

A Simple Method for Measuring the Amount of Immunoglobulin A Secreted onto the Skin Surface

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We developed a simple method for measuring the amount of the secretory form of immunoglobulin A (sIgA) present in sweat. A small disk (10 × 10 mm) made of cellulose membrane was attached to the skin surface for periods of 1 to 24 h. sIgA was absorbed to the membrane and accumulated during the period of application. Enzyme immunoassay using anti-sIgA and anti-secretory component (SC) antibodies revealed distinct dots on the disk that corresponded to the eccrine excretory ducts. A densitograph was used to determine the number and density of the dots, thus obtaining the amount of sIgA excreted to the surface of the skin (per mm²). The amount of skin sIgA excreted differed inter-individually as well as intra-individually. That is, it varied according to the region of the skin, and its distribution roughly reflected that of the sweat ducts. sIgA excretion was maintained at a certain level, regardless of the increased sweating produced by either heat or exercise, which raised the output of sweat 3- to 15-fold. Immunohistochemical studies revealed that fewer glandular cells expressed SC in their cytoplasm as the amount of sIgA decreased. Such an independence of the excretion of sIgA from that of sweat may be necessary to the local immune defenses of the skin. **Key words:** secretory IgA; human skin; sweat.

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The secretory form of immunoglobulin A (sIgA) is regarded as an immunological barrier that combines with antigens external to tissues, as in the lumen of the intestine or the respiratory tract, thus preventing them from adhering to the epithelial lining and entering the body (1, 2). Recent reports indicate the presence of sIgA in human sweat (3, 4). The expression of a secretory component (SC) in the cells of eccrine sweat glands has confirmed that sIgA is secreted, not exuded, into the sweat (4). Since the surface structures of microorganisms are bound to sIgA in the mouth and skin (5), sIgA in the sweat is likely to be involved in the local defense system of the skin. However, difficulties in measuring the amount and distribution of sIgA on the skin surface have presented an obstacle to understanding the functional role of this immunoglobulin in sweat. Accordingly, we developed a simple method for measuring sIgA in sweat by applying a cellulose membrane that absorbs immunoglobulins to the skin surface.

In the present study, we describe a procedure for determining the amount of sIgA excreted onto the surface of the skin. Since 50 healthy volunteers showed a wide range of the amount of skin sIgA, we further investigated 1) whether there were any regional differences in sIgA excretion, since the distribution of eccrine excretory ducts varies on the surface of the skin (6); 2) whether the amount of sIgA varied in accordance with sweating

elicited by internal and external heat stress; and 3) whether the excretion of sIgA might have clinical relevance.

MATERIALS AND METHODS

Subjects

A total of 50 healthy, unmedicated volunteers, 24 males and 26 females, age 12 to 42 years, mean age 27.4 years, participated in these studies. Informed consent was obtained from each subject before the study. We evaluated the amount of sIgA on the skin on the inner surface of the upper arm and on other regions, including the neck, chest, abdomen, back, arms, thighs, and legs. Six males (mean age 23.5 years) participated in a study in which sweating was increased by the application of heat or exercise. All participants lacked a history of frequent infection or of allergic disease. All laboratory data, including their serum levels of IgA, IgG, IgM and IgE, were within normal limits.

Disk for absorbing sIgA

A nitrocellulose membrane disk (10 × 10 mm) (Advantec, Tokyo, Japan), with a backing of filter paper (Whatman, Maidstone, U.K.), was affixed to the skin with a permeable adhesive tape (Transpore[®], 3M, Tokyo, Japan). During the application period, the immunoglobulins contained in sweat were absorbed by the disk, while the water content evaporated.

Application of the disk

In the following studies, the disk was preferentially applied to the inner surface of the upper arm where there is little distortion of the skin surface, and where moderate sweating would be expected during routine activities. The disk was left in place for 1 to 24 h to collect an amount of sIgA suitable for measurement. To reduce contamination by any immunoglobulins present on the skin surface, we applied the disk after cleansing the skin with soap. No sIgA was detected on the skin surface after such cleansing (see Fig. 2).

Induction of sweating

The disk was affixed to the inside of the upper arm for 3 h before and during heat or exercise. To induce heat stress, an exothermic pad (100 × 140 mm) that maintained the skin temperature at 42°C for 3 h (Hisamitsu Pharmaceuticals, Tosu, Japan) (7) was applied to the inside of the upper arm. To stimulate exercise-induced sweating, each subject ran up and down the stairs for 10 min three times during the collection period. Together with the measurement of sIgA, we also measured the amount of sweat produced next to the nitrocellulose membrane disk by affixing a 2 × 2 cm disk of a superabsorptive, sponge-like polymer made of polyvinyl alcohol (Bellclean[®], Kanebo, Tokyo, Japan), which absorbed and fixed water. Using this special sponge, we directly determined the total amount of sweat as an increase in weight of the sponge.

Assay of sIgA

The disks were collected, stored at -70°C, and processed at the same time to avoid artifacts. They were initially incubated with phosphate-buffered saline (PBS) (pH 7.2), containing 1% bovine serum albumin, to block non-specific reactions for 2 h. After thorough rinsing with 50 mM Tris buffer (pH 7.4), containing 150 mM sodium chloride and Tween 20[®] (Merck, Darmstadt, Germany), they were incubated for 1 h with a 1/1000 dilution of peroxidase-conjugated anti-human sIgA goat polyclonal antibody (Cappel, West Chester, PA, USA) (8). Specimens

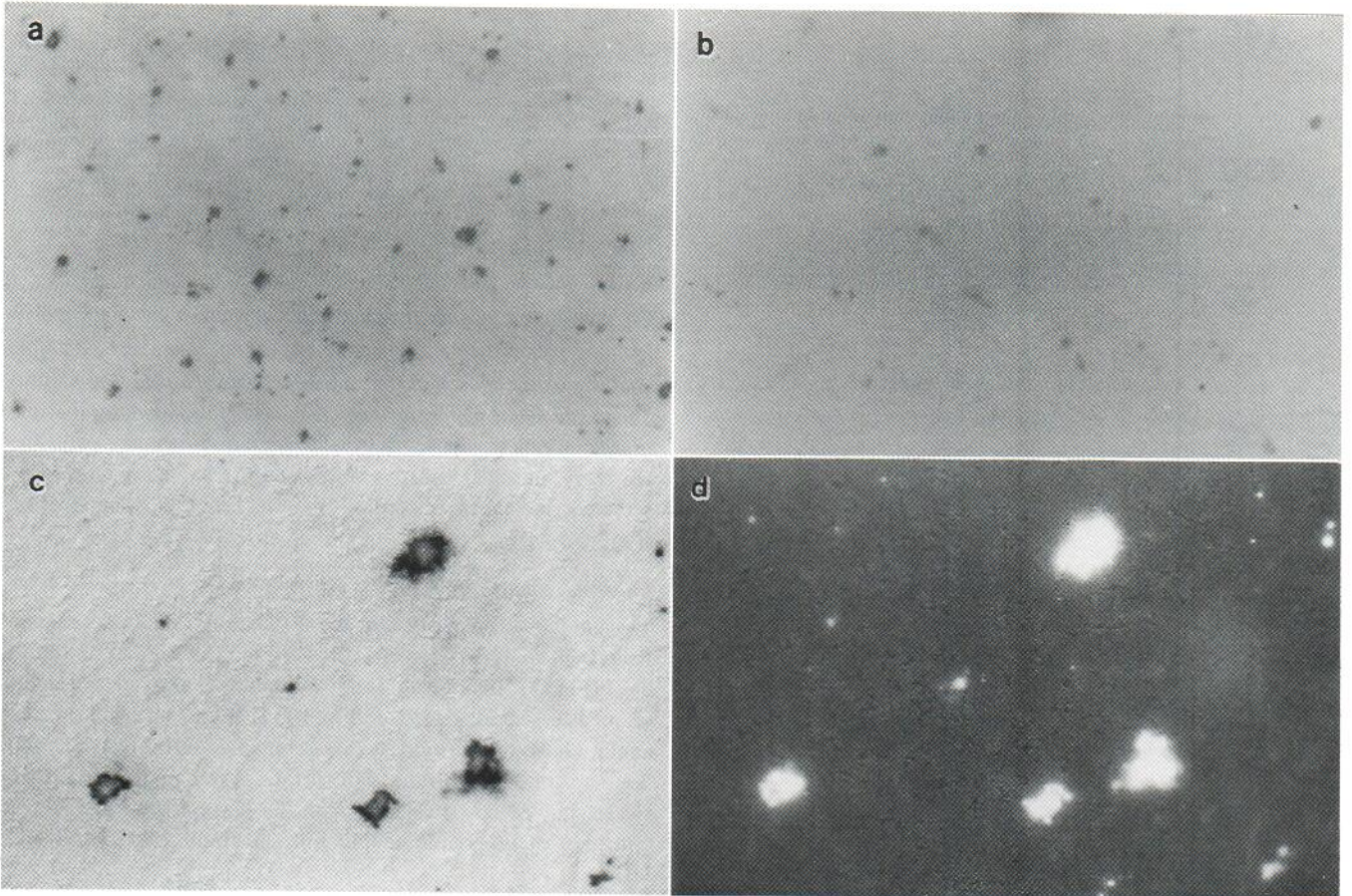


Fig. 1. Upper panels (a, b). Representative micrographs of disks obtained from the inner surface of the upper arm of a high sIgA secretor (a) and of a low sIgA secretor (b) ($\times 150$). Lower panels (c, d). Double staining of dots with anti-sIgA (c: peroxidase) and with anti-SC (d: FITC). Such staining demonstrated a similar pattern of distribution and amount of immunoreactive products on the disk, confirming the secretory form of IgA as that absorbed ($\times 250$).

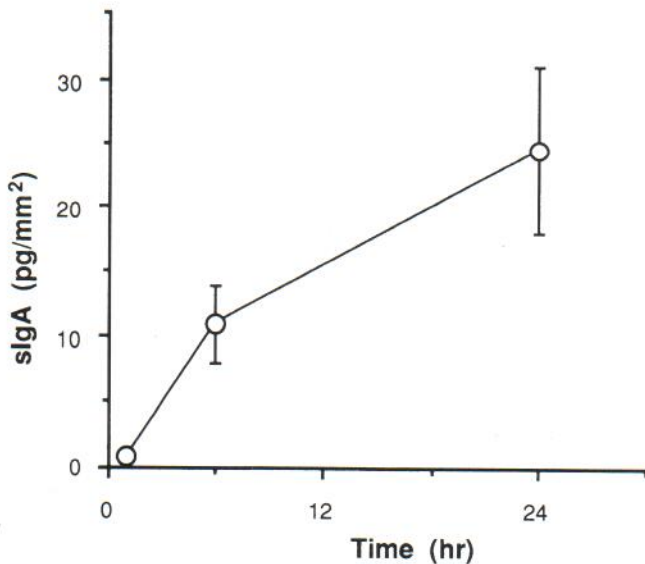


Fig. 2. Amount of sIgA (pg/mm²) collected over time in disks attached to the inner surface of the upper arm of a healthy subject. While no sIgA was detected on the disk after cleansing the skin, this substance gradually accumulated on the disk according to the length of application.

were again rinsed with Tris buffer solution and further incubated with 0.5 mg/ml of diaminobenzidine tetrahydrochloride (Nacalai Tesque, Kyoto, Japan) and 0.025% hydrogen peroxide in the same buffer solution for 3 min (9). This procedure revealed distinct dots of an immunoreactive product on the cellulose membrane disk (Fig. 1). The amount of sIgA was calculated as the product of the number and the density of the dots. The latter was determined with a densitograph (AE-6900, Atto, Tokyo, Japan). Densitograph scores showed a linear correlation between known amounts of sIgA on the cellulose membrane. The values of three different areas (2×2 mm) near the center of each disk (10×10 mm) were averaged to obtain the mean amount of sIgA per mm².

Negative controls were provided by omitting the primary monoclonal antibody. Anti-SC antibodies conjugated with peroxidase or fluorescein isothiocyanate (FITC) (DAKO, Santa Barbara, CA, USA) were used as the primary antibody for positive controls. The latter was also used to confirm that the secreted sIgA was IgA bound with SC.

Immunohistological study

Skin specimens were obtained from 8 subjects by biopsy of the skin from the inner surface of the upper arm, conducted under local anesthesia. The purpose was to demonstrate any differences in the number and/or density of eccrine glandular cells that expressed SC, and the number and/or density of plasma cells containing IgA around the eccrine sweat gland. Of the 8 subjects, 3 were high (> 30 pg/mm²/day), 2 were moderate ($10 <$ and < 20 pg/mm²/day) and 3 were low secretors of sIgA (< 5 pg/mm²/day) (see Fig. 3). One-half of each tissue block was fixed in liquid nitrogen. Sections $4 \mu\text{m}$ thick were cut and pro-

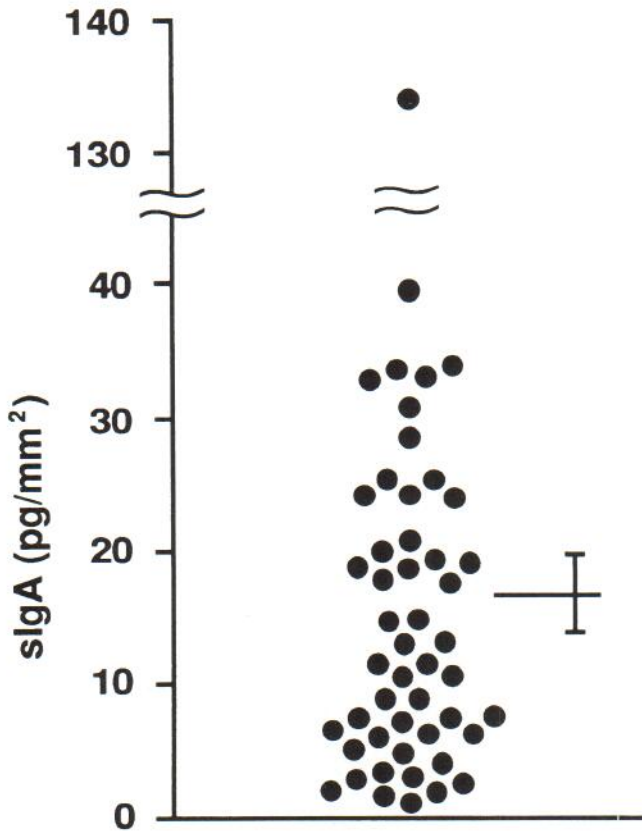


Fig. 3. Amount of sIgA (pg/mm²/day) present in disks attached to the inner surface of the upper arm of 50 healthy subjects. The amount ranged from 0.79 to 133.77 (mean: 16.79 ± 2.80) pg/mm²/day.

cessed immunohistochemically using the same anti-SC antibody and anti-human IgA polyclonal antibody (Cappel, West Chester, PA, USA). For routine paraffin sections, the remaining halves of the tissue blocks were fixed in 3% formaldehyde buffered with PBS, embedded in paraffin, sectioned at 4 µm thick, and stained with hematoxylin-eosin.

Statistical analysis

Data are reported as mean ± SD. Fisher's exact test (two-tailed), Wilcoxon's two-sample test and Mann-Whitney's U-test were used to establish the statistical significance of the differences between means. A level of $p < 0.05$ was accepted as statistically significant. Statistical computations were performed with a statistical package (SAS Institute Inc., Cary, NC, USA).

RESULTS

Basic studies

Enzyme immunoassay revealed distinct dots of varying number and density on the cellulose membrane disk (Fig. 1a, b). Double staining of the disk with anti-sIgA and anti-SC antibodies produced the same pattern of dots (Fig. 1c, d), confirming that the immunoglobulin detected by the present procedure was the secretory, not the exudative, form of IgA. The amount of sIgA secreted increased according to the length of application (Fig. 2).

Inter-individual differences

The amount of skin sIgA in the 50 healthy volunteers ranged from 0.79 to 133.77 pg/mm²/day (mean: 16.79 ± 2.80 pg/mm²/day) (Fig. 3). Interestingly, not all the eccrine excretory ducts seemed to secrete sIgA (see Fig. 1). This was particularly evident in those subjects whose disks contained little sIgA, i.e. who secreted little sIgA to the skin surface. The amount of sIgA in sweat showed no correlation with the plasma level of IgA, or with that of other immunoglobulins, including IgG, IgM and IgE.

Immunohistological studies

Histological studies demonstrated that, in the skin specimens obtained from the subjects who secreted high sIgA, two thirds of the eccrine glandular cells observed in the cross-sectional view of the secretory portion of the gland densely expressed SC within the cytoplasm. In the tissues of the low secretors of sIgA, only occasional cells expressed SC (Fig. 4). Since only a few plasma cells containing IgA were detected around the eccrine

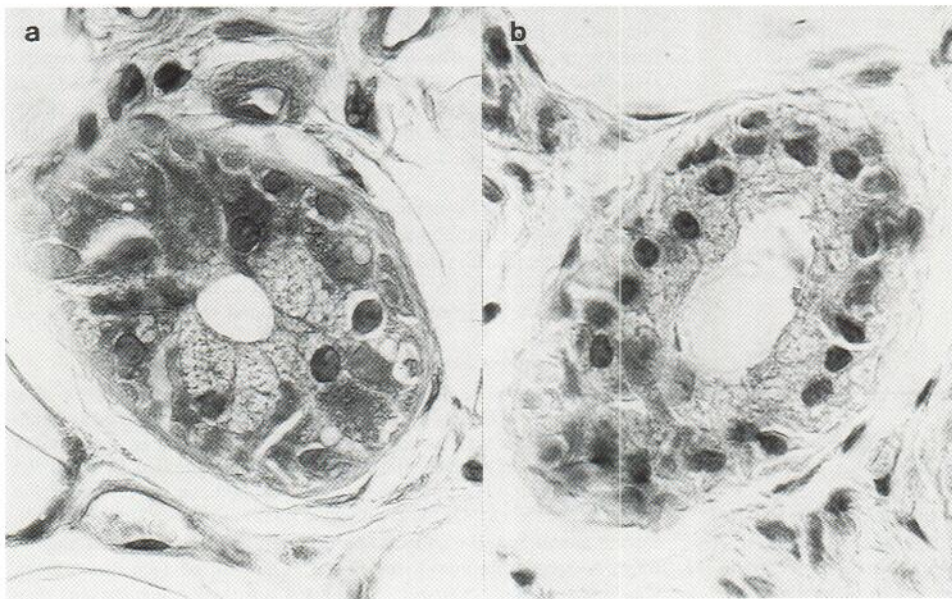


Fig. 4. Immunohistochemical demonstration of cells that expressed a secretory component (SC). In a high sIgA secretor, two thirds of the glandular cells of the eccrine sweat gland densely expressed SC within the cytoplasm (a), whereas those of the low sIgA secretor only did so occasionally (b) (× 860).

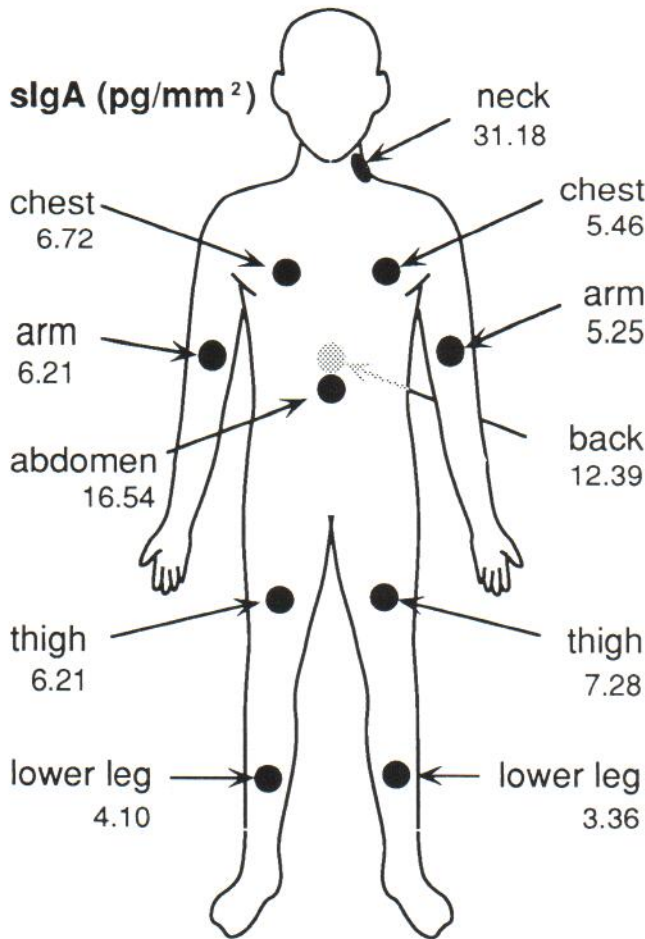


Fig. 5. Schematic representation of regional differences in secretion of sIgA (pg/mm²/day) in a healthy right-handed adult male. Right-handed subjects tended to secrete a greater amount of sIgA on the right side of their bodies.

sweat gland in the tissue sections, we could not infer any relationship between plasma cells and the amount of sIgA secreted.

Regional differences

The amount of sIgA excreted (per mm²/day) differed with skin region (Fig. 5). We also found that right-handed subjects tended to excrete a larger amount of sIgA on the right side of the body. This led us to measure the surface sIgA by applying the disk to the inner surface of the upper arm on the non-dominant side. This region (which showed a moderate amount of sIgA among the skin sites tested) is frequently used for performing patch tests, as it is usually protected from exogenous insults and is usually free of lesions, even in patients with atopic dermatitis (10).

Sweating experiments

The application of heat to the inner surface of the upper arm increased sweat output 3-fold. However, in this experiment, the amount of sIgA absorbed to the disk decreased, rather than increased, with an increase in sweating (Fig. 6). A similar discrepancy was observed during the sweating stimulated by

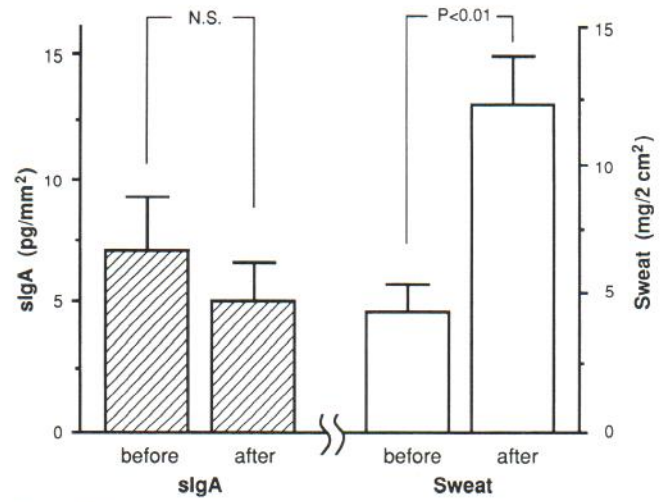


Fig. 6. Effect of heat on sweat production and sIgA secretion. Application of a heat pad produced an increase of nearly 300% in local output of sweat, but not in the amount of sIgA, which did not differ significantly in the stimulated vs. unstimulated conditions.

exercise. Even when the amount of sweat excretion increased 15-fold, the amount of sIgA increased non-significantly (Fig. 7).

During exercise, the disk, which was fixed to the skin with adhesive tape, sometimes moved slightly. While this artificially increased the number of dots on the disk, it produced little difference in the amount of sIgA absorbed per unit skin area, since the latter was the product of the number and the density of the dots per specified area of the disk.

DISCUSSION

We have shown that the amount of sIgA excreted on a specific area of skin could be determined by: 1) collecting it on an absorbent disk; 2) measuring it using an enzyme immunoassay; and 3) calculating the amount of sIgA as the product of the number and density of dots on the disk. Since the dots corresponded to the eccrine ducts open to the skin surface, we could

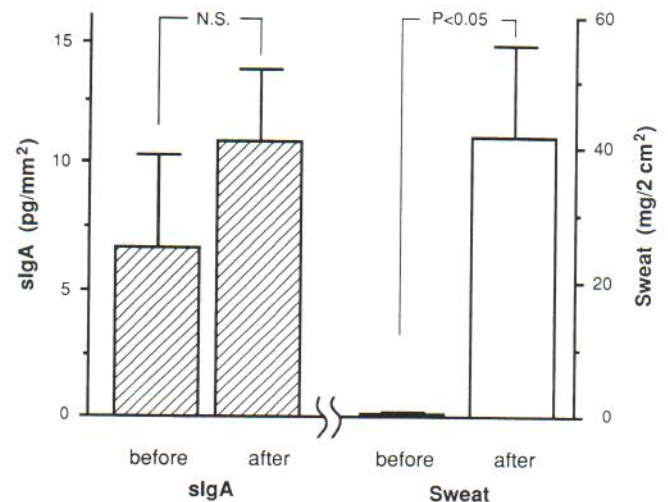


Fig. 7. Effect of exercise on sweat production and sIgA secretion. While exercise increased sweat production almost 15-fold, sIgA secretion did not differ significantly in the stimulated vs. unstimulated conditions.

also evaluate the sIgA-secreting function of the individual eccrine sweat glands *in vivo*. It was thus possible to identify subjects whose disks contained little sIgA, i.e. who secreted little sIgA to the skin surface. Although there are regional differences in their pattern and density, the eccrine excretory ducts are distributed relatively evenly on the surface of the skin. The number of eccrine ducts present on the inner surface of the upper arm is normally 1.17 to 2.03/mm² (6).

Using our method, we demonstrated that the amount of skin sIgA varied widely among healthy individuals, and further, that the sIgA excretion in each subject differed with body region, approximating the density of the distribution of the eccrine sweat ducts on the skin surface (6). A higher concentration of sIgA has been reported in the saliva of males (11). A similar difference has been shown for the IgA level in plasma (12). In the present study, however, we found no such difference, and there was no relationship between the sIgA level in sweat and the plasma levels of immunoglobulins, including IgA, IgG, IgM and IgE.

Immunohistological studies revealed that the difference in the number of eccrine glandular cells that expressed SC was consistent with that in the amount of sIgA excreted to the skin. This finding accounts for the fact that the endocytosis of IgA is proportional to the number of cell-surface SC. It is known that sIgA, which is derived primarily from plasma cells beneath the epithelium, is transported by epithelial cells via the SC into external secretions (13). The relatively constant excretion of sIgA, regardless of sweating, may be explained by the fact that only a limited number of glandular cells express SC, thus providing a limited capacity for transcytosis of IgA into the lumen. Since sweat production is regulated by the sympathetic and parasympathetic nervous systems so as to maintain the body temperature (14), the excretion of sIgA appeared to be independent of sweating.

These findings appear to support the hypothesis that sIgA on the skin surface may be involved in the first line of defense on the skin (5). Such independence of sweat production would be necessary to prevent invasion by foreign substances, while sharing the same secretory organ distributed over the body surface. We recently reported a reduced excretion of sIgA to the skin surface of patients with atopic dermatitis (15). This finding may support the possible functional role of sIgA in sweat, since patients with atopic dermatitis frequently develop bacterial and/or viral infections of the skin (16, 17). Also, the skin of such patients is usually colonized by bacteria, typically *Staphylococcus aureus* (18, 19). Nevertheless, the clinical significance of IgA remains controversial, since most patients with a selective IgA deficiency are asymptomatic (20). The presence, or lack of, clinical manifestations in such patients may be explained by a compensatory increase in other immunoglobulins (21). Most subjects with IgA deficiency show an increase in IgD- and IgM-secreting plasma cells in the nasal mucosa. The absence of this compensatory mechanism has been associated with frequent respiratory infections (22).

In a previous study using immuno-scanning electron microscopy, we showed that the cells that compose the wall of the eccrine sweat duct constitutionally express class II MHC molecules on their surfaces (23). Considering the proclivity of the

immunoglobulins and class II MHC molecules to bind to a variety of antigens (24), these substances may provide a first line of defense on the skin and enhance the immune reaction against foreign invaders.

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