Giant Axonal Neuropathy: A Conditional Mutation Affecting Cytoskeletal Organization

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ABSTRACT Giant axonal neuropathy (GAN) results from autosomal recessive mutations (gan-) that affect cytoskeletal organization; specifically, intermediate filaments (IFs) are found collapsed into massive bundles in a variety of different cell types. We studied the gan- fibroblast lines WG321 and WG139 derived from different GAN patients. Although previous studies implied that the gan- IF phenotype was constitutive, we find that it is conditional. That is, when cells were grown under the permissive condition of medium containing over 2% fetal calf serum, most cells had normal IF organization. IF bundles formed when gan- cells were transferred to the nonpermissive condition of low (0.1%) serum. Microtubule organization appeared normal in the presence or absence of serum. The effect of serum starvation was largely blocked or reversed by the addition of BSA to the culture media. We found no evidence that the gan- phenotype depends upon progress through the cell cycle. We discuss the possible role of serum effects in the etiology of GAN and speculate as to the molecular nature of the gan- defect.

Giant axonal neuropathy (GAN) is a rare, slowly progressive central–peripheral distal axonopathy (1, 40, 41); symptoms first appear in children between the ages of 3 and 5 yr. The identification of related siblings with GAN (21), parental consanguinity of some GAN patients (11, 18, 41), studies of a genetic disease with similar symptoms in inbred dogs (7, 8), and the persistence of aberrant intermediate filament (IF) phenotype in cultured cells derived from GAN patients (37–39; see below) indicate that GAN is a genetic disease, and results from an autosomal recessive mutation(s) (gan-). For the student of cell biology, GAN is of particular interest because it is one of only a handful of mutations known to affect cytoskeletal organization and function. Most of these mutations affect either actin or tubulin genes; they include mutations in Saccharomyces cerevisiae (35, 47), Schizosaccharomyces pombe (50), Aspergillus (34, 46), Drosophila melanogaster (23, 24), Caenorhabditis elegans (15), and cultured Chinese hamster ovary cells (4–6, 22). gan- is the only mutation known to affect IF organization specifically.

Diagnosis is of GAN is based upon the presence of large, focal accumulations of neuronal IFs that cause regions of nerve axons to "balloon." These IF bundles first appear distally in neurons that project into the periphery; as the disease progresses, central neurons that do not project into the periphery can also become affected (25, 40). Ultrastructural examination of gan- tissues reveals abnormal IF bundles not only in neurons, which express the neuron-specific triplet of IF subunit proteins; but also in astrocytes, Schwann cells, endothelial cells, and skin fibroblasts (40, 41), which express their own distinctive IF subunit proteins (see reference 36 for review). In addition, all but two of the GAN patients reported (20, 40) have had unusually kinky hair (1, 41), which is of interest because hair is composed of epithelial IF (10, 51).

To characterize the molecular defect underlying GAN, we have studied two independently isolated, gan- human fibroblast cell lines. In contrast to previous reports (37–39), we find that gan- mutations have a conditional, not a constitutive IF phenotype, and that this phenotype appears to be mediated by serum factors.

MATERIALS AND METHODS

Cell Culture: We studied the gan- skin fibroblast lines, WG321 and WG139, and the normal (GAN+) human foreskin fibroblast line, MCH7, which

1 Abbreviations used in this paper: anti-IF(1.4E9), monoclonal anti-intermediate filament antibody; anti-Mlg-Ft, fluorescein-conjugated, affinity-purified goat anti-mouse immunoglobulin antiserum; anti-Mlg-Rd, tetramethylrhodamine-conjugated, affinity-purified goat anti-mouse immunoglobulin antiserum; anti-MTOC, monoclonal anti-microtubule-organizing center; GAN, giant axonal neuropathy; gan-, autosomal recessive mutations; IF, intermediate filament; MEM, minimal essential medium; MTOC, microtubule-organizing center.
were obtained from the Repository of Mutant Human Cell Stains, deBelle Laboratory for Biochemical Genetics/McGill University–Montreal Children's Hospital Research Institute. Cells were grown in minimal essential media, Eagle’s modification (MEM), supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, and antibiotics, either penicillin/streptomycin or gentamicin (10% MEM). Alternatively, 10% Nu-Serum (Collaborative Research Inc., Waltham, MA), 6-20 mg/ml BSA, Cohn fraction V (Sigma Chemical Co., St. Louis, Mo.—lot 113F-0273, described as 96-99% albumin), or 10 mg/ml ovalbumin (Sigma Chemical Co., grade V—lot 33F-8110, described as electrophoretically pure and 99% pure) were substituted for fetal calf serum. The purity of the BSA used in our experiments was over 98% as determined by PAGE (30).

Cells were grown at 37°C in a humidified, 5% CO2 incubator, and were used at low passage number (<12) and low total cell doublings (estimated to be <40). Cells were passaged using trypsin and EDTA. For immunofluorescence microscopy, cells were grown on glass coverslips. Cells were fed every 3-4 d with fresh 10% MEM; experiments were performed 8-14 d after plating. When media conditions were changed, cultures were washed three times with warmed MEM containing HEPES, and then fed with medium containing appropriate additives. Cells could be maintained in 0-0.1% serum for over 5 d without apparent effects on viability. Occasionally we observed wholesale cell death in some colonies in response to repeated cycles of serum starvation; although some colonies died, the majority appeared normal. In addition, we observed the colonies to be an increase in the percentage of cell nuclei marking the positions of MTOC as determined by examination of cells under fluorescein optics (not shown). The percentage of cells containing detectable IF bundles was often <20% (Fig. 1 C). As reported by Pena et al. (39), microtubule organization appeared normal in all cells (Fig. 2).

We initially assumed that the variability in IF organization in gan· cells was due to difference between clones. We have observed apparent clonal variations in the organization of HeLa (31) and LCC-PK1 cell IFs (Klymkowsky, M. W., unpublished observations). To test the possibility that clonal variation was the cause of the discrepancy between our results and those of Pena, 200-400 WG321 or WG139 cells were plated into 60-mm diameter culture petri dishes; examination of these cultures within 24 h of plating confirmed that >80% of the cells were single, isolated colonies. Within 8-12 d colonies had grown up from these cells, and the presence of IF bundles was assayed using the Coomassie Blue-staining method; every colony contained some cells with and some without IF bundles (Figs. 1 C and 3 A). The percentage of cells with IF bundles varied among colonies within the same culture (Fig. 3 A).

Reasoning that variations in IF organization between clonally derived gan· cells could be due to their relative positions within the cell cycle, cells growing in colonies were synchronized by serum starvation (0-0.1% fetal calf serum). Within 24 h, the majority of serum-starved WG139 and WG321 cells contained IF bundles (Figs. 1 F and 3 A). Examination of serum starvation-induced IF reorganization by immunofluorescence microscopy revealed that the initial phases of IF

**RESULTS**

Pena first described the presence of large bundles of vimentin-type IFs in the cultured gan· fibroblast lines WG321, WG129, and WG791 (37, 38). These reports claimed that >90% of the cells contained IF bundles; no mention of variations in the percentage of cells containing IF bundles was made. We were therefore surprised to find in our initial immunofluorescence studies that both WG139 and WG321 cells showed either a normal or a "collapsed" IF phenotype (Fig. 1, A-C), and that the percentage of cells containing detectable IF bundles was often <20% (Fig. 1 C).

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![FIGURE 1 IF organization in WG321 cells grown continuously in 10% MEM (A–C) or 24 h after transfer to 0.1% fetal calf serum containing medium (D–F) was examined by indirect immunofluorescence microscopy (A and D, phase optics; B and E, rhodamine optics). Coomassie Brilliant Blue staining was used to examine the overall organization of IFs in gan· cells. C29 (anti-MTOC) and 14E9 (anti-IF) were used as immunogen and reacts with human, chick, and mouse vimentin (Klymkowsky, M. W., work in progress). Microtubule organization was visualized by using the monoclonal antitubulin antibody 4A1 (anti-tubulin) generated by Dr. M. Fuller (University of Colorado) and a monoclonal anti-mouse immunoglobulin antisera (anti-MIg-Rd) (1:100). Under these conditions, the anti-IF (1.4E9) and anti-MIg-Rd antibodies labeled the anti-MTOC (anti-Mlg-F1) complex, the small size of the microtubule-organizing center (MTOC) led to cell immune reaction to a small number of cells growing under bright-field conditions. Cells with IF bundles could be identified unambiguously (Fig. 1, C and F). Typically, between 40 and 50% of the cells (200-1,200 cells) within each of 10-14 independent colonies were examined for each experimental condition. In certain cases (see Figs. 3 B), all of the cells in the colonies examined were scored. Colonies were chosen for examination at random and once chosen no colony was discarded.

**Autoradiography:** Cells undergoing active DNA synthesis were labeled by incubating cultures for 24 h in 1 μCi [3H]thymidine (New England Nuclear, Boston, MA) per milliliter of media. After Coomassie staining and air-drying (see above), culture dishes were coated with Kodak NTB-2 emulsion diluted 1:1 with water. The emulsion was dried at room temperature, exposed at 0°C for 7-10 d, and developed with D19, as described by Stein and Yanishlevsky (48). Nuclei with >50 autoradiographic grains on them were scored as labeled; this value was chosen to provide an unambiguous index of labeling; lower numbers of autoradiographic grains were often obscured by the Coomassie Brilliant Blue staining of nuclei (see Fig. 1). Most nuclei (>80%) were very heavily labeled, i.e., >500 grains. In this series of experiments, background labeling was <1 grain per 100 μm².

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collapse were evident within 4 h after the removal of serum (not shown), and essentially complete in most cells within 24 h (Figs. 1, D–F and 3A). Parallel studies of colchicine-induced IF bundle formation in normal GAN+ MCH7 cells indicated that the time course of IF collapse in gan− cells is similar to that observed in response to microtubule depolymerization (not shown). In contrast to drug-induced IF collapse, microtubules remained intact within serum-starved, gan− cells (Fig. 2, C and D). Because both WG321 and WG139 cells showed similar conditional IF phenotypes (Fig. 3A), we chose to carry out most of our experiments using the WG321 cell line.

We have never observed a serum starvation-induced collapse of IF in normal MCH7 (GAN+) fibroblasts; their IF networks appeared normal by both immunofluorescence microscopy and Coomassie staining in the presence or absence of serum (not shown). Only rarely, i.e., in <0.1% of the cells, were small IF bundles found in MCH7 cells (not shown).

The collapse of IF was readily reversed by returning serum-starved cells to fetal calf serum-containing media (Fig. 3A). Normal IF organization as also found when Nu-Serum, a low-protein serum substitute, was used instead of fetal calf serum (Fig. 4A, a and g). Recovery of a normal IF phenotype was complete in most cells after 24 h in fetal calf serum or Nu-Serum (Figs. 3B and 4g). Titration of the fetal calf serum effect on IF organization revealed a threshold level of ~1% serum (Fig. 3B). There was a clear increase in the variability of IF phenotype between colonies at serum concentrations between 0.2% and 0.5% (Fig. 3B), suggesting that the gan− IF phenotype of different WG321 cell clones had different serum-threshold levels.

Immunofluorescence labeling of WG321 cells with antitubulin antibody revealed normal, intact microtubule systems...
in both the presence (Fig. 2, A and B) and absence (Fig. 2, C and D) of serum. IF bundles can often be identified under phase-contrast optics (Fig. 2 C—arrows), there seemed to be little interaction between the microtubule system and IF bundles in most cases (Fig. 2, C and D). The MTOC remained near the nucleus (Figs. 1 and 2 D) in most cells (although see Fig. 1 B), and sometimes appeared to associate with the periphery of the IF bundle (Fig. 1, B and E). Whether associations between IF bundles and MTOC were fortuitous or reflect significant interaction between the two remains to be determined.

Serum-induced recovery of normal IF organization occurred when DNA synthesis was inhibited by either 10 mM thymidine or 2 mM hydroxyurea (Fig. 4, h and i), which indicates that progress through the S phase of the cell cycle was not required for normal IF phenotype in gan+ cells. Autoradiographic analysis of gan+ cells recovering from serum starvation-induced IF collapse revealed no obvious correlation between DNA synthesis (mitotic index) and recovery of normal IF organization (Fig. 3 B). We have not attempted to determine whether protein synthesis was required for the reorganization of IF because protein synthesis inhibitors themselves have been reported to affect IF organization (45).

The absence of a correlation between the formation of IF bundles and progress through the cell cycle led us to suspect the involvement of serum factors in producing the gan+ IF phenotype. BSA at concentrations of 6 or 20 mg/ml prevented the formation of IF bundles in serum-deprived cells, and reversed the serum starvation-induced IF collapse (Fig. 4, b, c, e, and f). Ovalbumin added at 10 mg/ml to MEM did not prevent IF collapse (not shown), indicating that the effect of BSA was not due simply to the presence of protein in the media. The BSA used in these experiments was judged >98% pure (see Materials and Methods). Because at least 1% whole serum was required to produce the same IF phenotype, we consider it unlikely that the BSA effect was due to minor serum contaminants present in the BSA preparation. However, the effect of BSA on gan+ IF phenotype was less complete than that of either fetal calf serum or Nu-Serum; i.e., a greater percentage of cells contained IF bundles in the presence of BSA than in the presence of either fetal calf serum or Nu-Serum (Fig. 4). There may therefore be other factors present in serum that also contribute to the control IF phenotype in gan+ cells.

DISCUSSION

The gan+ mutations affect neuronal, glial, fibroblastic, and in some cases epithelial IF (40, 41). These IF are each composed of unique proteins coded for by separate genes (36, 51). Biochemical comparison of vimentin from normal, GAN+ and gan fibroblasts reveals no obvious difference in either electrophoretic mobility or level of posttranslational modification (38; Plummer, D. J., and M. W. Klymkowsky, work in progress) suggesting that the gan defect does not affect IF proteins themselves, but rather a widely distributed component involved in the control of IF organization. There is mounting evidence that normal IF organization depends upon interactions with microtubules. Linking structures between IF and microtubules have been observed by electron microscopy in neuronal (14) and non-neuronal cells (42), and disruption of interphase microtubules by any of a number of means (2, 3, 13, 16, 19, 44, 52) leads to the collapse of IF into bundles. However, in gan+ cells IF collapse occurs even though microtubule organization appears unaltered (Fig. 2). In this the phenotype of gan+ is similar to that produced by heat shock (49) and intracellularly injected anti-IF antibodies (12, 26-28, 33).

A number of compounds are known to induce neuronal IF disorganization when administered in vivo (reviewed in reference 29). Most of these agents cause the appearance of IF bundles in the proximal axon or within the perikaryon (29). In GAN, IF bundles form in the distal portion of axons that project into the periphery. The distal localization of IF bundles in GAN may be a result of the involvement of serum factors in the gan+ phenotype. The cell bodies of central neurons are shielded from direct interaction with serum factors by the “blood–brain barrier.” However, serum components are taken up by such neurons, and serum albumin has been shown to be the major retrogradely transported protein in sciatic nerve (9, 32). How serum factors influence IF phenotype of gan+ cells is unclear. Morphological changes in response to serum starvation have been noted in neuroblastoma cell lines; however, in these cases α1, α2, and β-globulin, not serum albumin, were effective substitutes for whole serum (43). In addition, it remains to be explained why the first symptoms of GAN do not appear until relatively late in childhood. Perhaps IF bundles are formed earlier, but fail to produce symptoms until other events, such as myelination, occur.

Although a number of models for how gan+ produces IF collapse are imaginable, we believe that the most likely is that gan+ alters the interaction between IF and microtubules. That is, when cells are maintained under permissive conditions, there are enough links between microtubules and IF to maintain normal IF organization in most cells. Under nonpermissive conditions, these links are broken and IF collapse. Assuming that gan+ causes IF collapse by disrupting the links between IF and microtubules, then a number of possible mech-

**FIGURE 4** WG321 cells were grown as colonies in 10% MEM for 10 d and then transferred to either 10% Nu-Serum (a), 20 mg/ml BSA (b), or 6 mg/ml BSA (c) containing serum-free MEM and assayed for the presence of IF bundles 24 h later by Coomassie staining. In a second experiment, cells in sister cultures were transferred first to 0.1% fetal calf serum-containing media for 48 h (d) and then returned to media containing 6 mg/ml BSA (e), 20 mg/ml BSA (f), 10% Nu-Serum (g), 10% MEM containing 10 mM thymidine (h), or 10% MEM containing 2 mM hydroxyurea (i). After 24 h these cultures were examined by Coomassie staining. The percentage of cells containing IF bundles is plotted with the mean and standard deviation.
anisms by which it could act are conceivable. Because of the role of serum and, by implication, cellular metabolism in the gαn" phenotype, we favor a model in which the effective concentration or binding affinity of linker protein(s) drops under nonpermissive conditions due to changes in either the relative rates of their synthesis, turnover, or posttranslational modification.

One hint as to molecular basis of the restrictive (i.e., serum minus gαn" phenotype may come from the recent report that a number of different cell types synthesize a specific ensemble of proteins during G0 arrest, and that a subset of these "G0 proteins" are also "heat shock" proteins (17). We are currently in the process of comparing heat shock and G0 protein in GαN+" and gαn" cells.

The experimental disruption of IF organization by intracellular injection of anti-IF antibodies has no recognizable affect on either cell morphology or behavior (12, 26, 28, 31, 33). Therefore, we suspect that the symptoms of GAN and other neuropathies displaying altered IF organization are not due to the loss of normal IF organization per se, but result either from side effects due to the presence of massive IF bundles within the confines of the axon or to metabolic alterations that only secondarily affect IF organization.

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