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Development of the immune system in children

Goro Mogi, Hideyuki Kawauchi and Yuichi Kurono, Oita, Japan

INTRODUCTION

Immaturity of the immune defense system is an underlying factor in infection-proneness, particularly in respiratory tracts. To clarify the development of systemic and local mucosal immunity, we conducted three experiments.

1. Since streptococcal cell wall (SCW) probably stimulates helper T cells in collaboration with macrophages, proliferative response of peripheral blood leukocytes (PBLs) of young ENT patients to SCW, was investigated.
2. We observed immunoglobulin forming cells of different classes in the nasal mucosa and tissue specimens of other organs of developing guinea pigs to make clear the development of local mucosal immunity.
3. Secretory IgA and serum type IgA, respectively, were measured in nasal secretions and the antibody activity of secretory IgA to SCW was investigated.

MATERIALS AND METHODS

I PROLIFERATIVE RESPONSES OF PERIPHERAL BLOOD LEUKOCYTES TO SCW

Separation of T cell subsets and macrophages

Peripheral blood cells were obtained from 48 patients, aged between 1 and 16, who visited our clinic. Patients with acute infectious disease were excluded. Mononuclear cells were separated from heparinized blood diluted with phosphate buffer saline (PBS) by centrifugation over Ficoll-Hypaque (Pharmacia, fine Chemicals, Uppsala, Sweden). One $\times 10^7$ peripheral blood Leukocytes (PBLs) were incubated for one hour at 37 °C in a humidified atmosphere at 5% CO_2 in air in a 35 mm diameter plastic dish containing 2 ml of RPM1-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat inactivated human AB blood type serum, 100 U/ml penicillin and 100 µg/ml streptomycin. After the incubation, non-adherent cells were gently removed from dishes by aspiration with a Pasteur pipette, followed by three more gentle washes. Adherent cells were removed by a rubber-policeman. These adherent cells were used as macrophages. A T cell enriched fraction was prepared by passing non-adherent cells through a

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nylon wool column. These cells were treated with anti-HLA DR antibody and rabbit complement to prevent contamination with B cells and macrophages. Separation of T cell subsets was carried out using anti-Leu 2a and anti-Leu 3a monoclonal antibodies and goat anti-mouse IgG antibody according to the method of Wysocki and Sato (1978).

Preparation of cell wall fraction of streptococcus pyogenes

SCW was kindly donated by Mr. Sugawara, Research Lab., of Chugai Pharmaceutical Co., Tokyo. Briefly, *streptococcus pyogenes* (Type III) *(S. Pyogenes)* were cultured in Todd-Hewitt medium at 37 °C for an appropriate period of time. The cells were harvested and washed three times in distilled water. The cells were suspended in PBS at a concentration of 25 mg (dry weight) /ml and disrupted by agitation with glass beads (0.10–0.11 ⌀) in Braun cell homogenizer at 4000 rpm for 6 minutes. The disrupted cells were digested with RNase (0.01 mg/ml) and DNase (0.01 mg/ml) at 37 °C for 2 hours. The suspension was centrifuged at 450 g for 10 minutes, and the supernatant was taken and centrifuged at 10000 g for 30 minutes. The precipitate was washed with PBS by centrifugation 4 times. This sample was digested with pronase E (0.6 mg/ml) at 37 °C for 24 hours, applied to a sucrose density gradient, and centrifuged at 1700 g for 24 minutes. A pooled cell fraction was washed in PBS by centrifugation at 10000 g, 4 times, of 30 minutes each. Finally, the pellet was filtered through a 0.45 μm millipore membrane, lyophilized and stored at 4 °C until use.

Proliferative response of PBLs to SCW

Proliferative response of PBLs to SCW was investigated using microculture method. Each culture well contained 2×10^5 PBLs and SCW (at various concentrations to determine the optimal SCW dose) in 0.2 ml of the same culture medium as used in the cell preparation. After six days incubation, tritiated thymidine (1 μCi/well) was added to the culture well for 18 hours. This assay was carried out quadruplicately. The stimulation index presents the ratio of uptake of ^{3}H-thymidine in SCW-containing well to that in SCW-free well.

II IMMUNOGLOBULIN FORMING CELLS IN THE NASAL MUCOSA OF DEVELOPING GUINEA PIGS

Forty-four healthy guinea pigs (6 to 10 months old) and 30 developing guinea pigs (under 1 month old) were used. Blood was drained from two developing guinea pigs. Rabbit antisera to guinea pig IgG_1, IgG_2, IgM, IgA, secretory component (SC), and lactoferrin (Lf) were made as described elsewhere (Mogi et al., 1980). These antisera were conjugated with fluorescein isothiocyanate (FITC) and employed for direct immunofluorescence study.

Two of the developing guinea pigs were killed immediately after birth and 2 each on postnatal days 1, 3, 5, 7, 9, 12, 15, 17, and 27. Five normal adult guinea pigs were

also killed. Nasal mucosae of the turbinates and septum were obtained from each of the 20 developing, and 5 normal adult, animals. A tissue specimen was also taken from the Eustachian tube, tympanic bulla, larynx, trachea, spleen, small intestine, and mesenteric lymph nodes. Specimens removed were fixed in cold ethanol (95%) and submitted to immunofluorescent observation.

III SECRETORY IgA AND SERUM TYPE IgA IN NASAL SECRETIONS

Nasal secretions were aspirated by Juhn tap (Xomed, Florida) from the nostrils of 57 normal control persons (14 were under 15 years old and 43 over 16 years old) and 55 patients with chronic sinusitis (18 were under 15 years old and 37 over 16 years old) and 53 patients with nasal allergy (38 were under 15 years old and 15 over 15 years old).

Levels of secretory IgA and serum type IgA in nasal secretions and of IgA in sera

Electroimmunodiffusion techniques (EID), elaborated by Tsukuda (1981) for analyses of secretory IgA, serum type IgA and SC, were adopted. Briefly, for analysis of secretory IgA, after completion of EID in an agarose gel plate containing rabbit anti-SC antibodies, the gel plate was thoroughly washed with PBS and treated with a solution of goat anti-IgA conjugated with horseradish peroxidase (HRP). The gel plate was again washed and stained for HRP. For analysis of serum-type IgA, two equal-sized gel layers were made on a glass plate. The gel on the cathodal side contained anti-SC antibodies, while the gel on the anodal side had anti-IgA antibodies. After the electrophoresis, the gels were washed, dried and stained with Coomassie brillant blue.

Secretory IgA and total IgA antibodies to SCW

To investigate this antibody activity in nasal secretions, an indirect enzyme-linked immunosorbent assay (ELISA) was employed. Each well of polystyrene microtiter plate was coated with SCW and then nasal secretion samples diluted 5 to 7 times with PBS were applied. After incubation and washing rabbit anti-SC antibodies conjugated with HRP were added. Incubation and wash were done, and o-phenylenendiamine (as substrate) solution was put into the wells and read by a spectrophotometer at 490 nm. For measurement of total IgA (secretory IgA + serum type IgA), wells coated by SCW were applied to samples, incubated, washed and then rabbit anti-IgA antibodies conjugated with HRP were added.

RESULTS

I PROLIFARETIVE RESPONSES OF PBLs TO SCW

Control studies

Since the highest proliferative response of PBLs to SCW was obtained on the 7th day of incubation, the culture was performed for 7 days in this experiment. The

optimal dose of SCW was 1 μg/well: thereafter, this dose was adopted for the experiment. PBLs were obtained from healthy adult subjects selected at random. Stimulation indexes of these PBLs were between 2.7 and 9.19 (Table 1). This finding categorized the subjects into two groups, high responder and low responder.

Table 1. Proliferative response to SCW of PBLs of healthy adult control.

responder cells		uptake of ^{3}H-thymidine (cpm) SCW		
initial	age	+	−	stimulation index
S.Y.	25	2554 ± 312	944 ± 55	2.70
K.Y.	26	14805 ± 2464	2944 ± 576	5.03
K.W.	30	13165 ± 1911	2661 ± 321	4.94
T.Y.	35	4561 ± 881	701 ± 54	6.51
M.W.	24	3263 ± 574	1071 ± 328	3.04
H.K.	29	19503 ± 1357	2126 ± 204	9.19
H.Y.	25	10311 ± 895	1470 ± 274	7.01

SCW: Streptococcal cell wall.
PBLs: Peripheral blood leukocytes.

As the PBLs sample of the subject HK showed the highest response, we used his PBLs as control in every experiment.

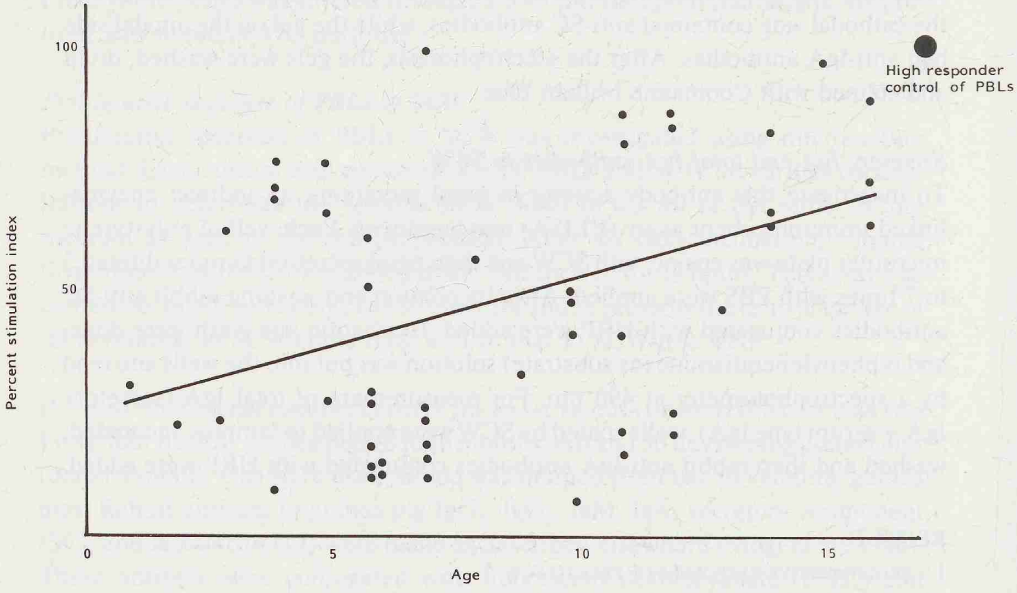

Figure 1. The change of PBLs response to SCW with aging.
PBL: Peripheral blood leukocytes.
SCW: Streptococcal cell wall.

The change of PBLs response to SCW with aging
As shown in Figure 1, there is a statistically significant correlation ($r = 0.655$) between proliferative response of PBLs to SCW and aging in young patients.

Requirement of macrophages for the proliferative response to SCW
Neither T cells nor macrophages, alone, did not respond to SCW. However, T cells responded in the presence of macrophages, as did PBLs (Table 2).

Table 2. Proliferative response of T cells and M to SCW.

	uptake of ^{3}H-thymidine (cpm)		
responder cells	SCW (+)	SCW (−)	Δcpm*
PBLs (1×10^5)*	32373	7037	25300
T cells (7×10^4)	4726	2823	1903
macrophages (7×10^3)	3338	2935	403
T cells + macrophages	20553	6209	14344

SCW: Streptococcal cell wall.
M: Macrophages.
* Cells were cultured with or without 1 μg of SCW antigen for six days and incubated with 2 μCi of ^{3}H-thymidine for 16 hours.
** Δcpm was calculated by subtracting from the SCW (+)(cpm) the SCW (−)(cpm).

Proliferative response of T cell subsets to SCW
Figure 2 exhibits the results. Neither Leu-2a positive T cells nor Leu-3a negative T cells responded to SCW in the presence of macrophages, while responses of Leu-2a negative T cells and Leu-3a positive T cells were high. Since Leu-2a negative and Leu-3a positive T cells are regarded as helper T cells, this result indicates that SCW antigen stimulates helper T cells in collaboration with macrophages.

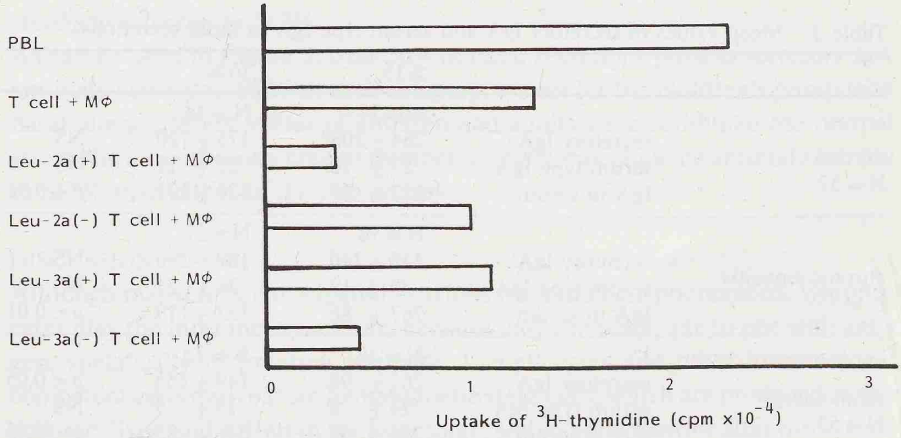

Figure 2. Proliferative response of T cell subsets to SCW.
SCW: Streptococcal cell wall.

II IMMUNOGLOBULIN FORMING CELLS IN THE NASAL MUCOSA OF DEVELOPING GUINEA PIGS

A very few IgA forming cells began to appear in the mucous membrane of the nose and intestine, and mesenteric lymph nodes 5 days postnatally. In the mucosa of Eustachian tubes, larynges, and tracheae, and in the spleens, a few IgA forming cells were first seen between 7 to 12 days postnatally. In normal adult animals, IgA forming cells were abundant in the subepithelial layer and interstitial connective tissue of upper respiratory tracts and intestine, except in the tympanic bulla and in the bony portion of the tube. Peripheral lymphoid organs contained IgA forming cells. There were few IgG_1 and IgG_2 forming in the nasal mucosa and in the rest of the respiratory tract of developing animals. In normal adult guinea pigs, however, both IgG_1 and IgG_2 forming cells were occasionally seen in the mucous membrane of the nose, in other parts of the respiratory tract and in the small intestine. The number of these forming cells was less than that of IgA forming cells. A very few IgM forming cells existed in the mucous membrane of the nose, larynx, trachea and intestine of 5 to 7-day-postnatal guinea pigs. SC and Lf were detected in glandular acinar cells negative for PAS-AB staining of the nasal mucosa, eustachian tube, and larynx of all postnatal and normal adult guinea pigs. The serum IgG_2 levels fell rapidly after postnatal day 7 and began to rise at postnatal day 23, while IgA and IgM levels, which were undetectable at birth and postnatal day 1, gradually increased with each day.

III SECRETORY IgA AND SERUM TYPE IgA IN NASAL SECRETIONS

Levels of secretory IgA and serum type IgA in nasal secretions and of IgA in serum

Results are described in Table 3. There was no statistically significant difference of secretory IgA and serum type IgA in nasal secretions between children and adults in groups of normal subjects, patients with chronic sinusitis, and patients

Table 3. Mean values of secretory IgA and serum type IgA in nasal secretions.

		≦15	16≦	
normal N = 57		N = 14	N = 43	
	secretory IgA	264 ± 208*	175 ± 120	NS
	serum type IgA	27 ± 10	22 ± 11	NS
	IgA in serum	222 ± 89	320 ± 103	$p < 0.01$
chronic sinusitis N = 55		N = 18	N = 37	
	secretory IgA	428 ± 240	189 ± 290	NS
	serum type IgA	29 ± 19	36 ± 21	NS
	IgA in serum	267 ± 86	353 ± 119	$p < 0.01$
nasal allergy N = 53		N = 38	N = 15	
	secretory IgA	261 ± 98	149 ± 155	$p < 0.05$
	serum type IgA	24 ± 8	19 ± 9	NS
	IgA in serum	285 ± 98	346 ± 83	$p < 0.05$

* Mean + SD mg/100 ml.
NS = no significance.

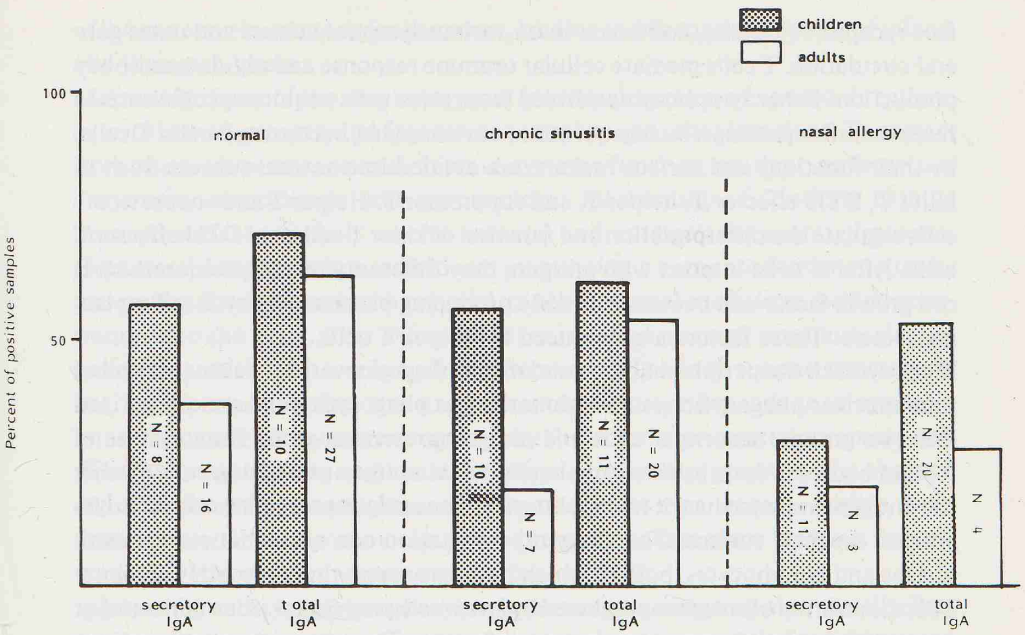

Figure 3. Antibody activity to SCW of secretory IgA and total IgA in nasal secretions. SCW: Streptococcal cell wall.

with nasal allergy. When values of secretory IgA and serum type IgA concentrations of children and adults were calculated together, mean levels of secretory IgA and serum type IgA of patients with chronic sinusitis exceeded significantly those of normal subjects, and patients with nasal allergy.

Antibody activities to SCW

As can be seen in Figure 3, over 50% of nasal secretions possess secretory IgA antibody activity to SCW in each category, except for the children's group with nasal allergy. When values of children and adults were combined the normal group had a significantly greater number of secretions, showing antibody activity to SCW, than either of the two groups.

DISCUSSION

Although many cells participate in various immunologic phenomena, lymphocytes play the most important role, because they can react, one to one with antigen, specifically in immune response. Lymphocytes and other immunologic competent cells derive from hematopoetic stem cells, which are produced in the yolk sac, liver, and spleen in the fetal stage, and in bone marrow after birth. Certain cell population of lymphocyte precursors derived from stem cells migrate to the thymus, differentiate to immunologically committed cells equipped with sur-

face receptors (T cells), and home-in on various lymphoid tissue and enter general circulation. T cells mediate cellular immune response and regulate antibody production. Other lymphocytes derived from stem cells acquire specific surface receptors after passing a bursa equivalent environment, becoming B cells. T cells, by their functions and surface makers, are divided into several subsets, such as killer T, DTH effector T, helper T, and suppressor T. Helper T and suppressor T cells regulate the differentiation and function of killer T cells and DTH effector T cells. After B cells interact with antigen, they differentiate and proliferate by B cell growth factor and become antibody producing plasma cells by T cell replacing factors. These factors are produced by helper T cells.

Monocytes in the peripheral blood and macrophages in various tissues are called mononuclear phagocytic system. Mononuclear phagocytic cells are categorized into two groups: scavenger cells and antigen presenting cells. Phagocytosis of foreign bodies is done by the former cells, while antigen presenting cells modify the antigen and introduce it to lymphocytes. The antigen presenting cells have IgA antigen on their surface. The antigen presentation can occur between macrophages and lymphocytes, both of which have to possess the same MHC antigen. There is an array of antigens possessed by *streptococcus pyogenes,* one of the major causative agents of upper respiratory infection. The most important antigen appears to be M protein that is one of the cell wall antigens. The M protein is essential for virulence, participating in its adherence to mucosal surface, thereby fostering its colonization. In the present study, we isolated SCW containing the M antigen, and investigated the antigen-specific T cell proliferative response of human peripheral lymphocytes *in vitro* against SCW. Since the results showed that T cells responded remarkably in the presence of macrophages, the immune response to SCW requires antigen-presenting macrophages.

Results of this study also found a high proliferative response to SCW of Leu 2a negative and Leu 3a positive T cells, indicating that SCW antigen stimulates helper T cells, since Leu 2a negative and Leu 3a positive T cells are regarded as helper T cells. Even though the number of normal adult subjects tested was small in this study, it is suggested that there are two groups: high responder and low responder to SCW antigen (Table 1). Sasatsuki et al. (1980) demonstrated that the low response of human T cell to SCW antigen is controlled by a single dominant gene which was closely linked to HLA.

The SCW antigen specific T cell proliferation varied somewhat in individual young patients. However, there is a statistically significant correlation between the proliferative response of T lymphocytes and aging. The antigen specific immunological defense system of individuals develops by repeating antigenic stimuli, even if there may be some genetic restriction. Therefore, our findings imply that low proliferative response of helper T cells, which act on differentation and proliferation of B cells to antibody producing cells, may cause the immaturity

of the systemic immune defense system, leading to infection-proneness of infants and young children.

Mucosal surfaces of the respiratory and intestinal tracts are exposed directly to external environments, and face various antigens and other invasions. To protect from these invasions, several defense systems function on their surfaces, i.e. enzymatic, mucociliary, and immunologic mechanisms. Secretions bathing mucosal surfaces contain several antiviral and antibacterial agents. Immunoglobulins, particularly IgA, provide an immunologic barrier to foreign matter by preventing absorption of such material into the mucosal epithelium, and its penetration into the body. This defense system is called mucosal immunity. IgA in secretions, called secretory IgA, differs from serum IgA in its subunit structure. It has been postulated that the mucosal immunity is independent of systemic immunity, since antigens encountered by the enteric, or respiratory, route can stimulate antibody response in the intestinal or respiratory tracts in the absence of an apparent systemic immune response. However, recent studies have demonstrated that important regulatory interactions take place between the systemic and mucosal immune systems (Strober, 1982).

IgA bearing cells, IgA B lymphocytes, become IgA-producing plasma cells. Precursors of IgA cells are abundant in special lymphoid tissue, called gut-associated lymphoid tissue (GALT) and/or bronchus-associated lymphoid tissue (BALT), while other peripheral lymphoid tissue contains a low percentage of IgA bearing cells. After sensitization with antigen in the GALT or BALT, precursors of IgA-producing cells enter circulation through the thoracic duct and eventually settle in mucosal tissues of the antigen-stimulated organ, as well as in distant secretory sites. The presence of IgA producing cells in distant mucosal tissues is considered evidence of a common mucosal immune system. In the present study we observed the appearance of immunoglobulin producing cells in the nasal mucosa and in other organs. IgA forming cells are the most predominant plasma cells in the mucosa of the intestinal and respiratory tracts. IgA forming cells began to appear between 5 and 15 days postnatally in the mucosa of the nose, larynx, trachea, and intestine of conventionally growing guinea pigs. The number of such cells increased and reached the adult level one month after birth. IgA forming cells were extremely rare in the tympanic mucosa of both developing and adult animals, even though antigenic challange to the tympanic cavity induced abundant IgA forming cells in its mucosa. This evidence may be attributed to the fact that, anatomically, the middle ear has less frequent opportunity to undergo antigenic stimulation than other areas of the upper respiratory, or alimentary canals. Antigenic stimulation accelerates the development of the IgA system. However, the exact reason for the slow maturation of the IgA system is still unclear. Our results suggest that the immaturity of the mucosal immunity is also a factor in the infection-proneness of infants and young children.

Although secretory IgA is the main immunoglobulin in external secretions, a certain amount of serum IgA is also present. It has not been reported that secretory IgA and serum type IgA are measured, respectively, in the same material sample. Tsukuda (1981) elaborated an EID technique which makes it possible to analyse quantitatively secretory IgA, serum type IgA, and SC, respectively. We adopted this technique and measured secretory IgA and serum type IgA in nasal secretions. Results failed to show a significant difference of values between children and adults of normal and patient groups. However, when values of secretory IgA and serum type IgA of children and adults were calculated together, mean values of secretory IgA and serum type IgA of patients with chronic sinusitis were significantly greater than those of normal subjects and patients with nasal allergy.

It is well known that secretory IgA plays an immunologic role in protection against certain viral diseases. However, little is known about the mode of action of secretory IgA in the protection from bacterial infection. The present investigation found secretory IgA antibody activity against the M protein of *S. pyogenes*. It is noteworthy that the percentage of nasal secretions, which possess the activity, was higher in normal subjects than in patients with chronic sinusitis and nasal allergy, even though the mean value of secretory IgA in patients with chronic sinusitis was greater than that in the normal subjects. This evidence suggests that normal nasal mucosae have a greater mucosal immunity preventing adherence of bacteria than do diseased mucosae.

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Goro Mogi, M.D.
Professor and chairman
Dept. of Otolaryngology
Medical College of Oita
1-1506 Idaigaoka
Hazama-cho
Oita 879-56, Japan