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Report

Silica Particles with Human Protein Corona Shows Sensitization Potential in the Human Cell Line Activation Test

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Nanoparticles are concerned to show adverse biological effects despite their unique functions. Their physicochemical properties and status are widely diverse; this makes safety analysis of nanoparticles complicated. Some reports showed that nanoparticles could disturb acquired immunity, while it is still unclear what is the inducer of that effects. Here, we tried to explore the relationship among nanoparticles' physicochemical properties and sensitizing potential by using the human cell line activation test based *in vitro* method; that uses in expression of CD86 and CD54 as an index of cellular activation. As a model of nanoparticles, we examined sensitization potential of silica particles with or without a human protein corona. Of the cells treated with silica particles (diameter: 50 or 300 nm) only, none of them showed activation. On the other hand, silica particles with human protein corona showed activation. Moreover, protein corona that forms around 50 nm silica nanoparticles have a higher sensitization potential than that of protein corona that forms around 300 nm silica particles. Our findings indicated that silica particles with human protein corona showed sensitization potential, and that sensitization potential could depend on the amount or kind of proteins within the corona.

Key words human cell line activation test, nanoparticle, protein corona, sensitization

INTRODUCTION

Recently, various materials are becoming smaller as the nanotechnology is developed; the particles of less than 100 nm in diameter are called nanoparticles.[1)] They are applied to various industries because of their unique functions along with their size decreasing, while concerns about their unexpected adverse biological effects have been emerged.[1)] For example, it is reported that some types of nanoparticles induced the activation of the innate immune response via NOD-, LRR- and pyrin domain containing protein 3 inflammasome; this pathway could lead to chronic inflammatory diseases.[2,3)] In addition to innate immunity-related biological effects, nanoparticles also have been concerned to disturb acquired immunity,[4,5)] which is activated subsequent to innate immunity. However, the key properties of nanoparticles to activate acquired immunity has not yet clear from their various physicochemical properties such as the size, the shape, or electron charges. Furthermore, it has been shown that the composition of the protein corona that forms around nanoparticles in biological matrices affects the biological response to the core nanoparticle, and that the composition of the protein corona changes depending on the physical characteristics of the core nanoparticle.[6,7)] Thus, further studies are needed to fully understand the activating potential of nanoparticles to disturb acquired immunity, particularly with respect to their physical characteristics.

In this regard, silica nanoparticles are reported to activate innate immune responses; the activation of innate immune cells are much higher around 50 nm.[8)] Mechanistically, it is pointed that size-dependent recognition by macrophages and size-dependent protein composition of protein corona could impact the degree of the innate immunity.[6,7)] Therefore, exploring the relationship among physicochemical properties of nanoparticles, their status (with or without protein corona), and their sensitization potential in acquired immunity is needed.

The human cell line activation test (h-CLAT) is an *in vitro* skin sensitization test that is included in Organization for Economic Co-operation and Development Test No. 442E (https://www.oecd.org/env/test-no-442e-in-vitro-skin-sensitisation-9789264264359-en.htm). In the h-CLAT, the sensitization potential in the development of acquired immunity of a chemical substance is evaluated by exposing human monocytic leukemia THP-1 cells to the test material and examining the changes in expression of the cell-surface markers CD86 and CD54, which is increased when the cells are activated.[9)] Here, we attempted to evaluate sensitization of silica particles and to explore the relationship among nanoparticles' size, their status, and sensitizing potential by using the h-CLAT.

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MATERIALS AND METHODS

Reagents Silica particles with diameters of 50 and 300 nm (nSP50 and SP300, respectively) were purchased from Micromod Partikeltechnologie (Rostock, Warnemünde, Germany). Before use, the suspensions were sonicated for 5 min at 400 W and mixed for 1 min with a benchtop vortexer. The hydrodynamic diameters of nSP50 and SP300 were tested by dynamic light scattering (Zetasizer Nano-S; Malvern Instruments., Malvern, UK) and were 44.39 ± 1.04 nm and 301.33 ± 3.10 nm, respectively. To induce protein corona formation around the silica particles, the suspensions of silica particles were mixed with an equal volume of human pooled serum (Kohjin Bio, Saitama, Japan) and incubated at 37°C for 30 min. Fluorescein isothiocyanate (FITC)–conjugated anti-human CD54 monoclonal antibody (mAb) and FITC-conjugated IgG1 isotype control were purchased from Dako (Glostrup, Denmark); FITC-conjugated anti-human CD86 mAb was purchased from BD Biosciences (San Jose, CA, USA).

Cell Line and Cell Culture Human monocytic leukemia THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), cultured in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 1% Antibiotic-Antimycotic (× 100) (Thermo Fisher Scientific), and 0.1% 2-mercaptoethanol (Thermo Fisher Scientific), and maintained at 37°C under an atmosphere of 5% CO_2 and > 95% humidity.

h-CLAT Based Cell Sensitization Test THP-1 cells (1.0×10^6 cells/well) were seeded in a 24-well flat-bottom cell culture plate (Iwaki, Shizuoka, Japan) and immediately treated with test substances, sodium dodecyl sulfate as a negative control, or 2,4-dinitrochlorobenzene as a positive control. Five concentrations with a geometric ratio of 1.2 were determined as applicable doses by conducting a cell viability test; these concentrations showed more than 75% cell viability and didn't affect flow cytometry analysis. This is because the expressions of CD86 and CD54 become stronger in along with cells dying,[9] and nanoparticles themselves scatter lights. A geometric ratio of 1.2 is applied according to the test guideline. After incubation under an atmosphere of 5% CO_2 at 37°C for 24 h, cell suspensions were divided into three groups, washed twice with phosphate-buffered saline containing 0.01% fetal bovine serum (FCM buffer), and then centrifuged at 250 *g* for 5 min at 4°C. Next, each cell group was labeled with FITC-conjugated anti-human CD54 mAb, FITC-conjugated anti-human CD86 mAb, or FITC-conjugated IgG1 isotype control for 30 min. After labeling, the cells were resuspended in FCM buffer containing propidium iodide (Sigma Aldrich, St. Louis, MO, USA), and the stained cells were analyzed by flow cytometry (FACS Aria Cell Sorter, BD Biosciences). The cells were gated first according to side- and forward-scattered light area and then according to propidium iodide staining to eliminate dead cells. The relative fluorescence intensity (RFI) of CD86 and CD54 was calculated by using mean fluorescence intensity (MFI) values and equation (1). A test substance was judged to have sensitization potential at a particular concentration if either of the following criteria was met: RFI of CD86 ≥ 150, RFI of CD54 ≥ 200.[9]

$$\mathrm{RFI} = \frac{MFI_{\text{test substance-treated cells}} - MFI_{\text{test substance-treated isotype cells}}}{MFI_{\text{solvent-treated cells}} - MFI_{\text{solvent-treated isotype cells}}} \times 100 \qquad (1)$$

RESULTS AND DISCUSSION

Silica Particles Incubated with Human Serum Showed Positive Sensitization Potential First, sensitization potential of negative control (sodium dodecyl sulfate) and positive control (2,4-dinitrochlorobenzen) was evaluated for quality control (Fig. 1a), and only 2,4-dinitrochlorobenzen indicated sensitization potential as reported.[10] Then, sensitization potential of silica particles with diameters of 50 and 300 nm (nSP50 and SP300, respectively) were evaluated to explore the relationship between sensitization potential and particles size. In the experiment, test substance was judged to have sensitization potential when either of the following criteria was met at more than 3 concentration points: the relative fluorescence intensity (RFI) of CD86 ≥ 150, RFI of CD54 ≥ 200. In cells treated with nSP50, RFI did not exceed either of the cut-off values for positive sensitization potential (RFI of CD86 ≥ 150, RFI of

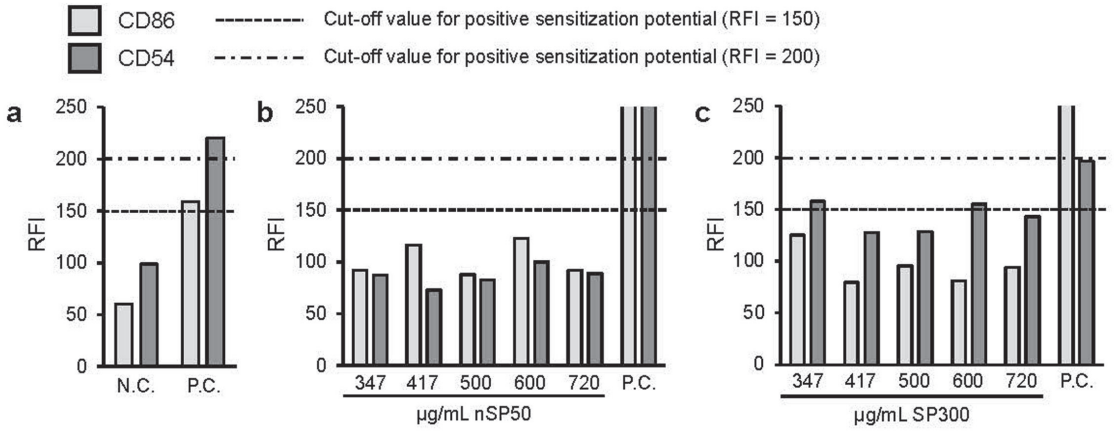

Fig. 1. Silica Particles of 50 nm and 300 nm didn't Show Sensitization Potential

THP-1 cells were treated for 24 h with (a) sodium dodecyl sulfate, 2,4-dinitochlorobenzen, or silica particles with a diameter of (b) 50 nm (nSP50), or (c) 300 nm (SP300). At the end of treatment, the expression levels of CD86 and CD54 were evaluated by flow cytometry. The relative fluorescence intensity (RFI) of each sample was calculated by using the solvent-treated group as a reference (N.C., negative control; P.C., positive control). The test materials were considered to be positive for sensitization potential when either of the following criteria was exceeded at more than 3 concentration points: RFI of CD86 ≥ 150 (dashed lines) or RFI of CD54 ≥ 200 (dash-dotted lines). Each test was conducted three times independently and similar results were obtained at least two times.

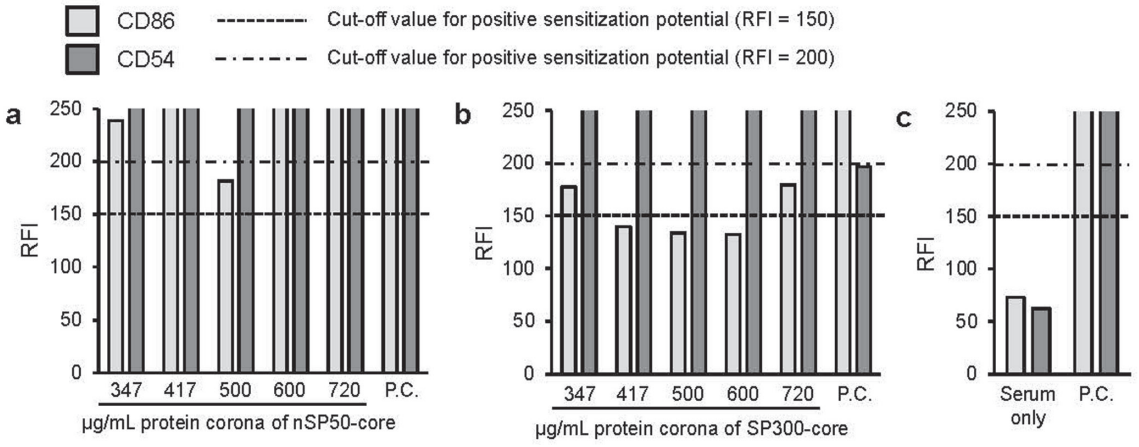

Fig. 2. Silica Particles Incubated with Human Serum Showed Positive Sensitization Potential

THP-1 cells were treated for 24 h with silica particles with a diameter of (a) 50 nm (nSP50) or (b) 300 nm (SP300) incubated with human serum to induce protein corona formation (protein corona of nSP50-core or its of SP300-core, respectively), or (c) human serum only. At the end of treatment, the expression levels of CD86 and CD54 were evaluated by flow cytometry. The relative fluorescence intensity (RFI) of each sample was calculated by using the solvent-treated group as a reference (P.C., positive control). The test materials were considered to be positive for sensitization potential when either of the following criteria was exceeded at more than 3 concentration points: RFI of CD86 ≥ 150 (dashed lines) or RFI of CD54 ≥ 200 (dash-dotted lines). Each test was conducted three times independently and similar results were obtained at least two times.

CD54 ≥ 200) at any of the concentrations examined (Fig. 1b). Besides, in cells treated with SP300, the RFI did not exceed either of the cut-off values for positive sensitization at any of the concentrations examined (Fig. 1c). This suggest that silica particles themselves wouldn't show sensitizing potential despite their particles size.

Nanoparticles including amorphous silica particles immediately form protein-nanoparticles complexes called 'protein corona' with surrounding proteins.[11] In any circumstances when we use such nanoparticles with *in vivo* experiment, they are assumed to form protein corona.[12] Moreover, protein corona is pointed that it affects cellular responses.[11] Therefore, we examined sensitization potential of protein corona that forms around nSP50 and SP300 to clarify the involvement of nanoparticles binding proteins with sensitizing. Protein coronas that form around nSP50 (protein coronas of nSP50-core) or protein coronas that form around SP300 (protein coronas of SP300-core) were respectively made by incubating nSP50 or SP300 with human serum. In cells treated with protein coronas of nSP50-core, the RFIs of CD86 and CD54 both exceeded the cut-off values at all concentrations examined (Fig. 2a). Moreover, in cells treated with protein coronas of SP300-core, the RFI of CD86 was exceeded the cut-off value at two of the concentrations and that of CD54 exceeded the cut-off value at all concentrations examined (Fig. 2b). At this time, the expression levels of CD86 and CD54 in cells treated only with human sera did not exceed these cut-off values (Fig. 2c), indicating that the protein coronas of nSP50-core and SP300-core has sensitization potential. Moreover, compared between expressions of protein corona treated cell surface markers, CD86 expression level in protein coronas of SP300-core treated cells did not exceed its criteria in three concentrations; in contrast, its level in protein coronas of nSP50-core treated cells exceeded in all concentrations. Together, these findings indicate that protein coronas of nSP50-core have a higher sensitization potential than those of SP300-core.

The present data indicate that silica particles activate THP-1 cells only when surrounded by a protein corona, providing evidence that it is the protein corona, not the core particle, that induces sensitization. However, it is not clear what the antigen could be. The amount of proteins binding to silica particles increases with decreasing particle size because of the corresponding increase in specific surface area. Together with the present findings that protein coronas of nSP50-core showed greater sensitization potential than did protein coronas of SP300-core, this suggests that sensitization potential could depend on the amount or kind of proteins within the corona. Furthermore, cellular uptake of nanoparticles differs depending on the nanoparticles' physicochemical properties and constituents of the protein corona,[13] meaning studies to explore the relationship among cellular uptake, the composition of protein corona, and sensitization ability is needed.

On the contrary, it is possible that the specific component of protein corona could become the trigger of the activation; this means it might gain antigenicity. The compositions of protein corona are reported to depend on the physicochemical properties of core nanoparticles.[14,15] For example, it is reported that fibrinogen hardly bind gold nanoparticles of more than 70 nm, which is 340 kDa in molecular weight and 45 nm in length.[16,17] In addition, proteins are denatured and may attain antigenicity by binding nanoparticles.[18,19] Together, it is possible the specific component of protein corona would play a critical role in the sensitization ability; meaning studies to identify the specific components of the protein corona and the responses of the adaptive immune system to those components are needed.

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Abbreviations MFI, mean fluorescence intensity; RFI, relative fluorescence intensity

Conflict of interest The authors declare no conflict of interest.

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