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VIROLOGY 189, 203–216 (1992)

Syncytium Formation Is Induced in the Murine Neuroblastoma Cell Cultures Which Produce Pathogenic Type G Proteins of the Rabies Virus

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Received January 9, 1992; accepted March 26, 1992

We investigated comparatively the interactions of host cells with two types of rabies virus G protein, an avirulent type G (Gln) and a virulent type G (Arg) protein, having glutamine and arginine at position 333, respectively. For this purpose, we established four types of cell lines (referred to as G(Gln)-NA, G(Arg)-NA, G(Gln)-BHK, and G(Arg)-BHK cells, respectively) by transfecting either the G(Gln)-cDNA or G(Arg)-cDNA into two kinds of cells, murine neuroblastoma C1300 (clone NA) and nonneuronal BHK-21. Both G(Gln)-NA and G(Arg)-NA cells produced G proteins when they were treated with 5 m*M* sodium butyrate, but only G(Arg)-NA cells formed syncytia at the neutral pH, which was suppressed by anti-G antiserum. The sodium butyrate-treated G(Arg)-NA cells fused also with sodium butyrate-treated NA cells under coculture conditions, but neither with untreated NA cells nor with BHK-21 cells. On the other hand, both G(Gln)-BHK and G(Arg)-BHK cells constitutively produced G proteins, but no syncytium was produced at the neutral pH. G(Arg)-BHK cells, however, formed syncytia with the sodium butyrate-treated NA cells when they were cocultured. These results suggest that only G(Arg) has a potential ability to produce syncytia of NA cells regardless of cell types by which G(Arg) protein was produced and also suggest that a certain cellular factor(s) is required for the syncytium formation, the factor(s) which is lacking in BHK-21 and untreated NA cells but is produced by the sodium butyrate-treated NA cells.

INTRODUCTION

Viral proteins on the surface of virions play important roles in the initial steps of viral invasion into host cells. They are also involved in determining the organotropism and/or the virulence of many types of viruses. Alteration or diminishment of the neurotropic nature and neurovirulence of some neurovirulent viruses has been found sometimes in association with the antigenic or structural changes of the viral proteins which constitute the surface of the virion, as reported for rabies virus (Dietzschold *et al.*, 1983; Spriggs *et al.*, 1983; Seif *et al.*, 1985; Davis *et al.*, 1986b; Prehaud *et al.*, 1988; Goodman and Engel, 1991), mumps virus (Löve *et al.*, 1985), murine hepatitis virus (Dalziel *et al.*, 1986), murine coronavirus (Fleming *et al.*, 1986), poliovirus (La-Monica *et al.*, 1987), Sindbis virus (Pence *et al.*, 1990), etc. For instance, several kinds of escape mutants of rabies virus have been isolated according to their acquired resistance to the neutralizing monoclonal antibodies directed to the viral glycoprotein (G) (Coulon *et al.*, 1982, 1983). A mutant having a one amino acid substitution at position 333 of G protein is one of such mutants of the altered virulence; that is, substitution of arginine at position 333 of G protein of the virulent strain by glutamine or isoleucine is known to be associated with the loss of pathogenic activity of the virus, and pathogenic revertants from the nonpathogenic mutants are shown to recover the arginine-333 (Dietzschold *et al.*, 1983; Seif *et al.*, 1985). Tuffereau *et al.* (1989) reported recently that a positively charged amino acid, such as arginine or lysine, at position 333 is essential for the virus to be virulent against adult mice.

Pathogenic and nonpathogenic viruses of rabies virus have been studied comparatively both in *in vivo* and *in vitro* infection systems. Dietzschold *et al.* (1985) showed that spread of the nonpathogenic virus within the mouse brain is slower than that of the pathogenic virus, and cell-to-cell spread of the nonpathogenic virus in the culture of mouse neuroblastoma C1300 cell (clone NA) is greatly inhibited when anti-rabies antiserum is added to the culture, while the virulent strains spread efficiently in the culture under the same conditions. But, it is still unclear how the amino acid at position 333 of G protein is involved in the process of viral spread and growth in the brain.

We have cloned and sequenced cDNA clones of the G protein gene (G-cDNA) of the nonpathogenic HEP-Flury strain (Morimoto *et al.*, 1989). We have also introduced the G-cDNA with an expression vector pZIP-NeoSV(X)1 into two kinds of cells, the murine neuroblastoma C1300 (clone NA) and the nonneuronal BHK-21 cell, from which several cDNA-transfected permanent cell lines have been obtained. The G-cDNA-

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0042-6822/92 $5.00

transfected permanent cell lines obtained from BHK-21 cell (G-BHK) constitutively produced G proteins, while those obtained from the NA cell (G-NA) produced G proteins only when they were treated with sodium butyrate (Morimoto *et al.,* 1992). By using these gene expression systems, we began comparative studies on the behaviors in the cell and some other properties of the virulent and avirulent types of rabies virus G protein, expecting to find a key for understanding the role of G protein in the neuropathogenesis of rabies virus at the molecular level.

In this report, we first produced by using a site-directed mutagenesis technique a point mutant from the G-cDNA of HEP strain which encoded the avirulent type G protein [G(Gln)] having glutamine at position 333 (Morimoto *et al.,* 1989). The mutated G-cDNA was made to encode a virulent type G protein [G(Arg)] having arginine, instead of glutamine, at position 333. And, we inserted these G-cDNAs into the retroviral expression vector, whereby we established four types of the G-cDNA-transfected cell lines from BHK-21 and NA cells [referred to as G(Gln)-BHK, G(Arg)-BHK, G(Gln)-NA, and G(Arg)-NA, respectively]. We found that, when G(Arg) protein was expressed in G(Arg)-NA cell cultures upon induction with sodium butyrate treatment, extensive syncytium formation was observed under the neutral pH conditions, but no such syncytium was observed in G(Arg)-BHK, G(Gln)-BHK, or G(Gln)-NA cell cultures. We also investigated other conditions required for the G(Arg)-induced syncytium formation.

MATERIALS AND METHODS

Virus and cell cultures

BHK-21 and the G-cDNA-transfected BHK-21 cells were cultured at 36° in Eagle's MEM supplemented with 10% tryptose phosphate broth (Difco) and 5% bovine serum. The clone (designated as NA) from the murine neuroblastoma C1300 strain (McMorris and Ruddle, 1974) and the G-cDNA-transfected NA cells were propagated at 36° in Eagle's MEM supplemented with 10% fetal calf serum. In the case of G-cDNA-transfected cells, 200 or 400 μg/ml G418 G-NA cells, 5 m*M* sodium butyrate (pH 7.4) was added to the culture medium as described in the text (the presence of 5 m*M* sodium butyrate in the culture medium did not decrease the pH of the medium below 7.0 during at least 4 days of incubation).

For preparing the lysate of infected cells to be used as a electrophoretic marker of G protein, cells were infected with the HEP-Flury strain of rabies virus (clone 2150-14, Kawai *et al.,* 1975), the strain which had also been used for cDNA cloning (Morimoto *et al.,* 1989).

G-cDNAs and oligonucleotide-directed mutagenesis

A cDNA clone (designated as pHP452; Morimoto *et al.,* 1989) of the G gene of rabies virus (HEP-Flury strain) and its point mutant (see below) were used in this study. They were transferred into the *Bam*HI site of expression vector pZIP-NeoSV(X)1 (Cepko *et al.,* 1984), as illustrated in Fig. 1.

Substitution of glutamine at position 333 by arginine was performed by a site-directed mutagenesis technique according to Carter *et al.* (1985). The cDNA insert in pBR322 was first cut with *Afl*II, and a *Bam*HI linker was ligated to the end of the *Afl*II cut (Fig. 1). After being cut with *Bam*HI, the negative strand of the G-cDNA was transferred into the *Bam*HI site of the M13mp19-am4 vector having an amber mutation in gene 4 of M13 (at 5237). The vector can grow only in the supE strain of *Escherichia coli.* A 20-mer (5′-AAGTCTGTCCGGACCTGGAA-3′) was used as the mutagenic oligonucleotide primer to induce a mutation in the G gene (at position 333 of G protein), which would result in a single amino acid change, from glutamine to arginine, at position 333 of G protein, as well as introduce a new cutting site for a restriction enzyme *Acc*III. Another mutagenic oligonucleotide primer, SEL 1 (5′-AAGAGTCTGTCCATCAC-3′, the selection primer), was also annealed to the vector to restore the glutamine codon at the amber mutated site in gene 4 of M13 phage. Accordingly, the mutated revertant phage vectors were made to grow in the nonsupressor strain. The revertant vectors obtained were first examined for the acquisition of the new *Acc*III site. The amino acid substitution in the mutant G protein was further checked by a DNA sequencing technique. The authentic HEP G-cDNA is referred to as G(Gln)-cDNA or nonpathogenic type G-cDNA, and the mutated G-cDNA is referred to as G(Arg)-cDNA or pathogenic type G-cDNA in this article. Then, the G-cDNA insert in M13 phage was cut out with *Bam*HI and transferred to the expression vector.

Transfection of the G-cDNA into BHK-21 and NA

The G-cDNAs inserted in pZIP-NeoSV(X)1 were transfected into BHK-21 and NA cells by the calcium phosphate method as described by Davis *et al.* (1986a). The cDNA-transfected cells were cultivated in the presence of 400 μg/ml of G418 from the 48th hour after the glycerol shock, and culture medium containing G418 (400 μg/ml) was changed at 3-day intervals until the isolation of G418-resistant colonies. The re-

sistant clones obtained were usually maintained in the presence of G418 (400 μg/ml).

Fluorescent antibody staining

Immunofluorescence studies on the G gene expression was performed as follows: cells grown on a coverslip were fixed with acetone (for detecting the internal antigen) or 3% paraformaldehyde (for detecting cell surface expression of the antigen) for 10 min at room temperature and were subjected to the indirect fluorescent antibody staining, where the rabbit immune serum against the rabies G protein (Naito and Matsumoto, 1978) was used as the first antibody and the fluorescein-conjugated anti-rabbit IgG goat antibody (Cappel) as the second antibody.

Immunoblot analysis

Cells were lysed with IP buffer (composed of 1% Triton X-100, 1% deoxycholate (DOC), 10 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml antipain, pH 7.4), and the nuclei were removed by low speed centrifugation. The same amount of the double-concentrated lysis buffer for SDS–PAGE was added to each lysate, which was then subjected to 10% gel SDS–PAGE (Laemmli, 1970). The proteins separated in the gel were then blotted onto the nitrocellulose membrane filter (BA85; Schleicher & Schuell) by the semidry method described by Kyhse-Andersen (1984). The filter was then incubated with the anti-G rabbit antiserum as the first antibody and then with the peroxidase-conjugated anti-rabbit IgG goat antibody as the second antibody. Color was developed by using 4-chloro-1-naphthol and hydrogen peroxide (Towbin *et al.*, 1979; Hawkes *et al.*, 1982).

Determination of the cell fusion index

Cells were grown on coverslips placed in 35-mm dishes and cultured in Eagle's MEM supplemented with 10% fetal calf serum. On the following day, 5 m*M* sodium butyrate was added to the culture medium. At 1927), and syncytium formation was examined under a light microscope. Cell fusion index was determined either by counting the frequency of multinucleated cells with more than four nuclei in 10 random fields, or by calculating the ratio of the number of nuclei in the multinucleated cells to the number of total nuclei in the photographs taken from the 10 randomly chosen fields, in which 300 nuclei or more were counted.

Quantification of G proteins expressed on the cell surface

Cells grown in 24-well plate or 35-mm dishes were fixed with 3% paraformaldehyde after incubation for various days in the presence of 5 m*M* butyrate. The cells were then washed with PBS and incubated with rabbit anti-G antiserum (at 1:100 dilution in PBS containing 3% BSA) for 1 hr on a rocking table at room temperature. They were washed with PBS and incubated with peroxidase-conjugated anti-rabbit IgG antibody (1:100 dilution) for 1 hr. After additional washes with PBS, the bound peroxidase was quantified by using a chromogenic substrate, 2,2′-azinobis-(3-ethylbenzthiazoline)-sulfate (ABTS) of the ELISA color reagent kit (Sumitomo Bakelite Co., Tokyo) and photospectrometric monitoring of the developed color at 420 nm.

pH determination

The pH value of the culture fluid was determined by using a pocket-size pH meter (FESTA pHBOY-C1, Shindengen Kogyo Co., Tokyo), which was equipped with a microelectrode of ion-sensitive field effect transistor. Culture dishes were taken out and immediately the electrode was dipped into the culture medium to determine the pH value within a few seconds.

RESULTS

Expression of G protein by cDNA-transfected cell lines

To investigate more precisely the possible roles of arginine at position 333 of rabies virus G protein, the arginine-333 which is essential for the virus to preserve its pathogenic nature, we prepared a mutant cDNA from the HEP G-cDNA by using the site-directed mutagenesis technique. The mutant cDNA was made to encode a pathogenic type G protein [G(Arg)] by substituting glutamine at position 333 by arginine (Fig. 1; see Materials and Methods). The mutated G-cDNA as well as the original HEP G-cDNA were transfected into BHK-21 and NA cells by using a retroviral expression vector pZIP-NeoSV(X)1 (Fig. 1). From both the G(Arg)-lines, which are referred to as G(Arg)-BHK, G(Gln)-BHK, G(Arg)-NA and G(Gln)-NA cells, respectively, in this paper.

Both G(Arg)-BHK and G(Gln)-BHK cells constitutively produced G protein, while G(Arg)-NA and G(Gln)-NA cells produced no or little G protein when they were cultured in the usual growth medium. We examined the effect of sodium butyrate on the G gene expression

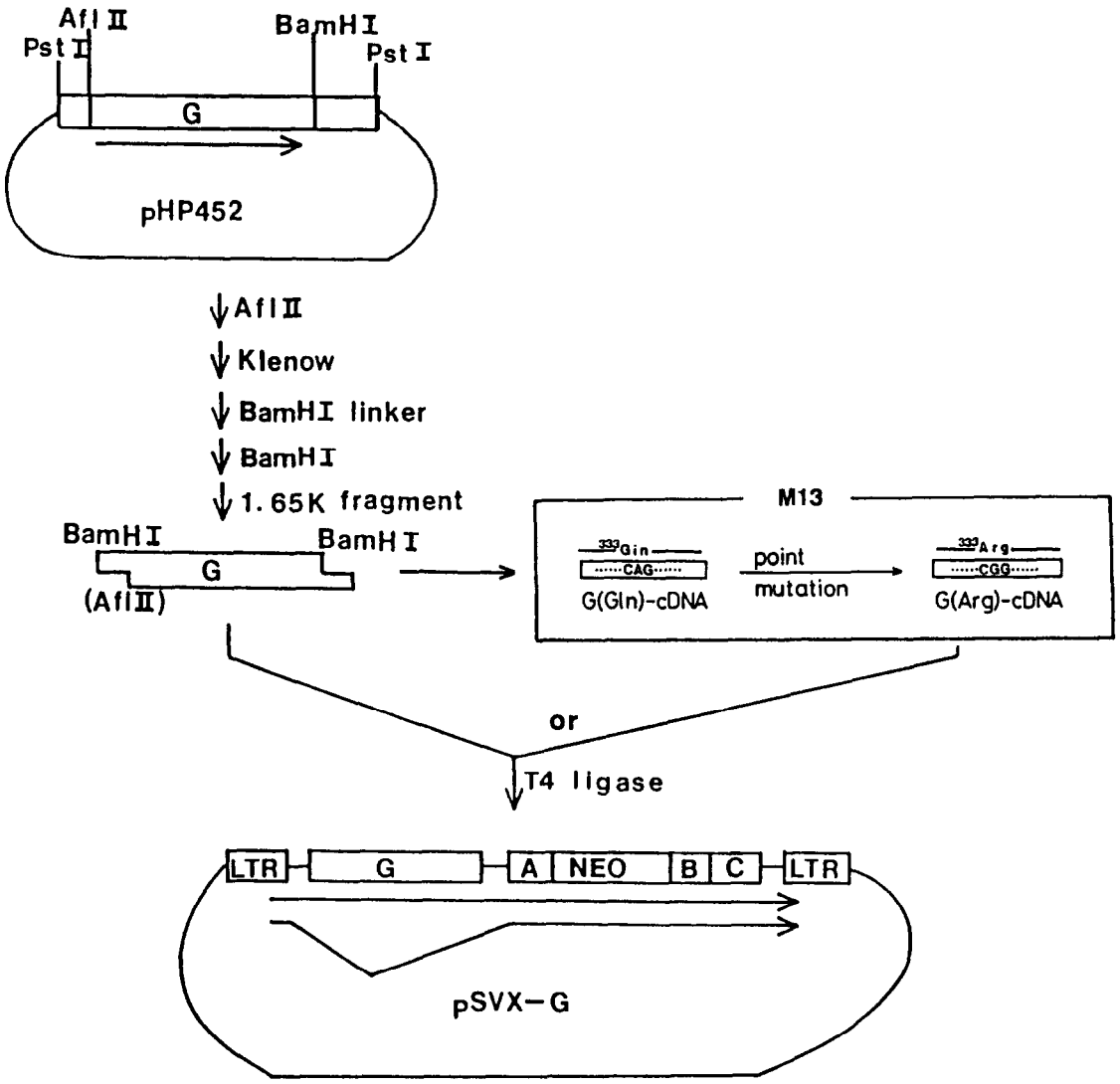


FIG. 1. Illustrations of construction of the expression vectors and site-directed point mutagenesis of the G gene (see text). The reconstructed vector is designated as pSVX-G. G, cDNA of the G protein; LTR, the long terminal repeat originated from the Moloney murine leukemia virus; A, 3′-splicing site; Neo, neomycin-resistant gene derived from a transposon Tn*5;* B, SV40 ori; and C, pBR322 ori. Arrows depicted below the genes indicate the direction of transcription and the bent arrow indicates a spliced transcript.

in NA cells, because the agent is known to induce the tissue-specific gene expression and morphological changes of murine C1300 neuroblastoma cells in culture and also to increase the expression of foreign genes transfected with certain kinds of expression

when they were treated with 5 m*M* sodium butyrate (studies on the optimal conditions of sodium butyrate treatment will be published elsewhere; Morimoto *et al.,* 1992). Dibutyryl cAMP, another agent known as a differentiation-inducing substance, did not show such G protein-inducing activity on either G(Arg)-NA or G(Gln)-NA cells (unpublished observations). Figure 2 shows the representative results of the production of G protein by G(Gln)-BHK, G(Arg)-BHK, G(Gln)-NA, and G(Arg)-NA cells. The time required for G(Gln)-NA and G(Arg)-NA cells to attain the maximum level of G protein synthesis after the sodium butyrate treatment somewhat varied from clone to clone of the cells.

G(Gln)-NA cells (Figs. 2C and 2D). Mobility of G proteins produced by G(Arg)-BHK cells was almost the same as those produced by G(Gln)-BHK cells, indicating that the amino acid substitution at position 333 did not affect the mobility of G protein in SDS–PAGE. As noted in our previous report (Morimoto *et al.,* 1992), however, the mobility of G proteins produced by G(Gln)-BHK as well as G(Arg)-BHK cells was quite

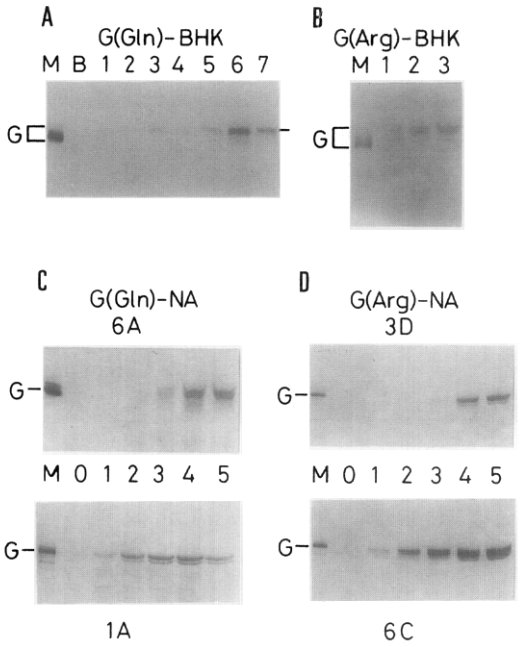


FIG. 2. Immunoblot analysis of the G protein synthesis by G-cDNA-transfected BHK-21 and NA cells. G(Gln)-BHK and G(Arg)-BHK cells were grown on 35-mm dishes (10^6 cells/dish) and incubated at 36° in the absence of sodium butyrate. Two days later the cells, which had formed confluent monolayers, were lysed with IP buffer. G(Gln)-NA and G(Arg)-NA cell clones were grown on 35-mm dish (10^6 cells/dish) and first incubated for 24 hr with the growth medium, and then 5 m*M* sodium butyrate was added to the cultures. The cells were collected at daily intervals and lysed with IP buffer. One tenth of the whole cell lysate from each plate was applied to the 10% SDS–PAGE and immunoblot analysis (see Materials and Methods). The rabbit immune serum against the G protein of rabies virus (HEP strain) was used for detecting the antigen. (A) Lane M: the lysate of the virus-infected BHK-21 cells (shown as a G protein marker); lane B: normal BHK-21 cells; lane 1: uncloned G(Gln)-BHK cells; lanes 2 to 7: G(Gln)-BHK cells, clones 2 to 7, respectively. (B) Lane M, the same as that described for the lane M of (A); lanes 1, 2, and 3: G(Arg)-BHK, clones A, D, and G, respectively. (C) Lane M: the lysate of the rabies virus-infected NA cells (shown as a G protein marker); lanes 0 to 5: cell lysates of G(Gln)-NA cells, clones 6A (upper) and 1A (lower), obtained on Days 0, 1, 2, 3, 4, and 5 after the sodium butyrate treatment, respectively. (D) Lane M: the same as that described for lane M of (C); lanes 0 to 5: cell lysates of G(Arg)-NA cells, clones 3D (upper) and 6C (lower), obtained on Days 0, 1, 2, 3, 4, and 5 after the sodium butyrate treatment, respectively.

slower than that of G proteins produced by the virus-infected BHK cells (Figs. 2A and 2B). We have also demonstrated that the difference in the mobility was originated from the differences in the numbers and structures of the oligosaccharide side chain moiety (Morimoto *et al.*, 1992). Mobility of G proteins produced by G(Gln)-NA and G(Arg)-NA cells was similar to those produced by the virus-infected BHK-21 and NA cells (lane M in Figs. 2C and D). To sum up, the different mobilities of G protein in SDS–PAGE were originated from the difference in the oligosaccharide side chain moiety, which was dependent on either the cell types or conditions (infection or cDNA transfection) of host cells that produced the protein and was not affected by substitution of glutamine-333 by arginine.

Expression of G protein on the surface of the cDNA-transfected cells

When G(Gln)-BHK and G(Arg)-BHK and the sodium butyrate-treated G(Gln)-NA and G(Arg)-NA cells were fixed with acetone, G antigen was detected throughout the cytoplasm by fluorescent antibody staining (Figs. 3A–3D). When fixed with paraformaldehyde, we could detect the antigen on the surface of the sodium butyrate-treated G(Arg)-NA and G(Gln)-NA cells (Figs. 3E and 3F) as well as on G(Arg)-BHK and G(Gln)-BHK cells (data not shown), indicating that G proteins were normally transported to the surface of these cDNA-transfected cells.

Next, we compared quantitatively the surface expression of G(Arg) and G(Gln) proteins of the cDNA-transfected cells by using a specific antibody against the G protein of rabies virus (HEP strain). Quantification was performed by detecting the antibody bound to the cell surface of the paraformaldehyde-fixed NA cells (see Materials and Methods). Figure 4A shows comparisons of the relative amounts of G proteins on the surface of several clones of G(Gln)-NA and G(Arg)-NA cells on Day 4 after the butyrate treatment. Amounts of G proteins expressed on the surface of all these G(Gln)-NA and G(Arg)-NA cell lines were almost comparable in average, although strength of the fluorescence in individual cells varied from cell to cell (Fig. 3). Similar results were obtained as to the expression of G protein on the surface of G(Arg)-BHK and G(Gln)-BHK cells (data not shown). Time course of G protein expression on the cell surface after induction with sodium butyrate was almost the same between G(Arg)-NA and G(Gln)-NA cell lines (Fig. 4B).

Morphological changes of G(Arg)-NA and G(Gln)-NA cells after treatment with sodium butyrate

In parallel to the induction of G protein synthesis, sodium butyrate treatment also induced morphological changes in G(Arg)-NA and G(Gln)-NA cells. The cell shape gradually changed from round to flat, with cytoplasmic protrusions, in a manner similar to that previously reported for the normal NA cell culture (Schneider, 1976).

In addition to these, we observed syncytium formation in the sodium butyrate-treated G(Arg)-NA cell cultures, which occurred under neutral pH conditions (Figs. 5B–5D). Frequency of syncytium formation was

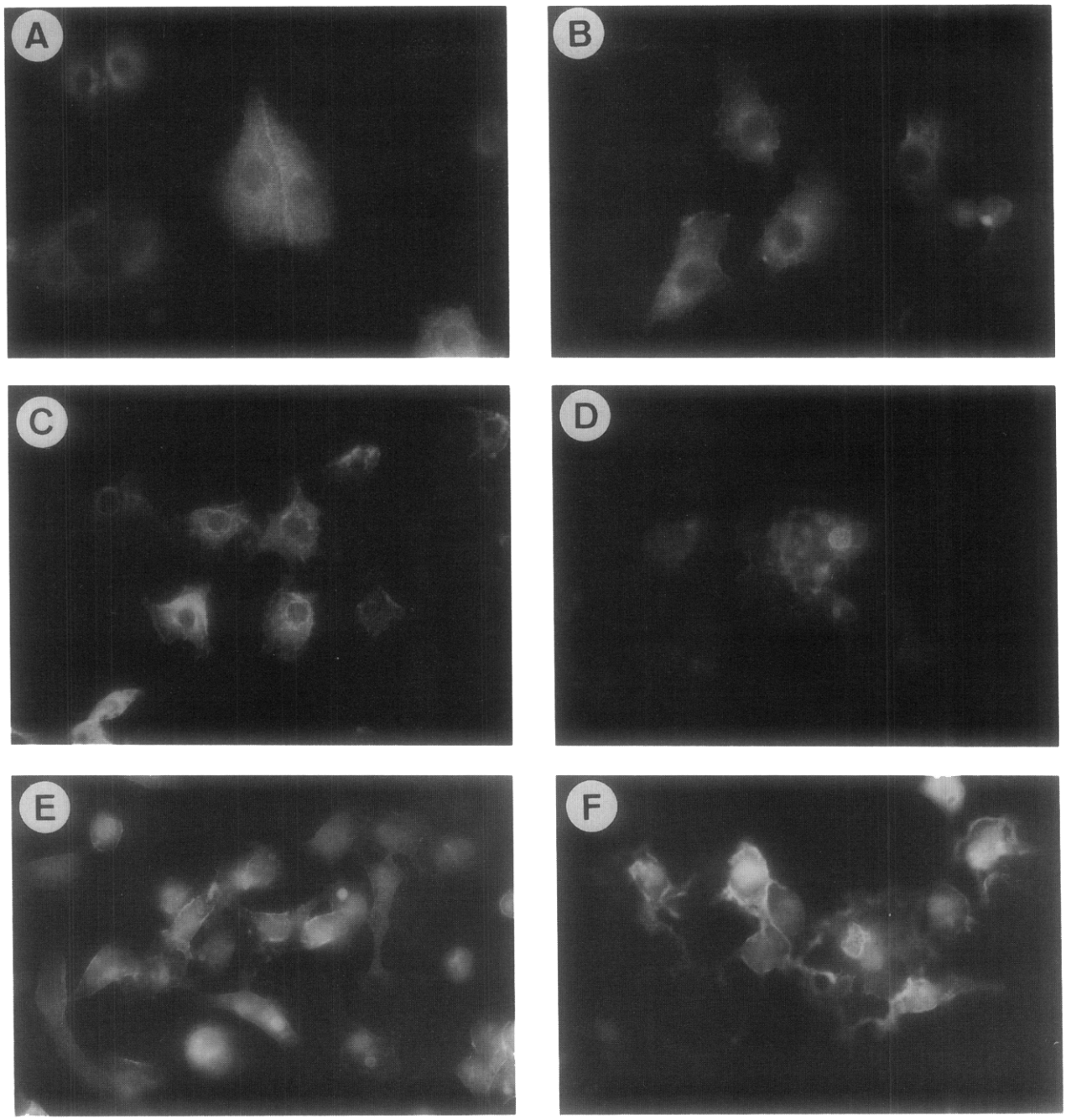


FIG. 3. Fluorescent antibody staining of G protein-producing BHK-21 and NA cells. G(Gln)-BHK (clone 6), G(Arg)-BHK (clone G), G(Gln)-NA (clone 1A), and G(Arg)-NA (clone 6C) cells were grown on coverslips placed in 35-mm dishes at about 10^5 cells/dish and incubated at 37° for 4 to 5 days. Sodium butyrate (final 5 m*M*) was added to the G(Gln)-NA and G(Arg)-NA cell cultures on Day 0. The cells were fixed with acetone on Day 5, or with 3% paraformaldehyde on Day 4, respectively. The fixed specimens were subjected to the indirect fluorescent antibody staining, for which we used the same stock of antiserum against the rabies virus G protein as described in Fig. 2 (see Materials and Methods). (A and B) Acetone-fixed G(Gln)-BHK and G(Arg)-BHK cells; (C and D) acetone-fixed G(Gln)-NA and G(Arg)-NA cells; (E and F) paraformaldehyde-fixed G(Gln)-NA and G(Arg)-NA cells. Syncytium formation is observed in (D) and (F).

greatly increased when the cell density of the G(Arg)-NA cells at the time of cell growth was increased up to at least 7.5×10^5 cells per 35-mm dish for closer cell-to-cell contact (we usually grew the cells at a lower cell density—ca. 10^5 cells/35-mm dish for the immunofluorescence studies of G protein synthesis). Under these improved culture conditions, however, no syncytium formation was observed in both the untreated G(Arg)-

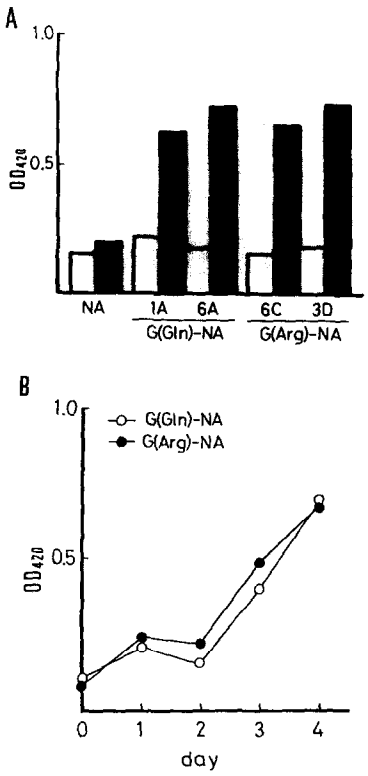


FIG. 4. Quantification of cell surface expression of G proteins. (A) Comparison of G protein expression on the surface of four G protein-producing NA cell clones. 7.5×10^5 cells grown on 35mm-dishes were incubated for 4 days in the presence or absence of 5 m*M* sodium butyrate and fixed with 3% paraformaldehyde on Day 4. Relative amounts of G protein on the cell surface of each clone were determined by using the anti-G anti-serum (rabbit) and peroxidase-conjugated anti-rabbit IgG antibody as described under Materials and Methods. Strength of the color developed was determined at 420 nm. NA, normal NA cell; 1A and 6A, G(Gln)-NA cell clones; 6C and 3D, G(Arg)-NA cell clones. Open rectangles, untreated control; closed rectangles, sodium butyrate-treated. (B) Kinetics of surface expression of G protein of G(Arg)-NA and G(Gln)-NA cells. The 2×10^5 cells in 24-well multiwell dish were incubated in the presence of 5 m*M* sodium butyrate, and a pair of cultures were fixed every day with 3% paraformaldehyde. Relative amounts of G protein on the cell surface were quantified as described above for (A). (O): G(Gln)-NA (clone 1A) cell; (●): G(Arg)-NA (clone 6C) cell.

NA cell cultures (Fig. 5A) and the sodium butyrate-treated G(Gln)-NA cultures (Fig. 5F). Very recently, rabies virus G protein (CVS strain) was reported to induce

ture fluids of G(Arg)-NA and G(Gln)-NA cells after the sodium butyrate treatment. During the experiments on the syncytium formation, the pH of the culture fluids did not decrease below 7.0 until the 5th day, even in the presence of 5 m*M* sodium butyrate. These observations suggest that only the pathogenic-type G(Arg) protein is involved in the syncytium formation at the neutral pH, and not G(Gln).

On the other hand, neither G(Arg)-BHK nor G(Gln)-BHK cells produced such multinucleated cells even when they were grown at higher cell densities and treated with sodium butyrate (data not shown), suggesting that production of G(Arg) protein itself is not enough for syncytium formation.

Correlation between G(Arg) protein synthesis and syncytium formation

The time required for syncytium formation seems to be correlated with the surface expression of the G protein (Figs. 4B and 5): expression of G protein on the surface of G(Arg)-NA (clone 6C) cells was much increased on Day 3 after the butyrate treatment (Fig. 4B), and the syncytium formation coincidentally became prominent on Day 3 (Fig. 5C).

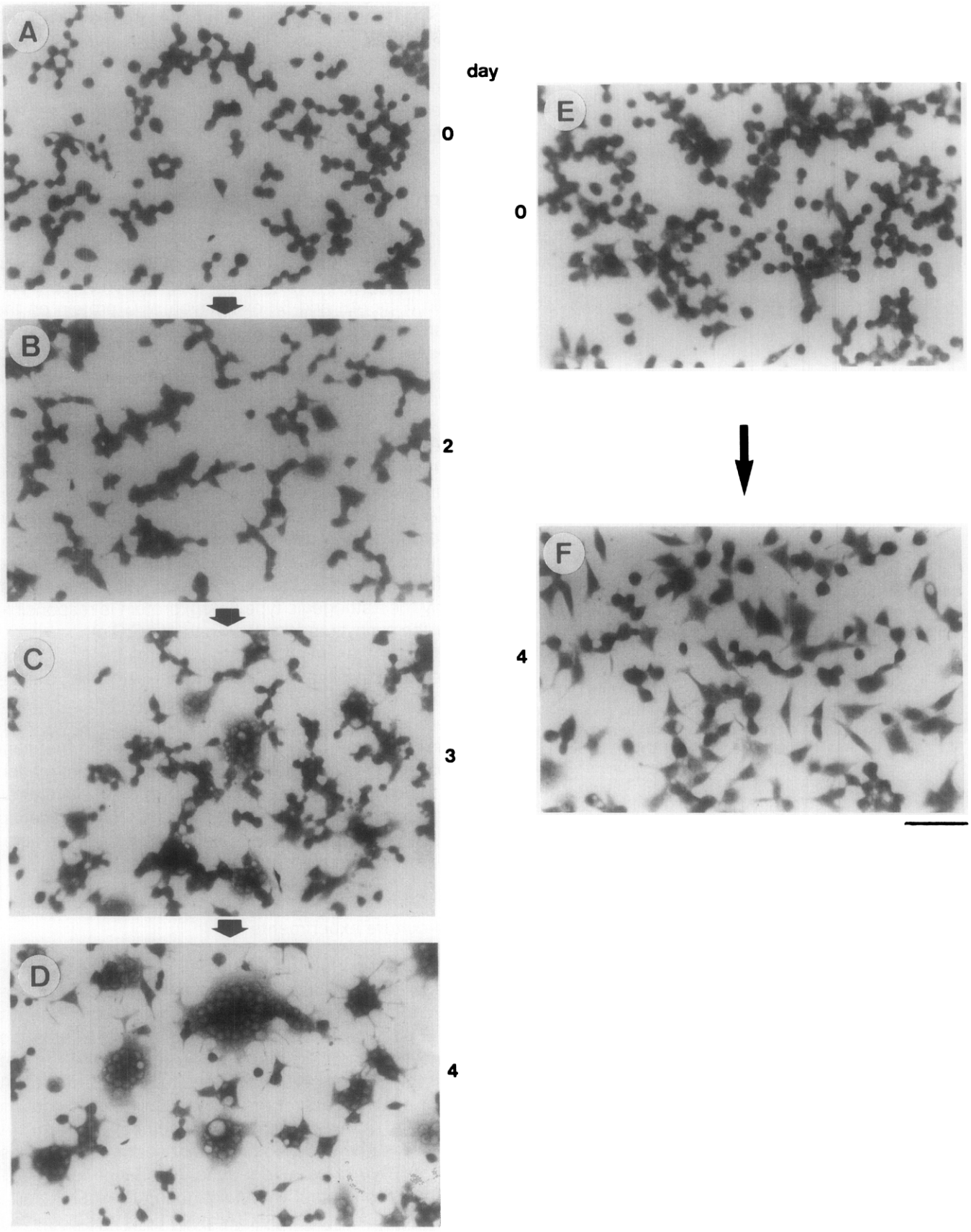
Since both G(Gln) and G(Arg) proteins produced in NA cells were normally transported almost equally to the surface of the cells (Fig. 4), it seems likely that the difference in the ability of syncytium formation between G(Arg)-NA and G(Gln)-NA cells did not originate from the quantitative difference in the expression of G protein on the cell surface, but was due to the qualitative difference of the G protein molecule. In other words, only the pathogenic type G(Arg) protein is responsible for the syncytium formation and not G(Gln).

Next we examined whether G proteins located on the surface of the butyrate-treated G(Arg)-NA cells are involved in the syncytium formation in culture. For this purpose, we tested a suppressive effect of the antiserum against the rabies virus G protein on the giant cell formation. As shown in Fig. 6, syncytium formation in the sodium butyrate-treated G(Arg)-NA cell cultures was completely inhibited by the antiserum, indicating that G proteins expressed on the cell surface are actually involved in the syncytium formation.

Co-culture experiments

We next performed coculture experiments to examine whether cellular factors are required for the syncytium formation and whether the different glycosylation

first, G(Arg)-NA cells were cocultured with either NA cells or BHK cells in the presence of 5 m*M* sodium butyrate. In this experiment, the number of G(Arg)-NA cells was decreased to one fifth that of the latter ones (NA or BHK-21 cells) to reduce the chance for G(Arg)-NA cells to contact with neighboring G(Arg)-NA cells. Syncytium formation by G(Arg)-NA cells was observed only when they were cocultured with NA cells, but not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with BHK cells (Fig. 7), suggesting again that production of G(Arg) protein itself is not enough for the giant cell formation, and that some cellular factor(s), which is lacking in BHK cells but is produced by the sodium butyrate-treated NA cells, is also required for the syncytium formation. The latter view was also obtained following coculture experiments.

To examine whether G(Arg) protein produced by BHK-21 cell is functional as a syncytium-forming factor, G(Arg)-BHK cells were cocultured with NA cells, which were either mock-treated or treated with sodium butyrate. As shown in Fig. 8A, we could observe massive syncytium formations in the cocultures of G(Arg)-BHK cells with NA cells only when they were treated with sodium butyrate (Fig. 8A), but not under the untreated conditions (data not shown). As already noted above, no syncytium was observed in both the control single cultures of G(Arg)-BHK cells (Fig. 8C) and the sodium butyrate-treated NA cells (Fig. 8D). When G(Arg)-BHK cells were cocultivated with the pretreated NA cells, syncytium formation was not so efficient probably due to rapid retraction of the sodium butyrate-induced host cell factor(s) of the cell during the cocultivation in the absence of the agent (the pretreated NA cells recovered its round shape soon after the elimination of sodium butyrate from the culture medium and began to propagate). In addition, no syncytium was observed in the cocultures of G(Gln)-BHK and sodium butyrate-treated NA cells (Fig. 8B). These results demonstrate that G(Arg) proteins synthesized by BHK-21 cells are as active in the syncytium formation as those synthesized by G(Arg)-NA cells, and that a cellular factor(s) expressed on the sodium butyrate-treated NA cells is required for syncytium formation. Although it was suggested that the G(Arg) proteins were differently glycosylated in NA and BHK-21 cells (Fig. 2), the difference does not seem to cause any difference in the syncytium-forming potency of G protein.

These results not only indicate that G(Arg) protein has an ability to produce syncytia of NA cells regardless of cell types by which the G protein was produced but also suggest strongly that some cellular factor(s) is also required for syncytium formation, the cellular factor(s) which is lacking in BHK-21 and untreated NA

DISCUSSION

In this report, we compared two types of rabies virus G protein, the nonpathogenic-type G(Gln) protein having glutamine at position 333 and the pathogenic-type G(Arg) having arginine-333, with regard to their interactions with host cells. For this purpose, we used two types of G-cDNAs, the original G-cDNA of the nonpathogenic type virus (HEP strain) (Morimoto *et al.*, 1989) and its point mutant, which was made to encode a pathogenic type G(Arg) protein having arginine, instead of glutamine, at position 333. With these G-cDNAs, four types of G gene-containing cell lines were obtained from BHK-21 and neuroblastoma NA cells. By using these cell lines, we found that only the pathogenic-type G(Arg) protein had an ability to induce the giant cell formation at a neutral pH, whereas the nonpathogenic-type G(Gln) protein did not display such an activity, although the amounts of G(Gln) proteins expressed on the cell surface were similar to those of G(Arg)-NA cells. During the incubation periods of these cultures, the pH of the culture fluids did not decrease below 7.0 even in the presence of 5 m*M* sodium butyrate. These observations show that arginine-333 is necessary for G protein to display the fusion activity at a neutral pH. The syncytium-forming ability of G(Arg) protein was independent of the cell types by which G(Arg) protein was produced. Syncytium formation was correlated with expression of G protein on the cell surface of G(Arg)-NA cells and was completely inhibited by anti-G antiserum, indicating that G(Arg) proteins which are expressed on the cell surface are involved in the syncytium formation.

It was also suggested that some specific cellular factor(s) is required for syncytium formation at a neutral pH, because syncytium formation was not observed in the cultures of G(Arg)-producing BHK-21 cells, whereas G(Arg)-BHK cells produced syncytia with the sodium butyrate-treated NA cells, but not with the untreated NA cells, under the coculture conditions. These observations also suggest that the host cell factor(s) is lacking or present in very small amounts in BHK-21 and untreated NA cells, but could be induced in NA cells by treatment with sodium butyrate.

FIG. 5. Syncytium formation in the sodium butyrate-treated G(Arg)-NA cell cultures. G(Gln)-NA (clone 1A) and G(Arg)-NA (clone 6C) cells were grown on 35-mm dishes at a cell density of 7.5×10^5 cells/dish. On the following day, 5 m*M* sodium butyrate was added to the cultures. On each day after the sodium butyrate treatment, a portion of dishes of each clone were fixed and stained with a Sellers' staining solution as noted under Materials and Methods. Morphology of the untreated control cells, which were also fixed and stained similarly, was the same as that of the cells fixed on Day 0 (data not shown). (A–D) G(Arg)-NA cells fixed and stained on Days 0, 2, 3, and 4, respectively; (E and F) G(Gln)-NA cells fixed and stained on Days 0 and 4, respectively. The cells on Day 0 were fixed and stained before incubation in the presence of sodium butyrate. A bar marker indicates 100 μm.

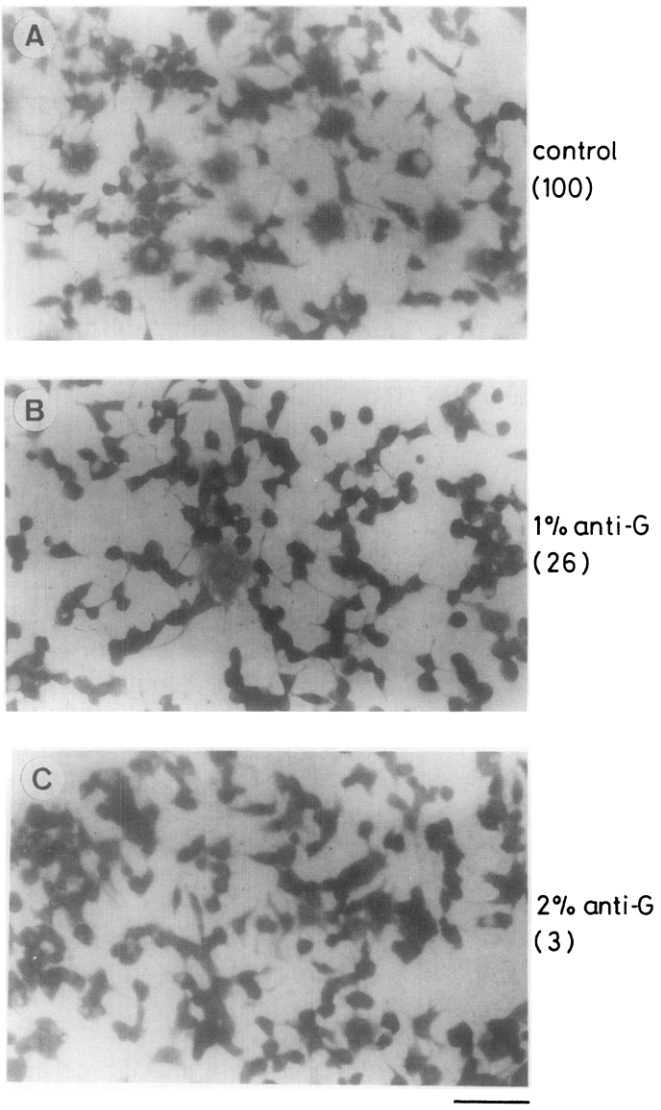

FIG. 6. Inhibition of anti-G antiserum of the syncytium formation induced in the sodium butyrate-treated G(Arg)-NA cell culture. G(Arg)-NA cells (clone 6C) were grown in 35-mm dish at a cell density of 7.5×10^5 cells/dish and were incubated for 24 hr in a growth medium, and then 5 m*M* sodium butyrate and various dilutions of anti-G antiserum were added to the cultures. On Day 4 after the sodium butyrate treatment, they were fixed and stained with a Sellers' staining solution. The grade of syncytium formation was determined by counting the numbers of multinucleated cells of more than four nuclei on the photographs taken from random 10 microscopic fields and was expressed as a percentage to the control (100%) in a parenthesis. Doses of antiserum: (A) 0 (control); (B) 1:100 dilution; (C) 1:50 dilution. The bar marker indicates 100 μm.

cessation of cell division and production of some neuronal cell-specific substances, which was followed by some morphological changes characteristic of neuronal cells. Accordingly, we assume that treatment of G(Arg)-NA cells with sodium butyrate not only induces production of G proteins but also induces concomitant synthesis of neuronal cell-specific substances including the host cell factor(s) required for the G protein-mediated syncytium formation.

Consistent with the results obtained from cDNA transfection experiments, we could also see the pH-independent cell fusion (syncytium formation at the neutral pH) in the virus infection system (unpublished observations). Only the pathogenic-type rabies virus (such as ERA strain and the neurovirulent revertants of HEP strain) induced the cell fusion in the sodium butyrate-treated NA cell cultures, but the nonpathogenic HEP strain did not. As expected, such pathogenic virus-induced pH-independent cell fusion could not be

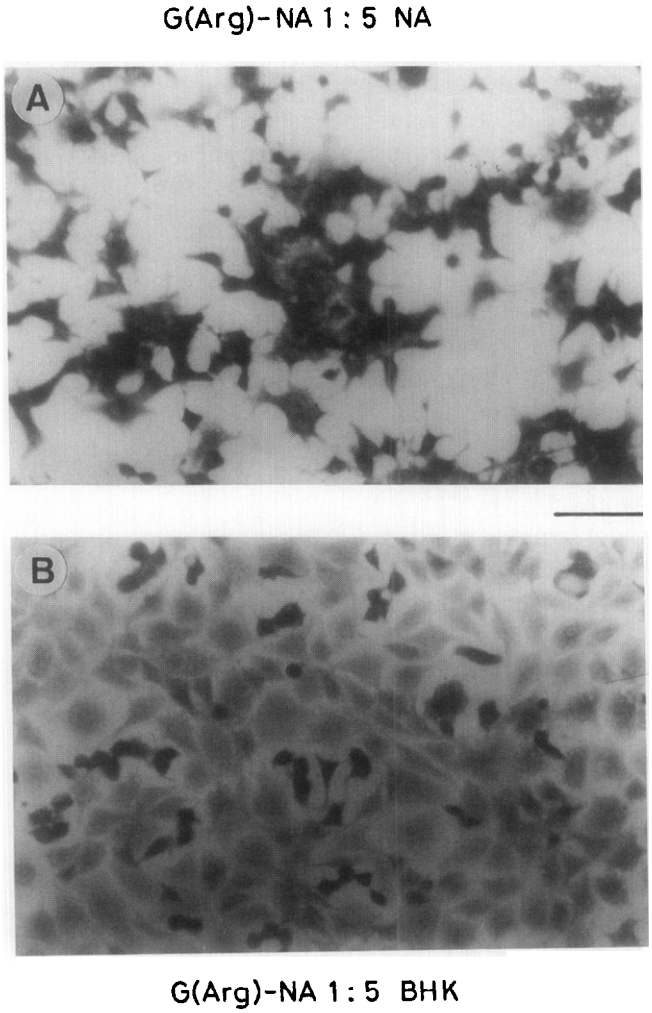

FIG. 7. Syncytium formation in the co-culture of G(Arg)-NA and NA cells. G(Arg)-NA (clone 6C) cells (1.25×10^5 cells) and NA or BHK-21 cells (6.25×10^5 cells) were mixed (the ratio was 1:5) and grown on 35-mm dishes. On the following day, 5 m*M* sodium butyrate was added to the cocultures. On Day 4 after the treatment, the cells were fixed and stained with a Sellers' staining solution. (A) G(Arg)-NA + NA cells; (B) G(Arg)-NA + BHK-21 cells. The bar marker indicates 100 μm.

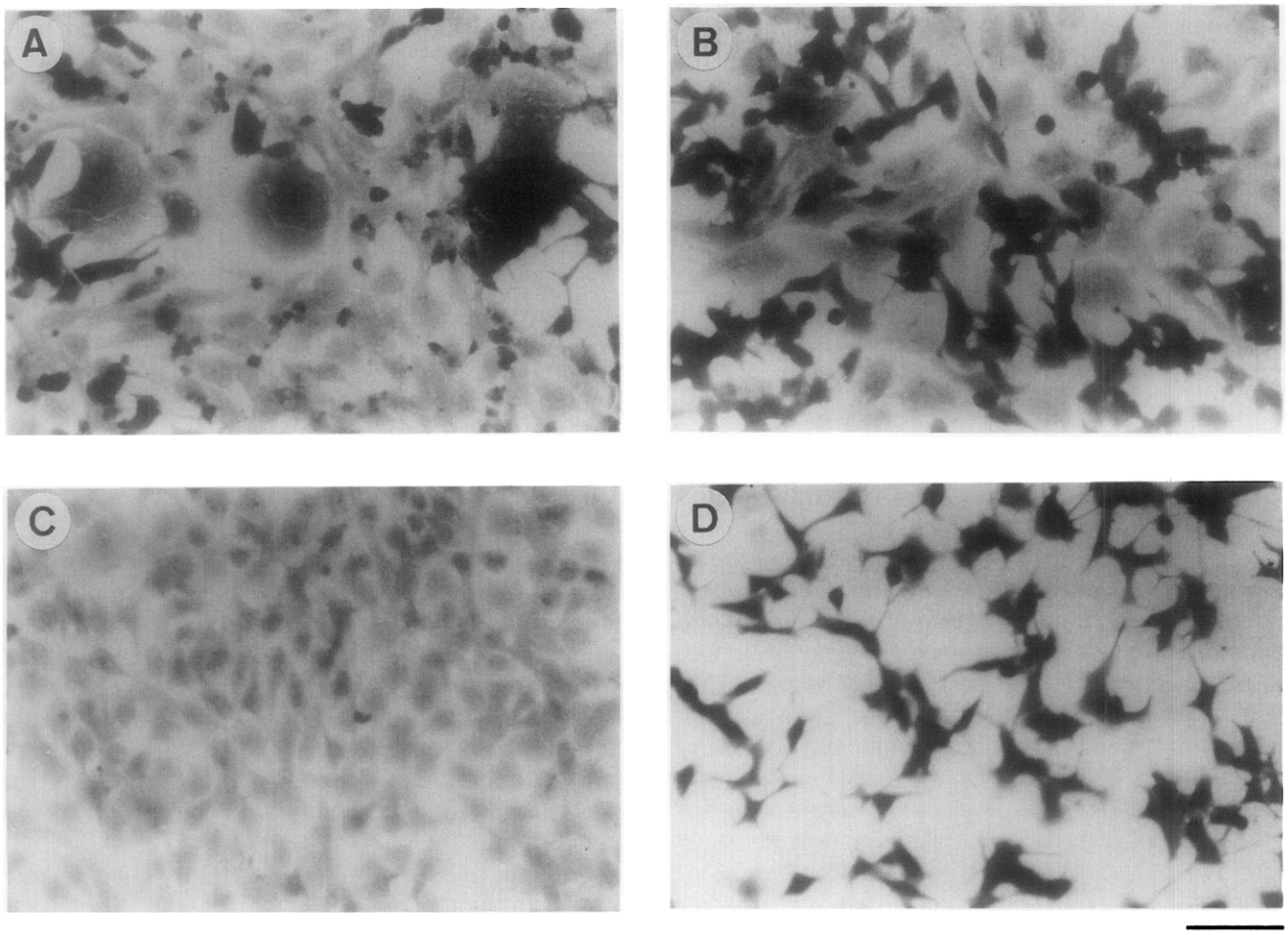

FIG. 8. Syncytium formation in the coculture of G(Arg)-BHK and NA cells. The same numbers of G(Arg)-BHK (clone G) or G(Gln)-BHK (clone 6) (3.75×10^5 cells) and normal NA cells (3.75×10^5 cells) were mixed and grown on 35-mm dishes. One day later, 5 m*M* sodium butyrate was added to the cocultures. On Day 3 after the treatment, the cells were fixed and stained with a Sellers' staining solution. Single cultures of each cell type (sodium butyrate-treated NA and G(Arg)-BHK cells; 7.5×10^5 cells/dish) were also treated in a similar manner. (A) G(Arg)-BHK + NA cells; (B) G(Gln)-BHK + NA cells; (C) G(Arg)-BHK cells; (D) NA cells. The bar marker indicates 100 μm.

seen in BHK-21 and untreated NA cell cultures (unpublished observations).

As reported by Mifune *et al.* (1982), rabies virus causes cell fusion at acidic pH, but no difference has been described between the pathogenic and nonpathogenic mutant viruses in their ability of low pH-dependent cell fusion (Wunner and Dietzschold, 1987). We also observed that the nonpathogenic virus (HEP strain) and the pathogenic virus (ERA strain and a pathogenic revertant of HEP strain) equally induced cell fusion in both BHK-21 and NA cell cultures when they were exposed to pH 5.0 (unpublished observations). Very recently, Whitt *et al.* (1991) reported that the rabies virus G protein (CVS strain) expressed on the cDNA-transfected HeLa cells induced giant cell formation under low pH conditions. Unexpectedly, however, we could not observe syncytium formation in the cultures of G(Arg)-BHK, G(Gln)-BHK and G(Gln)-NA cells even when they were exposed to acidic pH (5.1–5.7). We suppose that, although the amount of G(Arg) protein expressed on the surface of G(Arg)-NA cells was enough for the pH-independent cell fusion, such amount of G proteins expressed on the surface of G(Arg)-BHK, G(Gln)-NA and G(Gln)-BHK cells would not be enough for the low pH-dependent syncytium formation, and more abundant G protein should be expressed. Alternatively, other viral factor(s), such as another viral envelope component (the matrix protein) might be required in the case of HEP virus G protein. As for the former possibility, the estimated amounts of G protein produced by G(Gln)-BHK and the butyrate-treated G(Gln)-NA cells were at most 2 to 3% of those produced by the virus-infected BHK-21 and NA cells, respectively (Morimoto *et al.*, 1992).

Table 1 compares the properties of two types of rabies virus-induced cell fusion (syncytium formation): in

TABLE 1

COMPARISON OF THE TWO TYPES OF RABIES VIRUS-INDUCED CELL FUSION

Type of cell fusion	Cell fusion with rabies virus and types of virus and G protein: Pathogenic type, G(Arg)	Cell fusion with rabies virus and types of virus and G protein: Nonpathogenic type, G(Gln)	Requirement of neuronal cell-specific factor
Low pH-dependent	+	+	−
pH-independent	+	−	+

addition to the dependence of different pH, two other properties could be distinguished. First, syncytium formation at the neutral pH (low pH-independent cell fusion) was caused only by the pathogenic type rabies virus and G(Arg) protein, whereas the low pH-dependent cell fusion is caused by both the pathogenic and nonpathogenic type viruses and G proteins. Second, the G(Arg)-induced cell fusion at the neutral pH requires some cellular factor(s) which is specifically expressed on the cells of neuronal origin, but not on the nonneuronal BHK-21 cells. On the other hand, the low pH-dependent cell fusion of rabies virus does not seem to require such tissue-specific factor(s) (Mifune *et al.*, 1982; Whitt *et al.*, 1991). We suppose that the pH-independent fusogenic ability would be an *in vitro* marker of the pathogenic virus and would contribute to the efficient invasion of the virus into neuroblastoma cells in culture and possibly into neuronal cells *in vivo* (see below).

Many kinds of viruses, including paramyxoviruses and some members of human retroviruses like the human immunodeficiency virus (HIV), are known to display pH-independent cell fusion activity (the ability to cause cell fusion at a neutral pH) and are assumed to have a conserved fusogenic domain in the viral envelope glycoproteins (Richardson *et al.*, 1986). We supposed that the rabies virus G protein should also have a similar fusogenic domain for displaying the cell fusion activity at a neutral pH. Accordingly, we looked in the primary sequence of rabies virus G protein for a possible consensus sequence, such as F-X-G-X-V/I-I/L-G, which was found at the N-terminus of F_1 protein of the paramyxoviruses as well as of gp41 of HIV-1. After all, we found a homologous sequence, ranging from positions 360 to 366 (360-F-N-G-I-I-L-G-366) of the G protein, locating 27 amino acids downstream from the position 333, which was also connected downstream by a similar hydrophobic sequence as that found in F_1 protein of paramyxoviruses (Fig. 9). Accordingly, we assume that this homologous region found in rabies virus G protein may be a putative fusion domain (or a part of it).

Tuffereau *et al.* (1989) pointed out that the presence of a positively charged amino acid (arginine or lysine) at position 333 is necessary for a crucial step of viral invasion to the neuronal cells. Our present study strongly indicates that arginine at position 333 is essential for the G protein-induced cell fusion at a neutral pH. We think that the arginine-333-containing region is not a receptor-binding site, but would work for efficient interactions of G protein with a presumed neuronal factor(s) on the cell, which is essential for pH-independent membrane fusion on the surface of neuronal cells in collaboration with other regions on the G protein molecule. One such collaborating region may be a neurotoxin-like sequence, located at positions 189–214, and another one a putative fusogenic domain as mentioned above. The former region is a sequence that resembles the toxic loop of snake venom neurotoxins (the loop in the toxin is known to be involved in binding to the acetylcholine-binding site on AChR molecule; Lentz *et al.*, 1984). It is of much interest to determine whether and how these three regions of the G protein (the region from 189 to 214, the arginine-333-containing region, and a putative fusogenic domain) collaborate in giant cell formation and possibly in viral invasion into the neuronal cells.

Lentz *et al.* (1984) suggested that nicotinic acetylcholine receptor (nAChR) might serve as a rabies virus receptor *in vivo*, and the neurotoxin-like region of the rabies virus G protein might be involved in binding the virus to the nAChR-positive cells. We suppose that the neuronal cell-specific factor(s) induced in the sodium

FIG. 9. Comparison of the putative fusogenic domain of rabies virus G protein with that of the fusion proteins of other viruses. A hydrophobic region, a putative fusion domain, of the rabies virus G protein, ranging from positions 360 to 385, is compared with the presumed fusion domain (conserved hydrophobic region) of envelope proteins of the paramyxoviruses (F_1 protein) and human immunodeficiency virus (gp41 protein). Underlined are identical residues which are found both in rabies virus G protein and in either of four other envelope proteins listed. Hydrophobic amino acids are shaded. RV, rabies virus (Morimoto *et al.*, 1989); SV, Sendai virus (Blumberg *et al.*, 1985); MV, measles virus (Richardson *et al.*, 1986); RSV, respiratory syncytial virus (Collins *et al.*, 1984); HIV1, human immunodeficiency virus type 1 (Wain-Hobson *et al.*, 1985).

butyrate-treated NA cells supports the G protein to cause the cell fusion, probably by serving as a receptor for G proteins, and nAChR may be included in such neuronal factors. We also suppose that pH-independent fusogenic ability would contribute to the efficient invasion of the virus into neuroblastoma cells *in vitro* (in our preliminary experiments, we observed that the pathogenic ERA virus could infect to the sodium butyrate-treated NA cells at a neutral pH in the presence of NH_4Cl which completely blocked the endocytosis-mediated viral invasion) and possibly into neuronal cells in the brain where the factor(s) might be present. This assumption seems to be consistent with previous reports of comparative studies on the behavior of pathogenic and nonpathogenic viruses in *in vitro* and *in vivo* infections (Wunner *et al.*, 1984; Dietzschold *et al.*, 1985; Kucera *et al.*, 1985). Wunner *et al.* (1984) described that pathogenic and nonpathogenic viruses displayed no qualitative difference in the efficiency of the viral attachment to NA cells. In this case, however, NA cells were not pretreated with sodium butyrate. Dietzschold *et al.* (1985) reported that nonvirulent mutant viruses are deficient in cell-to-cell transmission in the mouse neuroblastoma cells. They also reported that the pathogenic viruses spread within the brain much more rapidly than the nonpathogenic viruses. Syncytium-forming ability at a neutral pH of the rabies virus G(Arg) protein in NA cell cultures may reflect such an efficient spread of the virulent type virus in the brain. On the other hand, Lafay *et al.* (1991) suggested recently that the pathogenic strain (CVS) of rabies virus should be able to bind several different kinds of receptors to penetrate neurons, while Avo1 (nonpathogenic virus) would be unable to recognize some of them. Accordingly, the requirement of the tissue-specific factor(s) for pH-independent fusogenic activity of the pathogenic virus may only reflect an aspect of the neurotropic nature of the virus. The details of this problem remain to be elucidated.

ACKNOWLEDGMENTS

We thank Dr. Mulligan for his kind permission of using a retroviral expression vector pZIP-NeoSV(X)1, Mr. S. Sakamoto for preparing the mutagenic oligonucleotide primers, and Ms. I. Kodera for assis-

director) from the Ministry of Education, Science and Culture, Japan, and in part by a research grant from the Chemo-Sero-Therapeutic Research Institute (Dr. J. Nonaka, director), Kumamoto, Japan.

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