inhibition of aggregation are calculated on the basis of control

RESULTS

Divalent Cation Requirement for Reaggregation

Cells of the teratocarcinoma stem cell line Nulli SCC-1 were harvested in CMF-PBS as a single-cell suspension and allowed to reaggregate in rotary culture for 30 min at 37°C. Aggregation was quantified by measuring the percent reduction in total particle number as described in Materials and Methods. The 30-min period was sufficient for near maximal aggregation (50–60%) under control conditions in Tricine-buffered MEM containing physiological levels of Ca^{++} and Mg^{++} (1.8 and 0.8 mM, respectively). When CMF-MEM was used to ensure that divalent cations would be effectively absent from the assay mixture, aggregation was reduced to ~40%, indicating a 32% inhibition of reaggregation (Fig. 1). Addition of EDTA to the
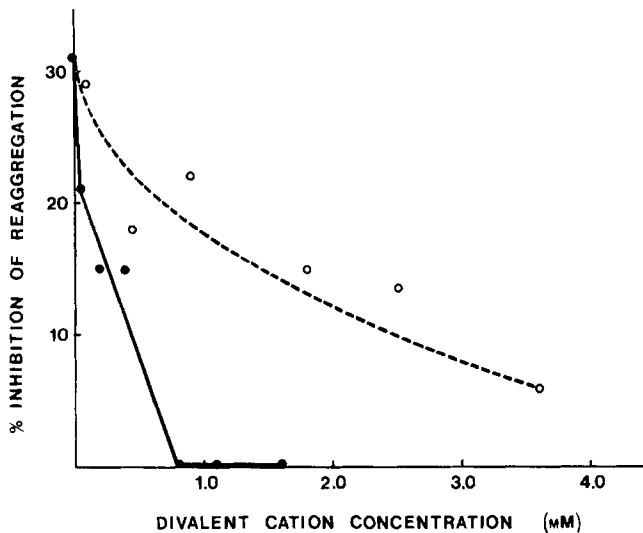

FIGURE 1 Stem cell reaggregation in the presence of Ca^{++} or Mg^{++}. Stem cells were dissociated in CMF-PBS and allowed to reaggregate for 30 min as described in Materials and Methods in CMF-MEM in the presence of either Ca^{++} (O) or Mg^{++} (●).

CMF-medium did not further inhibit aggregation (data not shown). That there was as much as 40% reaggregation in the absence of divalent cations indicated the existence of a divalent cation-independent system of intercellular adhesion. The increase over this baseline aggregation in the presence of divalent cations implied the existence of a divalent cation-dependent component. To further demonstrate this, we restored either Ca^{++} or Mg^{++} to the CMF-MEM assay mixture. Fig. 1 shows that in the presence of either of these divalent cations, reaggregation approached control levels (0% inhibition). Mg^{++} was more effective than calcium at promoting reaggregation.

Relationship between Lectin-dependent and Divalent Cation-independent Adhesion Systems

As noted in the Introduction, we have previously identified a lectin on the surface of teratocarcinoma stem cells that recognizes fucans and mannans. Evidence that this lectin plays a role in the intercellular adhesion of teratocarcinoma stem cells came from our study showing that mannans can interfere with the reassociation of stem cells in rotary cultures. Our goal here was to determine whether this lectin plays a role in the divalent cation-independent system or the divalent cation-dependent system. If the former were the case, one would expect that the effect of removing divalent cations and adding a lectin inhibitor would be additive, and that reaggregation could be completely inhibited. If, however, the lectin were involved in the divalent cation-dependent system of aggregation, then adding a lectin inhibitor to an assay mixture lacking Ca^{++} and Mg^{++} should not increase the extent of inhibition.

We first investigated the effect on the reaggregation of stem cells of several lectin inhibitors including fucoidan (a sulfated fucan of ~100,000 daltons [14] containing minor amounts [6%] of galactose, xylose, and uronic acid) in divalent cation-containing medium. Fig. 2a shows that fucoidan at concentrations of 50 μg/ml–1 mg/ml inhibited the reaggregation of stem cells to a maximum of 20%. We also found that mannans inhibited reaggregation up to 20% but at a 100-fold higher concentration than fucoidan (data not shown). Thus, fucoidan is a more potent inhibitor of reaggregation than mannans, as was the case for the rosetting and hemagglutination assays of lectin activity (8).

The Arbacia fucan, which contains only fucose and sulfate (15), also inhibited aggregation, producing 20% inhibition at a concentration of 250 μg/ml. The effectiveness of the Arbacia
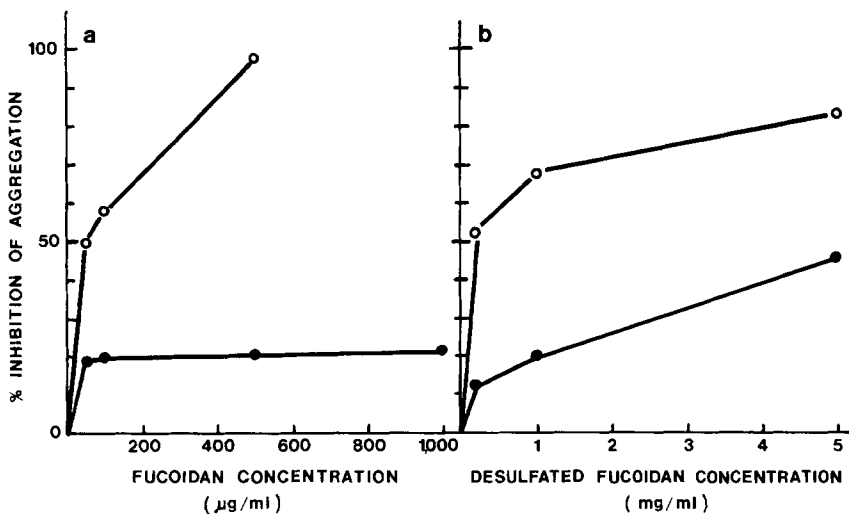

FIGURE 2 The effect of fucoidan and desulfated fucoidan on stem cell reaggregation in the presence and absence of divalent cations. Stem cells were reaggregated in control medium (●) or in control medium with 2 mM EDTA (O) as described in Materials and Methods in the presence of (a) fucoidan or (b) desulfated fucoidan. Percent inhibition is calculated on the basis of a divalent cation containing control for this figure and for Figs. 3 and 4.

fucan in this assay as well as in the rosette and hemagglutination assays (8) argues against the possibility that fucoidan-mediated inhibition is due to a contaminant rather than to the sulfated fucan. Fucoidan hydrolysis products obtained by mild acid treatment (0.02 N HCl, 60°C, 30 min) remained as effective on a mass basis as the intact molecule (data not shown). Desulfated fucoidan (95% sulfate removal, as determined by rhodizonate assay) retained its ability to block stem cell reaggregation, although 50–100-fold higher concentrations were required (Fig. 2b). Sulfation, therefore, appears to contribute to the activity of fucoidan. However, sulfation alone was not responsible for fucoidan's inhibitory effects, since several sulfated polysaccharides (chondroitin sulfate, dextran sulfate, and dermatan sulfate) were without inhibitory activity in the reaggregation assay as well as in the rosette and hemagglutination assays. Perhaps because of similar charge distribution and/or structural homology to fucans, xylan and heparin caused a 10–15% inhibition of reaggregation in divalent cation-containing medium (Fig. 3a). This observation was consistent with the fact that both of these glycoconjugates are effective rosette inhibitors, although only xylan is also a hemagglutination inhibitor (8). With all inhibitors tested, inhibition in the presence of divalent cations was partial, never exceeding 20–40%. Another series of glycoconjugates lacking fucose and activity in the hemagglutination and rosette assays (hyaluronic acid, fetuin, ovalbumin, and glycogen) did not inhibit aggregation at 1 mg/ml.

We next investigated the effect of lectin inhibitors upon stem cells reaggregated in the absence of divalent cations. These conditions were met by adding 2 mM EDTA or 10 mM EGTA to "control" aggregation medium that contained divalent cations or by using Ca^{++}- and Mg^{++}-free aggregation medium (Fig. 4). Consistent with the observations described above, all three of these conditions inhibited stem cell reaggregation (Fig. 4). When divalent cations were absent from the aggregation mixture, adding from 50 to 500 µg/ml fucoidan progressively inhibited stem cell reaggregation until it was almost completely blocked (Figs. 2a and 4). When aggregation was assayed in the presence of fucoidan (100 µg/ml) and physiological concentrations of either Ca^{++} or Mg^{++}, the extent of inhibition was similar to that in medium containing both divalent cations (Table I). Thus, adding either Ca^{++} or Mg^{++} markedly decreased the extent of inhibition by fucoidan.

Figure 4 The effect of fucoidan, EDTA, EGTA, and CMF-medium on stem cell reaggregation. Stem cell reaggregation assays were performed with CMF-PBS-harvested cells as described in Materials and Methods in control medium, control medium with EDTA at 2 mM, control medium with EGTA at 10 mM, or CMF-medium. Open bars represent percent inhibition of aggregation in the absence of fucoidan (in control medium without fucoidan there is no inhibition of aggregation), cross-hatched bars in the presence of 50 µg/ml fucoidan, and stippled bars in the presence of 500 µg/ml fucoidan. The values shown represent the means from three independent experiments. The error bars indicate SEM.

TABLE I

Effect of Ca^{++} and Mg^{++} on Fucoidan Inhibition of Stem Cell Reaggregation

Medium	Inhibition	Inhibition in the presence of 100 µg fucoidan/ml
	%	%
CMF-MEM	34 (±10.8)	60.3 (±4.4)
+Ca^{++}	18 (±2.6)	20.0 (±6.1)
+Mg^{++}	9.3 (±0.9)	32.3 (±5.4)
+Ca^{++} and Mg^{++}	0	25.0 (±7.0)

Cells were reaggregated in CMF-MEM with and without 1.8 mM Ca^{++} and/or 0.8 mM Mg^{++}. Percent inhibition was determined relative to cells reaggregated in control medium containing Ca^{++} and Mg^{++}. The means ± SEM were computed from three independent determinations.

The morphology of the aggregates formed under the various conditions is shown in Fig. 5. In the presence of divalent cations, large, tight aggregates were formed with indistinct cell-cell boundaries (Fig. 5a). In the presence of 500 µg/ml fucoidan the aggregates formed were generally smaller, but they retained their tight morphology (Fig. 5b). With divalent cations removed (2 mM EDTA) the aggregates appeared smaller and much looser, with individual cell boundaries now clearly discernible (Fig. 5c). When fucoidan (500 µg/ml) was added in the presence of EDTA, aggregation was almost totally blocked, leaving mostly single cells and small (2–3 cell) aggregates with individual cells distinguishable.

To determine whether the inhibition produced by fucoidan remained specific in the absence of divalent cations, we retested a series of polysaccharides under the same assay conditions. The inhibitory activity of desulfated fucoidan was promoted by EDTA, although once again, higher concentrations were required than for fucoidan (Fig. 2b). Although inhibition mediated by xylan was not significantly enhanced by the presence of EDTA, inhibition mediated by heparin was enhanced to the same extent as was inhibition mediated by fucoidan (Fig. 3b). The control substances, chondroitin sulfate, dextran sulfate, dermatan sulfate, hyaluronic acid, fetuin, oval-
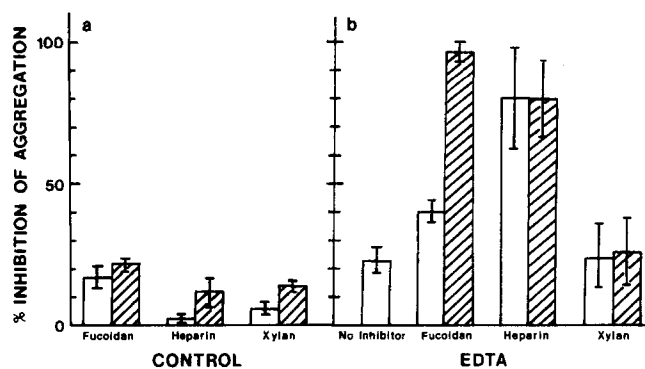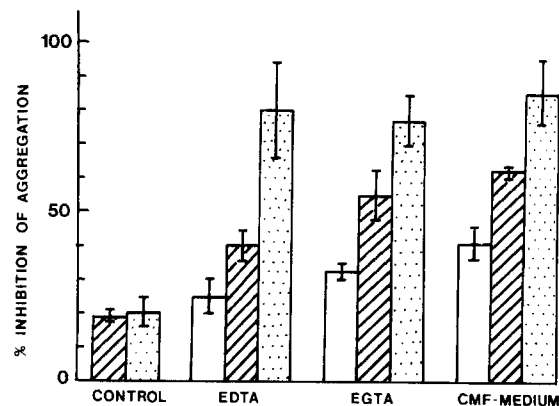
Figure 3 The effects of various glycoconjugates and EDTA on reaggregation of CMF-PBS-dissociated stem cells. The cells were reaggregated in control medium (no EDTA) (a) or with 2 mM EDTA (b) in the presence of varying concentrations of fucoidan, heparin, or xylan: open bars, 100 µg/ml; cross-hatched bars, 500 µg/ml. The values shown represent the means from three independent experiments. The error bars indicate SEM.
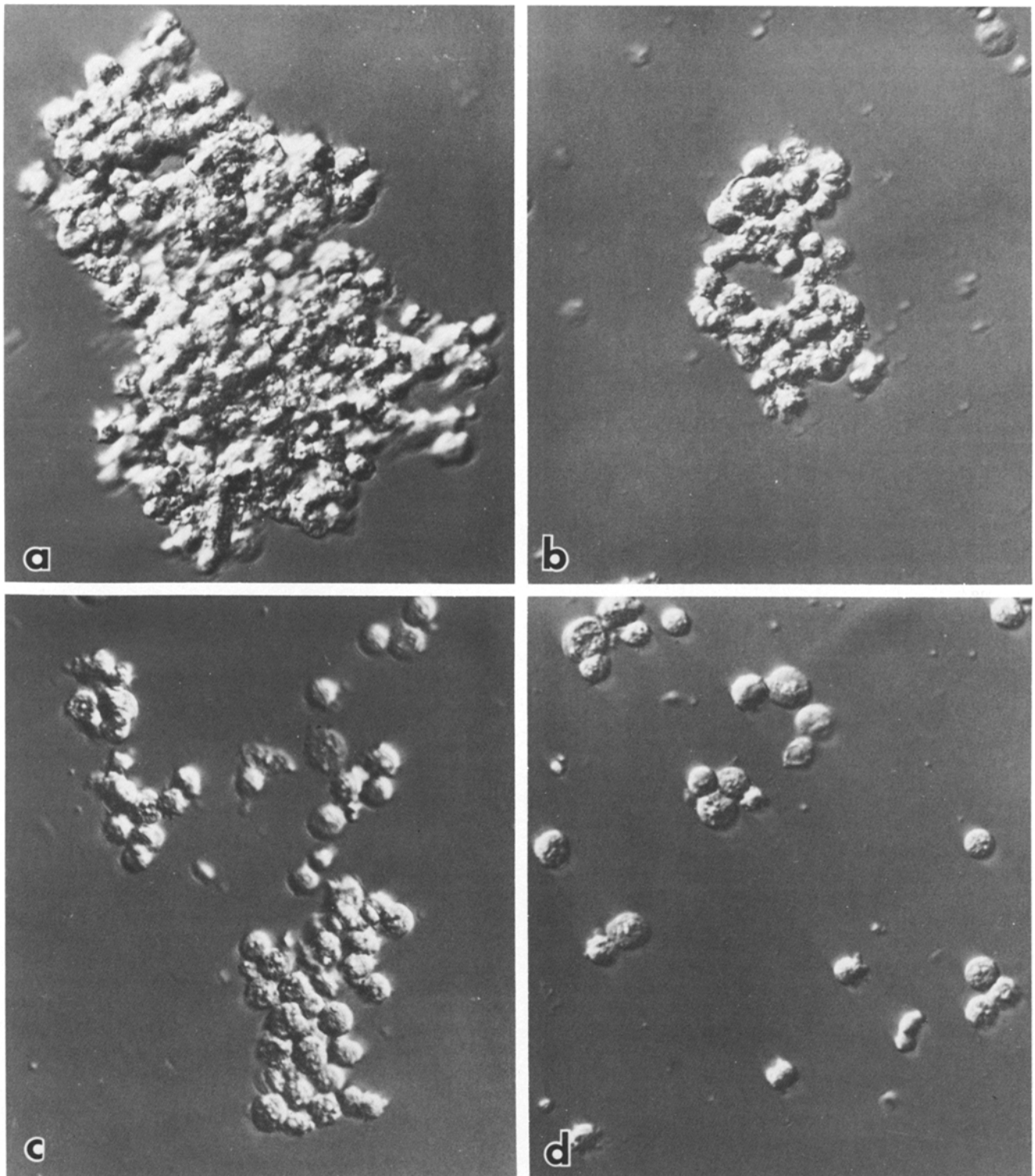
FIGURE 5 Morphology of stem cell aggregates in fucoidan and/or EDTA. Stem cells were harvested in CMF-PBS and after 30 min of aggregation in the presence of (a) MEM, (b) MEM with 500 μg/ml fucoidan, (c) MEM with 2 mM EDTA, or (d) MEM with 2 mM EDTA and 500 μg/ml fucoidan. Aggregates were fixed in 2.5% glutaraldehyde and observed by Hoffman-modulation contrast optics. Bar, 20 μm. × 500.

bumin, and glycogen at 1 mg/ml, however, still showed no inhibitory effect in the absence of divalent cations. Therefore, only those glycoconjugates that interacted with the lectin as defined by the rosette and hemagglutination assays could inhibit the reaggregation of stem cells. Charge alone did not

appear to be responsible for inhibition since the charged polysaccharides chondroitin sulfate, dextran sulfate, and hyaluronic acid were not inhibitory.

To demonstrate that fucoidan has no toxic effect upon the reaggregating stem cells, we determined that the incorporation

TABLE II

Effect of Fucoidan on the Reaggregation of Trypsin/Ca^{++}-harvested cells

Medium	Aggregation	Aggregation in presence of 100 μg/ml fucoidan
	%	%
CMF-MEM	15	17
+Ca^{++}	46	45
+Mg^{++}	25	30
+Ca^{++} and Mg^{++}	49	51

Ca^{++} and Mg^{++} were added to 1.8 and 0.7 mM, respectively.

of [^{3}H]leucine into trichloroacetic acid-precipitable material was not significantly decreased by adding fucoidan in either the absence or presence of EDTA (data not shown).

Reaggregation of Trypsin/Ca^{++}-harvested Cells

Takeichi et al. (4) have shown that teratocarcinoma stem cell lines (F9 and AT805), harvested with trypsin plus Ca^{++}, require the presence of Ca^{++} but not Mg^{++} for reaggregation. Nulli cells, when harvested by the trypsin/Ca^{++} procedure (4) also exhibit calcium-dependent adhesion. As shown in Table II there was a low baseline level of aggregation that was enhanced to a much greater extent by Ca^{++} than by Mg^{++}. This is in contrast with the results obtained for CMF-PBS-harvested cells, in which there was 30–40% aggregation in the absence of divalent cations, and either Mg^{++} or Ca^{++} enhanced this aggregation (see above). The aggregation of trypsin/Ca^{++}-harvested cells in the presence of calcium was not inhibited by fucoidan (Table II). Thus, neither the Ca^{++}/Mg^{++}-dependent system of CMF-PBS-harvested cells nor the Ca^{++}-dependent cell adhesion of trypsin/Ca^{++} cells was sensitive to fucoidan.

DISCUSSION

We report here evidence for the existence of two separable systems of intercellular adhesion in teratocarcinoma stem cells. One component requires divalent cations—either Ca^{++} or Mg^{++}. The other component is divalent cation-independent and appears to involve a fucan/mannan-specific cell surface lectin. This conclusion is based on the observation that either the presence of lectin inhibitors or the absence of divalent cations caused a partial reduction of reaggregation, whereas a combination of these two conditions caused complete inhibition of reaggregation.

Several laboratories have described dual calcium-dependent and calcium-independent components of intercellular adhesion for various cell types including fibroblasts (1), embryonic neural retina cells (2, 3), embryonic liver (16), and teratocarcinoma stem cells (4). Takeichi et al. (4) have investigated the calcium-dependent system of teratocarcinoma stem cells in detail. A 145,000-dalton cell surface protein (17) and a possibly related 84,000-dalton cell surface protein (18) have been implicated in this system. It has also been demonstrated that the same Ca^{++}-dependent aggregation system that plays a role in stem cell adhesion is involved in the adhesion of early mouse embryos (19). When Nulli cells are harvested according to the trypsin/Ca^{++} procedure of Takeichi et al. (4), we also observed Ca^{++}-dependent reaggregation. This aggregation was fucoidan insensitive and, therefore, did not depend on the fucan-specific lectin. The calcium-dependent component of cell adhesion exhibited by the trypsin/Ca^{++} cells was presumably related to

the divalent cation-dependent cell adhesion of CMF-harvested cells. However, there is one significant discrepancy in that reaggregation of CMF-PBS harvested cells was stimulated by either Mg^{++} or Ca^{++}, whereas reaggregation of trypsin/Ca^{++} cells was selectively stimulated by Ca^{++}. Perhaps the trypsin/ Ca^{++} cells required an additional Ca^{++}-dependent step to recover from the trypsinization and then, like the CMF-PBS harvested cells, would be able to aggregate in the presence of either divalent cation. We are presently investigating this possibility.

In terms of specificity, Takeichi et al. (4) present evidence that the Ca^{++}-dependent system of adhesion can mediate selective adhesion of stem cells in mixtures with fibroblasts. Brackenbury et al. (16), however, claim that, for neural retina cells and liver cells of the chick embryo, calcium-dependent adhesion is nonspecific, whereas the calcium-independent system is tissue-specific. It remains to be determined what role the divalent cation-independent adhesion system, which appears to depend on the fucan/mannan-specific lectin, plays in the selective adhesion of stem cells. It is of interest that lectins apparently mediate specific recognition events in a number of other systems including species-specific adhesion of slime molds (20), symbiosis of rhizobium and legume (21), and uptake of circulating glycoconjugates by the liver (22).

The effectiveness of fucans as inhibitors of a component of teratocarcinoma stem cell intercellular adhesion implies the existence of cell surface glycoconjugates that act as receptors for the CBP. Two recently described carbohydrate-containing antigens on teratocarcinoma stem cells are of particular interest as possible receptors for the lectin. First, Muramatsu et al. (23) have described the presence on the surface of teratocarcinoma stem cells of a family of large molecular weight, fucose-containing glycopeptides including the F9 antigen. Second, α-1-3 linked fucose, which is found in fucoidan, is a critical part of the recognition determinant of stage-specific antigen SSEA-1 detected on mouse embryos and teratocarcinoma stem cells (24, 25). Further studies will aid our understanding of the role of the stem cell lectin and its potential receptors in intercellular adhesion.

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