1. INTRODUCTION

Recent developments in biochemical analysis have revealed a close relationship between mental states of humans and secretory substances such as hormones and immunological substances [1]. For example, Jemmott III et al. reported low levels of an immune substance in subjects under chronic stress, which indicates a reduction in biophysical ability against some infectious disease [2]. This behavioral medicine study is a new methodology to investigate a relationship between human body and mind. Presently, these interdisciplinary fields of study are called psychoneuroendocrinology (PNE) or psychoneuroimmunology (PNI) [1] (hereafter, we use the term psychoneuroendocrine-immunology (PNEI) to indicate both PNE and PNI).

Previous PNEI studies focused particularly on mental stress in humans. Generally, mental stress is known to be a predisposition to various somatic disorders particularly with regard to the cardiovascular system. The studies focusing on mental stress have frequently been performed using statistical analyses of the relationship between the scores of questionnaires and behaviors. In contrast, a PNEI study is an attempt to evaluate mental stress by assessing secretory biomarkers. Therefore, it will help in obtaining an objective assessment of human stress. It is considered a valuable new methodology in broad fields of study that include not only psychophysiology or medical science but also ergonomics or Kansei engineering. For example, Deguchi et al. and Nakane et al. respectively introduced the use of salivary α-amylase and chromogranin A (CgA) for evaluating the mental workload experienced by drivers [3, 4].

However, PNEI is a relatively new field of study; therefore, there are numerous issues that require clarification: (1) most studies focus on temporal changes in the levels of a target substance (biomarker) introduced by experimentally controlled short-term stressor such as a 30 min calculation task; such studies have reported a transient increase in the levels of biomarkers. In contrast, changes in the levels of these biomarkers against long-lasting chronic stress or daily stress are not well understood [5]. (2) Several candidates for such biomarkers (or stress markers) have been found in previous PNEI studies [6]. However, the relationship or interaction between these biomarkers is little understood; this is because these biomarkers have frequently been assayed individually in previous studies.

Considering the 2 abovementioned points, we investigated the effects of chronic stress on changes in human physiology by assessing 4 biomarkers as described later in the text. Subsequently, we will describe the biological functions and properties of the 4 biomarkers introduced in this study.

2. POSSIBLE STRESS MARKERS AND THEIR BIOLOGICAL BACKGROUND

Several possible stress markers were found in previous PNEI studies. Almost all of these substances are secreted in various human secretory fluids such as serum, urine, breast milk, and saliva. The biomarkers detectable in saliva, in particular, are expected to be valuable stress markers; this is because saliva can be collected noninvasively in a less painful and stressful manner, unlike blood and/or
urine. In this study, we also focused on the biomarkers secreted by salivary glands, i.e., immunoglobulin A (IgA), cortisol, dehydroepiandosterone (DHEA), and CgA. The functions and properties of these biomarkers as biological substances and stress markers are described below.

### 2.1 IgA

IgA is one of the most important immune substances in the human immune system. It is present in the mucus of the mouth, respiratory tract, and intestinal tract. The IgA antibodies function nonspecifically by preventing bacteria from forming colonies, neutralizing toxins, and inhibiting the penetration of pathogenic viruses into epithelial cells. Thus, IgA, particularly salivary IgA, is frequently called the first line of defense against influenza or other upper respiratory tract infections (URTIs) [7].

On the other hand, with a psychological stress marker, IgA demonstrates a transient increase against short-term psychological stressors such as presentation and calculation task, while a decrease against long-lasting or chronic stress [5]. Tsujita et al. have referred to the 2 distinguishable responses of IgA in their review article: (1) an increase immediately after a short-term stress, which was termed as the “immediate stress effect” and (2) a decrease several days after stress, which was termed as the “delayed stress effect” [8]. However, the number of studies focusing on the latter effect remains small, and these studies have sometimes reported inconsistent results, while the former effect have been robustly observed [5].

### 2.2 Cortisol

The existence of 2 internal stress reaction pathways is known: (1) hypothalamic-pituitary-adrenal (HPA) axis and (2) sympatho-adrenal-medullary (SAM) system [9]. Cortisol, a steroid hormone released from the adrenal cortex, is considered to reflect HPA activity [10]. Salivary cortisol level also transiently increases against short-term stressors such as IgA; however, it is considered to respond via relatively strong and/or uncontrollable psychological stressors [9]. On the other hand, cortisol levels have been reported to increase in chronic stress or daily stressful events such as job strain [11], joblessness [12], and divorce [13]. In these studies, the diurnal change in cortisol levels, particularly the rapid increase during the first 30 min after waking up, termed as “morning cortisol”, has frequently been assumed to represent chronic stress. In contrast, a recent review article on cortisol indicated that the cortisol level was low in patients with chronic fatigue syndrome or other stress-related disorders [14].

### 2.3 DHEA

DHEA is also a steroid adrenal cortex hormone like cortisol. Thus it can be a biomarker for HPA axis. However, it is considered to function antagonistically with cortisol on the central nervous system and immune system [15]. Few studies have used DHEA to assess chronic stress; further, depression has been suggested to be associated with low DHEA levels [16, 17].

### 2.4 CgA

Because CgA is known to be released from the adrenal medulla into the blood with catecholamine, it is considered to be a possible biomarker of the SAM system [18]. Its level has been reported to transiently increase in response to short-term stressors such as a calculation test [4], white noise [19], and a cognitive test [20]. However, a recent study showed a transient increase in the CgA level after watching a comic video [21]. This study also indicated that the elevation in CgA levels was remarkable in the subjects who experienced lower daily stress.

### 2.5 Difficulty in performing PNEI studies on chronic stress

As described above, PNEI studies are categorized into 2 groups: one focuses on short-term stress, and the other on long-lasting chronic stress or daily stressful events. The results of these studies have shown transient increases in the levels of all biomarkers in short-term stress. Moreover, consistent results were obtained. However, the results of studies investigating the effect of chronic stress were less consistent or not well understood. Bosh et al. suggested in their review article on IgA that there were some methodological limitations for the studies focused on chronic stress such as (1) less control on subjects’ behaviors such as exercise, sleep, diet, alcoholism, and smoking, (2) difference in the timing of specimen collection, (3) difference in psychological stress measures, and (4) small number of subjects [5]. In addition, it can be easily assumed that the effect of chronic stress would depend on the duration of the stress. Thus, controlling the duration of chronic stress is preferable.

Considering the methodological difficulties described above, we investigated the effects of graduation examination on the biomarkers. In other words, we focused on the effect of relief from chronic stress. The graduation examination conducted in our institute, to which all subjects participating in this study belonged, is the final and the most difficult examination. It consists of an examination and a presentation (oral defense) of the graduation thesis. The presentation is considered equivalent to the final examination, and all undergraduate students begin preparing for it at least half a year before the presentation. Therefore, it is highly expected that most of the subjects are under chronic stress days before the presentation and relieved from the stress after the presentation. We then
assayed the salivary biomarkers before and after the day of presentation for the graduation examination. This experimental design could have an advantage in terms of controlling the psychological states under chronic stress. In addition, we assayed 4 salivary biomarkers to investigate the relationship and/or correlation among them.

3. METHODS

3.1 Subjects
The subjects were 20 healthy male undergraduate students (age, 21-27 years; \( \bar{AV} = 23.4, SD = 1.69 \) preparing for the graduation examination. It was confirmed that they did not have any health problem during the experimental period and that they were not being under any medication. They were instructed not to have any food and drink except for water, and exercise and smoke, for an hour before specimen collection.

This experiment was conducted in conformity with the Helsinki Declaration. All subjects were well informed regarding the purpose and contents of the experiment, and informed consent was obtained before their participation. They were also informed that they had the right to renounce their participation anytime.

3.2 Experiment
The saliva samples were collected approximately a week before and 2 weeks after the day of the presentation for the graduation examination (hereafter, the day of saliva collection before and after the presentation is denoted as day I and day II, respectively). It was taken by placing small cotton under the tongue for 3 min. Moreover, they were instructed to fill the profile of mood state (POMS) as the measure of psychological mood state after each saliva collection. POMS is a measure of 6 identified mood factors, i.e., tension-anxiety (T-A), depression-dejection (D), anger-hostility (A-H), vigor (V), fatigue (F), and confusion (C); it is commonly used for psychophysiological studies. All the experiments were conducted in the afternoon between 2 and 6 p.m. to minimize the effect of diurnal change in the biomarkers.

The small cotton containing saliva was centrifuged for 15 min at 1500 rpm for removing mucin or other impurities. Then it was stored in a freezing chamber at -20 °C before biochemical analysis. The levels of the biomarkers -IgA, cortisol, DHEA, and CgA- were determined by using the enzyme-linked immunosorbent assay (ELISA) (for IgA, cortisol, and DHEA: Salivary EIA Kit; Salimetrics, Co., Ltd., USA, and for CgA: YK070 Human Chromogranin A EIA kit; Yanaihara Institute Inc., JAPAN).

3.3 Statistics
We analyzed the changes in the mood factors of POMS and the levels of the biomarkers before and after the graduation examination by using the paired t-test. Also, the correlation coefficient was calculated among the concentration of biomarkers.

4. RESULTS

4.1 Physical condition and sleeping time
No subjects were suffering from any disease during any of the experiment days. The average sleeping time on experiment day I was 5.80 h (SD = 2.31) and that on day II, 6.83 h (SD = 1.43). The average sleeping time was shorter on day I than on day II. Nevertheless, there was no statistically significant difference in sleeping time on day I and day II. Considering diurnal changes in the levels of the biomarkers, the sleeping time could affect these levels. However, because there was no significant difference in sleeping time

![Figure 1: Scores of POMS on days I and II (error bars indicate the standard error)](image-url)
on days I and II, the effect of the sleeping time can be precluded from the analyses conducted in this study.

4.2 POMS scores (psychological states)
As expected before conducting this study, the psychological state clearly differed before and after the graduation examination as shown in Figure 1. The subjects marked higher scores on day I than day II for tension-anxiety (T-A) \((p<0.001)\), depression-dejection (D) \((p<0.01)\), anger-hostility (A-H) \((p<0.01)\), fatigue (F) \((p<0.001)\), and confusion (C) \((p<0.01)\). This result shows that the subjects were under strong stress before the presentation for the graduation examination.

4.3 The levels of biomarkers (physiological states)
Figure 2 (a)-(d) shows the concentration of each biomarker as determined by biochemical analysis. The average IgA concentration on day I was higher than that on day II \((I_{AV} = 182.0 \mu g/mL (I_{SD} = 55.4) \text{ and } II_{AV} = 134.1 \mu g/mL (II_{SD} = 66.6))\); the average cortisol level on day I was lower than that on day II \((I_{AV} = 0.204 \mu g/dL (I_{SD} = 0.154) \text{ and } II_{AV} = 0.273 \mu g/mL (II_{SD} = 0.168))\); the average DHEA level on day I was higher than that on day II \((I_{AV} = 331.7 \text{ pg/mL (I}_{SD} = 242.8) \text{ and } II_{AV} = 244.3 \text{ pg/mL (II}_{SD} = 225.6))\); and the average CgA level on day I was lower than that on day II \((I_{AV} = 5.89 \text{ pmol/mL (I}_{SD} = 2.70) \text{ and } II_{AV} = 6.04 \text{ pmol/mL (II}_{SD} = 4.00))\). Among these biomarkers, only IgA showed statistically significant difference in the levels before and after the examination \((p<0.001)\). With regard to the average values, only cortisol levels increased after the graduation examination.

On the other hand, Figure 3 shows the weight of the saliva of 3 min of specimen collection. This value is expected to represent the saliva flow rate in each subject. The average saliva flow rate on day I was lower than that on day II \((I_{AV} = 0.232 \text{ g/3min (I}_{SD} = 0.068) \text{ and } II_{AV} = 0.253 \text{ g/3min (II}_{SD} = 0.093))\); however, no significant difference was observed.

4.4 Correlation coefficient among biomarkers
With regard to the correlation among biomarkers, only DHEA-CgA showed a significant (positive) correlation as shown in Table 1. This result is rather intriguing assuming

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Figure 2: Concentration of salivary biomarkers on days I and II \((*** p<0.001, \text{ error bars indicate the standard error): (a) IgA, (b) cortisol, (c) DHEA, and (d) CgA, respectively.\)
that DHEA and CgA would be biomarkers reflecting different stress reaction pathways, i.e., HPA and SAM, respectively. Moreover, no significant correlation was observed between the saliva volume and the level of each biomarker as shown in Table 2. On the other hand, no significant correlation between IgA and saliva volume shows that the significant IgA change observed before and after the graduation examination cannot be responsible for the saliva flow.

Next, considering the effect of diurnal change in the levels of biomarkers, we calculated the correlation among the sleeping time, the time after waking, and the level of each biomarker. Nevertheless, no significant correlation was observed among them. Thus, the effect of diurnal change in biomarker levels could be less considerable in this experiment.

5. DISCUSSION

As expected, the result of assessing psychological mood states shows that the subjects were under greater stress before the presentation for the graduation examination than that after the examination. Also such a remarkable change of psychological states has occurred during the 2-3 weeks of this experiment days. All subjects had been engaged in preparations for the examination for at least half a year. Thus, the subjects were considered to be under chronic stress before the presentation. Therefore, the change in POMS as shown in our experiment could reflect a relief from the chronic stress. As already mentioned, controlling experimental conditions is fairly difficult in PNEI studies focusing on chronic or daily stress. Our experiment on chronic stress caused by graduation examination could be adequately controlled at this point.

With regard to the biomarkers, the IgA showed a significant decrease in concentration after the graduation examination. This result was inconsistent with the "delayed stress effect" reviewed by Tsujita [8]. However, to our knowledge, the number of IgA studies conducted on chronic stress is very small (about 10 studies until now). These studies have sometimes reported inconsistent results. Further, the results of some studies were not necessarily inconsistent with our results [22]. In addition, the methodologies followed in these studies were different, which could be a possible reason for inconsistent results. For example, Jemmott III et al. reported a decline in IgA levels during the academic examination period [2]. However, the baseline IgA data, which was used for comparing with IgA levels during the examination period, was obtained only 5 days before the examination. Therefore, if IgA levels were high under conditions of chronic stress as the current study showed, it is also possible that similar research is producing results which are consistent to this study. In either case, in order to discuss the possibility of assessing stress over the long-term, accumulating further basic information will be necessary in the future.

The levels of other biomarkers such as cortisol, DHEA, and CgA did not change significantly after the graduation examination. Nevertheless, the levels of cortisol and DHEA appeared to decrease and increase. As already mentioned, a recent review article on cortisol indicated that the cortisol level was low in patients with chronic fatigue syndrome or other stress-related disorders [14]. The relatively low cortisol level observed on day I might reflect a symptom accompanying the long-term preparation for the graduation examination. DHEA was indicated to act antagonistically with cortisol on the central nervous system and immune system [15]. Thus, the increase in DHEA levels observed in our experiment would reflect a decrease in cortisol levels. During the graduation examination, almost no change was observed in CgA. To our knowledge, no study has previously reported a relationship between CgA level and chronic stress, while several studies have reported a transient increase in CgA levels against an experimentally designed short-term stress such as a calculation task. Because such a task strongly acti-

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<th>Table 1: Correlation among biomarkers</th>
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<th>Table 2: Correlation between saliva and each biomarker</th>
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vates SAM system, CgA might be under strong control of SAM system.

Comparison and interpretation of the correlations among biomarkers observed in our study with those observed in other PNEI studies is difficult because no other study has assayed these biomarkers at the same time. The change in IgA levels can be suggested as the strongest candidate for chronic stress marker among these biomarkers. However, it is not necessary to assume that the other biomarkers could not function as chronic stress markers. In our experiment, each biomarker was assayed just before and after the graduation examination. Thus, the target period of our experiment was only 2-3 weeks. Therefore, if changes in the levels of biomarkers were considerably slower or faster than that of IgA, it is understandable that there were no marked differences between days I and II. Further experiments are required in this regard.

With regard to the other physiological indicators, the nervous system indicators, such as heart rate variability and peripheral blood pressure, have been frequently introduced for a number of past stress related studies. Generally, as nervous system indicators such as the autonomic nervous system are bioelectric signals, responsiveness toward the stress itself is quick (response initiation time). However, the effect of the body’s homeostasis is strong, and as a result, response is reflexive and the body quickly becomes accustomed. On the other hand, internal secretion indicators reflect internal “substance” changes, and while stress response (change in the production process) is gradual, it is possible that they may be suitable for stress assessment over a relatively long period of time.

On the other hand, the result of this study which is the remarkable decline of IgA just after accomplishing graduation examination gives us an interesting perspective. In our daily life, one might have an experience to have a cold or flu just after finishing some hard work rather than during being occupied with that work. Although this would not be an epidemiologically certain fact, the remarkable decline of secretory “immune” substance (IgA) just after finishing graduation examination which implies the attenuation of bodily defense at that moment might have some sort of connection with that experience. This perspective can be interesting and quite important in terms of management of mental health to maintain our bodily health. Thus our result could motivate a future epidemiological cohort study focusing on that point.

Finally, we have to note that there must be numerous mediators affecting the secretion of biomarkers, such as personality [23], and cognitive process [24]. For example, the subjects categorized in Type A, which is characterized by intense striving for achievement, competitiveness, urgency, and so on, showed a higher baseline for IgA [23]. Moreover, recent genetic research has revealed that the influence of life stress on depression differed in subjects having different types of a specific gene [25]. In addition, there are other biomarkers which have been studied in PNEI research, such as salivary α-amylase [26] and salivary free-3-methoxy-4-hydroxyphenylglycol (free-MHPG) [27]. These biomarkers were considered to respond to the activity of either the HPA axis or SAM system. Nevertheless, these substances have frequently been assessed individually. Therefore, a PNEI study that focuses on the possible mediators described above in which multiple factors are assessed should be conducted in future in order to gain a better understanding of the activities of and/or interaction between these 2 stress reaction pathways.

6. CONCLUSION

In this study, we investigated the effect of chronic stress on the physiological stress reaction pathway of humans using 4 salivary biomarkers. In conclusion, IgA was illustrated as the strongest candidate as chronic stress biomarker.

The “substance-based” stress detection has the highest scope for application in PNEI studies. More PNEI studies will provide reasonable criteria for psychological stress. Moreover, the results should be valuable for various field of study not only for stress management but also human error management, pleasant and un-pleasant evaluation, environmental health, functional food and flavor development, evaluation of therapeutic effect, complement medicine, or Kansei evaluation; however, it should not be thought that the change in human mental states is continuous in the time series and that the levels of these biomarkers could represent such a continuous change.

REFERENCES
EFFECT OF THE RELIEF FROM CHRONIC STRESS DURING GRADUATION EXAMINATION ON SALIVARY BIOMARKERS


