bacterial LPS acted as an adjuvant to induce CIA in mice [10]. Therefore, it has been suggested that microbial components influence the induction and severity of CIA.

β-Glucans are widely distributed in a variety of microbes, especially fungi. β-Glucans are adjuvants having immunopharmacological activity, levels of which vary with solubility in water, molecular mass, degree of branching and conformation [11–13]. Moreover, β-glucans possess the ability to activate leukocytes, stimulating phagocytic activity and the production of reactive oxygen and cytokines such as TNF-α [13–19]. β-Glucans also have the ability to enhance antibody production in mice [20]. We have recently reported particles containing only β-glucan, OX-CA, prepared from Candida albicans by oxidation with sodium hypochlorite (NaClO). Furthermore, we found that cell wall β-glucan from C. albicans could be solubilized by NaClO, followed by dimethylsulfoxide (DMSO). Such steps yielded a water-soluble fraction, Candida solubilized β-glucan (CSBG) [21]. These β-glucans are capable of activating an alternative pathway of complement, enhancing vascular permeability, having an adjuvant-type effect on the production of antibody and synthesis of interleukin-6 by macrophages in vitro, enhancing antitumor immunity, and so on [22]. Recently, a study using cDNA microarrays to analyze the mRNA expression in human PBMCs stimulated with Candida cell wall glucan, OX-CA and CSBG provided information on the biological activity [14]. CAWS is a water-soluble polysaccharide fraction released from C. albicans into the culture supernatant (Candida albicans water-soluble fraction: CAWS) and is thought to be similar to the free β-1,3-D-glucan actually present in the blood [23]. Recently, we clarified that CAWS exhibits various biological activities, affecting cytokine synthesis, showing lethal toxicity, causing the induction of coronary arteritis in mice, and so on [24–26]. Therefore, these reports suggested that Candida cell wall glucan activates the immune system and enhances the inflammation that exacerbates autoimmune disease.

In the present study, we investigated the ability of OX-CA as an adjuvant to induce autoimmune arthritis. The findings show that OX-CA derived from Candida cell wall acts as an adjuvant to induce collagen-induced arthritis, and the production of anti-CII antibodies. These results implicate β-glucans in the onset and exacerbation of autoimmune disease.

2. Materials and methods

2.1. Animals

Male C57BL/6, C57BL/10, BALB/c, C3H/HeN, DBA/2, and DBA/1 mice (6–7 weeks old) were purchased from Japan SLC Inc., Shizuoka, Japan, and housed in a specific pathogen-free facility.

2.2. Materials

Candida albicans IFO 1385 purchased from the Institute for Fermentation (Osaka, Japan) was maintained on potato dextrose agar (Difco, USA) at 27 °C and transferred once every 3 months. Sodium hypochlorite solution and sodium hydroxide were purchased from Wako Pure chemical. Co., Japan. Dimethylsulfoxide (DMSO) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Bovine Type II collagen solution was purchased from Chondrex LLC. Freund's complete adjuvant (FCA) was supplied by Difco, USA. FCA contained Mycobacterium butyricum at 5 mg/ml. Distilled water (DIW) and saline were from Otuka (Tokyo, Japan).

2.3. Media

A C-limiting medium originally described by Shepherd and Sullivan [27] was used to grow C. albicans. The medium contained (per liter) sucrose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 2 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 1 mg, $FeSO_4 \cdot 7H_2O$ 0.01 g and biotin 25 μg, with a final pH of 5.2. Five liters of medium was placed in the glass jar of a microferm fermentor (New Branswick Scientific, USA) and C. albicans was cultured at 27 °C with aeration at 5 L/min and stirring at 400 rpm.

2.4. Preparation of C. albicans β-glucan

Briefly, acetone-dried cells of C. albicans (2 g) were suspended in 200 ml of 0.1 M NaOH with NaClO (final concentration, 1%) for 1 day at 4 °C. After the reaction, the reaction mixture was centrifuged and the insoluble fraction (OX-CA) was collected and dried by washing with ethanol and acetone. OX-CA suspended in DMSO was ultrasonically disrupted and the resulting supernatant was designated CSBG [22]. OX-CA (5 mg) was suspended in 1 ml of saline and sonicated for 30 s. After centrifugation, the supernatant was removed and OX-CA was resuspended in saline. The suspension of OX-CA was kept in a refrigerator prior to use. CSBG was resoluble in 0.5 N NaOH and dialyzed against saline which was changed several times. A solution of CSBG was prepared at 5 mg/ml and kept refrigerated prior to use. Acetone-dried cells of C. albicans were suspended in saline at 5 mg/ml as an adjuvant.

2.5. Preparation of CAWS

CAWS was prepared from C. albicans strain IFO1385 according to conventional methods [23]. Briefly, 4 L of C-limiting medium was added to a glass incubator and cultured for 2 days at 27 °C with aeration at 5 L/min and stirring at 400 rpm. Following the culture, an equal volume of ethanol was added and after the mixture had stood overnight, the precipitate was collected. The precipitate was dissolved in 250 ml of distilled water, ethanol was added, and the mixture was allowed to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS.

2.6. Induction of arthritis by administration of type II collagen with OX-CA

The acetic acid solution of bovine type II collagen (CII) (2 mg/ml) was mixed with an equal volume of the OX-CA

suspension at 5, 2.5, 1.25, 0.625 or 0 mg/ml, and 100 μl of the mixture was injected subcutaneously (s.c.) into the tail of a DBA/1 mouse. Therefore, the animal was immunized with 100 μg of CII and OX-CA (250 μg). As a negative control, mice were immunized with 100 μg of CII mixed with an equal volume of saline. These procedures were repeated as a boost 21 days later.

2.7. Comparison of the arthritis induced with OX-CA to that induced with Freund's complete adjuvant

The solution of CII (2 mg/ml) was mixed with an equal volume of OX-CA suspension (5 mg/ml), and 100 μl of the mixture was injected subcutaneously (s.c.) into the tail of a DBA/1 mouse. As a positive control, DBA/1 mice were immunized s.c. with 100 μg of CII emulsified in an equal volume of FCA adjuvant, and as a negative control, mice were immunized with 100 μg of CII mixed with an equal volume of saline. These procedures were repeated as a boost 21 days later.

2.8. Comparison of injection methods

Solutions of CII with saline, FCA or OX-CA were prepared as described above. DBA/1 mice were injected with CII and adjuvant s.c. into the tail or intraperitoneally (i.p.). The injection of CII in saline was performed s.c. but not i.p. These procedures were repeated 21 days later.

2.9. Induction of arthritis by administration of CII with various components from C. albicans

The solution of CII (2 mg/ml) was mixed with an equal volume of various components from C. albicans (OX-CA, CSBG, CAWS and dried-cells) prepared as described above at 5 mg/ml. The mixture (100 μl) was injected s.c. into the tail of DBA/1 mice. These procedures were repeated 21 days later.

2.10. Induction of arthritis in different mouse strains injected with CII and FCA or OX-CA

Solutions of CII with saline, FCA or OX-CA were prepared as described above. Various strains of mice (DBA/1, DBA/2, BALB/c, C57BL/6, C3H/HeN and C57BL/10) were injected with CII and adjuvant s.c. via the tail. These procedures were repeated 21 days later.

2.11. Evaluation of arthritis

To evaluate the severity of arthritis, animals were assessed for redness and swelling of the limbs and a clinical score was allocated to each mouse weekly, for up to 56 days. The scoring system was based on that of Williams et al [28] where 0 = normal, 1 = slight swelling and/or erythema, 2 = extensive swelling and/or erythema and 3 = joint distortion and/or rigidity. The maximum score per mouse was 12.

2.12. Histology

Mice were killed at 42 days after the first immunization with CII and FCA or OX-CA. Control mice injected with CII and saline were killed similarly. Their paws were amputated, fixed in 10% neutral formalin and decalcified. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin—eosin.

2.13. Measurement of antibodies to CII

Individual serum samples were obtained at 21 days before the booster injection and at day 42 and frozen at -80 °C prior to use. A 96-well microtiter plate (Nunc 442404, F96 Maxiscorp) was coated with 50 μl of CII (1 μg/ml) in carbonated buffer (0.1 M, pH 9.6) and incubated overnight at 4 °C. The plate was washed with phosphate-buffered saline containing 0.05% Tween 20 (Wako Pure Chemical Co.) (PBST), and blocked with PBST containing 0.5% BSA (B-PBST) for 40 min at 37 °C. After further washing, the plate was incubated with a 1:1000 dilution of each serum sample for 40 min at 37 °C, washed again, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Zymed Laboratories Inc.) also for 40 min at 37 °C. After another wash, tetramethylbenzidine (TMB) microwell peroxidase substrate (Kirkegaard and Perry Laboratories Inc., MD, USA) was added to the wells. Color development was stopped with 1 N phosphoric acid and optical density was measured at 450 nm. The standard curves were constructed using the pooled sera of arthritic DBA/1 mice and the antibody titer was determined arbitrarily.

2.14. Statistics

Student's t-test was used to analyze the data statistically.

3. Results

3.1. Arthritis induced by immunization of type II collagen with OX-CA as an adjuvant

To examine the ability of OX-CA to induce arthritis as an adjuvant, type II collagen (CII) with OX-CA (0, 31.25, 62.5, 125, 250 μg) was injected s.c. into mice on day 0 and day 21. The arthritis score is shown in Fig. 1. Mice injected with CII and FCA developed arthritis in 4—5 weeks. However, only the mice injected with 250 μg/mouse of OX-CA developed arthritis (Fig. 1). The incidence of the disease is summarized in Table 1. The dose of 250 μg/mouse induced arthritis in more than 80% of animals. Therefore, the results show that OX-CA acts as an adjuvant for collagen-induced arthritis and a dose of 250 μg/mouse has the optimal effect.

To compare the ability of OX-CA as an adjuvant to induce arthritis with that of Freund's complete adjuvant (FCA), type II collagen (CII) with saline, CII emulsified with an equal volume of FCA or CII plus OX-CA was injected s.c. into mice on day 0 and day 21. The arthritis score is shown in Fig. 2A. Mice injected with CII and FCA developed arthritis in 4—5 weeks.
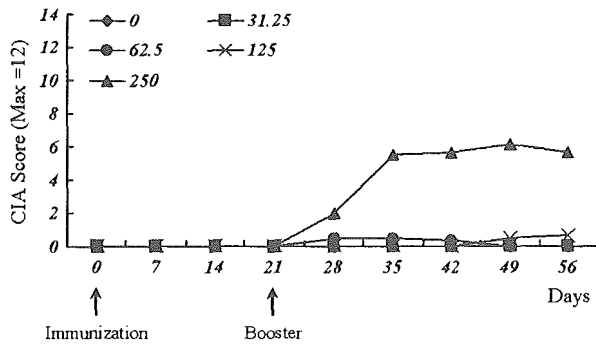
Fig. 1. Severity of the arthritis induced by immunization with bovine CII and OX-CA as an adjuvant. Mice were injected s.c. with CII and OX-CA (0—250 μg per mouse). The injections were repeated 21 days later. The severity of arthritis was determined every week after the injections. Results show means of the collagen-induced arthritis score (CIA score, maximum = 12 per mouse) over 56 days.

Table 1
The incidence of arthritis in mice administered CII with OX-CA

	OX-CA dose (μg/mouse)				
	0	31.25	62.5	125	250
Incidence	0/6	0/6	1/6	1/6	5/6
Incidence ratio (%)	0	0	16.7	16.7	83.3

Mice injected with CII and OX-CA developed arthritis with a similar time course. Fig. 2 B—D shows macroscopic views of representative arthritic paws at 42 days. The paws of mice immunized with CII plus OX-CA (D) developed arthritis as well as those of mice immunized with CII plus FCA (C). The injection of CII with saline did not induce inflammation in the joints (B). Furthermore, the arthritis induced by CII with OX-CA was more severe than that induced by CII with FCA. For example, mice injected with CII and OX-CA had inflammation of the joints in all limbs. These results show that OX-CA acts as an adjuvant for collagen-induced arthritis.

Mice with arthritis induced by the administration of CII with FCA or OX-CA were scarificed on day 42 and their paws were removed and processed for histological analysis of the joints. Mice treated with CII and saline (Fig. 3A) showed features of joint space (j), articular cartilage (c), bone (b), and bone marrow (m) seen in normal mice. However, the histopathology of mice injected CII plus FCA (Fig. 3B) or OX-CA (Fig. 3C) was similar. There was destruction of the articular cartilage and bone in both groups.

It is important for inducing arthritis that the anti-CII antibody is produced in serum. Therefore, anti-CII antibody levels were investigated in the sera of mice injected with CII and FCA or OX-CA on day 21 (Fig. 4A) and day 42 (Fig. 4B). The results show that CII administered with OX-CA induced the production of anti-CII antibody as effectively as CII with FCA. Thus, CIA mice treated with OX-CA as an adjuvant produce the anti-CII antibody.

3.2. Effect of route of injection

In this series of experiments, the injection of CII emulsified with FCA into the proximal region of the tail induced severe inflammation and necrotized the tail. Therefore, the collection of blood from a tail vein and booster injection was difficult. To examine whether the route of injection influences the onset of
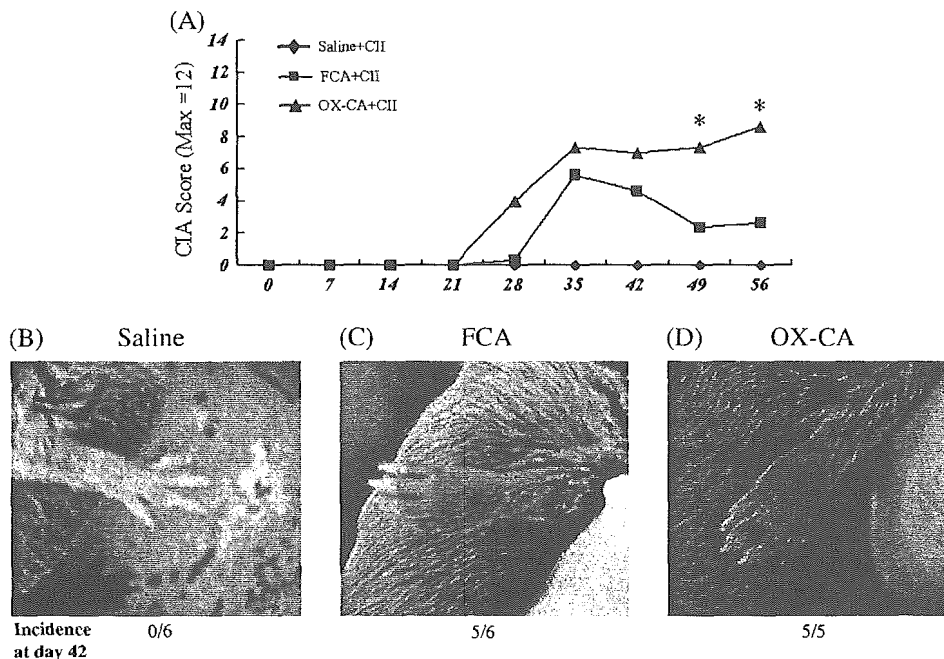

Fig. 2. Comparison of arthritis induced with OX-CA to that induced with Freund's complete adjuvant. Mice were injected s.c. with bovine CII on day 0. FCA or OX-CA was used as an adjuvant and saline was used as a control. The injections were repeated 21 days later. The arthritis score was determined on day 56 after the first injection (A). (B—D) a representative image of a paw in each group (B: saline; C: FCA; D: OX-CA as an adjuvant) and incidence of disease. *$p < 0.05$, OX-CA versus FCA.

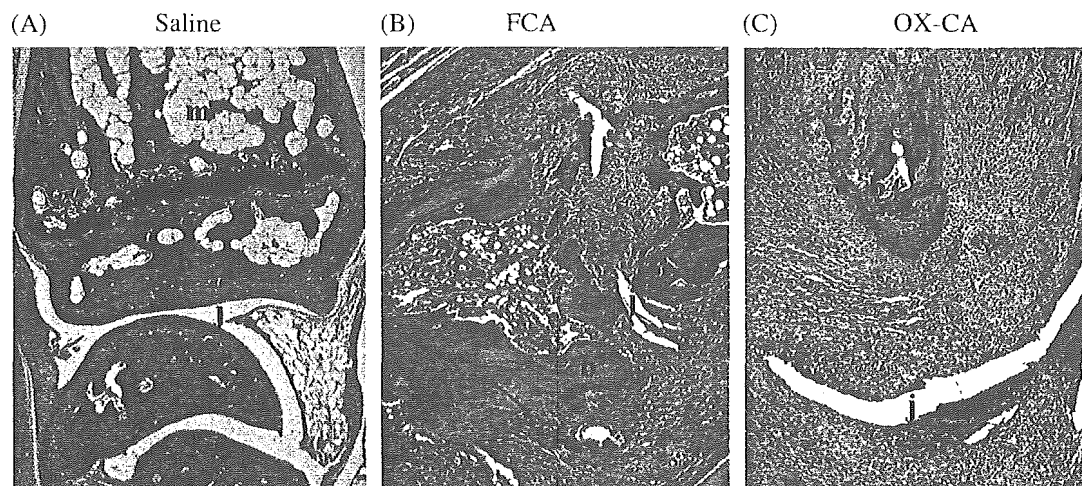(A)   Saline   (B)   FCA   (C)   OX-CA

Fig. 3. Histological changes in distal interphalangeal joints of mice with arthritis. Mice were injected with CII and adjuvant on day 0 and the injection was repeated 21 days later. On day 42, mice were killed and the paws removed and processed for histological analysis. (A) A non-arthritic joint from a mouse immunized with CII and saline. The joint space (j), articular cartilage (c), bone (b), and bone marrow (m) are indicated. (B) A joint from a CIA mouse injected with FCA as an adjuvant. (C) A joint from a CIA mouse injected with OX-CA as an adjuvant. Original magnification: ×100.

CIA, we injected CII with adjuvant, subcutaneously (s.c.) or intraperitoneally (i.p.). DBA/1 mice were injected with CII plus FCA or CII plus OX-CA into the proximal region of the tail or intraperitoneally. All groups developed arthritis on day 42 after the first immunization except for the control mice treated with CII plus saline (Fig. 5A). However, the severity of the arthritis differed with the route of injection when FCA was used as an adjuvant. Although mice treated with CII emulsified with FCA intraperitoneally developed arthritis, the severity was milder than that following subcutaneous injection, e.g., only one digit was swollen. On the other hand, when OX-CA was used as an adjuvant, there was no difference between the routes of injection. Both routes induced an equally severe arthritis in mouse limbs. The anti-CII antibody levels were investigated, but no difference was observed between the methods of immunization (Fig. 5B). Although the severity of the arthritis was mild, the antibody titer was the same value in the sera from mice treated with CII emulsified with FCA intraperitoneally.

3.3. Effect of components of C. albicans on the induction of arthritis

Various components (OX-CA, CSBG, CAWS and Acetone-dry cells) from C. albicans were tested for their ability to induce arthritis. According to a similar protocol, mice were injected with CII plus the components of C. albicans on day 0 and day 21. The results show that the administration of OX-CA, CSBG and cells with CII induced inflammation of the joints (Fig. 6A), and the anti-CII antibody was produced in sera from mice treated with those adjuvants (Fig. 6B). The OX-CA and cells had a comparable effect in terms of severity, and the severity was milder in mice treated with CII plus CSBG. However, none of the mice immunized with CAWS plus CII developed arthritis. Furthermore, the anti-CII antibody titer was much lower than for the other adjuvants. These results show that β-glucan from C. albicans has the ability to induce arthritis, which differs in degree of onset, severity and antibody production.

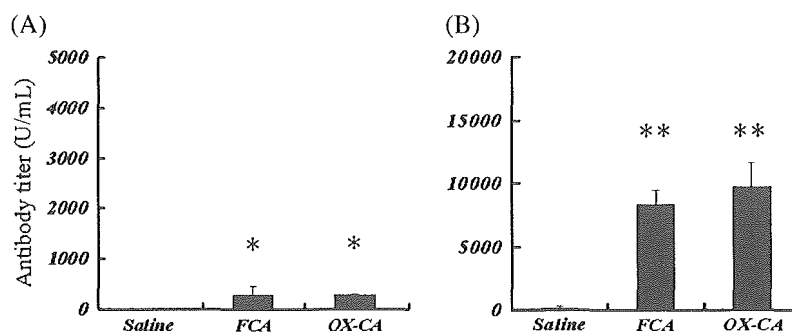(A)   (B)

Fig. 4. Anti-CII antibody production on injection of CII with OX-CA on day 21 and day 42. Mice were injected s.c. with CII and adjuvant. FCA or OX-CA was used as an adjuvant and saline was used as a control. The injections were repeated 21 days later. On day 21 and day 42, blood was collected and anti-CII antibody levels in serum were determined by using ELISA (A: day 21; B: day 42). Error bars show the standard deviation. $*p < 0.05$ versus saline, $**p < 0.01$ versus saline.
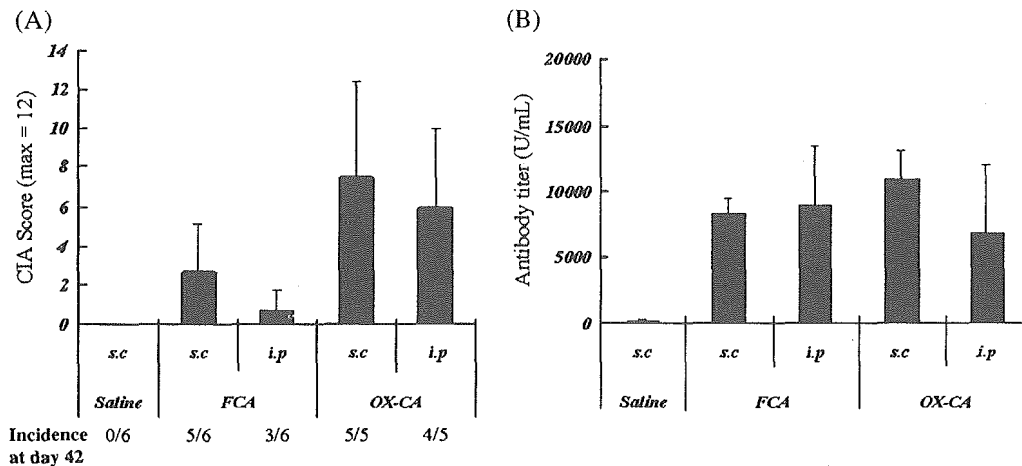
Fig. 5. Comparison of route of injection for collagen-induced arthritis. Mice were injected s.c. or i.p. with CII and adjuvant. FCA or OX-CA was used as an adjuvant and saline was used as a control. The injections were repeated 21 days later. The arthritis score was determined on day 42 and the incidence of disease was calculated (A). At the same time, serum was collected from the mice and the anti-CII antibody titer was measured (B). Error bars show the standard deviation.

3.4. Comparison of anti-CII antibody production in serum from various strains of mice immunized with CII and adjuvant

Susceptibility to CIA is linked to MHC-class II molecules, and the two strains of mice (DBA/1 and B10.RIII) most commonly used are highly susceptible to CIA. To examine whether susceptibility to CIA exists when OX-CA is used as an adjuvant, various strains of mice (DBA/1, DBA/2, BALB/c, C57BL/6, C3H/HeN and C57BL/10) were immunized with CII and FCA or OX-CA on day 0 and day 21. None of the strains except for the DBA/1 mice suffered from arthritis regardless of which adjuvant was used (data not shown). Antibody production was measured in serum on day 21 (Fig. 7A) and day 42 (Fig. 7B). The antibody titer varied with the strain and adjuvant. On day 21, anti-CII antibody was produced in sera from DBA/1, C57BL/6 and C3H/HeN mice treated with CII and FCA, but not produced in C57BL/6 and C3H/HeN mice treated with CII and OX-CA (Fig. 7A). Only DBA/1 mice produced the antibody on injection of CII and OX-CA. DBA/2, BALB/c and C57BL/10

mice did not produce it after either treatment. On day 42, a remarkable secondary response to CII was observed (Fig. 7B). Although antibody was produced in C57BL/6 and C3H/HeN mice treated with CII and FCA, the titer was lower than in DBA/1 mice. Furthermore, only DBA/1 mice exhibited increased production of the anti-CII antibody on day 42 when OX-CA was used as an adjuvant. The results show that in the CIA model using OX-CA as an adjuvant, not only MHC-class II molecules but also susceptibility to OX-CA is important to induce arthritis.

4. Discussion

The activation of autoreactive cells is required for the development of autoimmune disease. Infectious agents, for example, peptides from microbial proteins that have sufficient structural similarity with self-peptides and microbial superantigen have been considered possible factors involved in the activation of autoreactive T cells [29]. In the CIA model used in this research, the fact that arthritis was not induced
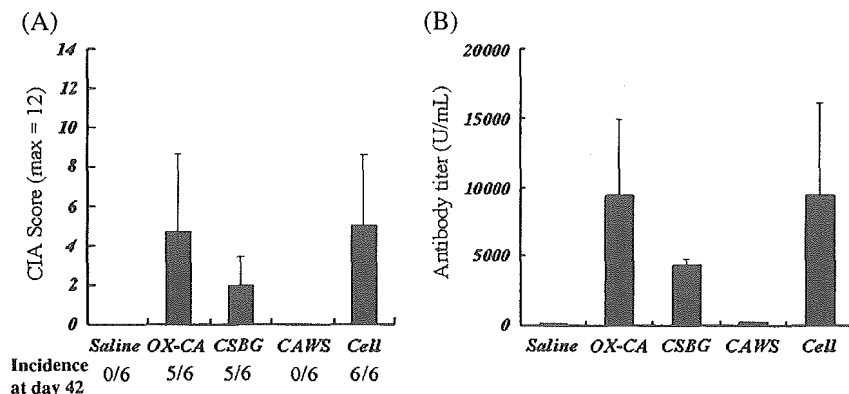

Fig. 6. Induction of arthritis by injection of CII with components of Candida albicans. Mice were injected s.c. with CII and various components from C. albicans (OX-CA, CSBG, CAWS and dead cells) as an adjuvant. The injections were repeated 21 days later. The arthritis score was determined on day 42 and the incidence of disease was calculated (A). At the same time, serum was collected from mice and the anti-CII antibody titer was measured (B). Error bars show the standard deviation.
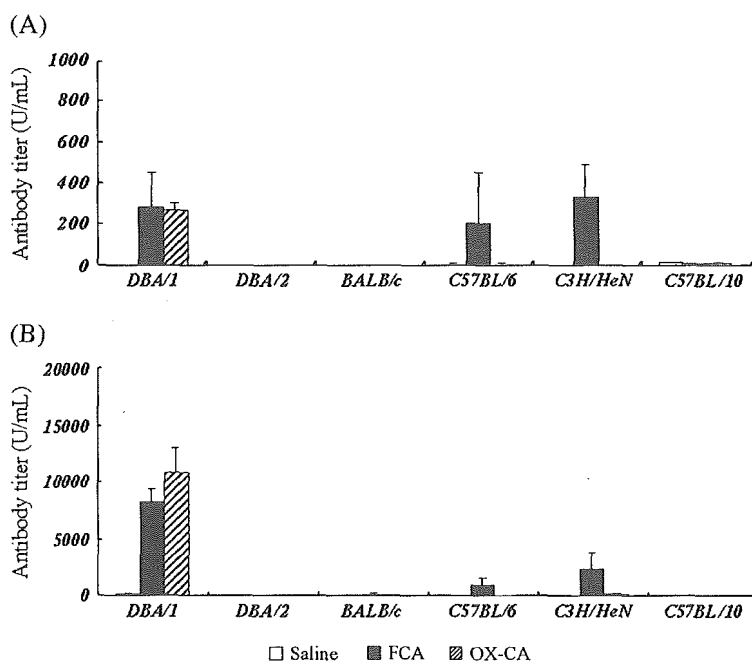
Fig. 7. Comparison of anti-CII antibody production in serum from different strains of mice injected with CII and adjuvant. Mice (DBA/1, DBA/2, BALB/c, C57BL/6, C3H/HeN and C57BL/10) were injected s.c. with CII and FCA or OX-CA as an adjuvant. The injections were repeated 21 days later. On day 21 (A) and day 42 (B), serum was collected from the mice and the anti-CII antibody titer was measured. Error bars show the standard deviation.

by immunization with antigen only shows the importance of microbial components of FCA to the induction. The dead-cells of *Mycobacterium tuberculosis* in FCA act as an adjuvant and enhance the production of anti-CII autoantibodies. In this study, we have shown that β-glucans derived from *Candida albicans* act as an adjuvant for autoimmune arthritis in mice (Fig. 1). The arthritis that developed when β-glucan was used as an adjuvant was more severe than that when FCA was used (Fig. 2A). Histological changes (Fig. 3) and anti-CII autoantibody production (Fig. 4) were observed similarly in the mice immunized with CII and FCA or OX-CA, particles of β-glucan. The results suggested that β-glucan acts as an adjuvant, producing similar symptoms and levels of antibody production such as FCA.

Various infectious agents, including viruses and bacteria, have been suspected to be causative agents of autoimmune diseases such as RA. For example, lipopolysaccharide (LPS), a Gram-negative bacterial component, plays a role in some diseases in which autoantibodies or antigen-specific T cells are involved [9,30,31]. The inflammation that results from a microbial infection leads to the local activation of antigen-presenting cells and can result in the enhanced processing and presentation of self-antigens. That process can result in the activation of autoreactive T cells and spreading of epitopes. The fact that β-glucan acts as an adjuvant in the induction of arthritis suggested that the mechanism mentioned above can be conceived in the case of not only bacterial and viral infections but also fungal infections.

CIA is purportedly restricted to mice bearing the MHC class II H-2q or H-2r haplotypes. However, Campbell et al. reported that mice derived from the C57BL/6 background developed CIA with a high incidence and sustained severity

when a modified immunization procedure was used whereby the dose of *M. tuberculosis* in FCA was increased [32]. The report supported the concept that the susceptibility to CIA may reflect conditions involved in the change of adjuvant. Therefore, we compared the strain difference for induction of CIA using OX-CA as an adjuvant. Although symptoms such as swelling of the paws and joints, and local inflammation, did not develop in the mice injected with CII plus FCA or OX-CA except DBA/1 mice, anti-CII antibody production differed for each strain. Interestingly, C57BL/6 and C3H/HeN mice immunized with CII and FCA produced anti-CII antibody, but mice injected with CII and OX-CA did not (Fig. 7). The results suggested that the mechanism behind the effect of OX-CA and FCA is different, although the symptoms of arthritis in DBA/1 mice administered CII with OX-CA were similar to those in mice treated with FCA. FCA is one of the most commonly used adjuvants in research. It also contains killed *M. tuberculosis*. The *Mycobacterium* in FCA attracts macrophages and other cells to the injection site, which enhances the immune response. However, the mechanism of the adjuvant's effect is complicated when whole *Mycobacterium* is used. On the other hand, OX-CA used in this study is a pure component composed of β-linked glucose. Recently, research into the cellular receptor and molecular mechanism of β-glucan has advanced significantly [33]. For example, a number of receptors have been identified, including complement receptor 3 (CR3) [34], lactosylceramide [35], scavenger receptors [36] and Dectin-1 [37,38]. Dectin-1 was shown to be the major receptor for β-glucan on macrophages and to mediate cellular responses to particulate β-glucan, including the production of proinflammatory cytokines [38,39]. Furthermore, Hong et al. speculated that β-glucan might act as an adjuvant

in immunotherapy for cancer using monoclonal antibody [40,41]. They suggested that the role of β-glucan involves CR3, enhancing the effect of anti-tumor mAbs. Therefore, the effect of β-glucan on the induction of autoimmunity also may involve the production of proinflammatory cytokines dependent on a cellular receptor such as Dectin-1 that activates antigen-presenting cells and the enhancing the effect of anti-CII autoantibody that is important for collagen-induced arthritis, via CR3.

Furthermore, the production of anti-CII antibody differed between DBA/1 and DBA/2 mice administered CII with OX-CA (Fig. 7). Recently, we reported that a β-glucan from *Sparassis crispa* fruiting bodies (SCG) induced the production of interferon-gamma (IFN-γ) in splenocytes derived from both DBA/1 and DBA/2 mice [42]. Furthermore, not only IFN-γ, but also tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-12 (IL-12) were produced by splenocytes from DBA/2 mice stimulated with SCG [43]. This shows that DBA/1 and DBA/2 are high responders to β-glucan. However, in the present study, DBA/1 mice injected with CII plus OX-CA produced anti-CII antibody and developed arthritis, but DBA/2 mice did not produce antibody and did not develop arthritis (Fig. 7). The results suggested that not only the sensitivity to β-glucan as an adjuvant but also genetic background including MHC, complement system and so on are important for the induction of arthritis when using OX-CA as an adjuvant.

During the induction of CIA there were technical failures, for example, the death of mice due to mistakes with the administration of the emulsion, and necrosis of the tail caused by excessive inflammation in mice injected with CII and FCA. The main disadvantage of FCA is that it can cause granulomas, inflammation at the inoculation site, and lesions. The use of Bacillus Calmette—Guerin (BCG) as a vaccine can lead to complications such as keloids, lupus vulgaris, and granulomas [45]. Therefore, we changed the route of administration and examined whether the intraperitoneal injection of CII with an adjuvant can induce arthritis. When FCA was used as the adjuvant, arthritis developed but the severity was less than that induced by subcutaneous injection. On the other hand, with OX-CA, there was no difference irrespective of the method of injection (Fig. 5). Thus, there are significant advantages to using OX-CA as an adjuvant to induce arthritis.

In this study, we investigated the ability of various glycans derived from *C. albicans* to induce arthritis. The results showed that acetone dried cells, OX-CA and CSBG acted as an adjuvant, but CAWS did not (Fig. 6). Acetone-dried cells contain both mannan and β-glucan as well as constituent. Either mannan or β-glucan might act as an adjuvant. OX-CA and CSBG have a similar primary structure composed mainly of β-(1—3)- and β-(1—6)-D-glucosidic linkages [21], but differ in being particulate or soluble. Such characteristics influence their biological activity. For example, β-glucan activated leukocytes significantly more effectively in a particulate than solubilized form in terms of production of TNF-α by RAW 264.7 cells, production of hydrogen peroxide by murine PEC, and

production of IL-8 by human PBMC [13]. A difference in biological activity was also observed in this study. The score for arthritis and the anti-CII antibody titer were higher in DBA/1 mice injected with CII plus OX-CA than CSBG (Fig. 6). CAWS is mainly composed of a mannoprotein-β-glucan complex [23]. CAWS has various biological effects in mice causing lethal toxicity and severe vasculitis [44]. Such activities may be regulated by signals mediated by mannose residues. The composition of CAWS is considerably different from that of OX-CA or CSBG. The fact that arthritis did not develop in the mice injected with CII and CAWS may result from those differences. The effect of the adjuvant on the induction of arthritis in DBA/1 mice may depend on β-glucan. In future, it will be necessary to perform research with fungal components containing various structural β-glucans as an adjuvant.

In conclusion, β-glucan derived from *C. albicans* acted as an adjuvant and injection of CII with β-glucan resulted in induction of arthritis with anti-CII autoantibody production. These symptoms resembled those when FCA was used as an adjuvant. The results suggest that fungal infection may induce and exacerbate autoimmune diseases such as RA.

Acknowledgments

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Polyglutamylation of Tubulin during Differentiation of Neural Precursor Cells

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Summary

Polyglutamylation is a major post-translational modification of tubulin in neurons, and a change in glutamylated tubulin level has been reported during differentiation of neurons. In this study, we produced monoclonal antibodies (mAbs) with different specificities for α- and β-subunits of tubulin and investigated polyglutamylation of tubulin during differentiation of neural precursor cells (neurospheres) using these antibodies.

We raised a mAb to carp brain tubulin (K9) and two mAbs to the synthetic peptide A (corresponding to the polyglutamylation site of α-tubulin) (E10 and PG1). K9 reacted with tubulin carrying more than one unit of polyglutamylated chains while PG1 and E10 reacted with tubulin carrying more than two and three chains, respectively. Using ELISA and immunoblot analysis, we revealed that K9 recognizes a conformational change of the conservative region between the two subunits of tubulin. We also found that PG1 specifically recognizes α-tubulin under high NaCl concentrations, and reacts with both subunits under low NaCl concentrations. Using this property of PG1, we observed a change in the localization of α-tubulin during differentiation of neural precursor cells prepared from rat embryonic brain. Polyglutamylated tubulin was not detected in neurospheres, but was detected in cells cultured for one day in medium containing retinoic acid. Polyglutamylated β-tubulin was detected in cells cultured for 5 days; polyglutamylated α-tubulin was localized in the cell body while β-tubulin was seen in neurites. After 7 days, localization of polyglutamylated species changed. Polyglutamylated α-tubulin was localized in both the cell body and neurites. These results indicate that polyglutamylation is important in neuronal differentiation and that each polyglutamylated tubulin subunit plays a different role in differentiation of neurons.

Key words

Polyglutamylation, monoclonal antibodies, tubulin, neurosphere, neuronal differentiation, multi-tubulin hypothesis

Introduction

Microtubules (MTs) are essential for a large variety of functions such as cell division, cell morphology, intracellular transport, and cell motility. In neurons, the complex MT network is important for neurite outgrowth and axonal transport (Black et al., 1989; Cambray-Deakin and Burgoyne, 1987). This functional diversity correlates with a high level of heterogeneity in the main component of MT protein, tubulin (Wolff et al., 1982). There are two subunits of tubulin, and both α- and β-subunits display a large isoform polymorphism due to the expression of multiple genes and several post-translational modifications. This heterogeneity is thought to allow MTs to undergo specialized functions as conferred by the "multi-tubulin hypothesis" (Cleveland, 1987). This idea has been supported by several observations in the last few decades (Edde et al., 1983; Edde et al., 1987; Gard and Kirschner, 1985; Kreitzer et al., 1999). Tubulin is a good substrate for various modification enzymes, and seven different types of post-translational modification (acetylation/deacetylation, tyrosination/detyrosination, generation of $\Delta 2$-tubulin, polyglutamylation, polyglycylation, palmitoylation, and phosphorylation) have been reported so far (Westermann and Weber, 2003). Polyglutamylation (Edde et al., 1990) is one of the major modifications of brain tubulin, consisting of the sequential addition of glutamate units. The added unit is linked by an isopeptidic bond to the γ-carboxyl of a glutamate residue of the main polypetidic chain. Addition of the other units occurs through peptide bonds and leads to formation of polyglutamyl chains of

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various lengths. This modification occurs in two α-tubulin isotypes (α1 and α2) and four β-tubulin isotypes (β2, β4, β5, and β6). It is catalyzed by tubulin polyglutamylase and forms at least three major protein complexes (32, 50, and 80 kD subunits) (Regnard et al., 2003). Regnard et al. (2003) reported that the 32 kD subunit, PGs1, has no catalytic activity on its own. In addition, polyglutamylase has been partially purified from mouse brain (Regnard et al., 1998).

Accumulating data suggest that polyglutamylation can differentially influence the interaction between tubulin and MT-associated proteins (MAPs) such as tau and kinesins. These proteins show strong affinity for MT composed of tubulin modified by the addition of glutamyl chains to a length of three residues (they show a lesser affinity for longer side chains) (Larcher et al., 1996; Boucher et al., 1994). Tubulin isoforms with short (<3 units) polyglutamyl chains have a high affinity for MAP2, while the affinity of tubulin for long polyglutamyl chains (>4 units) is drastically reduced. In contrast, the binding of MAP1B to MTs remains strong even for tubulin with long side chains (Bonnet et al., 2001).

Polyglutamylated tubulin is abundant in neurons, and developmental regulation of polyglutamylation of both tubulin subunits in mouse brain neurons has been reported (Audebert et al., 1994). Most of the neurons found in the mature central nervous system originate from precursor cells in the ventricular zone of the fetal brain (McConnell, 1988; McKay, 1989). However, the existence of polyglutamylated tubulin in neural precursor cells is unknown. In addition, the localization of polyglutamylated tubulin in neurons has not been investigated because the monoclonal antibodies (mAbs) reported so far do not specifically react with each subunit. In this article, we describe the use of mAbs with different specificities for polyglutamylated tubulin, in order to investigate polyglutamylation of tubulin and localization of polyglutamylated α-tubulin during differentiation of neural precursor cells.

Materials and Methods

Materials

Tubulin was isolated as MT protein from carp and Wistar rat brains. MTs were polymerized according to the method of Shelanski et al. (1973) with some modifications (Arai, 1983). For protein chemical experiments, tubulin was further purified using a phosphocellulose column.

E3B8, an anti-α-tubulin mAb, and E1, an anti-β-tubulin mAb, were produced in our laboratory (Arai and Matsumoto, 1988; Ohuchi et al., 2000). DM1A (a mAb specific for α-tubulin), JBR.3B8 (for β1- and β2-tublin), and SDL.3D10 (for β3-tublin) were purchased
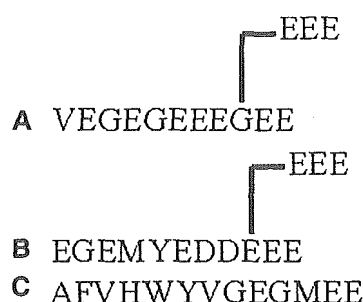
Fig. 1. Structure of synthetic peptides. A) The synthetic peptide A corresponds to residues 440-450 of the α1-tubulin, carrying three lateral glutamyl units. The first glutamyl unit is linked via an amide bond to the γ-carboxylic group of the lateral chain of ^{445}E, and the second and third units are linked to the former via a α-peptidil bond. B) The synthetic peptide B corresponds to residues 443-453 of the β3-tubulin, carrying three lateral glutamyl units. C) Peptide C corresponds to residues of 403-415 of α-tubulin. The residue is highly conserved in the regions 393-405 of β-tubulin.

from Sigma Aldrich Fine Chemicals, (St. Louis, MO, USA) and Rat401 for nestin was from BD pharmingen (San Diego, CA, USA). Anti-serum against neurofilament-H subunit (NF-H) and horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin G (IgG) (H +L) polyclonal antibody were obtained from Sigma Aldrich Fine Chemicals and from Zymed Laboratories (South San Francisco, CA, USA), respectively. Alexa 488-labeled anti-mouse IgG and Alexa 594-labeled anti-mouse IgM polyclonal antibodies were purchased from Molecular Probes (Eugene, OR, USA). AMCA (7-Amino-4-methylcoumarin-3-acetic acid)-labeled streptavidin was obtained from Vector Laboratories (Burlingame, CA, USA).

Three peptides were chemically synthesized (Fig. 1). Peptides A and B mimic the polyglutamylated sites of α- and β-subunits of tubulin, respectively, and peptide C corresponds to the region from ^{401}D to ^{421}E in α-tubulin. This region is highly conserved between both subunits. For immunization and immunochemical experiments, these peptides were conjugated with KLH (Keyhole Limpet Hemocyanin) (Wako Chemicals, Osaka, Japan).

Production of monoclonal antibodies

Production of mAbs was performed as described previously (Yoshida et al., 1995; Kuroiwa et al., 2000). In brief, BALB/C female mice (Sankyo Labo Service, Tokyo, Japan) were immunized with KLH-conjugated peptides and carp brain tubulin by the conventional and footpad procedures. Three days after the final injection, spleens and lymph nodes were removed and cells prepared from these organs were fused with the myeloma cell line PAI using 50% (w/w) polyethylene glycol (Roche Diagnostics, Indianapolis, IN, USA) or

by the electrofusion method. The fused cells were plated on 96-well culture plates (BD Pharmingen) and cultured in HAT (hypoxantine, aminopterin, and thymidine) selection medium (Invitrogen, Carlsbad, CA, USA).

Hybridoma-producing anti-tubulin antibodies were screened by the enzyme-linked immunosorbent assay (ELISA) using 96-well polyvinyl chloride microtiter plates (BD pharmingen) coated with KLH-peptides A and B or rat MT proteins as described previously (Arai and Matsumoto, 1988). Reactivity of anti-tubulin antibodies with polyglutamylated tubulin was characterized by immunoblotting procedures.

Culture and differentiation of neurospheres

Neurospheres were prepared as described by Wohl and Weiss (1998) with some modifications. Briefly, telencephalons were dissected from embryonic day 14 (E14) brains of Wistar rats (Sankyo Labo Service) and mechanically dissociated in Hank's buffered saline solution (HBSS) with a micropipette. The cells were then suspended in serum-free medium and plated on poly 2-hydroxy-ethlmethacrylate (HEMA) (Sigma Aldrich Fine Chemicals)-coated 75-cm^2 tissue culture flasks in the presence of 20 ng/ml of bFGF (basic fibroblast growth factor) (Pepro Tech, Rocky Hill, NJ, USA). The basic culture medium for neurospheres was composed of DMEM (Dulbecoo's modified Eagle's medium)/F12 (1:1) (Sigma Aldrich Fine Chemicals) supplemented with 5 mM Hepes buffer, 0.6% glucose, 3 mM sodium bicarbonate, and 2 mM gulutamine. A defined hormone and salt mixture composed of 25 μg/ml insulin, 100 μg/ml transferrine, 20 nM progesterone, 60 μM putrescine, and 30 nM sodium selenite was used in place of serum.

After 7 days *in vitro*, primary spheres in flasks were removed, spun down, and dissociated into single cells. The cells were then plated on poly-D-Lysine coated-75-cm^2 tissue culture flasks or 8 well culture slides (BD Pharmingen) for 6 days at 1.4 × 10^5 cells/cm^2 in basic medium containing 0.5% FBS (fetal bovine serum) and 0.5 mM retinoic acid (Sigma Aldrich Fine Chemicals). After 6 days, the medium was replaced with basic medium, and the cells were cultured for a further 3 to 21 days.

Proteolytic digestion of tubulin

For limited digestion, phosphocellulose-purified tubulin (PC-tubulin) was dissolved in 0.1 M MES buffer (pH6.4) containing 0.5 mM MgCl$_2$ and 2 mM EGTA and digested by 1% subtilisin (Sigma Aldrich Fine Chemicals) at 30 °C for 30 min. Digestion was stopped by the addition of 1 mM PMSF (phenylmethanesulfonyl fluoride) (Sigma Aldrich Fine Chemicals). For

isolation of epitope peptides, PC-tubulin was dissolved in 50 mM Tris-HCl buffer (pH9.0) and digested with AP1 (Sigma Aldrich Fine Chemicals) at an enzyme-to-substrate ratio of 1:25 at 37 °C overnight (Masaki et al., 1981). Digested peptides were separated by HPLC (high-performance liquid chromatography).

Analysis of polyglutamylated subunits and isoforms by immunoblot procedure

To study polyglutamylated subunits, developing rat brains or cultured cells were homogenized in PBS containing 1 mM PMSF (Roche Molecular Biochemicals), 10 μg/ml leupepsin (Sigma Aldrich Fine Chemicals), and 10 μg/ml pepustatin (Sigma Aldrich Fine Chemicals); homogenates were centrifuged at 1,200 × g for 5 min. Tubulin subunits in these crude extracts were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electrically transferred to polyvinlidene floride (PVDF) microporous membrane (Millipore, Billerica, MA, USA) according to the method of Towbin et al. (1979).

To study polyglutamylated isoforms, tubulin preparations from developing brains were separated by isoelectric focusing (IEF) using Immobiline and MutiphorII Electrophosis Systems (Ammersham Biosciences, Buckinghamshire, UK) according to O'Farrel's method with some modifications (1975). Proteins were diluted in a sample buffer containing 2.5% Pharmalyte (mixed at a ratio of 1 (pH4.2-4.9): 3 (pH4.5-5.4): 1 (pH5.0-6.0)), 2% Nonidet P-40, 8 M Urea, 5% 2-mercaptoethanol, and Bromophenol Blue. The samples were electrofocused for 5 hr at 3,500 V, 10 mA, and 20 W. Proteins on gels were transferred to PVDF (polyvinylidene fluoride) membrane using 0.7% acetic acid as the transfer solution according to Albaugh's (1989) method.

Tubulin subunits and isoforms transferred on membranes were analyzed by immunoblot procedure as described previously (Arai and Matsumoto, 1989). Immunoreactive proteins were visualized with a peroxidase-conjugated anti-rabbit antibody and an enhanced chemiluminescence detection ECL Kit (Ammersham Biosciences) after exposure to Hyperfilm (Ammersham Biosciences).

Observation of polyglutamylated tubulin by immunofluorescence procedure

Neurospheres were fixed with 4% paraformaldehyde at 4 °C for 3 hr according to the method of Wohl and Weiss (1998). After being washed with PBS, the samples were dehydrated with increasing concentrations of ethanol (Wako Chemicals); samples were shaken in 50% ethanol (15 min), followed by 70% ethanol (15

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min), 80% ethanol (30 min), 90% ethanol (30 min), 95% ethanol (45 min), and 100% ethanol (60 min), at room temperature. After dehydration, they were cleared by toluene (Wako Chemicals). The samples were then embedded in paraffin (Wako Chemicals) and sectioned by microtome (Erma, Tokyo, Japan), and sections were placed on glass slides (Matsunami Glass, Osaka, Japan). The slides were dried in a hybridization oven (Tokyo Rikakikai) overnight.

The cells cultured on poly D-Lysine-coated 8-well culture slides (BD Pharmagen) were fixed with 4% paraformaldehyde at 4 °C for 1 hr, rinsed with phosphate buffer saline containing Ca^{2+} and Mg^{2+} (PBS(+); Invitrogen, Carlsbad, CA, USA), and blocked with 1% skim milk in PBS(+) at 4 °C as described previously (Ohuchi et al., 1994).

Primary antibodies were added and incubated for 1 hr at 4 °C. After being washed with PBS(+), culture slides were incubated with secondary antibodies for 1 hr at 4 °C. In the case of double and triple labeling, appropriate secondary antibodies were added together. Localization of tubulin in neurospheres and cells was observed with fluorescence microscopy (BH2-RFCA, Olympus, Tokyo, Japan), and processed with processing software; ARUGUS 50 image processor (Hamamatsu Photonics, Hamamatsu, Japan).
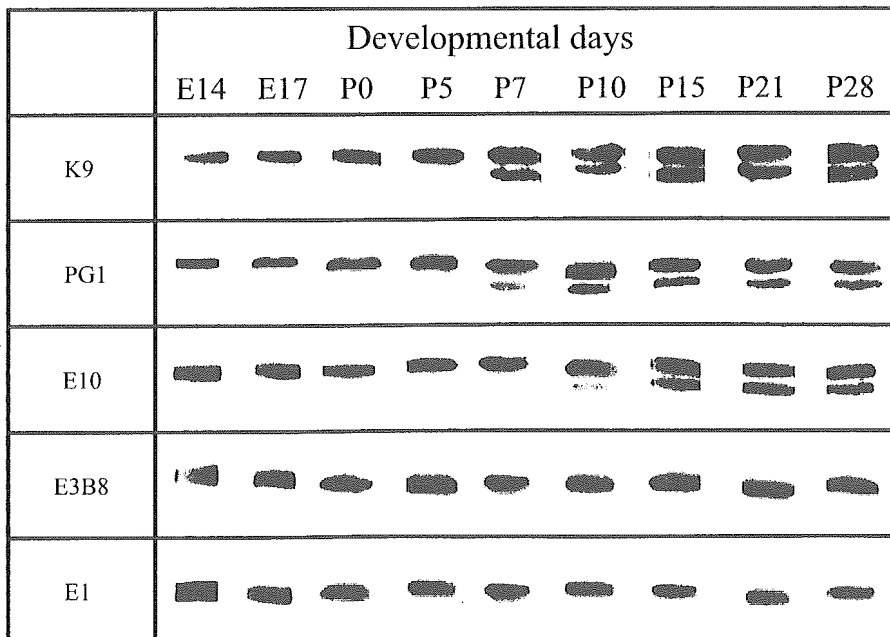
Results

Production of mAbs against anti-polyglutamylated tubulin

Mice were immunized with carp brain tubulin and KLH-peptides (Fig. 1) that mimic the polyglutamylated site of α- and β-subunits of tubulin, and three mAbs were produced reacting with both subunits (Fig. 2). K9, an IgG2 mAb raised from carp tubulin, and E10, an IgM mAb raised from the synthetic peptide A, reacted almost equally with both subunits. PG1, another IgM mAb raised from peptide A, reacted with β-tubulin to a lesser extent than α-tubulin.

It is well known that α-tubulin is polyglutamylated in the newborn brain while β-tubulin is modified (polyglutamylated) later at birth (Audebert et al., 1994). Extracts from developing rat brains were submitted to SDS-PAGE, and reactivity of these mAbs with polyglutamylated tubulin subunits was confirmed by immunoblot analysis (Fig. 3). These antibodies mainly reacted with α-tubulin in rat brains of E14 to postnatal day 5 (P5) while they reacted with both subunits in postnatal day 10 (P10) and with adult brains, demonstrating that these mAbs recognize polyglutamylated tubulin. It is noted that K9 and PG1 reacted with P7 β-tubulin while E10 did not.

Fig. 2. Reactivity of mAbs with tubulin from adult rat brain. Tubulin preparation (0.2 mg/ml) was submitted to SDS-PAGE, and the reactivity of mAbs with tubulin subunits was investigated by immunoblot analysis.

	Developmental days								
	E14	E17	P0	P5	P7	P10	P15	P21	P28
K9									
PG1									
E10									
E3B8									
E1									

Fig. 3. Reactivity of mAbs with tubulin from developing rat brains. Extracts (1 mg/ml) from embryonic and postnatal brains were submitted to SDS-PAGE, and the reactivity of mAbs was investigated by immunoblot analysis.
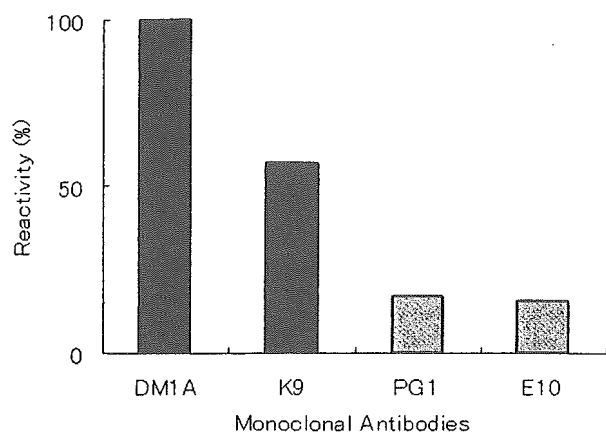
Fig. 4. Reactivity of mAbs with subtilisin-digested tubulin. PC-tubulin was digested with 1% subutilisin for 30 min. Change in reactivity of mAbs with tubulin by the limited digestion was measured by ELISA using micro plates coated with the digested tubulin.

Studies of binding regions of tubulin for anti-poly-glutamylated tubulin mAbs

During limited subtilisin digestion, cleavage by the protease occurs at position of ^{438}D of α-tubulin and at position ^{433}Q of β-tubulin (Serrano et al., 1984; Redeker et al., 1992) and C-terminal regions of both subunits including polyglutamylated sites. The regions from 426 to 430 of α-tubulin are important for reactivity of DM1A, an anti-tubulin mAb (Breitling and Little, 1986). This suggests that mAbs recognizing the polyglutamylated region become non-reactive after limited digestion, while DM1A remains reactive. To obtain further information about the binding sites of mAbs on tubulin, the effect of limited subtilisin digestion on binding of mAbs was studied by ELISA using DM1A as a control antibody and microtiter plates coated with intact and digested tubulin as antigens (Fig. 4). Binding of both PG1 and E10 was dramatically reduced following subtilisin digestion, while binding of K9 was partly affected. These results suggest that the important region for binding of K9 is not included in the C-terminal region, which is removed by subtilisin digestion.

To analyze the epitope region of K9, tubulin was digested by Ap-1 and a K9 binding peptide, corresponding to ^{403}R-^{430}K of α-tubulin, was obtained by RP-HPLC. This region is highly conserved between both subunits. K9 also reacts with both subunits, leading us to postulate that the epitopes of α- and β-subunits are ^{403}AFVHWYVGEGMEE and ^{393}AFLHWYTGEGMDE, respectively. We synthesized the epitope candidate peptide of α-tubulin, peptide C. Reactivity of K9 with peptide C was investigated by ELISA using microtiter plates coated with the synthetic peptide (Fig. 5). The immunoreactivity observed was dependent on the K9 concentration added to each well
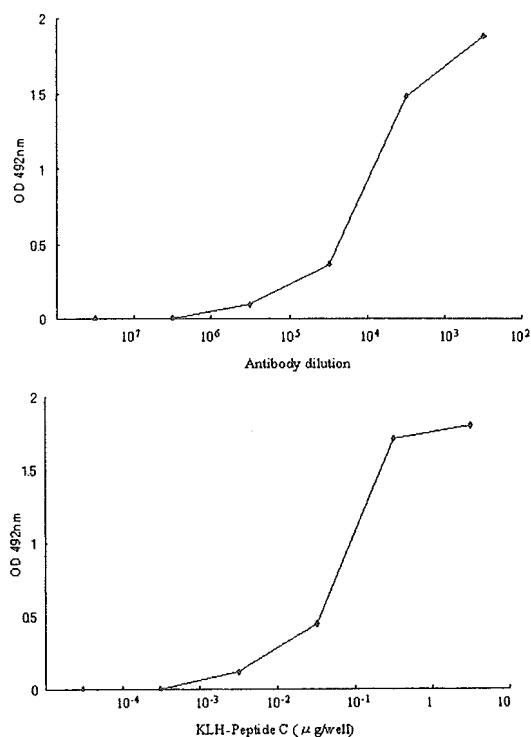
Fig. 5. Reactivity of K9 with synthetic peptide C. A) Ascites of K9 was diluted at indicated concentrations with PBS containing 10% FBS, and reactivity of the mAb was investigated by ELISA using micro plates coated with the KLH-peptide C (0.5 μg/ml). B) Ascites of K9 was diluted 4,000-fold with PBS-10% FCS, and reactivity of this antibody was investigated by ELISA using micro plates coated with indicated concentration of KLH-peptide C.

(Fig. 5A) and on peptide concentration coated on the plates (Fig. 5B), indicating that peptide C corresponds to the K9 epitope of α-tubulin. This suggests that K9 recognizes the conformational change that occurs in the conserved regions, and that the C-terminal regions removed by the limited subtilisin digestion might regulate the conformation of the epitope regions.

Post-translational modification causes a large number of electrophoretic variants, which can be easily resolved by IEF (Wolff et al., 1982). To study the number of polyglutamylated chains added on tubulin recognized by mAbs, PC-tubulin was separated by IEF and reactivity of anti-polyglutamylated tubulin antibodies was studied by immunoblot analysis (Fig. 6). In P0 rat brain, two bands were detected by SDL.3D10, a mAb against β3-tubulin. The minor band, corresponding to the isoform carrying one glutamylated chain, was detected by K9 while the major band, the non-modified isoform, was not. In the case of α-tubulin, K9 reacted with all isoforms except the most basic one (Fig. 6A). These results show that K9 recognizes tubulin species carrying more than one unit of polyglutamylated chains. Reactivity of K9 was compared to PG1 and E10, indicating that PG1 and E10 recognize tubulins carrying polyglutamylated chains of more than two and

A

B (-)   (+)
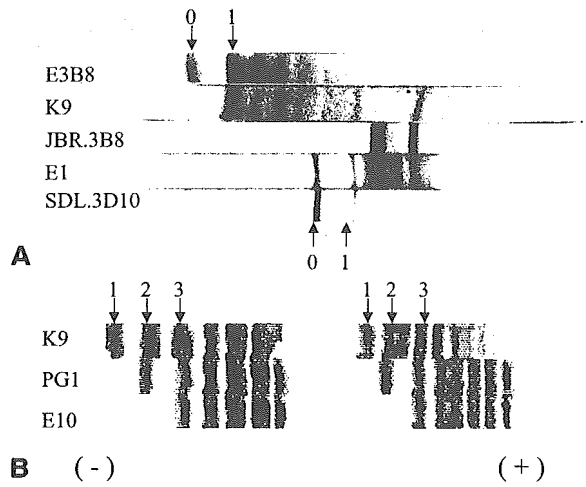
Fig. 6. Identification of polyglutamylated tubulin isomers recognized by mAbs. MT proteins from newborn (A) and adult (B) rat brains were separated by IEF, and tubulin isomers recognized by mAbs were investigated as described in Materials and Methods. Numbers 0 to 3, indicate numbers of polyglutamate units added on tubulin isoforms.

three units, respectively. As shown in Fig. 3, K9 and PG1 reacted with P7 β-tubulin while E10 did not. Speculating from the specificity of these mAbs, the number of polyglutamylated chains carried by P7 β-tubulin is one or two.
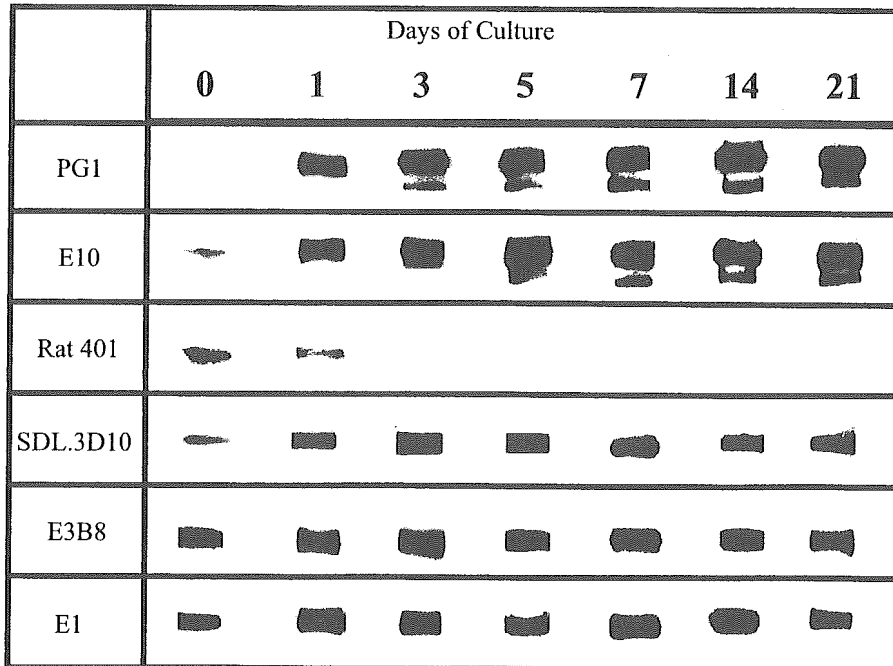
Properties of the three anti-tubulin mAbs obtained in this study are summarized in Table 1.

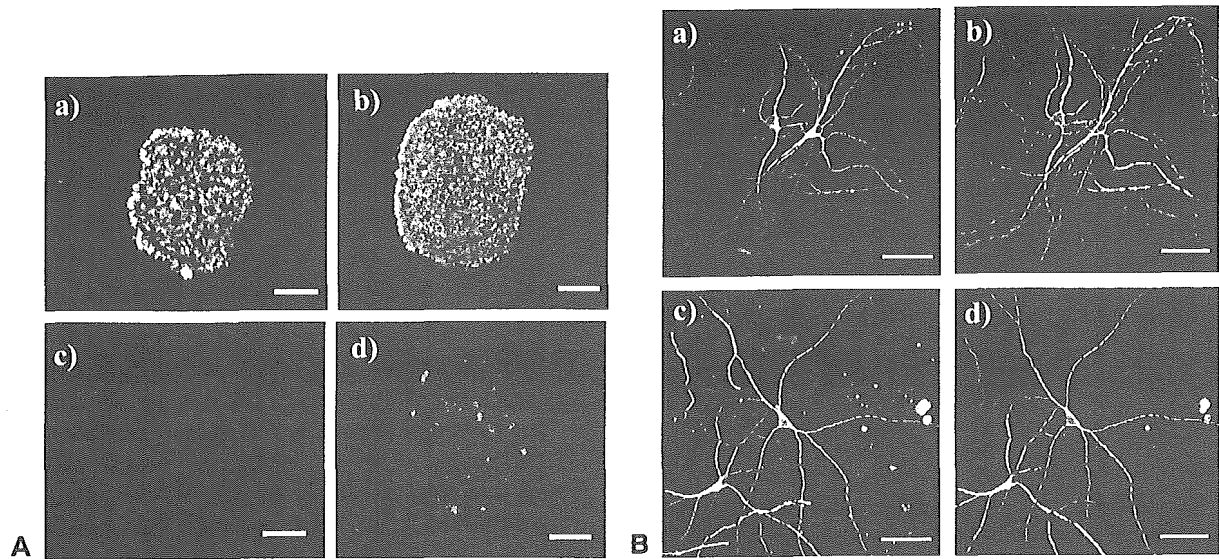## Polyglutamylation of tubulin in neural precursor cells and neurons

The polyglutamylation of tubulin was initially identified in adult brain and neurons (Edde et al., 1990; Audebert et al., 1994; Przyborski and Cambray-Deakin, 1997). We investigated polyglutamylation in neural precursors using neurospheres differentiated by the addition of retinoic acid (Fig. 7). First, we studied the presence of polyglutamylated tubulin in extracts from the differentiated cells by immunoblot analysis. Rat 401, an anti-nestin mAb, and SDL.3D10 were used as markers of neural precursor cells and neurons, respectively. PG1

Table 1. Properties of anti-polyglutamylated tubulin mAbs produced

mAbs	Immunogens	Isotypes	Regions recognized	Comparative Reactivity
K9	Carp brain tubulin	IgG2	^{403}A to ^{415}E (α) ^{393}A to ^{405}E (β)	$\alpha = \beta$
PG1	Peptide A	IgM	Polyglutamylated sites ($\geqq$2 units)	$\alpha > \beta$
E10	Peptide A	IgM	Polyglutamylated sites ($\geqq$3 units)	$\alpha = \beta$

	Days of Culture						
	0	1	3	5	7	14	21
PG1							
E10							
Rat 401							
SDL.3D10							
E3B8							
E1							

Fig. 7. Polyglutamylation of tubulin during differentiation of neurospheres. Neurospheres were cultured for 1 to 21 days in the presence of retinoic acids as described in Materials and Methods. At the indicated day, extracts (1 mg/ml) from cultured cells were submitted to SDS-PAGE, and reactivity of mAbs and anti-sera with tubulin subunits was investigated by immunoblot analysis.

Fig. 8. Observation of polyglutamylated tubulin in neurosphere and neurons differentiated from neurospheres. A) Neurospheres were embedded in paraffin and serially sectioned. The sections were immunostained with E3B8 (a), E1 (b), PG1 (c), and E10 (d). B) Neurospheres were suspended to single cells and cultured for 7 days in the presence of retinoic acid. Cells were double-stained with SDL.3D10 (a) and PG1 (b) or SDL.3D10 (c) and E10 (d). Scale bar = 50 μm.

did not react with tubulin bands in neurospheres (cells at day 0). Greater reactivity is seen with E10 than PG1; this reactivity with E10 is probably due to the presence of a small number of differentiated neurons in neurospheres. At day 1 after the addition of retinoic acid, polyglutamylated α-tubulin was detected by both PG1 and E10. PG1 and E10 detected polyglutamylated β-tubulin from day 3 and day 5, respectively.

Next, we looked at the presence and localization of polyglutamylated tubulin by immunostaining (Fig. 8). In neural precursor cells, PG1 did not stain neurospheres, while a small number of cells were stained by E10 (Fig. 8A). In differentiated cells, double-immunostaining with SDL.3D10/E10 and with SDL.3D10/PG1 show that both PG1 and E10 stain the entire neuron (Fig. 8B).

Use of PG1 for specific probe of polyglutamylated α-tubulin

As shown in Fig. 2, PG1 recognizes both subunits of tubulin, but its reactivity to β-tubulin is much weaker than to α-tubulin. We found that reactivity of PG1 to β-tubulin is dependent on NaCl concentration (Fig. 9). The mAb reacted equally with both subunits in low NaCl concentration (100 mM) while it specifically recognized α-tubulin at high NaCl concentrations (180 mM). Reactivity of PG1 is not affected by pH, and neither NaCl concentration nor pH affects reactivity of E10.

Using PG1 as a specific probe for α-tubulin, we observed localization of polyglutamylated α-tubulin in two types of neurons cultured with retinoic acid for 5
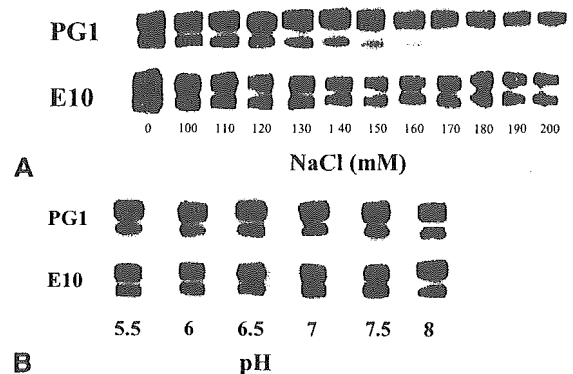

Fig. 9. Effect of NaCl concentration and pH on reactivity of mAbs. Tubulin preparation was submitted to SDS-PAGE, and reactivity of mAbs with tubulin subunits was investigated under indicated concentration of NaCl (A) and indicated pH (B).

days, by immunostaining (Fig. 10). As shown in Fig. 10, PG1 did not stain neurites at 180 mM NaCl while it stained both the cell body and neurites at 100 mM NaCl. This result suggests that polyglutamylated α-tubulin localizes in the cell body.

Change in localization of polyglutamylated α-tubulin during differentiation of neurospheres

The neurofilament (NF), a major neuronal intermediate filament expressed in most neurons, is the most abundant cytoskeletal element in axons (Fliegner and Liem, 1991; Nixon and Shea, 1992; Pant and Veeranna, 1995). NFs are heteropolymers composed of three kinds of subunits, NF-H, NF-M, and NF-L. NF-L and NF-M are coexpressed in embryonic rat brain, while

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100mM NaCl　　　180mM NaCl

SDL.3D10　　　PG1　　　SDL.3D10　　　PG1
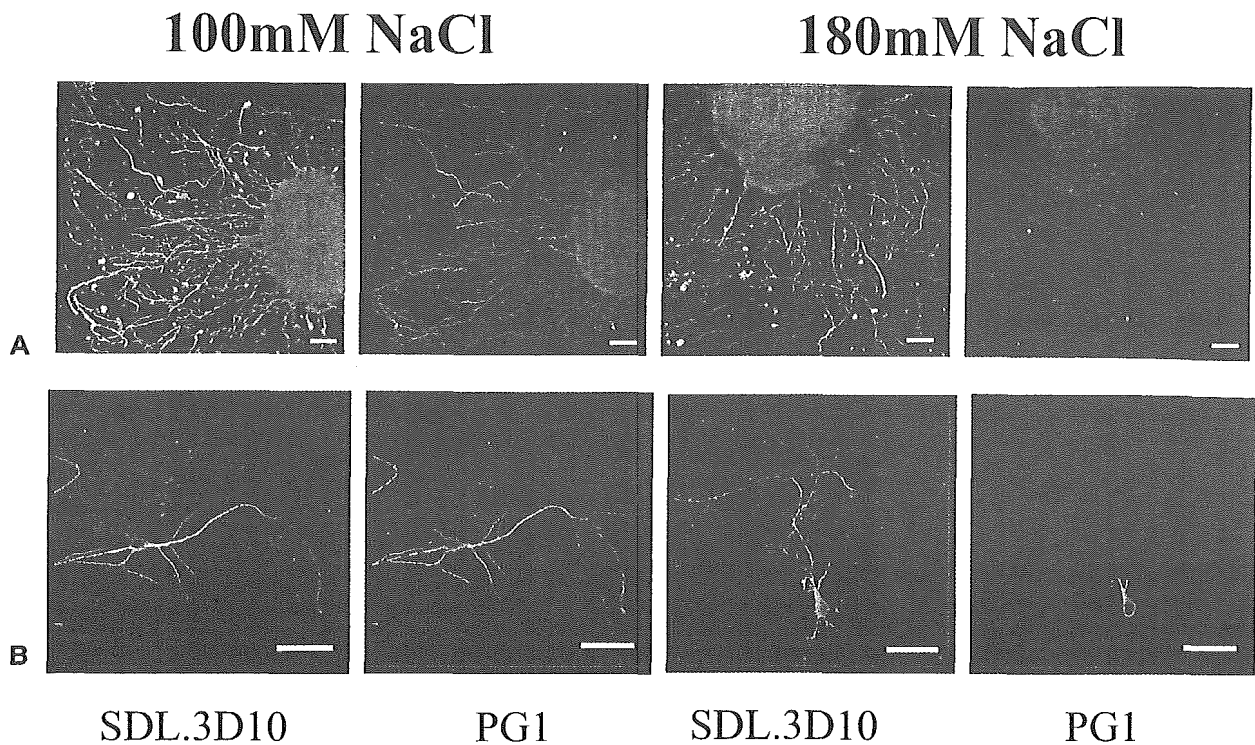
Fig. 10. Localization of polyglutamylated α-tubulin in neurons differentiated from neurospheres. A) Neurospheres were cultured in the presence of retinoic acid for 5 days without suspension into single cells. B) Neurospheres were suspended to single cells and cultured in the presence of retinoic acid for 5 days as described in Materials and Methods. These cells were double-stained with SDL.3D10 and PG1 at 100 mM and 180 mM NaCl. Scale bar = 50 μm.

the NF-H is expressed in a very late embryogenetic or postnatal period (Shaw and Weber, 1982; Schlaepfer, 1987). We observed localization of polyglutamylated α-tubulin during differentiation of neural precursor cells using NF-H as a marker for mature neurons. Neurons cultured for a varying number of days were triple-stained with SDL.3D10, PG1, and NF-H. In day 1 and 3 cultures, PG1 stained both the cell body and neurites at 100 mM NaCl while it stained only the cell body at 180 mM NaCl. NF-H was observed in neither the cell body nor the neurites of these cells (Fig. 11A and B). In day 5 culture, the observation shown in Fig. 10 was confirmed and slight expression of NF-H was observed (Fig. 11C). In day 7 and 14 cultures, PG1 stained both the cell body and neurites at 180 mM NaCl as well as at 100 mM. In these cells, significant levels of NF-H were expressed (Fig. 12A and B).

Discussion

It has been reported that polyglutamylation of α-tubulin occurs in P0 rat brain while β-tubulin is polyglutamylated after birth (Audebert et al., 1994). In Fig. 3, β-tubulin is polyglutamylated in P7 brain and the level increases to P21. The figure also shows that most of polyglutamylated tubulin isoforms in P7 and P10 brains are mono- and di-glutamylated proteins,

respectively, while polyglutamylated tubulin in P15 and older brains has more than three glutamylated units. Using neurospheres as neural precursor cells, we confirmed that no polyglutamylated tubulin is present in neurospheres, indicating that polyglutamylation occurs after induction into neurons. Moreover, polyglutamylated α- and β-subunits of tubulin accumulated during the differentiation of neurospheres (Fig. 7). Since polyglutamylation of β-tubulin is low in young neurons and increases progressively throughout differentiation, it may play a role in maturation of neurons. Neuronal differentiation is accompanied by an increase in MT stability and by changes in both α- and β- subunits (Black et al., 1986; Lim et al., 1989). However, acetylation and detyrosination of α-tubulin do not result in an increase in MT stability (Schulze et al., 1987; Schulze and Kirschner, 1987; Khawaja et al., 1988; Webster et al., 1990), indicating that modification of β-tubulin may play an important role in changing MT stability. Two types of polyglutamylase, catalyzing tubulin polyglutamylation, are proposed. Type I would be present in proliferative cells and would be responsible for the polyglutamylation of β-tubulin and nucleosome assembly proteins (NAPs). Type II would be responsible for α-tubulin in neurons and axonemes (Regnard et al., 2003). Taken together, these facts suggest that polyglutamylated α- and β-tubulin may
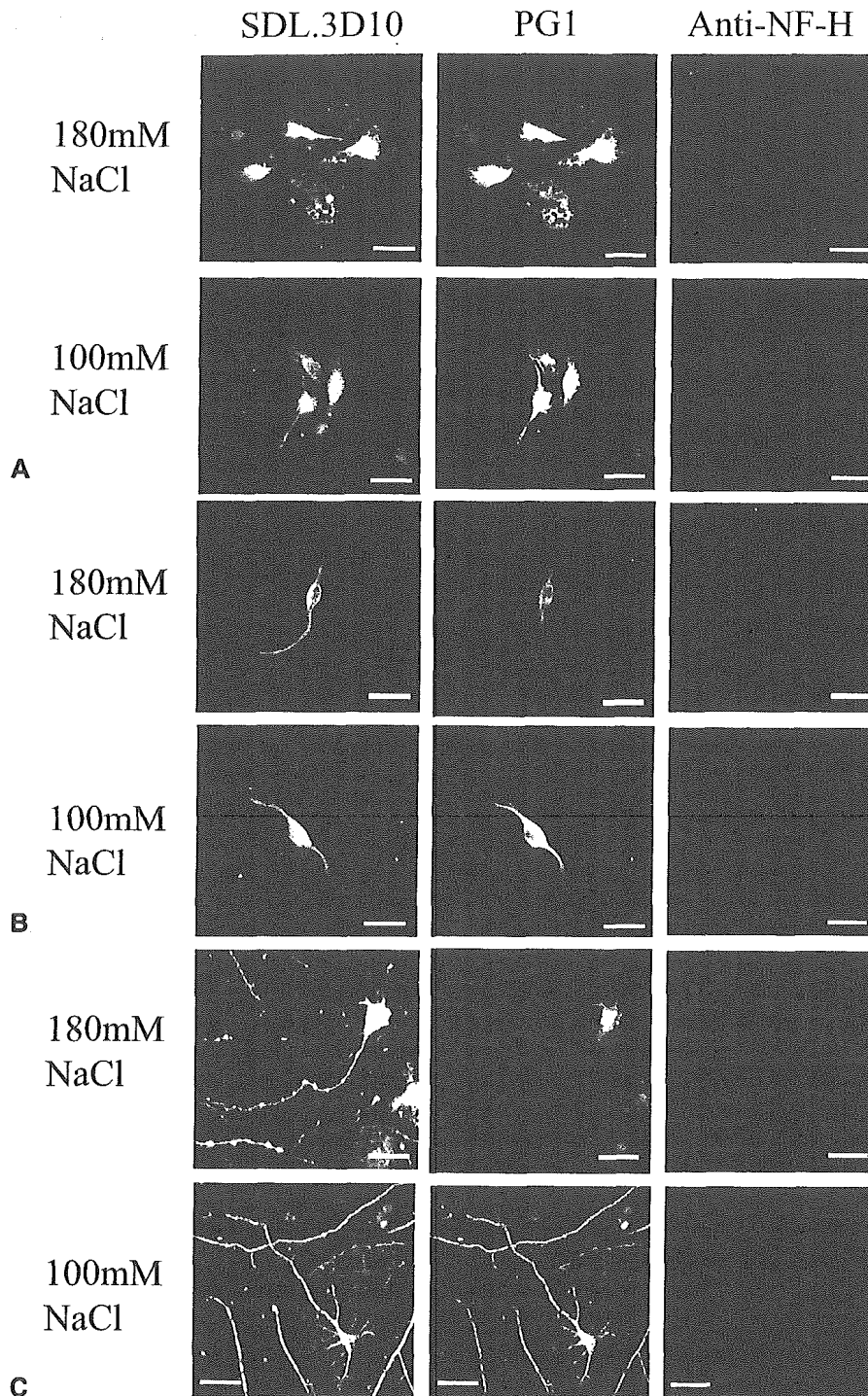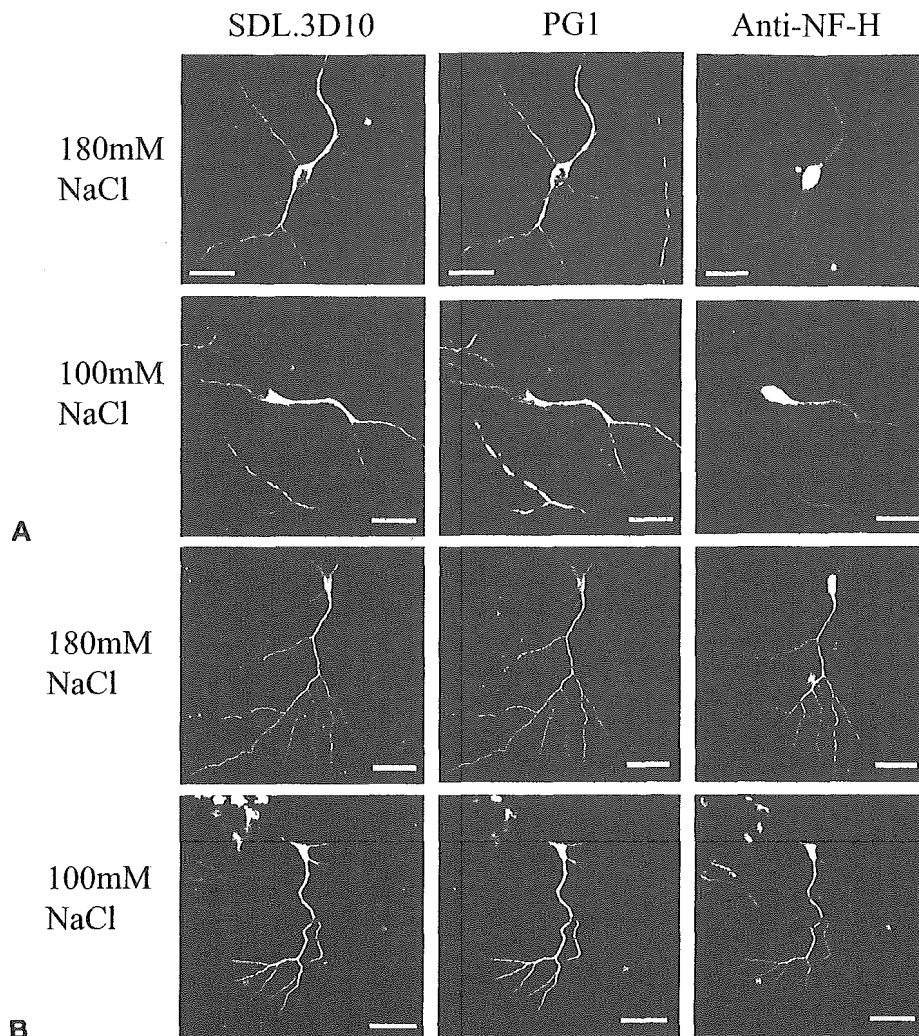
Fig. 11. Localization of polyglutamylated α-tubulin in neurons cultured within 5 days. Neurospheres were suspended into single cells and cultured for 1 day (A), 3 days (B), and 5 days (C). Cells were triple-stained with SDL.3D10, PG1, and anti-NF-H at 100 mM and 180 mM NaCl. Scale bar = 50μm.

play a distinct role in neurons and that the regulation of polyglutamylase responsible for β-tubulin is more important in differentiation and maturation of neurons.

Using PG1 as a specific probe for polyglutamylated α-tubulin, we first revealed that polyglutamylated α-

tubulin was localized in cell bodies (Fig. 10). Furthermore, we found that this localization is observed in the case of NF-H negative neurons and NF-H positive neurons, which are differentially matured, and have polyglutamylated α-tubulin in both neurites and cell

SDL.3D10   PG1   Anti-NF-H

Fig. 12. Localization of polyglutamylated α-tubulin in neurons cultured more than 7 days. Neurospheres were suspended into single cells and cultured for 7 days (A) and 14 days (B). Cells were triple-stained with SDL.3D10, PG1, and anti-NF-H at 100 mM and 180 mM NaCl. Scale bar = 50μm.

bodies. It is reported that the carboxyl-terminal tail domain of NF-H interacts with MTs and NF-H stimulates MT polymerization and forms networks with MTs (Minami et al., 1982; Minami and Sakai, 1983; Minami et al., 1984). Moreover, phosphorylation and dephosphorylation are thought to have an effect on the interaction between MTs and NF-H (Minami and Sakai, 1985; Hisanaga and Hirokawa, 1990). Polyglutamylation of tubulin may be affected by this interaction.

For studying the functions of various proteins, mAbs are very useful probes. In this study, we obtained two interesting mAbs, K9 and PG1. K9 is a conformation-sensitive mAb while reactivity of PG1 is dependent on NaCl concentration. The epitope of K9 is a conservative region between both subunits. However, the region is non-reactive with this antibody when tubulin is not polyglutamylated, indicating that K9 is sensitive to

conformational change in this region; this antibody could prove to be a useful probe for conformational change of this conservative region. Specificity of PG1 for β-tubulin changes dramatically (Fig. 9). Under normal conditions (PBS containing 140 mM NaCl), reactivity of PG1 for α-tubulin is more specific than for β-tubulin. In low NaCl concentration (less than 120 mM), its reactivity for both subunits is almost equal, while it recognizes only α-tubulin in high NaCl concentrations (more than 170 mM). However, reactivity of E10 does not change. These result suggest that PG1 could be used as a specific probe for α-tubulin: using this antibody, we observed localization of α-tubulin in neurons (Figs. 10-12).

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