*Journal Homepage: -www.journalijar.com*

# INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

## RESEARCH ARTICLE

## THROMBIN ENHANCES OVALBUMIN-SPECIFIC IGA IN MUCOSAL SURFACES BY INTRANASAL ADMINISTRATION IN MICE.

### Kazuyoshi Kaminaka[1] and Chikateru Nozaki[2].

1.  Development department, kikuchi research center, km biologics co., ltd., 1314-1 kyokushi kawabe, kikuchi-city, kumamoto 869-1298, japan.
2.  Department of medical technology, kumamoto health science university, 325 izumi-machi, kita-ku, kumamoto-city, kumamoto 861-5598, japan.

………………………………………………………………………………………………………....

| *Manuscript Info* | *Abstract* |
|---|---|
| …………………….. | ……………………………………………………………… |
| | Thrombin plays a central role in blood coagulation. In addition, thrombin is an important inflammatory mediator that activates T lymphocytes and monocytes and induces the release of proinflammatory cytokines in vitro. However, it has not been clarified whether thrombin can induce the production of antibodies against antigens in vivo; i.e., it is not known whether thrombin has an adjuvant effect to enhance immune responses. In the present study in mice, intranasally administered thrombin plus ovalbumin enhanced not only antigen-specific IgG in blood but also antigen-specific IgA on mucosa in the nasal cavity and intestine. IgA plays a major role in protecting against infection by preventing pathogens from invading the body through mucous membranes. This study provides new information regarding thrombin as an adjuvant of mucosal immunity. |

………………………………………………………………………………………………………....

## Introduction:-

Thrombin is a trypsin-like serine protease that converts fibrinogen to fibrin and triggers fibrin crosslinking by activating Factor XIII (Lorand and Konishi, 1964; Takagi and Doolittle, 1974). It also promotes coagulation by activating Factors V and VIII, which are cofactors of Factors IX and Xa, respectively (Nesheim and Mann, 1979; Hoyer and Trabold, 1981). These functions are essential for the maintenance and protection of life, such as the formation of hemostatic plugs and wound healing (Fenton, 1988). On the other hand, when thrombin binds to thrombomodulin on vascular endothelial cells, its substrate specificity changes to promote anticoagulation by activating protein C (Esmon, 1995).

In addition to these fundamental roles in hemostasis, thrombin plays a role as an important inflammatory mediator (Grand et al., 1996). For example, thrombin activates T lymphocytes and monocytes and induces the release of proinflammatory cytokines such as interleukin (IL)-6 and IL-8 (Naldini et al., 1993; Johnson et al., 1998). In addition, thrombin promotes T lymphocyte recruitment (Kaur et al., 2001). Immunologically, thrombin suppresses the production of IL-12, a cytokine that acts on monocytes, and induces a Th1 type immune response in vitro, skewing toward a Th2 type immune response (Naldini et al., 2003). This in vitro effect of thrombin suggests it might induce antibody production against antigens in vivo; i.e., it may have an adjuvant effect to enhance immune responses.

**Corresponding Author:-Kazuyoshi Kaminaka.**
Address**:-**Development department, kikuchi research center, km biologics co., ltd., 1314-1 kyokushi kawabe, kikuchi-city, kumamoto 869-1298, japan.

Although many kinds of vaccines are marketed, adjuvant-containing vaccines are limited (Christensen, 2016), and adjuvants that can induce IgA on mucosal surfaces are uncommon. It is well known that mucosal IgA plays a major role in protecting against infection by preventing pathogens from invading the body through the mucous membranes (Woof and Mestecky, 2005). However, there are no clinically useful strategies to enhance IgA. Therefore, adjuvants inducing IgA are desired for mucosal vaccines (Boyaka, 2017).

In our present study, the adjuvant effect of thrombin in mice was investigated. We found that administering thrombin with ovalbumin intranasally enhanced not only ovalbumin-specific IgG in sera but also ovalbumin-specific IgA on mucosal surfaces.

## Material and methods:-
### Ovalbumin and thrombin
Ovalbumin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Human thrombin produced by recombinant DNA technology was prepared as described previously (Yonemura et al., 2004). Briefly, an expression cassette in which the human prothrombin gene was linked downstream of the chicken β-actin promoter was introduced into Chinese hamster ovary (CHO) cells, and cells expressing human prothrombin were selected. The genetically modified CHO cells were cultured, and human prothrombin was purified from the supernatant. This was converted to thrombin (α-thrombin) using ecarin, which is a protease derived from snake venom obtained by recombinant DNA technology (Yonemura et al., 2004), and further purified for these experiments. The resultant thrombin was indistinguishable from the plasma-derived material.

### Mice
Male C57BL/6 mice (7 weeks of age) were obtained from Japan SLC (Hamamatsu, Japan). The mice were bred and housed under specific pathogen-free conditions prior to the experiments. After one week of acclimatization, mice were subjected to the immunization studies.

All animal experimentation procedures were approved by our institutional animal care and use committee and performed according to the animal experimentation regulations instituted under the Guidelines for Proper Conduct of Animal Experiments in Japan.

### Immunizations and blood sample collection
Mice were divided into four groups of five to six individuals each (Table 1). Ovalbumin with and without thrombin was administered intranasally six times (10 μL/mouse each time) at intervals of one week. Blood samples were collected from the tail vein on the day before immunization ("pre") and on the day of every $3^{rd}$ to $6^{th}$ immunization just prior to the administration ($2^{nd}$ to $5^{th}$ immunization samples). One week after the $6^{th}$ immunization, mice were sacrificed, and blood, nasal, and intestinal lavage samples were collected. All mouse procedures were performed under anesthesia.

### Nasal and intestinal lavage fluids
Nasal lavage fluids were collected from nostrils by injecting 0.5 mL of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich) through a catheter that was inserted from the trachea at the throat and leading to the nasal cavity. Then, 5 μL of Protease Inhibitor Cocktail Set III (EMD Millipore, Billerica, MA, USA) was added to each lavage fluid sample.

Intestinal lavage fluids were collected with reference to the method reported by Elson et al. (Elson et al., 1984). Briefly, mouse intestine was removed from the pyloric region of the stomach to just before the cecum so as not to damage the intestinal tract. Then, the inside of the intestinal tract was washed with 2 mL of ice-cold 50 mM EDTA (Dojindo, Kumamoto, Japan) containing 0.2 mg/mL soybean trypsin inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The lavage fluid was collected into a dish containing 1 mL of the same ice-cold washing solution. Debris in the lavage fluid was removed by centrifugation after adjusting the total volume to 3 mL and adding 30 μL of 100 mM phenylmethylsulfonyl fluoride. $NaN_3$ (1%) (Katayama Chemical Industry, Osaka, Japan) and Protease Inhibitor Cocktail Set III, corresponding to 1% volume of the supernatant, were added to 1 mL of the supernatant, followed by the addition of 50 μL of fetal bovine serum (GE Healthcare Japan, Tokyo, Japan). The nasal and intestinal lavage fluids were stored at -80°C until analyzed.

**Determination of the anti-ovalbumin IgG titer in serum**
The anti-ovalbumin IgG titer in serum was determined by an enzyme-linked immunosorbent assay (ELISA). Ovalbumin was immobilized on a 96-well plate (Maxisorp, Thermo Fisher Scientific). After blocking with Block Ace (Sumitomo Dainippon Pharma, Osaka, Japan) in phosphate-buffered saline, hyper-immunized mouse sera (standard antisera) and antisera serially diluted with Block Ace were added. Ovalbumin-specific IgG titers were measured with horseradish peroxidase-conjugated anti-mouse IgG (American Qualax, San Clemente, CA, USA) and the chromogenic substrate solution TMB (Agilent Technology, Santa Clara, CA, USA). Antigen-specific IgG titers were determined by measuring the absorbance at 450 nm (OD450).

**Determination of anti-ovalbumin IgA titers in nasal and intestinal lavage fluids**
Total IgA titers in lavage fluids were determined according to the protocol of a Mouse IgA ELISA Quantitation Kit (Funakoshi, Tokyo, Japan). Ovalbumin-specific IgA titers in five-fold-diluted nasal lavage fluids and twenty-fold-diluted intestinal lavage fluids were also determined with an ELISA using horseradish peroxidase-conjugated anti-mouse IgA (Funakoshi). To standardize the variance of IgA recovery and compare ovalbumin-specific IgA titers among immunization groups, the OD450 value of ovalbumin-specific IgA and the total IgA value (converted to mg/mL) obtained from the ELISA were used to calculate the anti-ovalbumin IgA titer with the following formulas:

anti-ovalbumin IgA titer in the nasal lavage fluid

$$= \frac{\text{OD450 value of ovalbumin-specific IgA} \times 5}{\text{total IgA titer (mg/mL)}}$$

anti-ovalbumin IgA titer in the intestinal lavage fluid

$$= \frac{\text{OD450 value of ovalbumin-specific IgA} \times 20}{\text{total IgA titer (mg/mL)}}$$

**Statistical analyses**
Statistical analyses were performed using analysis of variance followed by Dunnett's test by BellCurve for Excel (Social Survey Research Information, Tokyo, Japan). A p value of $< 0.05$ was considered statistically significant.

# Results and discussion:-
Table 1 shows the components (vaccine formulations) that were administered intranasally to each immunization group. The anti-ovalbumin IgG titers in sera before immunization and after the 2nd to 6th intranasal immunizations are shown in Figure 1. The results confirmed that anti-ovalbumin antibodies appeared at an early stage, and high antibody titers were induced according to the administered dose of thrombin compared to the group immunized with ovalbumin alone. When 20 and 40 µg of thrombin per mouse were added with ovalbumin, specific antibodies appeared at the same time. In addition, specific antibody titers in both thrombin groups were about the same and were significantly higher relative to the group immunized with ovalbumin alone at sampling points after the 3rd immunization. Because the thrombin used for mouse immunization was deduced to contain less than $10^{-2}$ endotoxin units, the observed effect could not be attributed to endotoxin contamination. Therefore, it was considered that intranasally administered thrombin had an adjuvant effect, elevating IgG titers against ovalbumin as an immunogen, and that 20 µg of thrombin was sufficient to exert this effect.

One week after the 6th immunization, nasal and intestinal fluids were collected and used to measure the anti-ovalbumin IgA titer. The dose of thrombin that exerted an adjuvant effect on the IgG titer in sera also induced mucosal IgA against ovalbumin significantly (Fig. 2). These results clearly demonstrated that intranasally administered thrombin can enhance not only anti-ovalbumin IgG in sera but also anti-ovalbumin IgA in the nasal cavity and intestine.

Thrombin is synthesized in the liver and plays a central role in blood coagulation. In addition, it is now considered that coagulation components, including thrombin, are closely associated with the immune system and are essential for effective immune responses to control infection (Antoniak et al., 2018). In fact, inhibition of thrombin, the major blood coagulation factor used in this study, increases the risk and severity of viral infection (Antoniak et al., 2013). Given these facts and its stimulatory effects on immune cells (Naldini et al., 1993, 2003; Johnson et al., 1998; Kaur et al., 2001; Antoniak et al., 2017), it is not surprising that thrombin has an adjuvant effect in vivo to enhance IgG in sera. However, it is noteworthy that thrombin can also enhance mucosal IgA by mucosal administration.

The results of the current study help to clarify the mechanism of the adjuvant effect of thrombin. Various cellular actions of thrombin are mediated by specific G-protein-coupled receptors, namely protease-activated receptors, and require its protease activity to activate the receptors (Naldini et al., 2003, 2006; Tordai et al., 1993; Vu et al., 1991; Ishihara et al., 1997). Furthermore, it was reported that human thrombin exhibits enzymatic activities in mice and on murine cells (Antoniak et al., 2017; Hansson et al., 2016). Therefore, it is conceivable that a protease-activated receptor-mediated pathway is also involved in the adjuvant effect of thrombin.

The adjuvant effect of thrombin clarified in this study, especially the induction of mucosal IgA by intranasal administration, is important for host defenses against pathogens, such as the influenza virus, which invades from the mucosal surface. Thus, thrombin may be useful as a safe adjuvant for intranasal vaccines. Indeed, no abnormalities such as inflammatory reactions at the inoculation sites were found in mice receiving thrombin in this study. However, the use of thrombin as an adjuvant may be limited because it requires more protein than the vaccine antigen and increases the cost. By dissecting the mechanism of mucosal IgA induction in this study and elucidating the target molecule(s) that mediate the adjuvant effect of intranasally administered thrombin, the development of new adjuvants that can induce mucosal IgA effectively may be facilitated.

### Acknowledgments:-

### Conflicts of interest
Both authors declare no conflicts of interest on this study.

**Table 1:-**Details of immunization groups

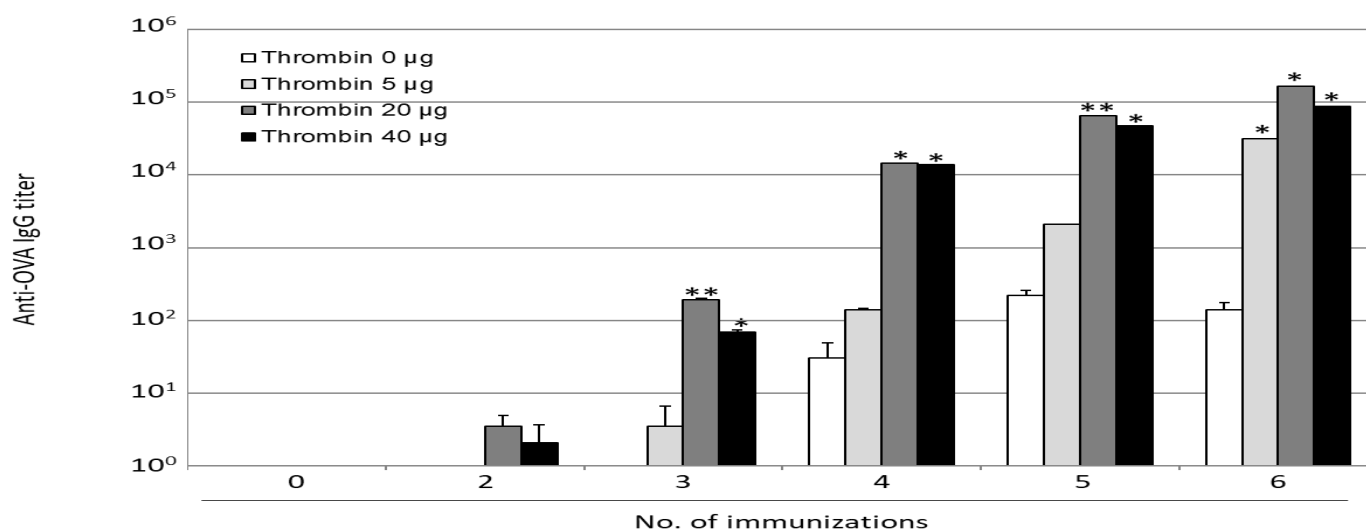| Group | No. of mice | Administered component (µg/mouse) | |
|---|---|---|---|
| | | OVA (Immunogen) | Thrombin |
| 1 | 5 | 5 | 0 |
| 2 | 5 | 5 | 5 |
| 3 | 6 | 5 | 20 |
| 4 | 6 | 5 | 40 |



**Fig 1:-**Anti-ovalbumin (OVA) IgG titers in sera after intranasal immunization with OVA plus thrombin.

Male C57BL/6 mice were divided into four groups. OVA with 0 to 40 µg of thrombin was administered intranasally as described in Material and Methods. After 0-6 immunizations, blood samples were collected, and anti-OVA IgG

titers were determined. The geometric mean of the anti-OVA IgG titers in each group ± SE is indicated. *p < 0.05 and **p < 0.01 vs. the group immunized with OVA alone.
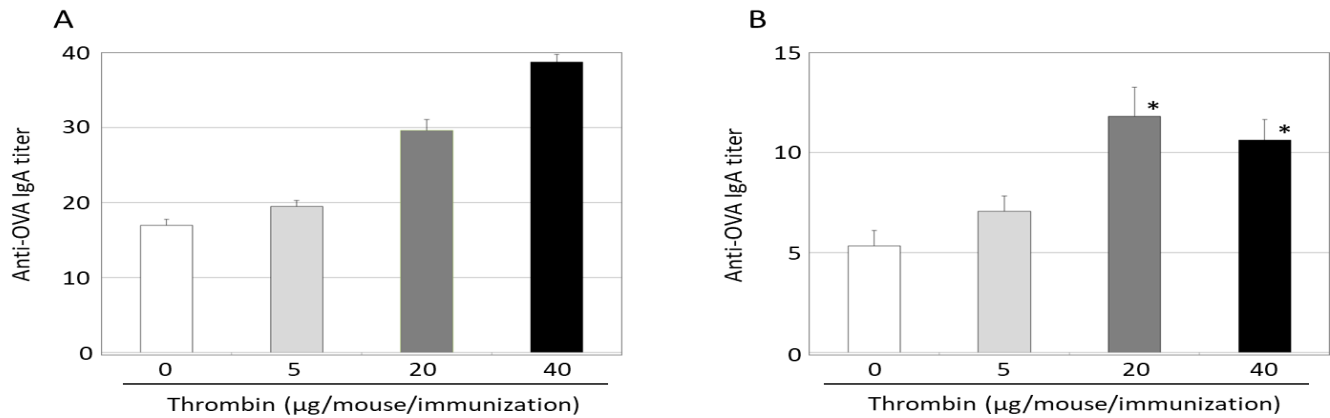


**Fig 2:-**Anti-ovalbumin (OVA) IgA titers in mucosal lavage fluids after intranasal immunization with OVA plus thrombin.

After the last (6th) immunization, nasal (A) and intestinal (B) lavage fluids were collected and anti-OVA IgA titers determined as described in Material and Methods. The geometric mean of the anti-OVA IgG titers in each group ± SE is indicated. *p < 0.05 vs. the group immunized with OVA alone.

## References:-

1.  Antoniak, S. (2018): The coagulation system in host defense. Res. Pract. Thromb. Haemost., 2: 549-557.
2.  Antoniak, S., Owens, A.P. 3rd, Baunacke, M., Williams, J.C., Lee, R.D., Weithäuser, A., Sheridan, P.A., Malz, R., Luyendyk, J.P., Esserman, D.A., Trejo, J., Kirchhofer, D., Blaxall, B.C., Pawlinski, R., Beck, M.A., Rauch, U. and Mackman, N. (2013): PAR-1 contributes to the innate immune response during viral infection. J. Clin. Invest., 123: 1310-1322.
3.  Antoniak, S., Tatsumi, K., Bode, M., Vanja, S., Williams, J.C. and Mackman, N. (2017): Protease-activated receptor 1 enhances poly I:C induction of the antiviral response in macrophages and mice. J. Innate Immun., 9: 181-192.
4.  Boyaka, P.N. (2017): Inducing mucosal IgA: a challenge for vaccine adjuvants and delivery systems. J. Immunol., 199: 9-16.
5.  Christensen, D. (2016): Vaccine adjuvants: why and how. Hum. Vaccin. Immunother., 12: 2709-2711.
6.  Elson, C.O., Ealding, W. and Lefkowitz, J. (1984): A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. J. Immunol. Methods, 67: 101-108.
7.  Esmon, C.T. (1995): Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB J., 9: 946-955.
8.  Fenton, J.W. 2nd. (1988): Regulation of thrombin generation and functions. Semin. Thromb. Hemost., 14: 234-240.
9.  Grand, R.J.A., Turnell, A.S. and Grabham P.W. (1996): Cellular consequences of thrombin-receptor activation. Biochem. J., 313: 353-368.
10. Hansson, K.M., Lindblom, A., Elg, M. and Lövgren, A. (2016): Recombinant human prothrombin (MEDI8111) prevents bleeding in haemophilia A and B mice. Haemophilia, 22: 453-461.
11. Hoyer, L.W. and Trabold, N.C. (1981): The effect of thrombin on human factor VIII. Cleavage of the factor VIII procoagulant protein during activation. J. Lab. Clin. Med., 97: 50-64.
12. Ishihara, H., Connolly, A.J., Zeng, D., Kahn, M.L., Zheng, Y.W., Timmons, C., Tram, T. and Coughlin, S.R. (1997): Protease-activated receptor 3 is a second thrombin receptor in humans. Nature, 386: 502-506.
13. Johnson, K., Choi, Y., DeGroot, E., Samuels, I., Creasey, A. and Aarden, L. (1998): Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. J. Immunol., 160: 5130-5135.
14. Kaur, J., Woodman, R.C., Ostrovsky, L. and Kubes, P. (2001): Selective recruitment of neutrophils and lymphocytes by thrombin: a role for NF-κB. Am. J. Physiol. Heart Circ. Physiol., 281: H784-H795.
15. Lorand, L. and Konishi, K. (1964): Activation of the fibrin stabilizing factor of plasma by thrombin. Arch. Biochem. Biophys., 105: 58-67.

16. Naldini, A., Aarden, L., Pucci, A., Bernini, C. and Carraro, F. (2003): Inhibition of interleukin-12 expression by α-thrombin in human peripheral blood mononuclear cells: a potential mechanism for modulating Th1/Th2 responses. Br. J. Pharmacol., 140: 980-986.

17. Naldini, A., Carney, D.H., Bocci, V., Klimpel, K.D., Asuncion, M., Soares, L.E. and Klimpel, G.R. (1993): Thrombin enhances T cell proliferative responses and cytokine production. Cell Immunol., 147: 367-377.

18. Naldini, A., Morena, E., Filippi, I., Pucci, A., Bucci, M., Cirino, G. and Carraro, F. (2006): Thrombin inhibits IFN-gamma production in human peripheral blood mononuclear cells by promoting a Th2 profile. J. Interferon Cytokine Res., 26: 793-799.

19. Nesheim, M.E. and Mann, K.G. (1979): Thrombin-catalyzed activation of single chain bovine factor V. J. Biol. Chem., 254: 1326-1334.

20. Takagi, T. and Doolittle, R.F. (1974): Amino acids sequence studies on factor XIII and the peptide released during its activation by thrombin. Biochemistry, 13: 750-756.

21. Tordai, A., Fenton, J.W. 2nd, Andersen, T. and Gelfand, E.W. (1993): Functional thrombin receptors on human T lymphoblastoid cells. J. Immunol., 150: 4876-4886.

22. Vu, T.K., Hung, D.T., Wheaton, V.I. and Coughlin, S.R. (1991): Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell, 64: 1057-1068.

23. Woof, J.M. and Mestecky, J. (2005): Mucosal immunoglobulins. Immunol. Rev., 206: 64-82.

24. Yonemura, H., Imamura, T., Soejima, K., Nakahara, Y., Morikawa, W., Ushio, Y., Kamachi, Y., Nakatake, H., Sugawara, K., Nakagaki, T. and Nozaki, C. (2004): Preparation of recombinant α-thrombin: high-level expression of recombinant human prethrombin-2 and its activation by recombinant ecarin. J. Biochem., 135: 577-582.