Involvement of Microtubules in Cytopathic Effects of Animal Viruses: Early Proteins of Adenovirus and Herpesvirus Inhibit Formation of Microtubular Paracrystals in HeLa-S3 Cells

By T. EBINA, M. SATAKE AND N. ISHIDA

Department of Bacteriology, Tohoku University School of Medicine,
2-1, Seiryo-machi, Sendai 980, Japan

(Accepted 10 October 1977)

SUMMARY

In order to examine the involvement of microtubules in the virus-induced cytopathic effect (c.p.e.), the effect of virus infection on the formation of microtubular paracrystals (PC) induced by 10 μg/ml of vinblastine sulphate in HeLa-S3 cells was examined by phase-contrast microscopy. In poliovirus-infected cells, c.p.e. (cell rounding) and the inhibition of PC formation proceeded in parallel, starting 4 h post-infection. In Sendai virus-infected cells, however, PC formation was not inhibited even 24 h post-infection when most infected cells clearly showed c.p.e. (syncytial formation).

In adenovirus-infected cells, the inhibition of PC formation was observed 9 h before the appearance of c.p.e. Cytosine arabinoside (ara C) did not block the inhibition of PC formation in infected cells, but blocked the appearance of late c.p.e. (nuclear alteration). Cycloheximide blocked both the inhibition of PC formation and the induction of late c.p.e. These results suggest that an early protein synthesized de novo by adenovirus is required for direct or indirect inhibition of the microtubular PC formation. Furthermore, on ultraviolet (u.v.) inactivation of adenovirus both activities (induction of early c.p.e. shown by shrinkage of cytoplasm, and inhibition of PC formation) followed the same inactivation curve and were inactivated at a slower rate than viral infectivity and the activity leading to late c.p.e. The u.v. light sensitive target responsible for the induction of early c.p.e. and the inhibition of PC formation is about 20% of that for infectivity and is in accord with the genome size of the early functioning virus genes.

In herpes simplex virus (HSV)-infected cells, the inhibition of PC formation, the appearance of c.p.e. (cell rounding and disappearance of nucleoli) and the synthesis of V antigen proceeded in parallel. These three functions of HSV were not blocked in infected cells even when the de novo synthesis of virus DNA was inhibited by ara C or phosphonoacetic acid (PAA), whereas these three functions were blocked by cycloheximide, suggesting that a protein coded by the input virus genome early after infection inhibits the microtubular PC formation and is responsible for c.p.e. From the u.v. inactivation curve of HSV, it was confirmed that only one-tenth of virus genome was responsible for both activities (induction of c.p.e. and inhibition of PC formation).
INTRODUCTION

Interactions between viruses and susceptible cells in tissue cultures result in cellular alterations referred to as cytopathic effect (c.p.e.) (Enders, 1954). The present understanding of c.p.e. is morphological alteration of cells, usually resulting in death. There are various types, of which the following are three typical examples. With adenovirus the cells shrink and aggregate in grape-like clusters (Pereira, 1958); with poliovirus they round up and then shrink (Barski, Robineaux & Endo, 1955); with Sendai virus they fuse, producing large syncytia (Okada, 1958). However, little is known about the precise mechanisms producing cytopathic effects, and how viral products may interact with cellular organelles (reviewed by Pereira, 1961; Westwood, 1963; Bablanian, 1975).

It is from the following three aspects that the authors are interested in the involvement of the microtubules in cytopathic effects caused by virus infection. First, Dales (1975) has reported from electron microscopic observations that both adenovirus type 5 and reovirus type 3 bind to microtubules during their replication. Luftig & Weihing (1975) also reported that adenovirus binds to rat brain microtubules in vitro. Secondly, it is known that substances which interact with microtubules, such as colchicine and vincristine sulphate (VLB) cause cell rounding (Seeds et al. 1970), suggesting the involvement of microtubules in morphological changes. Thirdly, the treatment of cultured cells with VLB results in the formation of large microtubular paracrystals (PC) (Bensch & Malawista, 1969; Krishan & Hsu, 1969), which are easily detectable under the phase-contrast microscope. The test was found to be extremely simple for studying microtubular changes inside cells (Ebina & Ishida, 1975).

In order to study microtubular involvement in c.p.e., the effect of polio-, Sendai, adenovirus and herpesvirus infections on PC formation in HeLa cells was examined at various times after virus infection. The present paper reports the following findings: (1) the alteration of microtubules is associated with c.p.e. (shrinkage of cytoplasm and cell rounding) in polio- and herpesvirus-infected cells but not in Sendai virus-infected cells (syncytial formation); (2) early proteins of adenovirus and herpesvirus seem to affect microtubules in the cytoplasm.

METHODS

Cell culture. A clonal line with a mean chromosome number of 68 derived from HeLa-S3 cells (Ebina et al. 1969) was cultivated in Eagle’s minimum essential medium (MEM) supplemented with 10% calf serum. For most of the experiments, Lab-Tek chamber slides, which are subdivided by a plastic partition into eight chambers on a standard microscope slide (Lab-Tek Products, Naperville, Illinois, U.S.A.), were used in a CO2 incubator. Before infection, the growth medium was replaced with MEM containing 2% calf serum.

Viruses. The viruses used and original infectivity titres in HeLa cells are as follows: the Adenoid 71 strain of human adenovirus type 1, $10^8$ TCD50 per ml; the Miyama strain of herpes simplex virus type 1 (HSV-1), $10^7$ TCD50 per ml; the Lsc, 2ab strain of poliovirus, $10^{7.5}$ TCD50 per ml; the Z strain of Sendai virus, 3200 haemagglutinin (HA) units per ml.

Determination of PC-positive cells. The method of determination of PC-positive cells has been detailed previously (Ebina & Ishida, 1975). Briefly, monolayer chamber slide cultures of HeLa cells inoculated with various viruses were treated with 10 μg/ml of vincristine sulphate (VLB, Exal, Shionogi Pharmaceutical Co., Osaka, Japan) at 37 °C for 2 h. The cells were then fixed with 100% acetone and prepared as wet mounts in one drop of 0.01 M-phosphate buffered saline of pH 7.2 (PBS). The percentage of cells with distinct
Involvement of microtubules in c.p.e.

intracellular PC was calculated by counting 300 cells under a phase-contrast microscope (oil immersion, 1000 × magnification).

**Determination of c.p.e.-positive cells.** Virus-infected cells were washed with PBS, fixed with ethanol for 15 min and stained with May–Grünwald solution for 5 min, followed by staining with 10% Giemsa solution for 15 min. The percentage of cells with definite morphological changes was calculated by examining 300 cells at 400 × magnification.

**Detection of V antigens.** For poliovirus, herpesvirus and adenovirus, immunofluorescent staining was used. Lab–Tek chamber slide cultures of HeLa cells were washed with PBS, dried in air, fixed with acetone for 10 min at room temperature and stained with fluorescent antibody by the indirect method.

For Sendai virus, the haemadsorption test was used. Chamber slide cultures of HeLa cells were washed three times with PBS and were reacted with a 1% chicken red blood cell suspension in PBS. They were left at 4 °C for 20 min and unadsorbed erythrocytes were removed by washing with PBS. Haemadsorbing cells were observed microscopically.

**Ultraviolet irradiation.** The virus suspension (2–5 ml) in Petri dishes (90 mm diam.) was exposed to ultraviolet (u.v.) light (Toshiba germicidal lamp, two GL10) at a distance of 20.4 cm (900 μW/cm²/s) for varying times. Irradiated preparations, as well as the non-irradiated, were assayed for their ability to induce c.p.e., inhibit PC formation, and infect and adsorb to HeLa cells.

**Infectivity titrations.** Infectivity titres were measured by the tube titration method. Tube cultures of HeLa cells received a series of 10-fold dilutions of the sample, and 50% tissue culture infectious doses (TCID₅₀) were determined by the appearance of cytopathic change on day 14 according to the method of Reed & Muench (1938). Three tubes were used per dilution.

**Adsorbing capacity.** One ml of the adeno- and herpesvirus suspension was added to HeLa cells and incubated for 2 h at 37 °C with occasional shaking. After sedimenting the cells by centrifugation, the amount of virus left unadsorbed in the supernatant was measured by the complement fixation test with specific standard sera.

**Inhibitor experiments.** In order to inhibit DNA synthesis, 10 μg/ml of cytosine arabinoside (ara C) were used for adenovirus-infected cells, and 20 μg/ml of ara C (Buthala, 1964; Feldman & Rapp, 1966) or 200 μg/ml of phosphonoacetic acid (PAA, Abbott Laboratories, Illinois) were used for HSV-infected cells (Overby et al. 1974). In order to inhibit protein synthesis, 10 μg/ml of cycloheximide (Tanabe Pharmaceutical Co., Osaka, Japan) were used (Watanabe, Kudo & Graham, 1967). HeLa cells were treated with each drug beginning 2 h before virus infection; at the doses used these drugs were not cytotoxic for HeLa cells nor did they inhibit PC formation.

**RESULTS**

Two-day-old HeLa cells treated with 10 μg/ml of VLB showed a gradual accumulation of PC in the cytoplasm. VLB-induced crystals under phase-contrast microscopy appeared as shiny, 3 to 10 μm long, 1 μm thick structures contrasted by a surrounding bright halo (Fig. 1 a). Fig. 2 gives the time course of PC formation in HeLa cells. PC formation was detected in 10% of the cells 30 min after VLB treatment and the percentage reached a maximum approaching 95% at 2 h. In the following study, therefore, at various times after virus infection, cells were treated with VLB for 2 h, fixed and examined under the phase-contrast microscope.
Fig. 1. PC formation in HeLa cells after VLB treatment (10 μg/ml) for 2 h. (a) Uninfected HeLa cells. Note dense PC. (b) Poliovirus-infected cells 10 h post-infection. Note few PC. (c) Sendai virus-infected cells 12 h post-infection. Note dense PC. (d) Adenovirus-infected cells 24 h post-infection. Note few PC. (e) Adenovirus-infected cells in the presence of 10 μg/ml of ara C. Note few PC. (f) Adenovirus-infected cells in the presence of 10 μg/ml of cycloheximide. Note dense PC. (g) HSV-infected cells 24 h post-infection. Note no PC. (h) HSV-infected cells in the presence of 20 μg/ml of ara C. Note few PC. (i) HSV-infected cells in the presence of 200 μg/ml of PAA. Note few PC. (j) HSV-infected cells in the presence of 10 μg/ml of cycloheximide. Note dense PC. Phase-contrast, 400× magnification.
Involvement of microtubules in c.p.e.

Effect of poliovirus infection on PC formation in HeLa cells

Poliovirus produced initial cell rounding and late retraction of HeLa cells. When the time course of cell rounding and PC formation in HeLa cells after poliovirus infection at a multiplicity of infection (m.o.i.) of 40 TCD₅₀ per cell was examined, the results shown in Fig. 3 were obtained. Cell rounding was first detected at 4 h after infection and all cells showed cytological change by 10 h post-infection. In accordance with appearance of c.p.e., the inhibition of PC formation was detected in 20% of the cells at 4 h post-infection and detected in almost all cells by 10 h post-infection (Fig. 1 b). This parallelism suggests that the inhibition of PC formation might be closely related to cell rounding by poliovirus infection.

Effect of Sendai virus infection on PC formation in HeLa cells

When HeLa cells were infected with Sendai virus at an m.o.i. of 32 HA units per 10⁶ cells (m.o.i. of 32), infection was first detected by positive haemadsorption of chicken erythrocytes at 6 h post-infection and syncytial formation appeared in 40% of cells at 12 h after infection (Fig. 4). On the other hand, PC formation was not inhibited even at 24 h post-infection when almost 80% cells were clearly involved in syncytial formation (Fig. 1 c).
Fig. 2. Time course of PC formation in uninfected HeLa cells. The monolayer cultures were treated with 10 μg/ml of VLB, fixed with acetone and the number of PC-positive cells was counted at the indicated times.

Based on these results, it seems probable that microtubules in the cytoplasm might not be involved in fusion of Sendai virus-infected cells.

Effect of adenovirus type 1 infection on PC formation in HeLa cells

When HeLa cells were infected with adenovirus type 1 at an m.o.i. of 60 TCD₅₀ per cell (Fig. 5), cytopathic changes appeared at 12 h after infection but the inhibition of PC
Involvement of microtubules in c.p.e.

Fig. 4. Time course of PC formation and c.p.e. in HeLa cells after Sendai virus infection at an m.o.i. of 32 HA units per 10⁶ cells. At the indicated times, PC formation (○—○), cells showing c.p.e. (●—●) and haemadsorptive cells (HAD) were counted.

Fig. 5. Time course of PC formation and c.p.e. in adenovirus-infected cells and effects of ara C and cycloheximide (CH) on PC formation and c.p.e. Monolayer cultures of HeLa cells were infected with adenovirus type 1 at an m.o.i. of 60 TCD₅₀ per cell and PC formation (○—○) and cells showing c.p.e. (●—●) were examined at the indicated times. At 2 h prior to virus infection, 10 µg/ml of ara C or 10 µg/ml cycloheximide was added to the cultures of HeLa cells and at different times after virus infection, PC formation (△—△, with ara C; ■—■, with cycloheximide), c.p.e. (▲—▲, with ara C; ●—●, with cycloheximide) and V antigen (Vag) in infected cells were quantified.

Formation began by as early as 3 h post-infection (Fig. 1d). Thus it seems probable that the inhibition of PC formation by adenovirus infection is not the consequence of cytopathic changes.

In the next experiment, the effect of metabolic inhibitors on PC formation in infected cells was examined. It has been established that inhibitors of DNA synthesis such as ara C
prevent the synthesis of late virus proteins (structural proteins) and inhibitors of protein synthesis such as cycloheximide prevent the synthesis of both early and late virus proteins in adenovirus-infected cells. It has also been found that the cytopathic action of adenoviruses in HeLa cell culture results in two distinct effects: shrinkage of cytoplasm (early c.p.e.), and typical nuclear alteration (late c.p.e.) (Pereira, 1958).

The effects of ara C and cycloheximide on PC formation, V antigen synthesis and c.p.e. in HeLa cells inoculated with adenoviruses were analysed (Fig. 5). Ara C blocked the appearance of late c.p.e. and the synthesis of V antigen, but did not block the inhibition of PC formation in almost all cells (Fig. 1 e). Also, in 15% of cells it did not block the induction of early c.p.e. In the presence of cycloheximide, however, the inhibition of PC formation was blocked in 85% of cells (Fig. 1 f) and early c.p.e. was observed in about 15% of cells. Both V antigen synthesis and the induction of late c.p.e. were completely blocked. It has been established that a high concentration of virions or of penton antigen itself (cell detaching factor) initiates early c.p.e. without replication of infectious virus as a toxic effect (Everett & Ginsberg, 1958; Pettersson & Hoglund, 1969). Thus pentons in the input virus preparation are a likely cause of the early c.p.e. in 15% of the infected cells in the presence of cycloheximide. However, in the presence of cycloheximide, the early gene function of adenovirus which inhibited PC formation in 85% of cells was completely blocked.

Effect of ultraviolet inactivation of adenovirus on inhibition of PC formation and induction of c.p.e.

Another approach to compare the relationship between PC formation and early c.p.e. was made by ultraviolet (u.v.) irradiation of adenovirus. In Fig. 6, the u.v. inactivation rates of infectivity, ability to induce early and late c.p.e., inhibitory activity on PC formation and adsorbing capacity were calculated as percentage activity of the non-irradiated virus. The viral adsorptive capacity was not reduced after u.v. irradiation for up to 3 min. Both infectivity and ability to induce late c.p.e. were the most sensitive to inactivation and they followed the same single hit target curve. The activity to induce early c.p.e. and to inhibit PC formation followed a parallel two hit target curve and was inactivated at a slower rate than the activity to induce late c.p.e. and virus infectivity. Therefore, it is possible that two proteins are responsible for the induction of early c.p.e. and the inhibition of PC formation. The target responsible for the induction of early c.p.e. and the inhibition of PC formation was about 20% of that for infectivity. This genome size is almost in accord with one determined to encode for the early viral functions (Fujinaga & Green, 1970). These results suggest that in addition to pentons themselves, an early protein coded by the early genome is responsible for both the inhibition of PC formation and the induction of early c.p.e. (shrinkage of cytoplasm). In contrast with early c.p.e., the expression of the whole virus genome may be required for the induction of late c.p.e. (nuclear alteration).

Effect of herpes simplex virus infection on PC formation in HeLa cells

Experiments similar to those conducted with adenovirus-infected cells were also carried out with herpesvirus-infected cells. When HeLa cells were infected with herpes simplex virus type 1 (HSV-1) at an m.o.i. of 60 TCD$_{50}$ per cell, both cell rounding and nucleolar alteration (Barski & Robineaux, 1959) appeared 6 h after infection. At the same time, the inhibition of PC formation and the synthesis of V antigen were detected as illustrated in Fig. 7. Next, effects of ara C, PAA and cycloheximide on the induction of c.p.e. and the inhibition of PC formation by HSV infection were examined. Although the de novo synthesis of virus
Involvement of microtubules in c.p.e.

DNA was blocked by both ara C and PAA, c.p.e. and the inhibition of PC formation appeared in these treated cells as well as in the untreated cells (Fig. 1 g, h, i). Moreover, immunofluorescent staining detected the synthesis of virus antigen in infected cells in the presence of PAA or ara C, although the intensity was not so pronounced as in untreated cells. On the other hand, cycloheximide completely blocked both the induction of c.p.e. and the inhibition of PC formation (Fig. 1 j).

Effect of ultraviolet inactivation of HSV-1 on its ability to induce c.p.e. and to inhibit PC formation

After u.v. irradiation of HSV for different periods, viral functions including activities for inducing c.p.e., inhibiting PC formation and infectivity were assayed in order to compare each genome size. As shown in Fig. 8, the infectivity of HSV-1 was the most sensitive
Fig. 7. Time course of PC formation and c.p.e. in HSV-infected cells and effects of ara C, PAA and cycloheximide (CH) on PC formation and c.p.e. Monolayer cultures of HeLa cells were infected with HSV type I at an m.o.i. of 60 TCD₅₀ per cell and PC formation (O--O) and c.p.e. (●--●) were examined at the indicated times. At 2 h prior to virus infection, 20 μg/ml of ara C or 200 μg/ml of phosphonoacetic acid (PAA) or 10 μg/ml of cycloheximide was added to the cultures of HeLa cells and at different times after infection, PC formation (△---△, with ara C; \( \nabla \rightarrow \nabla \), with PAA; ■--■, with cycloheximide), c.p.e. (▲--▲, with ara C; ▼--▼, with PAA; ■--■, with cycloheximide) and V antigen (Vag) in infected cells were quantified.

to u.v. irradiation among the activities tested. Both activities, induction of c.p.e. and inhibition of PC formation, followed the same inactivation curve and were more resistant. The cell-adsorbing capacity was most resistant and was not reduced at all after 3 min irradiation. These results indicate that about one-tenth of HSV virus genome is responsible for the induction of c.p.e. and the inhibition of PC formation.

DISCUSSION

In adeno- and herpesvirus-infected HeLa cells, a molecular alteration of microtubules detectable by the inhibition of PC formation was suggested as an early event, and the time course study indicated that this kind of alteration correlates with the appearance of c.p.e. The estimated genome size to cause c.p.e. and to inhibit the PC formation is also in good agreement.

Although the mechanism of PC formation is not yet completely understood, the following scheme has been proposed by Owellen et al. (1974). When low concentrations (1 μg/ml) of VLB are used, 1 mol of this alkaloid binds 2 mol of tubulin, the subunit of microtubules of 120000 mol. wt., forming a soluble, stable dimer complex. As a result, normal polymerization into microtubules cannot follow. When higher concentrations of VLB (10 μg/ml) are used, there is additional binding of the alkaloid to the tubulin molecule, resulting in polymerization of tubulin into paracrystals (PC) at a VLB:tubulin ratio of 1:1. The metabolic inhibitors used in this experiment did not affect PC formation in HeLa cells.
Involvement of microtubules in c.p.e.

Fig. 8. U.v. inactivation curve of HSV type I. The u.v. inactivation rates for infectivity (■—■), induction of c.p.e. (▲—▲), inhibition of PC formation (●—●) and cell-adsorbing capacity (○—○) for HeLa cells were calculated as percentage activity of non-irradiated virus.

With these considerations in mind, we have observed the following phenomena in adenovirus-infected cells: (1) the inhibition of PC formation was found at 3 h post-infection, 9 h before the appearance of characteristic c.p.e.; (2) the presence of ara C which might allow the expression of early viral functions did not block the inhibition of PC formation, but the presence of cycloheximide which might inhibit translation of the virus genome blocked the inhibition of PC formation (Fig. 5); and (3) the virus genome size of the activity inhibiting PC formation was almost equal to that of the activity inducing early c.p.e. (shrinkage of cytoplasm), corresponding to 20% of the size for infectivity (Fig. 6). This genome size is in accord with that reported for the early functioning portion of the virus genome (Fujinaga & Green, 1970). On the basis of these observations and considerations, it is suggested that an early protein of adenovirus coded by an early functioning portion of the genome binds or in some way affects microtubules, resulting in the prevention
of assembly of tubulins into PC on one hand and the induction of shrinkage of cytoplasm known as early c.p.e.

We observed the following phenomena in HSV-infected cells: (1) the inhibition of PC formation and c.p.e. (cell rounding and nucleolar alteration) were initiated at 6 h and proceeded in parallel; (2) in the presence of ara C or PAA, neither the inhibition of PC formation nor the appearance of c.p.e. was blocked, whereas in the presence of cycloheximide both of the activities were blocked (Fig. 7); and (3) the genome size of the activity leading to inhibition of PC formation was shown to be as small as that for inducing c.p.e. (Fig. 8). On the basis of these observations, it is suggested that a protein coded by the input virus genome early after infection affects microtubules, resulting in both the inhibition of assembly of tubulins into PC and cell rounding.

An important question is which of the non-capsid or capsid protein(s) is responsible for the inhibition of PC formation and the induction of c.p.e. in HSV-infected cells. There was also evidence that some products of poliovirus may affect microtubular proteins, resulting in cell rounding (Fig. 3). However, microtubules are not affected during syncytial formation by the same HeLa cells after Sendai virus infection (Fig. 4). Although numerous studies have indicated that microtubules have an important cytoskeletal role (reviewed by Olmsted & Borisy, 1973), the type of virus-specified protein(s) which are responsible for impairment of microtubules and how they interact with microtubules have not been determined.

Virus cytopathic effects which may lead to the death of cells have been recognized for more than 20 years. The following mechanisms of action have been considered: (1) the inhibition of host macromolecular synthesis, reviewed by Bablanian (1975); (2) the alteration of lysosomes reviewed by Allison (1967); (3) the alteration of cell membranes (Okada, 1958; Watkins, 1964); and (4) cytotoxic factors associated with virus particles (Pereira, 1958). The present results show that the alteration of microtubules in the cytoplasm was circumstantially associated with some types of early virus c.p.e., such as cell rounding and the shrinkage of cytoplasm.

Little is known about the biological functions of the early proteins specified by adenoviruses. Most workers have used complement fixation or immunofluorescence to detect new antigens early after virus infection. The most prominent of these appears to be T antigen, which reacts with sera from tumour-bearing hamsters (Pope & Rowe, 1964). The early proteins of adenovirus have been studied by SDS-polyacrylamide gel electrophoresis of infected cell extracts after labelling with 35S-methionine. By this method several prominent bands, which are absent in uninfected cells, have been observed early after infection (Russell & Skehel, 1972; Saborio & Oberg, 1976). However, the biological activity of these components or T antigen has not been reported. As for herpesviruses, the exact number and function of proteins coded for by virus DNA but which are not part of the virus particle are unknown. Only two enzymes in infected cells (thymidine kinase and DNA polymerase) have been clearly identified as virus specified (Keir, 1968). The present results show that one of the biological functions of an early protein(s) of adenovirus and probably herpesvirus is to affect microtubules, resulting in the inhibition of PC formation and causing morphological changes (shrinkage of cytoplasm and cell rounding). Thus two large DNA viruses possess an early functioning portion of the genome responsible for the coding of a protein which affects microtubules. We plan to attempt to isolate an early protein which might inhibit PC formation.

Recently, Brinkley et al. (1975) and Edelman & Yahara (1976) using tubulin antibody immunofluorescence suggested that the virus transformed cell line (SV40-3T3) is impaired in its ability to assemble cytoplasmic microtubules. Therefore, there is the possibility that
Involvement of microtubules in c.p.e.

one of the main targets of early protein or T antigen of oncogenic DNA viruses may be microtubules. The investigation of this possibility should be the subject of future research. Moreover, in order to ascertain directly that an early protein of adenovirus affects the microtubular protein system in infected cells, an immunofluorescent experiment is needed. This is being carried out.

This work was supported in part by the research grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare, Japan.

REFERENCES


(Received 14 June 1977)