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COLLOIDAL GOLD PARTICLES DETECTED ON HIGHLY STRUCTURED SURFACES OF LARGE SAMPLES BY BACKSCATTERED ELECTRONS IN THE SCANNING ELECTRON MICROSCOPE

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Abstract

Colloidal gold labelled surface antigens on large, highly structured samples were unambiguously visualized at high magnification with backscattered electrons at 25 kV in a field emission scanning electron microscope (SEM). Secondary electron (SE) and backscattered electron (BSE) images of large and highly structured specimens are frequently disturbed by charging effects. By optimizing the thickness of the conducting carbon layer and the intensity of the incident electron beam, this problem can be overcome. The midgut of the lepidopteran larvae of Spodoptera litoralis was used as specimen. The activated δ-endotoxin of Bacillus thuringiensis coupled to colloidal gold binds to the microvilli exposed to the midgut lumen.

Key Words: Surface labelling, Colloidal Gold, Backscattered Electrons, Bacillus thuringiensis δ-endotoxin, Spodoptera litoralis, Pieris brassicae.

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Introduction

Colloidal gold has proved to be an excellent marker for surface antigens in the SEM (Horisberger and Rosset, 1977; Trejdosiewicz et al., 1981; Walther et al., 1983; de Harven et al., 1984; Walther et al., 1984). The smaller the gold markers are, the more amenable to verification by additional information they have to be. The BSE-signal allows unambiguous detection of even 5 nm gold markers (Walther and Müller, 1985).

The surface of small objects, e.g. red blood cells, yeast cells (Walther et al., 1983) and leucocytes (de Harven et al., 1984) are well depicted in SEM-images when encapsulated with 10 nm carbon.

To avoid severe charging effects during recording images of large highly structured surfaces, as the microvilli of midgut cells, the carbon coating and the intensity of the electron beam have to be adjusted.

Our target of interest is the larvae of Pieris brassicae, the cabbage butterfly. Biological pest control is carried out by applying Bacillus thuringiensis δ-endotoxin formulations. The toxin destroys the midgut of the larvae (Lüthy and Ebersold, 1981). We suggest that the first step in the mode of action of the activated toxin (Huber and Lüthy, 1981) is the binding to the microvilli. Binding studies with toxin gold particles were performed. However, after formaldehyde fixation (Tokuyasu and Singer, 1976) of the pieris midgut only few cells remain intact. Spodoptera litoralis which should function as a negative control (the larvae are not susceptible to the toxin) show fine structural preservation of the midgut cells after the same fixation.

Materials and Methods

The protein-gold complexes

15 nm gold particles were obtained according to Frens (1973). Activated B. thuringiensis δ-endotoxin (Huber and Lüthy, 1981) was directly coupled to the gold particles in carbonate buffer (0.05 m) at pH 9 and protein-A was coupled as described by

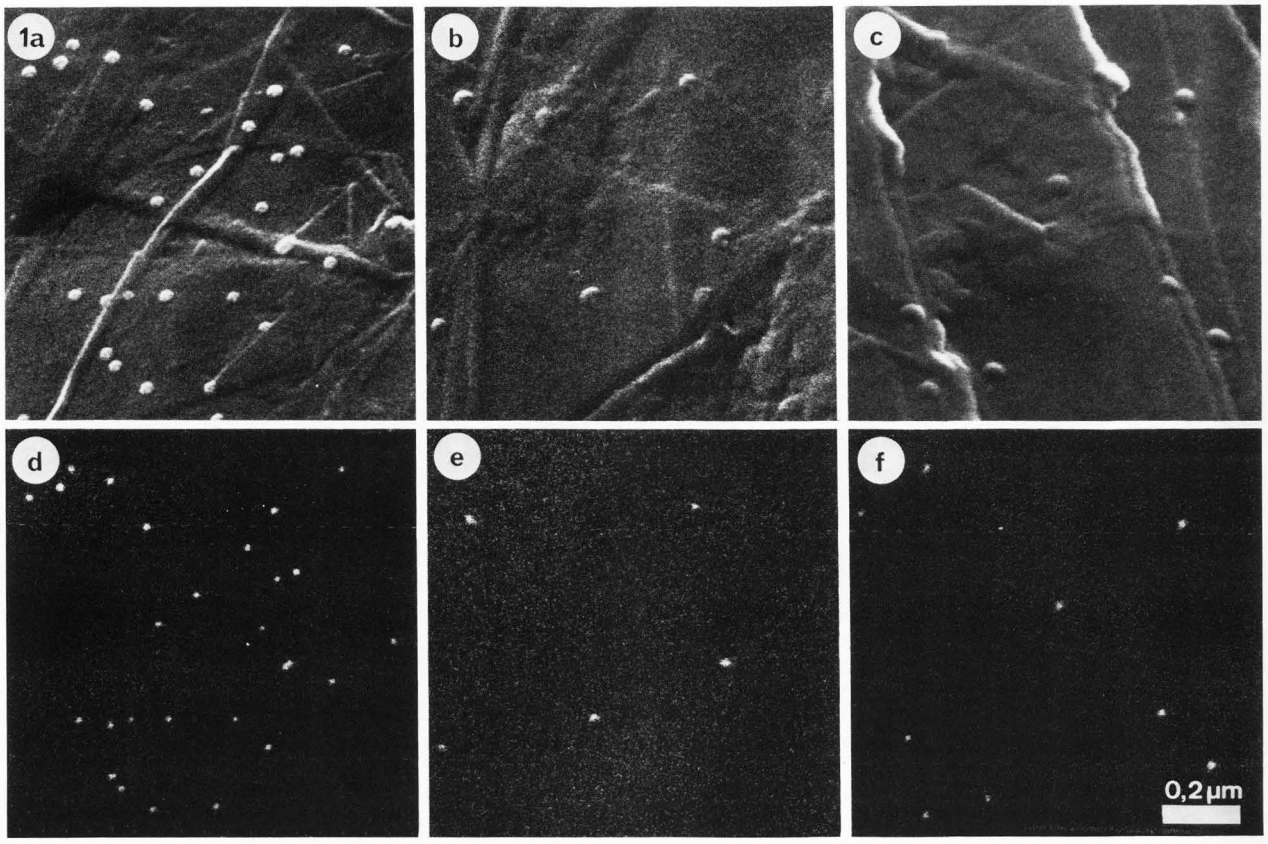

Fig. 1: Gold particles adsorbed on a carbon foil and coated with 10 nm (a, d), 20 nm (b, e), or 40 nm (c, f) carbon. SE-images illustrate how the coating becomes thicker (a-c). BSE-images (d-f) are not affected by the thickness of the carbon coating. Figs. a-f are at same magnification.

Roth (Roth et al., 1980). The protein-gold sols were stabilized with 0.1% polyvinylpyrolidone.

Gold particle examination

Gold particles were visualized by adsorbing a droplet of the gold sol onto glow discharged carbon platelets (3 x 2 x 0.1 mm; Goodfellow Metals, Cambridge, England). The platelets were air-dried and coated with 10, 20 or 40 nm carbon on a rotary tilting stage (Müller et al., 1975).

Labelling of midguts

Living Spodoptera litoralis larvae were fixed in a solution of 2 % formaldehyde and 0.2 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 1 hour at 4 °C. Prior to the immersion the fixative was injected into the larval midgut. The guts were cut using a razor blade, washed with cacodylate buffer and preincubated with 0.5 % ovalbumin in the same buffer for one hour at room temperature. Omitting an additional washing step the midguts were incubated for one hour with the toxin gold or protein-A gold sols in cacodylate buffer. The used gold concentration was 1/10 of the original concentration (O.D. = 1.2 at 520 nm).

SEM Preparation

The labelled samples were rinsed with bi-distilled water, then dehydrated with 2,2-dimethoxypropane (Muller and Jacks, 1975). After three washings with acetone the samples were critical point dried from CO_2 (Anderson, 1951). Dried specimens were coated with a 10, 20 or 40 nm thick carbon layer by using a rotary tilting stage. The thickness of the carbon coating was measured with a quartz crystal thickness monitor (QSG 201, Balzers). The monitored value has to be divided by a factor of π to know the real thickness of the coating (e.g. to get a 10 nm thick coating, the measured thickness has to be 31.4 nm), because the stage is rotating and tilting whereas the quartz is mounted perpendicular to the evaporation direction.

Microscopy

A Hitachi field emission SEM S-700 modified for improved resolution as described by Nagantani and Okura (1977) was used. Secondary and backscattered electrons were recorded simultaneously at a measured beam current from 8 to 40 x 10^{-12} A. The current was set by changing the excitation of the condenser lens. A 300 µm condenser and a 100 µm objective aperture were used. The acceleration voltage was 25 kV. The working distance was 5-10 mm. Images were usually recorded at a primary magnification of 50,000 x. Backscattered electrons were detected with an annullar yttrium-aluminum-garnet single crystal detector (Autrata, 1978), as described by Walther et al. (1984).

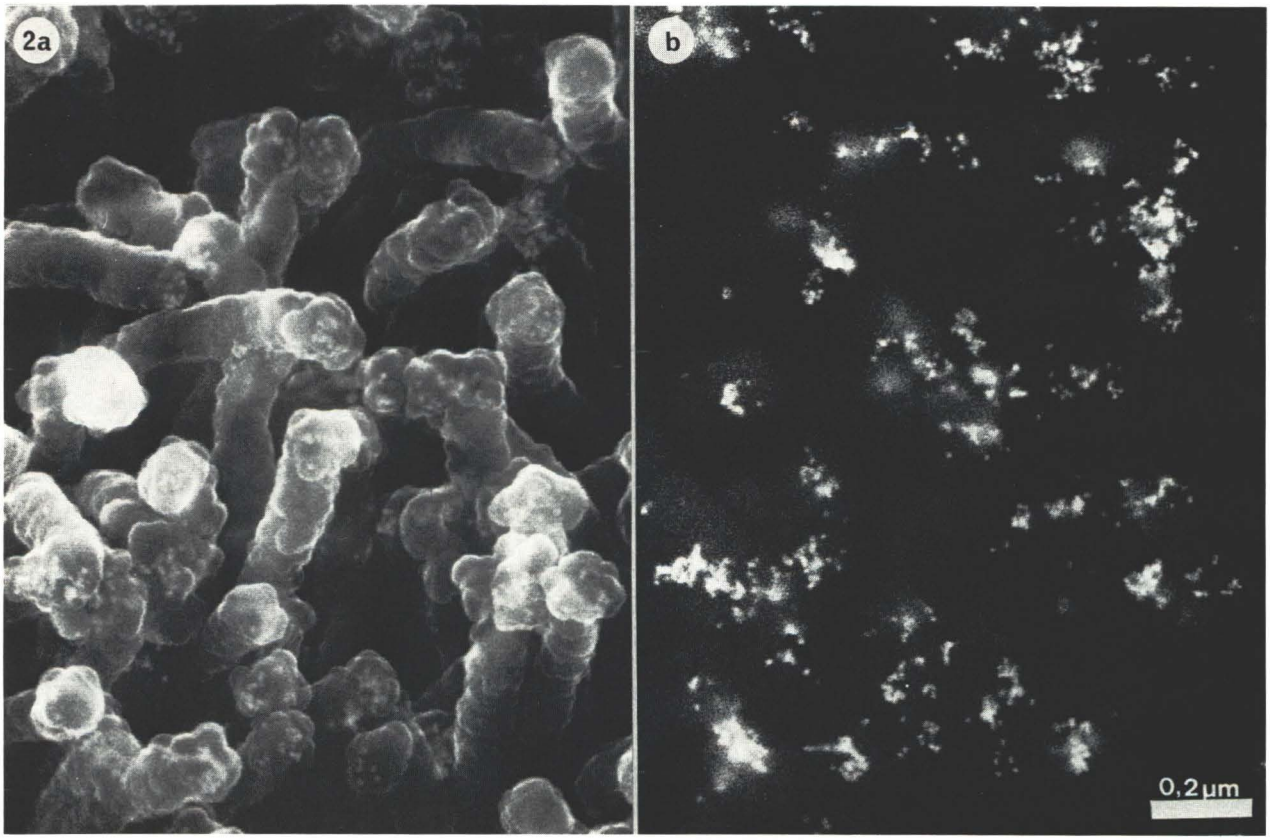

Fig. 2: Microvilli coated with 40 nm carbon and recorded at a beam current of 40 pA. The SE-image (a) shows the surface structure and the toxin gold particles. Unambiguous gold detection is revealed in the BSE-image (b). Figs. a-b are at same magnification.

Results and Discussion

Critical point dried midguts of Spodoptera litoralis encapsulated with 10 nm carbon as used by Walther (Walther et al., 1983) and de Harven (de Harven et al., 1984) show severe charging artifacts when recorded at a beam current of 40 x 10^{-12} A. The microvilli move and the images seem to be out of focus. By reducing the beam current to 8 x 10^{-12} A occasionally no charging effects occur, but the images appear noisy. Because of this unsatisfactory results we tried to enhance the carbon coat thickness. To be sure that colloidal gold particles of 15 nm diameter are also detected when coated with more than 10 nm carbon we adsorbed (15 nm) gold particles to carbon platelets and encapsulated them with 10, 20 or 40 nm carbon. All micrographs of Fig. 1 have the same magnification. The SE-images show how the size of the bumps increased, when the carbon coating becomes thicker. The bright spots in the middle or below the bumps indicate the presence of colloidal gold particles. As shown in Fig. 1, the BSE-signal of the 15 nm gold particles is not affected even by a carbon layer of 40 nm thickness.

Based on these findings we coated midguts with 40 nm carbon and examined them with a beam current of 40 x 10^{-12} A in the microscope. Under these conditions the samples rarely show charging effects and it was possible to record images shown in Fig. 2. In the SE-image (Fig. 2a) the topography is adequately depicted and a lot of gold particles are easily identified. Fig. 2b shows the corresponding BSE-image. When examining the gold particles on the microvilli (Fig. 2a), the measured 40 nm carbon coat appears much thinner than the 40 nm carbon coat of the gold particles adsorbed to the platelets (Figs. 1a, b, c). This is due to the 40-fold increase of the midgut surface, assuming that approximately 30 microvilli of 2 µm length cover an area of 1 μm^2. Uniform coating of the whole surface would result in a 1 nm thick carbon layer, although 40 nm of carbon were evaporated. We think, however, that the thickness of the carbon layer variation is large. The important feature of this carbon film is its sufficient conductivity.

Although we are able to demonstrate toxin-gold labelled, carbon coated midguts, we still have severe difficulties in obtaining relevant

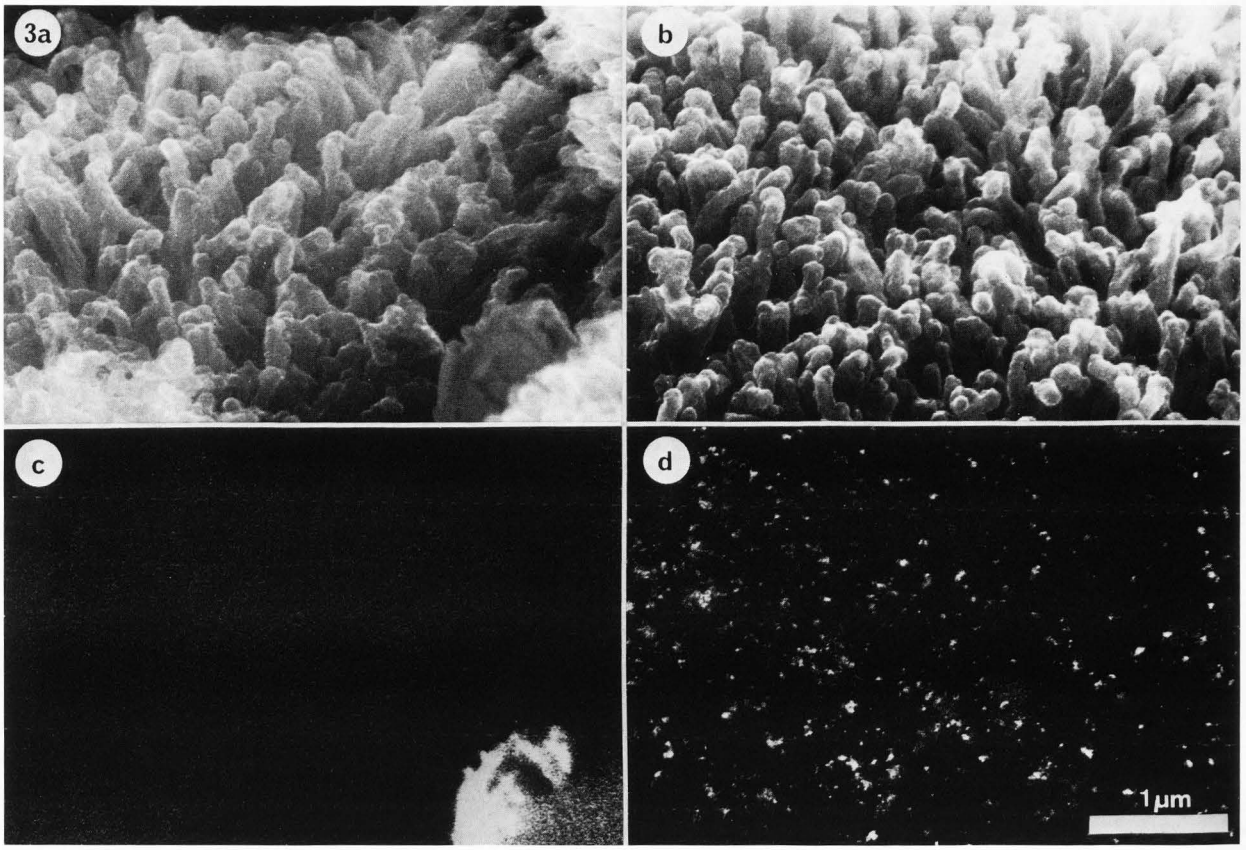

Fig. 3: SE-images of single midgut cells labelled with protein-A gold (a) and toxin gold (b) respectively, show the surface of well preserved microvilli. BSE-images show no binding of protein-A gold on microvilli (c: only an aggregate is visible), but the binding of hundreds of toxin gold particles (d). Figs. a, b, c, d are at same magnification.

biological information. Fig. 3 shows the midgut of Spodoptera litoralis incubated with protein-A gold (Figs. 3a, c) or toxin gold (Figs. 3b, d). Only toxin gold binds to the midgut. Because Spodoptera litoralis is not susceptible to the toxin this result per se is only of methodological interest. We hope to get similar results with Pieris brassicae after having improved the fixation of the gut. 3 % glutaraldehyde fixed midguts are well preserved but any binding properties are lost. However, the few intact cells after formaldehyde fixation show labelling with toxin gold and almost no labelling with protein-A gold.

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