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Evolutionary conservation of key structures and binding functions of neural cell adhesion molecules

(cell–cell adhesion/adhesive specificity/morphogenesis/cell surface proteins/brain development)

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ABSTRACT **The neural cell adhesion molecule N-CAM is a sialic acid-rich, cell surface glycoprotein that mediates cell adhesion by a homophilic mechanism. Its binding function has been implicated in both morphogenesis and histogenesis; during development it changes in amount at the cell surface and perinatally it undergoes a decrease in sialic acid content (embryonic → adult conversion) with an increase in binding efficacy. In the present study, salient aspects of the structure and the mutual binding specificities of N-CAMs from a variety of vertebrate species were examined to determine whether (N-CAM)-mediated adhesion mechanisms have been conserved during evolution. N-CAM immunoreactivity was detected in a series of polypeptides of characteristic molecular weight extracted from brain tissues of all vertebrate species tested, including mammals, birds, reptiles, amphibia, and bony and cartilaginous fish. Adhesion mediated by N-CAM occurred across species lines as indicated by the co-aggregation of chicken and mouse neural cells. By using a quantitative membrane vesicle aggregation assay, the efficacy of cross-species brain membrane vesicle adhesion in various pairings (chicken–mouse, chicken–frog, mouse–frog) was found to be similar to the efficacy of intra-species adhesion. Effective cross-species aggregation of brain membrane vesicles also occurred in embryonic–embryonic, adult–adult, and embryonic–adult pairings. In a control experiment, embryonic chicken liver membrane vesicles (which do not contain N-CAM) did not co-aggregate with embryonic chicken brain membrane vesicles. Cross-species co-aggregation could be inhibited by Fab′ fragments of antibodies to N-CAM and was most effectively inhibited in the presence of mixtures made from the Fab′ fragments of specific antibodies prepared against the N-CAMs from each of the animal species constituting a co-aggregating pair. These results suggest that, in accord with the proposed role of N-CAM as a regulator of morphogenesis, both the specificity of the binding region of the molecule and its basic chemical structure have been highly conserved during evolution.**

The histologic differences and differences in form that distinguish vertebrate species probably result from covariant alterations (1) during evolution in the relative rates of two or more of the primary morphogenetic processes of cell adhesion, cell migration, cell proliferation, cell differentiation, and cell death. Work on cell adhesion molecules or CAMs (2, 3) provides one opportunity to test the hypothesis (1) that pattern formation may be mediated in part through the regulatory effects on other primary processes of a relatively small number of CAMs of different specificities, provided that their adhesive efficacies could be graded over a wide range by various forms of surface modulation (3), including differential expression and chemical modification. This hypothesis suggests that major changes in vertebrate form attributable to adhesion would most likely have arisen by alterations during evolution of various regulatory genes for CAMs rather than by major changes in the structure and specificity of the CAMs themselves.

Previous studies have indicated that N-CAM, the neural cell adhesion molecule, mediates cell–cell adhesion in chicken (4–6), rodent (7, 8), human (9), and frog (10) neural tissues. The importance of N-CAM in morphogenesis is suggested by sequential changes in its distribution during early development (11, 12). N-CAM undergoes alterations in prevalence at the cell surface during critical periods of primary and secondary induction in a fashion that is spatio-temporally coordinated with changes in the prevalence and distribution of another non-neural adhesion molecule, liver CAM (L-CAM) (12). These observations raise the possibility that embryonic induction and CAM modulation may be causally linked (1).

N-CAM is also involved in later histogenetic processes (3), including highly patterned events such as the formation of the retinotectal projection (10). During this developmental epoch, N-CAM converts from a sialic acid-rich embryonic (E) form having a microheterogeneous electrophoretic distribution to several adult (A) forms, which contain approximately one-third as much sialic acid and which migrate on $NaDodSO_4$ gel electrophoresis as discrete components of M_r 180,000, 150,000, and 120,000 (13–15). E → A conversion occurs gradually, is exhibited to varying degrees and at varying rates in different brain regions (16), and is delayed in the cerebellum of the neurological mutant *staggerer* (14). The possible functional significance of these changes is suggested by analyses (6) of the rates of vesicle aggregation that indicate that the efficacy of N-CAM to N-CAM (homophilic) binding is inversely related to the sialic acid content of the participating molecules. The predominance of modulation mechanisms and the distribution patterns of N-CAM raise the question whether N-CAM specificity is conserved in vertebrate evolution.

In the present study, an estimate of the degree of conservation of N-CAM structure and function was made across a wide range of vertebrate species by employing a series of immunological, electrophoretic, and aggregation assays. The two main findings are (*i*) N-CAM polypeptides of characteristic electrophoretic behavior and immunological cross-reactivity can be detected in descendants of early vertebrates such as the shark and in all higher species tested and (*ii*) within the sensitivity range of present assays, cross-species aggregation of membrane vesicles containing N-CAMs prepared from mouse, chicken, and frog brains occurs as readily as intra-species vesicle aggregation.

MATERIALS AND METHODS

Animals. White Leghorn chickens and BALB/c and NCS mice were obtained from a variety of sources. Adult frogs

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Abbreviations: CAM, cell adhesion molecule; N-CAM, neural CAM; L-CAM, liver CAM; E and A, embryonic and adult forms of N-CAM.

and stage 55 tadpoles (*Xenopus laevis*) were obtained from Nasco (Fort Atkinson, WI). Other species were obtained from the JBJ Pet Shop (New York).

Antibodies. Rabbit anti-chicken N-CAM (17), anti-mouse N-CAM (7), and anti-frog N-CAM (10) IgG and Fab' fragments (18) were prepared following published procedures.

Cross-Species Cell Adhesion. Embryonic mouse (16 day) and embryonic chicken (9 day) cells were prepared by using 0.002% trypsin in the presence of 1 mM EDTA (19). Mouse cells, labeled with diacetyl fluorescein (19), and chicken cells, labeled with rhodamine isothiocyanate (20), were co-incubated (4×10^7 cells from each species in 1.5 ml of minimal essential medium for suspension culture, 30 min, 37°C, 90 rpm) in the presence of either 1 mg of nonimmune Fab' or a mixture of 500 μg each of anti-(mouse N-CAM) and anti-(chicken N-CAM) Fab'.

Quantitative Measurement of Membrane Vesicle Aggregation. To prepare brain membrane vesicles, brains in calcium/magnesium-free medium (17)/Trasylol (Mobay Chemical, New York) (200 units/ml)/deoxyribonuclease I (Worthington) (0.5 mg/ml) were homogenized using a Polytron (Brinkmann) (five 2-sec bursts at setting 5) followed by a tight-fitting steel Dounce homogenizer (10 strokes). The homogenate was mixed with 2 vol of 70% (wt/vol) sucrose, layered in the bottom of SW27 ultracentrifuge tubes (Beckman), overlaid with 42%, 26%, and 10% sucrose in phosphate-buffered saline, pH 7.4 (P_i/NaCl), and centrifuged 2 hr at 4°C, at 25,000 rpm. The turbid material at the 26%/42% sucrose interface was collected, washed, and resuspended in P_i/NaCl. Liver membrane vesicles were prepared as described (21) and material at the 37%/41% and 41%/45% sucrose interfaces was pooled.

Membrane vesicle aggregation experiments were performed and monitored as described (6) with the following exceptions: (*i*) vesicles were passed through 0.6-μm pore-size filters (Nucleopore) before each experiment and (*ii*) the experiment shown in Fig. 3*B* was done in L-CAM assay buffer (21) containing Ca^{2+}, which is required for the aggregation of the liver membrane vesicles. Rates of membrane vesicle aggregation varied <10% between duplicate experiments.

RESULTS

In addition to criteria previously established (7, 9, 17), in the present studies we used several means to test for evolutionary conservation of N-CAM structure and function: (*i*) immune cross-reactivity among a broad range of species detected by using polyclonal anti-(N-CAM) antibodies, (*ii*) similar electrophoretic migration patterns of the adult forms of N-CAM from these species, and (*iii*) (N-CAM)-mediated adhesion between cells of different species followed by quantitative assessment of the relative efficacy of cross-species and intra-species aggregation of membrane vesicles.

Conservation of Characteristic N-CAM Antigenic Determinants and Electrophoretic Patterns Among Vertebrates. N-CAM was originally isolated from chicken neural tissue and several monoclonal antibodies were prepared that recognized the molecule. One of the antibodies, 15G8, was used to immunoaffinity purify N-CAM from human (9), rodent (7), and frog (10) brains. The following members of subphylum vertebrata were surveyed for the existence of N-CAM immunoreactive material using a mixture of rabbit antibodies to mouse, chicken, and frog N-CAM to maximize potential cross-reactivity: human, mouse, rat (class Mammalia); chicken, canary (class Aves); turtle, snake, lizard (class Reptilia); frog, salamander, newt (class Amphibia); goldfish, blue fish (class Osteichthyes); and shark (class Chondrichthyes). Immunoblot analysis of these adult brain extracts indicated that some or all of the characteristic (13–15) adult-form N-CAM polypeptides (M_r 180,000, 150,000, and 120,000) were present in all of the species tested. Results from several representative species are shown in Fig. 1. In every case, the M_r 180,000 polypeptide was predominant. These polypeptides were also detected by immunoblot analysis using monoclonal antibody 15G8 (7, 14, 16), although the relative intensities of the components differed from those obtained with rabbit anti-(N-CAM) antibodies (data not shown).

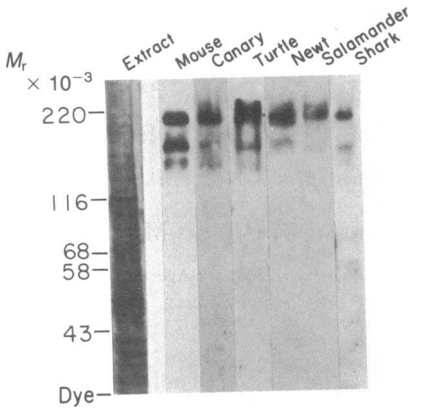

FIG. 1. Polypeptides of characteristic molecular weight from a variety of species react with anti-(N-CAM) antibodies. Nonidet P-40 extracts (100 μg protein) of crude brain membrane vesicles were prepared from adults of the indicated species and analyzed by immunoblotting using a mixture of rabbit anti-mouse, chicken, and frog N-CAM antibodies (50 μg each), and the bound antibodies were detected as described (16). The migration of reference proteins is indicated by their $M_r \times 10^{-3}$. The leftmost lane shows that a typical brain extract (in this case, salamander) is composed of many proteins detectable by amido black staining. The right lanes are autoradiographs showing the polypeptides that react with anti-(N-CAM) antibodies. Note that the only polypeptides recognized by the antibodies corresponded in molecular weight to the characteristic adult-form N-CAM polypeptides (M_r 180,000, 150,000, and 120,000).

N-CAM Mediates Cross-Species Neural Cell Adhesion. To determine qualitatively whether cross-species neural cell adhesion occurs and is mediated by N-CAM, fluorescein-labeled mouse brain cells and rhodamine-labeled chicken brain cells were co-incubated in the presence of non-immune Fab' fragments or a mixture of Fab' fragments prepared against mouse N-CAM and chicken N-CAM. A comparison of Fig. 2 *a* (phase contrast), *b* (fluorescein epifluorescence), and *c* (rhodamine epifluorescence) clearly indicates that composite aggregates containing both mouse and chicken cells were formed in the presence of non-immune Fab'. In the presence of the mixture of anti-(N-CAM) Fab' fragments, however, no aggregation occurred (Fig. 2*d*). Dramatic examples of co-aggregation were also obtained when mouse and chicken cells were first allowed to self-aggregate before being used in a cross-species adhesion experiment. During co-incubation, large aggregates formed (Fig. 2*e*), which contained blocks of both mouse (Fig. 2*f*) and chicken (Fig. 2*g*) cells.

Although these results provided qualitative evidence that mouse and chicken neural cells can co-aggregate by an (N-CAM)-dependent mechanism, the relative affinity of mouse–chicken, mouse–mouse, and chicken–chicken cell interactions could not readily be compared by these methods. To evaluate the degree of cross-reactivity in N-CAM binding among species, a quantitative co-aggregation assay was therefore devised.

Quantitative Measurement of Co-Aggregation. In a previous study (6), we devised a quantitative vesicle aggregation assay in which rates of aggregation of native membrane vesicles and reconstituted lipid vesicles containing purified N-CAM were monitored and then expressed in terms of accumulation of vesicle aggregates larger than a given threshold

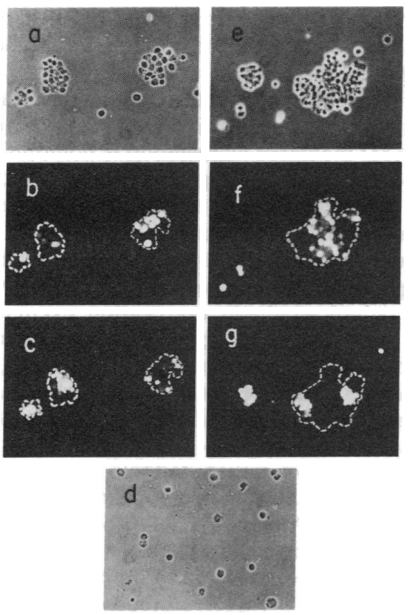

FIG. 2. Co-aggregation between mouse and chicken brain cells. Co-aggregates formed in the presence of non-immune Fab' (*a* and *e*, phase-contrast microscopy) and were composed of both mouse cells (*b* and *f*, fluorescein optics) and chicken cells (*c* and *g*, rhodamine optics). In the presence of anti-(N-CAM) Fab', no aggregation occurred (*d*, phase-contrast microscopy). The dashed white lines indicate the contours of co-aggregates determined by comparison of these fields with *a* and *e*. (×80.)

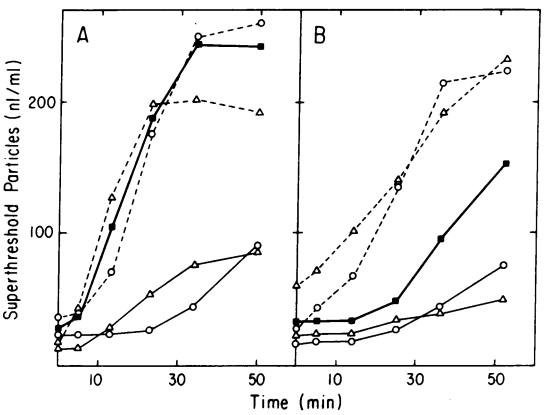

FIG. 3. Assay demonstrating co-aggregation and independent aggregation between mixed membrane vesicles. The time course of membrane vesicle aggregation was analyzed, and in each panel, the bold curve represents the aggregation of a mixture of two different vesicle preparations, whereas the other four curves are controls representing the aggregation of different concentrations of the individual preparations. Vesicle concentrations are expressed below in μg of protein per ml (22). (*A*) Co-aggregation of embryonic (E) and adult (Ad) chicken brain membrane vesicles. —○—, E, 37 μg/ml; —△—, Ad, 30 μg/ml; --○--, E, 74 μg/ml; --△--, Ad, 60 μg/ml; and ■, E, 37 μg/ml plus Ad, 30 μg/ml. (*B*) Independent aggregation of embryonic chicken brain (B) and liver (L) membrane vesicles. —○—, B, 40 μg/ml; —△—, L, 260 μg/ml; --○--, B, 80 μg/ml; --△--, L, 520 μg/ml; and ■, B, 40 μg/ml plus L, 260 μg/ml. Note that the aggregation of the mixture of aliquots of embryonic and adult chicken brain membrane vesicles in *A* (■) is comparable to the aggregation of a double aliquot of each individual preparation (--○--, --△--), indicating effective co-aggregation. In contrast, the aggregation of the mixture of aliquots of embryonic chicken brain and liver membrane vesicles in *B* (■) is only comparable to the sum of the aggregation of single aliquots of each individual preparation (—○—, —△—), indicating independent aggregation.

(1.5 μm^3). Analyses of plots of the superthreshold aggregate accumulation revealed two key properties of these vesicle preparations: (*i*) there was an apparent lag before the commencement of aggregation probably because the earliest aggregation events do not generate superthreshold particles and (*ii*) following this lag, the accumulation of superthreshold particles proceeded at a rate proportional to the second power of the initial vesicle concentration.

The quantitative co-aggregation assay devised for the present work takes advantage of the apparent second-order dependence on vesicle concentration of the rate of superthreshold aggregate formation, using it to discriminate between pairs of vesicle preparations that co-aggregate and those that do not. For example, if two vesicle preparations that do not co-aggregate are mixed, the rate of aggregation of the mixture will simply be the sum of the rates of aggregation of each component as if those rates had been estimated in separate tubes at similar dilutions. On the other hand, mixing of two vesicle preparations from different species that fully co-aggregate should yield results that are no different than those obtained after mixing two aliquots of a vesicle preparation from one species—i.e., doubling the vesicle concentration by adding two equal aliquots of vesicles will quadruple the initial rate of vesicle aggregation.

Prior to a co-aggregation experiment, dilutions of each vesicle preparation were tested to determine the concentration that yielded a plateau level of aggregation of about 200 nl/ml of superthreshold particles. To assess the degree of co-aggregation, the aggregation of five samples was compared: the predetermined dilution of each preparation as described above, one-half that concentration for each preparation, and, finally, a mixture of the lower concentrations of each preparation. This assay readily distinguishes between pairs of membrane vesicle preparations that do co-aggregate (e.g., embryonic and adult chicken brains; Fig. 3*A*) and those that do not (e.g., embryonic chicken brain and liver; Fig. 3*B*).

Cross-Species Co-Aggregation of Brain Membrane Vesicles. To evaluate whether the efficacy of the cross-species binding between chicken and mouse neural cells obtained in the qualitative experiment shown in Fig. 2 was comparable to the efficacy of their intra-species binding, the co-aggregation of membrane vesicles prepared from chicken and mouse brains was analyzed (Fig. 4). The experiments indicated that mouse and chicken brain membrane vesicles co-aggregated as effectively as they self-aggregated. The degree of co-aggregation was found to be similar when the membrane vesicles were derived from embryonic chicken and embryonic mouse (Fig. 4*A*), embryonic chicken and adult mouse (Fig. 4*B*), or adult chicken and adult mouse (Fig. 4*C*). These results are consistent with previous studies (6), which showed that the loss of sialic acid from N-CAM that occurs during development increases the rate of N-CAM to N-CAM interactions but does not alter the specificity of binding.

Cross-species co-aggregation of brain membrane vesicles is not limited to the chicken–mouse pair. Co-aggregation was equally effective in several additional pairings with frog brain membrane vesicles. These included embryonic mouse–tadpole (Fig. 5*A*) and embryonic chicken–tadpole, adult frog–embryonic chicken, adult frog–adult chicken, and adult frog–adult mouse (data not shown).

Anti-(N-CAM) Fab' Fragments Inhibit Cross-Species Aggregation. Fab' fragments of antibodies against purified N-CAM from mouse, chicken, and frog inhibited the rate of within-species aggregation of vesicles from embryonic mouse, embryonic chicken, and tadpole brains, respectively, >90% at very low titers [as low as 7 μg of anti-(mouse N-CAM) Fab' vs. 30 μg of embryonic mouse brain vesicle membrane protein]. Significant functional cross-reactivity was also observed in certain antibody–vesicle pairings; for example, anti-(chicken N-CAM) Fab' fragments blocked the aggregation of embryonic mouse brain vesicles at a titer only

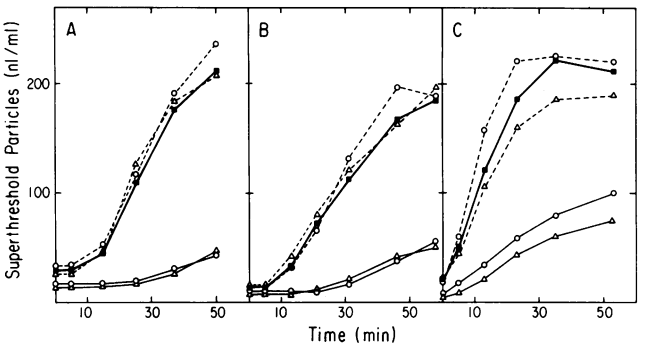

FIG. 4. Co-aggregation of embryonic and adult chicken and mouse brain membrane vesicles. Assay conditions as in Fig. 3. (*A*) Co-aggregation of embryonic chicken (EC) and embryonic mouse (EM) brain membrane vesicles. —○—, EC, 38 μg/ml; —△—, EM, 41 μg/ml; --○--, EC, 76 μg/ml; --△--, EM, 82 μg/ml; and ■, EC, 38 μg/ml plus EM, 41 μg/ml. (*B*) Co-aggregation of embryonic chicken (EC) and adult mouse (AM) brain membrane vesicles. —○—, EC, 37 μg/ml; —△—, AM, 32 μg/ml; --○--, EC, 74 μg/ml; --△--, AM, 64 μg/ml; and ■, EC, 37 μg/ml plus AM, 32 μg/ml. (*C*) Co-aggregation of adult chicken (AC) and adult mouse (AM) brain membrane vesicles. —○—, AC, 35 μg/ml; —△—, AM, 30 μg/ml; --○--, AC, 70 μg/ml; --△--, AM, 60 μg/ml; and ■, AC, 35 μg/ml plus AM, 30 μg/ml. Note that, in *A–C*, the aggregation of the mixture of aliquots from two brain membrane vesicle preparations (■) is comparable to the aggregation of a double aliquot of either individual preparation (--○--, --△--), indicating effective co-aggregation.

four times that required for the inhibition of embryonic chicken brain vesicle aggregation. On the other hand, there was little functional cross-reactivity observed with antibodies to mouse N-CAM and to frog N-CAM. The concentration of anti-(frog N-CAM) antibodies necessary to inhibit embryonic mouse brain vesicle aggregation was at least 50-fold higher than the concentration necessary to inhibit equally

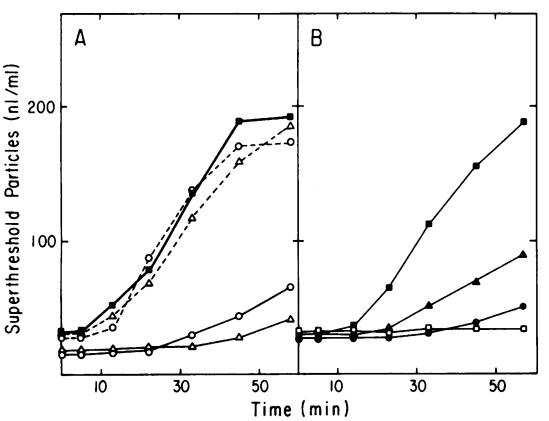

FIG. 5. Co-aggregation of embryonic mouse (EM) and tadpole (T) brain membrane vesicles and inhibition by anti-(N-CAM) Fab' fragments. Assay conditions as in Fig. 3. (*A*) Demonstration of co-aggregation. —○—, EM, 37 μg/ml; —△—, T, 32 μg/ml; --○--, EM, 74 μg/ml; --△--, T, 64 μg/ml; and ■, EM, 37 μg/ml plus T, 32 μg/ml. Note that the aggregation of the mixture of aliquots of embryonic mouse and tadpole brain membrane vesicles (■) is comparable to the aggregation of a double aliquot of each individual preparation (--○--, --△--), indicating effective co-aggregation. (*B*) Inhibition by non-cross-reactive anti-(N-CAM) Fab' fragments. Each curve represents the time course of co-aggregation of embryonic mouse brain membrane vesicles (37 μg/ml protein) plus tadpole brain membrane vesicles (32 μg/ml protein) in the presence of the following Fab' fragments: ■, normal rabbit, 140 μg/ml; ▲, anti-(frog N-CAM), 140 μg/ml; ●, anti-(mouse N-CAM), 140 μg/ml; and □, anti-(frog N-CAM), 70 μg/ml plus anti-(mouse N-CAM), 70 μg/ml. Note that, although the total Fab' concentration is identical in each case, aggregation is more effectively inhibited by a mixture of antibodies to mouse and frog N-CAM (□) than by either antibody alone (●, ▲).

tadpole brain vesicle aggregation; anti-(mouse N-CAM) antibodies were similarly ineffective in inhibiting tadpole brain vesicle aggregation.

This lack of cross-species functional inhibition was used to test further the previous conclusion (3, 5, 6) that the mechanism of (N-CAM)-mediated aggregation is homophilic—i.e., N-CAM to N-CAM. If co-aggregation of embryonic mouse–tadpole brain vesicles is mediated by cross-species interactions, then antibodies to mouse N-CAM should inhibit mouse–mouse aggregation events and should partially inhibit mouse–tadpole aggregation events but should have almost no effect on tadpole–tadpole aggregation events. The reverse should hold for anti-(frog N-CAM) antibodies. It follows, then, that to inhibit mouse–tadpole co-aggregation most effectively, it would be necessary to include antibodies to both mouse and frog N-CAM to inhibit mouse–mouse vesicle aggregation and tadpole–tadpole vesicle aggregation, and, above all, to inhibit mouse–tadpole vesicle aggregation optimally by perturbing both sides of the cross-species and the intra-species N-CAM to N-CAM bonds. The observed inhibitions of embryonic mouse–tadpole brain vesicle aggregation by antibodies to mouse and frog N-CAM were found to fit this model (Fig. 5*B*). Each antibody alone inhibited aggregation only partially; in contrast, a mixture containing one-half as much of each antibody completely inhibited aggregation.

DISCUSSION

A number of observations made in both the present and previous studies indicate a high degree of conservation of N-CAM structure among various species. (*i*) N-CAM from embryonic human (9), mouse (7), rat (7), chicken (17), and frog (unpublished data) migrated on $NaDodSO_4$ gels in a broad zone of apparent M_r 200,000–250,000. Their microheterogeneous appearance upon electrophoresis and their recognition by monoclonal antibody 15G8 suggest that embryonic N-CAMs from all of these species contain unusual sialic acid-rich oligosaccharides. (*ii*) N-CAMs from adult organisms of all vertebrate species tested (from shark through human) showed three distinct components of M_r 180,000, 150,000, and 120,000. (*iii*) N-CAM has been found to convert from the embryonic to adult forms during the period of brain histogenesis in several different species (ref. 16; unpublished data). (*iv*) Amino acid compositions of N-CAMs from different species are similar (7, 9, 17). (*v*) Antigenic cross-reactivity is observed between N-CAMs from different species. (*vi*) As shown here, in addition to mediating the self-aggregation of brain membrane vesicles from chicken, mouse, and frog, N-CAM mediates cross-species aggregation between any pair of these species with equal efficacy. These results suggest that the tertiary structure and binding specificity of the domain of N-CAM involved in cell adhesion have been highly conserved during evolution. The actual degree of conservation of N-CAM primary structure and of its post-translational modifications remains to be determined by more precise structural studies and gene cloning techniques (23).

The existence of tissue-specific cross-species cell aggregation was observed many years ago (24), but its significance could not be assessed in detail because the molecular mechanisms underlying the adhesion had not been analyzed. The current observation that mouse and chicken neural cells co-aggregate by an (N-CAM)-dependent mechanism led us to design a quantitative assay to compare the relative efficacy of intra-species vs. cross-species adhesion. By using this assay, which was based on previous studies (6), it was determined that membrane vesicles from any pair of mouse, chicken, and frog brains co-aggregated as effectively as they self-aggregated, whereas control membrane vesicles from embryonic chicken brain and liver did not co-aggregate.

Cross-species co-aggregation occurred equally effectively with embryonic membranes, adult membranes, or mixtures of embryonic and adult membranes, in accord with previous results indicating that the E → A conversion in N-CAM structure increases the rate of membrane vesicle aggregation but does not alter its specificity.

When mouse and chicken neural cells were coincubated, co-aggregates formed that frequently contained clumps of two to four cells from the same species (Fig. 2 *b* and *c*). At present, we cannot distinguish whether this effect is due to occasional incomplete dissociation of the parent tissue, to species-specific adhesive mechanisms mediated by molecules other than N-CAM, or to a higher intra-species N-CAM binding affinity. More sensitive assays than the vesicle co-aggregation assay might, in fact, reveal fine differences in cross-species aggregation that were not detected in the present studies.

The current observation that cross-species vesicle aggregation is most effectively inhibited when antibodies against N-CAM from both species are present further supports the conclusion that N-CAM mediates adhesion by a homophilic mechanism—i.e., N-CAM on one cell binds to N-CAM on a second cell (6). In view of the symmetry requirements of homophilic binding and the impossibility of mirror symmetry in proteins made of L-amino acids, it would seem that two different complementary sites or subsites ought to exist within each N-CAM binding domain to mediate N-CAM to N-CAM interactions. Mutations altering either of these sites would, by symmetry, affect binding of the complementary site; those that diminished binding would therefore be particularly deleterious. The observed conservation of the specificity of N-CAM homophilic binding suggests that the structure of each of these complementary binding sites has been evolutionarily conserved.

Characteristic N-CAM polypeptides appeared in all of the vertebrate species examined here, including the cartilaginous fish, suggesting that (N-CAM)-like precursors were present on ancestor species arising at least 470 million years ago (25). The fact that mouse and frog N-CAMs can bind effectively to each other even though the ancestors of these species diverged about 400 million years ago (25) indicates that the conservation of N-CAM function is almost equally ancient. Such observations do not rule out the possibility that N-CAM analogues exist in invertebrate species that arose much earlier; indeed, in view of the relatively ancient origin of neural tissue, we expect such molecules to be found.

The results of the present study are consistent with the idea that (N-CAM)-mediated morphogenetic events are fundamental in developmental regulation and suggest that over relatively long periods of evolutionary time little variation in the specificity of those regions of N-CAM that mediate binding and cell adhesion has occurred. In view of occasional large differences in morphology within nervous systems of the same and different animal classes, this, in turn, suggests that differences in schedules of CAM gene expression (1) and CAM modulation (3) are likely to be found both within and across different vertebrate classes, accounting in part for their differences in form.

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