Journal of Biochemistry and Molecular Biology, Vol. 31, No. 1, January 1998, pp. 106–110

JOURNAL OF
Biochemistry
and
Molecular Biology

Short Communication

Properties of the Endonuclease Secreted by Human B Lymphoblastic IM9 Cells

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Received 13 October 1997

We have employed a DNA-native-polyacrylamide gel electrophoresis (DNA-native-PAGE) assay system to characterize the enzyme activity of the endonuclease secreted by human B lymphoblastic IM9 cells. Experimental results clearly demonstrated that the endonuclease activity of IM9 cell culture medium is distinct from that of DNase I in the DNA-native-PAGE assay system. Immunoprecipitation analysis using anti-DNase I antibody showed that the secreted endonuclease is not recognized by the antibody. The secreted endonuclease was isolated from the cell culture medium by native-PAGE elution technique, and the enzyme activity was estimated using supercoiled plasmid DNA as a substrate. The pH optimum required for the catalytic activity was determined to be in the range of pH 6.6–7.4. No significant difference in the endonuclease secretion was observed by stimulation of the IM9 cells with interferon-γ or interleukin-1β.

Keywords: DNA-native-PAGE, Endonuclease, IM9 cell, Secretory enzyme.

Introduction

Deoxyribonucleases from tissues of human (Yasuda *et al.*, 1990; Yasuda *et al.*, 1992; Ribeiro and Carson, 1993) and other animals (Ball, 1974; Cordis *et al.*, 1975; Wadano *et al.*, 1979; Stratling *et al.*, 1984; Tanuma and Shiokawa, 1994; Torriglia *et al.*, 1995) have been characterized and all of them were shown to exist in multiple forms. Because of the marked heterogeneity of human serum DNase I (Love and Hewitt, 1979; Kishi *et al.*, 1990), urine deoxyribonuclease (Yasuda *et al.*, 1990), and other nucleases of different tissue types (Yasuda *et al.*, 1992; Ribeiro and Carson, 1993), it is unclear how the enzymes are released into body fluids and how they function. The enzyme has also been identified and partially characterized in human serum (Love and Hewitt, 1979; Kishi *et al.*, 1990). However, it is not known whether several different endonucleases are present in serum and various cell types. Our recently established endonuclease assay system using DNA-native-PAGE is suitable for identification of the secreted endonuclease activity in human B-lymphoblastic IM9 cells (Kwon and Kim, 1997). The Mg^{2+}-dependent endonuclease activity that we have found from the human immune cell line (Kwon and Kim, 1997) appears to be different from the nucleases reported so far in several aspects.

In this communication, we show that the particular endonuclease in IM9 cell culture medium is clearly distinguished from DNase I which is the typical endonuclease secreted by pancreas (Ito *et al.*, 1984).

Materials and Methods

Cell culture and treatment Human B-lymphoblastic IM9 cell lines were obtained from the American Type Culture Collection ([ATCC] Rockville, MD). Cells were maintained at $4–9 \times 10^5$ cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in an atmosphere of 95% air and 5% CO_2 environment. Cell number and viability were periodically estimated by trypan blue exclusion in a hemocytometer during culture time. Lymphocyte viability was greater than 95% at the beginning of each experiment. Interferon-γ (IFN-γ, Genentech, Inc.) and Interleukine-1β (IL-1β, Genentech, Inc.) were suspended in culture medium and added to the cells at the concentrations of 10 units/ml. IM9 cell culture medium was obtained by centrifugation for 5 min at 1,500 rpm to remove cells and/or cell debris. Culture medium was stored at −70°C for using the further experiments.

Determination of endonuclease activity in DNA-native-PAGE Modified native polyacrylamide gel assay was performed according to the method of Kwon and Kim (1997). Briefly, sodium dodecylsulfate-free 7% polyacrylamide gels were

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co-polymerized with supercoiled plasmid DNA (pGEM-T vector, 3.0 kb, Promega) at a final concentration of 150 μg/ml. Protein samples (10 μg/well) of culture medium or cell lysates were loaded and electrophoresed at 4°C. After electrophoresis, the gels were washed three times in distilled water, and placed in a reaction buffer consisting of 20 mM Tri-HCl (pH 7.0), 10 mM $MgCl_2$ (TM buffer) at 37°C for the different time interval with gentle shaking. Then, the gels were stained in TM buffer containing 1 μg/ml ethidium bromide at 37°C for 30 min and photographed on 302 nm transilluminator. The position of nuclease activity was detected as a dark band on orange background of the gel. Bovine pancreatic DNase I (RNase-free $10-50 \times 10^3$ units/ml) from Boehringer Mannheim was used as a standard for the endonuclease activity.

Immunoprecipitation analysis of endonuclease activity Sprague-Dawley (SD, 150–200 g) rats were bled from the tail to obtain preimmune serum. Bovine DNase I (Sigma) was further purified by separating in 7% native-PAGE for immunization. DNase I band on native-PAGE was fragmented into pieces in sterile PBS, and used for vaccination. SD rats were immunized with 100 μg of protein according to a standard protocol (Harlow and Lane, 1988). Animals were bled 10 days after the last injection, and samples were tested for antibody titer and specificity. Anti-DNase I antibody was affinity purified by ImmunoPure *plus* protein A/G IgG purification kit (Pierce) and bovine DNase I conjugated-Sepharose CL 4B. Immunoprecipitation was performed by mixing medium (50 μl) or human serum (5 μl serum diluted to 50 μl PBS) with anti-DNase I antibody (3 μg)-conjugated protein A-Sepharose CL 4B beads (10 μl) followed by incubation at 4°C for 6 h. The supernatant of immunoprecipitated sample was collected and the endonuclease activity was estimated by DNA-native-PAGE assay.

Optimal pH for the enzyme activity For partial purification of the endonuclease from IM9 cell culture medium, enzyme was eluted from the activity gel band after the electrophoresis as described above. Elution was achieved by cutting out the protein band corresponding to the nuclease activity after intensive washing of the gel, fragmentation of the band into pieces, transfer of the pieces into Eppendorf microtubes, and elution of the protein in 20 mM Tris-HCl (pH 7.0) by overnight shaking at 4°C. The sample was centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was separated into 20 μl aliquots. Recovered sample containing 20 ng of eluted protein was incubated at 37°C for 10 min with 100 ng of supercoiled plasmid DNA (pGEM-T vector, 3.0 kb, Promega) in 20 μl of 20 mM MOPS buffer containing 10 mM $MgCl_2$ at different pH. Reaction was stopped by transferring the sample to ice and adding DNA sample buffer (TE buffer containing 30% glycerol, 0.5% bromophenol blue, and 0.5% xylene cyanol). The reaction product was resolved on a 1% agarose gel containing ethidium bromide (0.5 μg/ml).

Results and Discussion

Enzyme activity of DNase I and secreted endonuclease by IM9 cells It was not successful to detect the secreted endonuclease activity of IM9 cells by the conventional DNA-SDS-PAGE assay method (Rosenthal and Lacks, 1977). Figure 1 illustrates selective detection of the enzyme activity in conditioned cell culture medium by the DNA-native-PAGE nuclease assay system developed in our laboratory. Approximately as much as 10 μg total protein of the cell culture medium gave an intense activity band corresponding to the position of the endonuclease under the experimental condition. When the DNA-native-PAGE gel was incubated in 20 mM Tris-HCl, pH 7.0 buffer containing 10 mM $MgCl_2$ at 37°C for 1 h, the enzymatic activity was detected only in the secreted endonuclease band (Fig. 1A). With this major endonuclease activity, a fast-migrating minor nuclease band corresponding to DNase I also gave a detectable activity when the reaction was extended to 4 h (Fig. 1B). The fast-migrating minor activity band of the culture medium in Fig. 1B was identified to be DNase I by comparing the activity band with that of commercial bovine DNase I. These results clearly indicate that the endonuclease found in IM9 cell culture medium has different reactivity and electrophoretic mobility from DNase I.

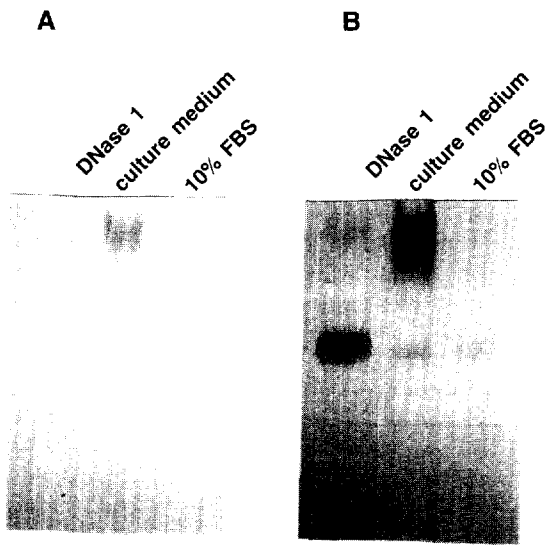

Fig. 1. Endonuclease activities of DNase I and IM9 cell culture medium were analyzed by DNA-native-PAGE. Culture medium and bovine DNase I were electrophoresed in DNA-native-PAGE as described under "Materials and Methods". Reaction was performed in 20 mM Tris-HCl (pH 7.0) buffer containing 10 mM $MgCl_2$ at 37°C for 1 h (A) or 4 h (B) with gentle shaking.

Immunoprecipitation analysis of the endonuclease activity To further characterize that the endonuclease of the human immune cell line is a distinct species from DNase I, we prepared a polyclonal antibody in SD rats against bovine pancreatic DNase I. Figure 2A displays that the purified anti-bovine DNase I antibody cross-reacts not only with FBS DNase I, but also with human serum DNase I. The preimmune serum was not able to recognize

any of the above DNase I. Further analysis was carried out with this antibody to show that the secreted endonuclease is a different enzyme species from DNase I (Fig. 2B). The anti-DNase I antibody was used to remove immunoprecipitable endonuclease activity of the IM9 cell culture medium. After the immunoprecipitation, the enzyme activity of the supernatant was analyzed by DNA-native-PAGE. The immunoprecipitated supernatant was shown to be free of the fast-migrating minor DNase I activity. On the other hand, the secreted endonuclease activity band was not immunoprecipitated by the anti-DNase I antibody. These results are concrete evidence showing that the secreted endonuclease by IM9 cell is immunochemically distinguished from DNase I.

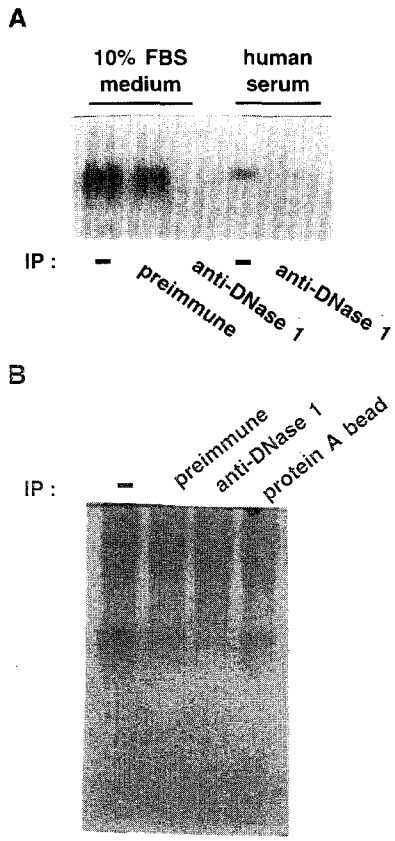

Fig. 2. Immunoprecipitation of endonuclease activity by anti-bovine DNase I antibody. Endonuclease activity in supernatant after the immunoprecipitation (IP) was estimated at indicated anti-serum treatment by the DNA-native-PAGE as described under "Materials and Methods". A, 10% FBS medium and human serum; B, IM9 cell culture medium.

Optimal pH for enzyme activity In order to investigate the pH-dependent catalytic activity of the endonuclease secreted by IM9 cells, the enzyme was isolated from the electrophoresed gel band corresponding to the endonuclease, and the enzyme activity was measured using supercoiled plasmid DNA as a substrate. The endonuclease

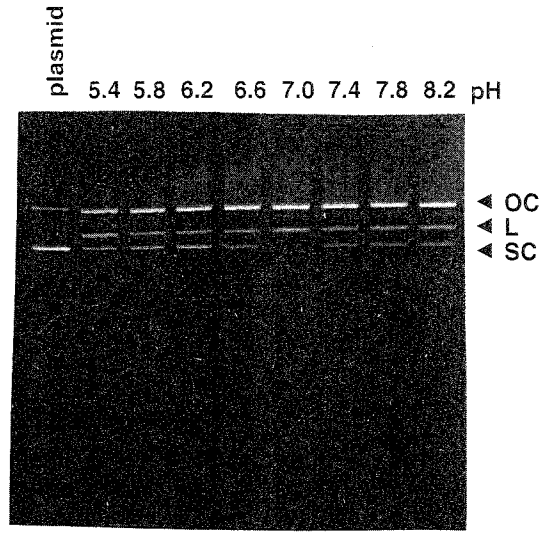

Fig. 3. Optimal pH for the endonuclease activity. The endonuclease was isolated from IM9 cell culture medium as described in "Materials and Methods" and the enzyme activity was determined by resolving the reaction products on a 1% agarose gel containing ethidium bromide. Activity is reflected by the conversion of supercoiled (SC) DNA to open coiled (OC) and linear (L) forms. The enzyme was reacted with 100 ng of plasmid DNA for 10 min at 37°C in the indicated pH of 20 mM MOPS buffer containing 10 mM $MgCl_2$.

activity can be estimated by analyzing the conversion of the supercoiled DNA into linear fragment via nicked circular form. As shown in Fig. 3, the enzyme activity is displayed over the relatively broad pH range in this assay condition. The optimal pH condition required for the catalytic activity of the endonuclease was determined to be in the range of pH 6.6–7.4.

Effect of cytokines on the endonuclease secretion IFN-γ possesses a wide range of biological properties including macrophage/monocyte activation and induction of expression of class I and II major histocompatibility complex molecules, immunoglobulin receptor, and other molecules (Adams *et al.*, 1984). In addition to its effects on cellular immunity, IFN-γ can either stimulate or inhibit IgM secretion by activated B cells (Leibson *et al.*, 1984; Cowdery *et al.*, 1992; Chace *et al.*, 1993). IL-1β also promotes B-cell maturation and the clonal expansion of B cells following antigen-induced activation (Jandl *et al.*, 1988; Fotedar and Diener, 1988). It was investigated in this work to evaluate whether IFN-γ or IL-1β influences on the endonuclease secretion by IM9 cells. Experimental results indicated that either IFN-γ or IL-1β has no significant effect on the degree of endonuclease secretion as demonstrated in Fig. 4. The signals for proliferation and differentiation of IM9 cells are provided by extracellular stimuli such as phorbol ester and lipopolysaccharide. We were not able to observe any significant changes in the

endonuclease secretion after treating the cell with the above stimulating agents (data not shown). However, based on our unpublished data obtained with another cell line, it appears that the expression of certain intracellular endonuclease is regulated by the signalling events associated with differentiation. Therefore, it is interesting to speculate that the expression and secretion of the endonuclease in IM9 cells may be controlled by extracellular stimulation(s).

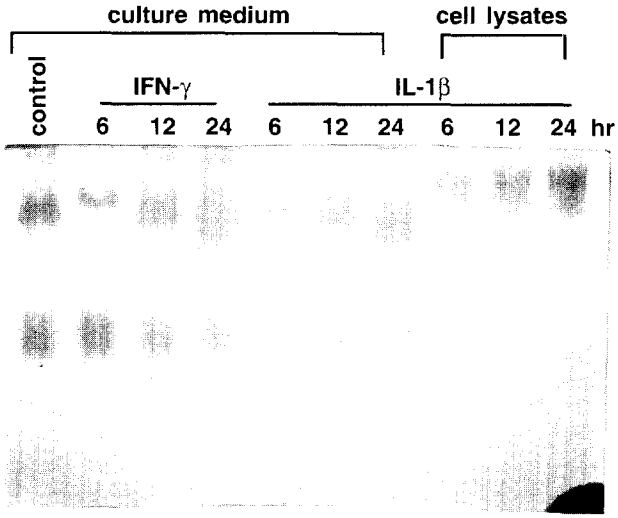

Fig. 4. Endonuclease activity of IFN-γ or IL-1β treated IM9 cell culture medium. The endonuclease activity in cytokine (10 units/ml)-treated IM9 cell culture medium was analyzed at indicated culture period by the DNA-native-PAGE as described under "Materials and Methods". IM9 cells were cultured for 12 h in RPMI1640 medium containing 10% FBS, and then endonuclease activity of the medium was analyzed as a control.

We have demonstrated in this communication that the particular Mg^{2+}-dependent endonuclease identified in IM9 cell culture medium is a different enzyme species from DNase I. Although the physiological function and significance of the endonuclease that we have investigated in the present study are still in question yet, the enzyme itself differs from any of the reported Mg^{2+}-dependent endonucleases (Kawabata *et al.*, 1993; Sun and Cohen, 1994; Anzai *et al.*, 1995), Ca^{2+}/Mg^{2+}-dependent endonucleases (Stratling *et al.*, 1984; Ribeiro and Carson, 1993; Pandey *et al.*, 1997), DNase I (Peitsch *et al.*, 1993), DNase II (Barry and Eastman, 1993; Torriglia *et al.*, 1995), and NUC18 (Gaido and Cidlowski, 1991). The Mg^{2+}-dependent endonuclease activity that we have analyzed in this work appears to be distinct from the nucleases reported so far in several aspects including optimal pH required for catalysis, cation dependence for enzyme activity and electrophoretic mobility in native-PAGE. Since the enzyme is produced and secreted by IM9 cells, an immune cell line of human origin, it is possible to hypothesize that the endonuclease may play a role associated with the defense mechanism. Further biochemical and molecular biological studies in more detail on the IM9 cell endonuclease will provide useful information in understanding the functional significance of the enzyme.

References

Adams, D. O. and Hamilton, T. A. (1984) The cell biology of macrophage activation. *Annu. Rev. Immunol.* **2**, 289–318.

Anzai, N., Kawabata, H., Hirama, T., Masutani, H., Ueda, Y., Yoshida, Y., and Okuma, M. (1995) Types of nuclear endonuclease activity capable of inducing internucleosomal DNA fragmentation are completely different between human $CD34^+$ cells and their granulocytic descendents. *Blood* **86**, 917–923.

Ball, W. D. (1974) Isolation of salivary ribonuclease, deoxyribonuclease and amylase from the parotid gland of the rat, *Rattus norvegicus* albinus. *Biochim. Biophys. Acta.* **341**, 305–317.

Barry, M. A. and Eastman, A. (1993) Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch. Biochem. Biophys.* **300**, 440–450.

Chace, J. H., Abed, N. S., Adel, G. L., and Cowdery, J.S (1993) Regulation of differentiation in $CD5^+$ and conventional B cells. Sensitive to LPS-induced differentiation and interferon-gamma-mediated inhibition of differentiation. *Clin. Immunol. Immunopathol.* **68**, 327–332.

Cordis, G. A., Goldbaltt, P. J., and Deutscher, M. P. (1975) Purification and characterization of a major endonuclease from rat liver nuclei. *Biochemistry* **14**, 2596–2603.

Cowdery, J. S. and Fleming, A. L. (1992) *In vivo* depletion of CD4 T cells increases B cell sensitivity to polyclonal activation: the role of interferon-gamma. *Clin. Immunol. Immunopathol.* **62**, 72–77.

Fotedar, R. and Diener, E. (1988) The role of recombinant IL-2 and IL-1 in murine B cell differentiation. *Lymphokine Res.* **7**, 393–402.

Gaido, M. L. and Cidlowski, J. A. (1991) Purification and characterization of a major endonuclease from rat liver nuclei. *Biochemistry* **14**, 2596–2603.

Harlow, E. and Lane, D. (1988) Immunization. In *Antibodies, A laboratory manual*, Harlow, E. and Lane, D. (eds.) pp. 53–138, Cold Spring Harbor, New York

Ito, K., Minamiura, N., and Yamamoto, T. (1984) Human urine DNase I: immunological identity with human pancreatic DNase I, and enzymic and proteochemical properties of the enzyme. *J. Biochem.* **95**, 1399–1406.

Jandl, R. C., Flanagan, R. G. and Schur, P. H. (1988) Inter-leukin-1 stimulation of human B-lymphoblast differentiation. *Clin. Immunol. Immunopathol.* **46**, 115–121.

Kawabata, H., Anzai, N., Masutani, H., Hirama, T., Yoshida, Y., and Okuma, M. (1993) Detection of Mg^{2+}-dependent endonuclease activity in myeloid leukemia cell nuclei capable of producing internucleosomal DNA cleavage. *Biochem. Biophys. Res. Commun.* **191**, 247–254.

Kishi, K., Yasuda, T., Ikehara, Y., Sawazaki, K., Sato, W. and Iida, R. (1990) Human serum deoxyribonuclease I (DNase I)

polymorphism: pattern similarities among isozymes from serum, urine, kidney, liver, and pancrease. *Am. J. Hum. Genet.* **47**, 121–126.

Kwon, H. J. and Kim, D. S. (1997) Identification of an endonuclease secreted by human B lymphoblastic IM9 cells. *Int. J. Biochem. Cell Biol.* (in press).

Leibson, H. J., Gefter, Z., Zotnik, A., Marrack, P., and Kappler, J. W. (1984) Role of gamma-interferon in antibody-producing responses. *Nature* **309**, 799–801.

Love, J. D. and Hewitt, R. R. (1979) The relationship between human serum and human pancreatic DNase I. *J. Biol. Chem.* **254**, 12588–12594.

Pandey, S., Walker, P. R., and Sikorska, M. (1997) Identification of a novel 97 kDa endonuclease capable of internucleosomal DNA cleavage. *Biochemistry* **36**, 711–720.

Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G., and Tschopp, J. (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degration during apoptosis (programmed cell death). *EMBO J.* **12**, 371–377.

Ribeiro, J. M. and Carson, D. A. (1993) Ca^{2+}/Mg^{2+}-dependent endonuclease from human spleen: purification, properties, and role in apoptosis. *Biochemistry* **32**, 9129–9136.

Rosenthal, A. L. and Lacks, S. A. (1977) Nuclease detection in SDS-polyacrylamide gel electrophoresis. *Anal. Biochem.* **80**, 76–90.

Stratling, W. H., Grade, C., and Horz, W. (1984) Ca^{2-}/Mg^{2+}-dependent endonuclease from porcine liver. *J. Biol. Chem.* **259**, 5893–5898.

Sun, X. M. and Cohen, G. M. (1994) Mg^{2+}-dependent cleavage of DNA into kilobase pair fragments is responsible for the initial degradation of DNA in apoptosis. *J. Biol. Chem.* **269**, 14857–14860.

Tanuma, S.-I. and Shiokawa, D. (1994) Multiple forms of nuclear deoxyribonucleae in rat thymocytes. *Biochem. Biophys. Res. Commun.* **203**, 789–797.

Torriglia, A., Chaudun, E., Francois, C.-F., Jeanny, J.-C., Courtois, Y., and Counis, M.-F. (1995) Involvement of DNase II in nuclear degeneration during lens cell differentiation *J. Biol. Chem.* **270**, 28579–28585.

Wadano, A., Hobus, P. A., and Liao, T.-H. (1979) Isolation and characterization of multiple forms of ovine pancreatic deoxyribonuclease. Chromatographic behavior of the enzyme on concanavalin A-agarose and carboxymethylcellulose columns. *Biochemistry* **18**, 4124–4130.

Yasuda, T., Awazu, S., Sato, W., Iida, R., Tanaka, Y., and Kishi, K. (1990) Human genetically polymorphic deoxyribonuclease: purification, characterization, and multiplicity of urine deoxyribonuclease I. *J. Biochem.* **108**, 393–398.

Yasuda, T., Nadano, D., Awazu, S., and Kishi, K. (1992) Human urine deoxyribonuclease II (DNase II) isoenzymes: a novel immunoaffinity purification, biochemical multiplicity, genetic heterogeneity and broad distribution among tissues and body fluids. *Biochim. Biophys. Acta.* **1119**, 185–193.