RELEASE OF SUPEROXIDE ANION FROM POLYMORPHONUCLEAR LEUKOCYTES STIMULATED BY RUBELLA VIRAL ANTIGEN-ANTIBODY COMPLEX *IN VITRO*

H. KIMURA[1], H. MINAKAMI[2], M. OHBUCHI[1], N. YAMAKI[1], S. TSUCHIDA[1], K. KANAZAWA[1], Y. HARA[1], J. ASAHINA[3], O. ABE[3], Y. IKE[4], S. KURASHIGE[5], K. OTSUKI[1]

[1]Gunma Prefectural Institute of Public Health and Environmental Sciences, 3-21-19 Iwagami, Maebashi 371; [2]Department of Obstetrics and Gynecology, Jichi Medical School, Tochigi; [3]Health Management Division, Department of Public Health and Environment, Gunma Prefectural Government, Maebashi; [4]Department of Microbiology, Gunma University School of Medicine, Maebashi; [5]College of Medical Care and Technology, Gunma University, Maebashi, Japan

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Summary. – Using a highly sensitive and specific chemiluminescence (CL) method, we detected the release of superoxide anion ($O_2^{\cdot-}$) from human polymorphonuclear leukocytes (PMNLs) stimulated by rubella viral antigen-antibody complex (VAAC) adsorbed on latex particles. The amount of superoxide anion produced by PMNLs was proportional to the amount of VAAC. Neither rubella virus alone nor antibody alone, adsorbed on latex particles, stimulated production of superoxide anion by PMNLs. It is likely that rubella virus requires the presence of antibody in order to be recognized by PMNLs, and that the superoxide anion is somehow involved in the disease process of rubella.

Key words: superoxide anion; leukocytes; rubella virus

Introduction

The reaction of phagocytic cells (PMNLs, monocytes and macrophages) with certain viruses leads to the release of active oxygen species. Influenza viruses are capable of activating the production of active oxygen species in phagocytic cells in the absence of a specific antibody (Peterhans, 1980; Mills *et al.*, 1981). In contrast, herpesviruses, e.g. respiratory syncytial virus and canine distemper virus require, serum containing a specific antibody for activation of production of active oxygen species in phagocytic cells (Weber and Peterhans, 1983; Faden *et al.*, 1983; Bingham *et al.*, 1985; Burge *et al.*, 1989). However, it is not known whether rubella virus stimulates phagocytic cells to produce active oxygen species directly in the absence of a specific antibody.

We have developed a highly sensitive and specific CL method for detecting small amounts of superoxide anion (Nishida *et al.*, 1989; Kato *et al.*, 1991a; Kimura *et al.*, 1995). We conducted this study to determine whether superoxide anion is released during the reaction between PMNLs and rubella virus in the absence or presence of a specific antibody.

Abbreviations: CL = chemiluminescence; DMSO = dimethylsulfoxide; EDTA = ethylenediamine tetraacetate; FMLP = N-formyl-methionyl-leucyl-phenylalanine; HBSS = Hanks' balanced salt solution; MCLA = 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazin-3-one; PBS = phosphate-buffered saline; VAAC = viral antigen-antibody complex; PMNL = polymorphonuclear leukocyte

Materials and Methods

Reagents. A 10% (v/v) suspension of latex (polystyrene) particles (mean diameter of 0.78 μm) was obtained from Sekisui Chemicals (Tokyo). Phosphate-buffered saline (PBS) without calcium and magnesium (PBS) and Hanks' balanced salt solution (HBSS) were purchased from Nissui Pharmaceutical Co. (Tokyo) and dissolved in bidistilled water. HBSS was adjusted to pH 7.4 with disodium phosphate (Nishida *et al.*, 1989). Dimethylsulfoxide

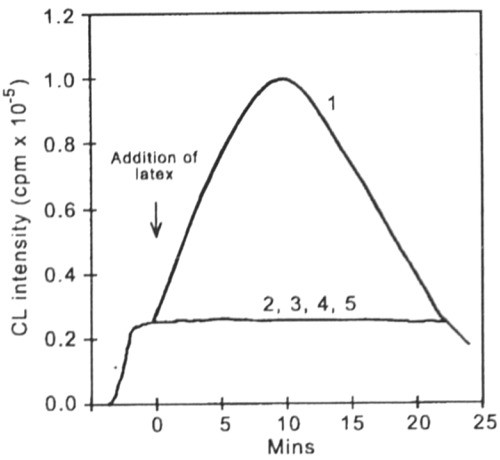

Fig. 1
MCLA-dependent release of superoxide anion (CL) from PMNLs
All samples (2 ml) contained 10^6 PMNLs, 1 µmol/l MCLA and variously coated 0.05% latex in HBSS. The reaction was initiated by addition of the latex. Latex with VAAC (curve 1), latex with virus (curve 2), latex with antibody (curve 3), latex with virus plus non-specific antibody (curve 4), and latex with VAAC plus 0.5 µmol/l superoxide dismutase (curve 5).

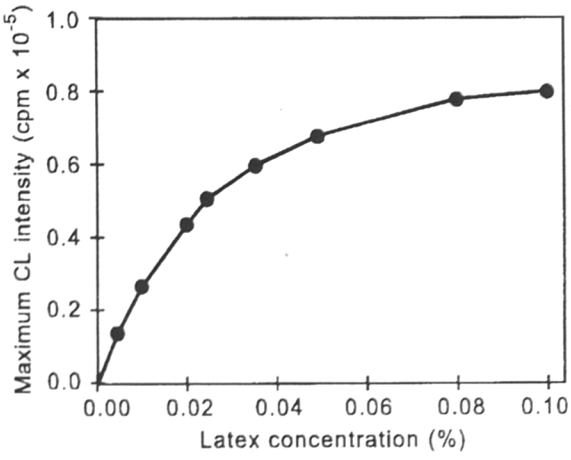

Fig. 2
Dependence of maximum CL intensity on the concentration of latex coated with VAAC
The reaction mixture (2 ml) contained 10^6 cells, 1 µmol/l MCLA, and 0.0 – 0.1% latex coated with VAAC in HBSS.

(DMSO) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were obtained from Sigma. Aliquots of 5 mmol/l FMLP in DMSO:HBSS (1:1, v/v) were stored at -80°C. Immediately before use, FMLP was diluted with HBSS to appropriate concentration. Other chemicals were of reagent grade and were purchased from Wako Pure Chemicals (Tokyo).

Cells and virus. Rubella virus (Baylor strain) was grown in BHK-21 cell monolayers. Virus-infected cells were frozen and thawed three times and centrifuged at 500 x g for 30 mins. The supernatants were layered onto a discontinuous gradient of 25 – 50% sucrose in TNE buffer (0.01 mol/l Tris.HCl, 0.1 mol/l NaCl, 1 mmol/l ethylenediamine tetraacetate (EDTA) pH 7.5) and centrifuged at 85,000 x g for 2 hrs at 4°C. The virus band was dissolved in TNE buffer (Best and O'Shea, 1989a). Protein concentration, determined by Lowry's method, served as a measure of the quantity of virus.

Antibody to rubella virus was titrated by the haemagglutination-inhibition test (Best and O'Shea, 1989b).

Preparation of rubella antibody-rich and poor sera. Pooled sera that showed a high (>256) and a low titer (<8) of rubella virus antibodies, respectively, were collected from 5 healthy adults with a history of rubella infection and from 3 healthy adults, respectively. Each pooled serum was diluted 20-fold with PBS, passed through a molecular filter, (Advantec Co, Tokyo) with cut-off value of molecular mass of 1.5 x 10^5 to remove substances of low molecular mass, and concentrated to the original volume. To inactivate the complement, the sera were incubated at 56°C for 30 mins.

VAAC preparation. A 1% (v/v) suspension of latex particles was washed three times with 10 volumes of PBS. A 200 µl aliquot of rubella virus in TNE buffer, containing 1.25 mg of protein, was mixed with 2 ml of the latex suspension and the mixture was incubated at room temperature for 2 hrs, and then at 4°C for 15 hrs. After incubation, the latex with adsorbed virus was washed twice with 10 volumes of PBS. Five hundred µl of the rubella antibody-rich serum, containing 6.3 mg of protein, was then added to the suspension of latex coated with virus, the mixture was incubated at 37°C for 1 hr, and washed three times with 10 volumes of PBS. The resulting product was designated VAAC. The same procedure was carried out with the rubella antibody-poor serum and the resulting product was regarded as the latex coated both with the virus and the non-specific antibody.

Preparation of PMNLs. Human peripheral blood from a healthy volunteer was collected into a heparinized syringe, mixed with a quarter volume of 6% dextran and kept for 30 mins at room temperature. The leukocyte-rich plasma aspirated from this mixture was layered onto a Conray-Ficoll gradient and centrifuged at 400 x g for 30 mins at 25°C (Kato *et al.*, 1991b). The PMNL-rich fraction was washed twice with HBSS. More than 95% of the collected cells were PMNLs. The viability of PMNLs, as determined by the trypan blue exclusion test, was more than 97%. PMNLs were suspended in HBSS (10^7 cells/ml) and kept on ice no longer than for 3 hrs prior to use.

Detection of superoxide anion by CL. The method for detecting superoxide anion by CL was described by Nishida *et al.* (1989). Briefly, a CL probe, an analogue of *Cypridina* luciferin, 2-methyl-6-(*p*-methoxyphenyl)-3, 7-dihydroimidazo [1,2-a] pyrazin-3-one (MCLA), was synthesized as previously described (Nishida *et al.*, 1989). MCLA was dissolved in bidistilled water and its concentration was calculated by using the ε value of 9600 mol/l. The reaction mixture contained 10^6 PMNLs, 1 µmol/l MCLA, 0.005 to 0.1% latex coated with VAAC, or 10^{-6}mol/l FMLP in HBSS, in a total volume of 2.0 ml. Control experiments were performed with 0.05% latex coated with the virus only, the antibody only, or both the virus and the non-specific antibody instead of VAAC. All components except for the latex were pre-incubated

for 3 mins at 37°C. CL was measured with BLR-2 a Luminescence Reader (Aloka Co., Tokyo). During the measurement of CL, the incubation mixture was agitated by rotation at 37°C. The maximum light intensity of MCLA-dependent CL was corrected for each control.

Results

The addition of the latex coated with VAAC to a mixture containing PMNLs promptly elicited MCLA-dependent light emission (Fig. 1, curve 1). The CL was completely abolished by the addition of 0.5 μmol/l superoxide dismutase, suggesting that the CL was produced by superoxide anion (Fig. 1, curve 5). The latex coated with the virus only, the antibody only, or both the virus and non-specific antibody, did not affect the production of superoxide anion (Fig. 1, curves 2, 3, 4). When the relationship of maximum CL to VAAC concentration was investigated (Fig. 2), maximum CL was enhanced by an increasing VAAC concentration. The intensity of maximum CL elicited by 0.1% VAAC corresponded to approximately one-eighth of the CL elicited by 10^{-6} mol/l FMLP (data not shown). A non-specific metal-catalyzed CL of MCLA did not influence the superoxide anion-dependent CL.

Discussion

We demonstrated that PMNLs released superoxide anion after reacting with latex particles coated with rubella VAAC, but not with latex particles coated with rubella virus only, the antibody only, or both the virus and non-specific antibody.

Latex particles made of polystyrene can themselves stimulate PMNLs (Babior, 1978). Indeed, untreated latex particles used in the present study stimulated PMNLs (data not shown). However, the latex used in this study was not effective in inducing the release of superoxide anion from PMNLs when it had been pretreated with rubella virus alone, rubella antibody-rich serum alone, or both rubella virus and rubella antibody-poor serum. It is unlikely that the latex pretreated with both rubella virus and rubella antibody-rich serum regained its original ability to stimulate PMNLs. The enhanced superoxide anion release from PMNLs after the addition of latex treated with both rubella virus and rubella antibody-rich serum should be ascribed to VAAC but not to the latex particles themselves, suggesting that rubella virus requires the presence of the (rubella) antibody in order to be recognized by PMNLs.

An antigen-antibody complex activates phagocytic cells by binding to Fc receptors expressed on the cell surface (Unkeless *et al.*, 1981). Bovine PMNLs generate active oxygen species in the presence of both herpes virus and anti-herpesvirus antibody by a mechanism dependent on the antibody Fc portion, as $F(ab')_2$ fragments bound to antigen are unable to stimulate PMNLs (Weber and Peterhans, 1983). The superoxide anion release from PMNLs stimulated by VAAC observed in the present study may be due to stimulation of Fc receptors on the PMNLs surface.

Active oxygen species, including superoxide anion released from phagocytic cells, play a pivotal role in bactericidal mechanisms (Babior, 1978). Although viruses bound to a specific antibody are considered to be non-infective (Roitt, 1991), studies on viruses suggest that active oxygen species, produced through an interaction between viral antigen and phagocytic cells in the presence or absence of an antibody, are involved in the pathogenesis of viruses (Faden *et al.*, 1983; Burge *et al.*, 1989; Oda *et al.*, 1989; Akaike *et al.*, 1996). In the antibody-dependent cytotoxicity mediated by mouse macrophages, active oxygen species are known to contribute to target cell killing (Nathan and Cohn, 1980) and to toxic effects on bystander cells exposed to human macrophages (Sagone and Rinehart, 1984). To be pathogenic, some viruses apparently require the presence of specific antibodies.

At present it is not known whether rubella-induced tissue injuries seen in clinical rubella are associated with an antibody-dependent superoxide anion production by PMNLs. Additional studies are needed to define mechanisms of cytotoxicity of rubella virus.

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