INDUCTION AND PROPERTIES OF CYTOPLASMIC FACTOR(S) WHICH ENHANCE NUCLEAR NONHISTONE PROTEIN PHOSPHORYLATION IN LYMPHOCYTES STIMULATED BY ANTI-Ig*

By YOSHIO NISHIZAWA, TADAMITSU KISHIMOTO, HITOSHI KIKUTANI, and YUICHI YAMAMURA

(From the Third Department of Internal Medicine, Osaka University Medical School, Fukushimaku, Osaka, Japan)

The receipt and translation of the signals responsible for the activation of B cells to immunoglobulin (Ig)-producing cells is largely a cell surface phenomenon and the specificity of B-cell response resides in cell surface-associated Ig molecules [see references (1) and (2)]. However, in many experiments the signals given through Ig receptors by anti-Ig antibodies have not been stimulative but often served as negative signals for differentiation of B cells to plasma cells unless they were accompanied or followed by some T-cell activities (3–6). A series of our previous experiments (7–9), in which a sequential stimulation of rabbit lymphocytes with anti-Ig antibody (anti-Ig) and T-cell-derived soluble products (SF)[1] induced IgG production, have suggested that the signals given through Ig receptors were essential for the activation of B cells.

Receptor-ligand interaction on the surface of lymphocytes induces several morphological and biochemical changes in lymphocytes, such as capping (10), movement (11), increased transport of amino acids (12), changes of Ca^{++} influx (13), and increase or decrease of intracellular level of cyclic nucleotides (14, 15). However, the molecular mechanism with which the signals given through Ig receptors are transduced across membranes so as to induce proliferation and/or differentiation of B cells is entirely unknown. So far many experiments have been done to explore the mechanism of the transmission of membrane-mediated signals. For example, the experiments done by Edelman and his collaborators (16) showed that microtubules were intimately involved in the movement of cell surface-associated receptors. The important role of the esterase activation in the induction of movements of B cells with anti-Ig was shown by Becker and Unanue (17). It should be noted, however, that the relation of the modulation of cell surface receptors or lymphocyte movements to B-cell activation is entirely unknown.

Recently, it has been shown in several experiments that nonhistone nuclear proteins have an important role in regulating the gene expression of eukaryotic cells (18). In lymphocytes, Johnson et al. (19) showed an increase of the amounts and phosphorylations of nonhistone nuclear proteins by stimulation with mitogens. Johnson and Hadden (20) also showed that cyclic GMP induced an increased phosphorylation of nonhistone proteins as well as proliferation of lymphocytes, suggesting that the phosphorylation of nonhistone nuclear proteins may be involved in the activation process of lymphocytes.

* Supported by a Grant-in-Aid for immunological research from the Ministry of Education, Science and Culture, Japan.

[1] *Abbreviations used in this paper:* ATP, adenosine triphosphate; NHP, nonhistone nuclear proteins; SF, T-cell-derived soluble products.

In our preceding paper (21), it was shown that the stimulation of rabbit lymphocytes with anti-Ig or SF induced an increase of amounts and phosphorylations of nonhistone nuclear proteins in nuclei. Furthermore, the addition of dibutyryl cyclic AMP showed the same biphasic effect on the changes of nonhistone nuclear proteins as well as on IgG production induced with anti-Ig and SF. The result suggested strongly that the changes of amounts or phosphorylations of nonhistone nuclear proteins were intimately involved in the differentiation processes of B cells. As the signals given on the surface of membranes induced the changes of nonhistone nuclear proteins in nuclei, it will be reasonable to suppose that there might be some substances in the cytoplasm of anti-Ig-stimulated cells which are responsible for the transmission of membrane-mediated signals to nuclei. In the present experiment, we have found that cell extracts from anti-Ig-stimulated cells induced an increased phosphorylation of nonhistone nuclear proteins in the quiescent nuclei isolated from nonstimulated cells. A partial characterization of the active substance, which may be a possible candidate for the signal transmission from membranes to nuclei, has been performed.

Materials and Methods

Reagents and Antibody. Magnesium chloride ($MgCl_2 \cdot 6H_2O$), calcium chloride ($CaCl_2 \cdot 12H_2O$), sucrose, ammonium sulfate, Triton X-100, and diphenylamine were obtained from Wako Pharmaceutical Co., Osaka, Japan. DNA, histone from calf thymus, trypsin from bovine pancreas, soybean trypsin inhibitor, and adenosine triphosphate (ATP) were purchased from Sigma Chemical Co., St. Louis, Mo. The purified goat antibody against rabbit IgG (anti-Ig) was the same preparation as that used in our previous experiment (21).

Stimulation of Lymphocytes with Anti-Ig. Lymphocytes were obtained from mesenteric lymph nodes of a normal rabbit. After washing lymphocytes three times with Hanks' balanced salt solution, 100 million nucleated cells were suspended in 5 ml of Eagle's minimum essential medium for suspension culture (Microbiological Associates, Bethesda, Md.) supplemented with 20% fetal calf serum (Microbiological Associates) and 100 U/ml each of a mixture of penicillin and streptomycin. Cells were cultured with or without 25 μg/ml of anti-Ig for appropriate periods of time in plastic culture dishes (60 × 15 mm; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 37°C.

Preparation of Lymphocyte Nuclei and Isolation of Nuclear Proteins. Preparation of lymphocyte nuclei and differential extractions of nuclear proteins were done according to the method of Levy et al. (22) and Teng et al. (23), respectively. The details of the methods were described in our previous article (21).

Preparation of Cell-Free Extracts from Lymphocytes. 100 million nucleated cells in 5 ml of culture medium were stimulated with 25 μg/ml of anti-Ig. After 2 h incubation at 37°C, cells were washed three times with cold saline and resuspended in 1 ml of cold 10 mM Tris-HCl buffer, pH 8.0. After 10 min, cells were homogenized with 60 strokes in a tight fitting Dounce homogenizer and ultracentrifuged at 150,000 *g* for 60 min at 4°C. The supernate was recovered and adjusted at a final concentration of 0.1 M Tris-HCl, pH 8.0, containing 0.25 M sucrose, 0.025 M NaCl, and 0.01 M $MgCl_2$. The supernate was free from cell membranes or nuclear contamination as judged by phase-contrast microscopy and used as cell-free extracts.

Activation of Normal Nuclei with Cell-Free Extracts. Normal nuclei were isolated from nonstimulated cells and washed three times with the reaction buffer (0.25 M sucrose containing 0.01 M $MgCl_2$, 0.025 M NaCl, and 0.1 M Tris-HCl buffer, pH 8.0) to remove detergents. Nuclear pellets were resuspended in 1 ml of the reaction buffer. 1 ml of nuclear suspension containing 10^8 nuclei was mixed with 1 ml of the cell-free extracts of anti-Ig-stimulated cells and incubated for appropriate periods of time at 37°C. After incubation, nuclei were centrifuged at 900 *g* for 10 min at 4°C and washed with the reaction buffer to remove the cell-free extracts. Activated nuclei were used for in vitro phosphorylation. For the activation of 100 × 10^6 nuclei, the cell-free extracts

obtained from 10×10^6 cells were used, since this ratio of nuclei to extracts was optimum for the activation of nuclei.

In Vitro Phosphorylation of Nuclei. In vitro phosphorylation of nuclei was performed by the method of Rickwood et al. (24). 100 million nuclei suspended in 1 ml of 0.25 M sucrose, 0.01 M $MgCl_2$, 0.025 M NaCl, 0.1 M Tris-HCl buffer, pH 8.0, and 20 μmol γ-^{32}P-ATP (15 μCi/mmol) were incubated for 15 min at 37°C. The reaction was stopped by cooling in iced water and adding 5 ml of cold ethanol.

Tryptic Treatment of Cell-Free Extracts. 1 ml of cell-free extracts obtained from 10×10^6 anti-Ig-stimulated cells was incubated with 50 μg of trypsin at 37°C. After 20 min incubation, 150 μg of soybean inhibitor was added to stop the reaction. As a control experiment, cell-free extracts were incubated with the mixture of 50 μg of trypsin and 150 μg soybean inhibitor for 20 min at 37°C.

Chemical Analysis. DNA was determined by the diphenylamine reaction as modified by Burton (25) using calf thymus DNA as a standard.

Results

Induction of an Increased Phosphorylation of Nonhistone Nuclear Proteins (NHP) in Anti-Ig-Stimulated Nuclei. In the preceding paper (21), we have shown that the stimulation of lymphocytes with anti-Ig for 4–6 h induced an increased phosphorylation of NHP in nuclei. The result suggested that the stimulation of lymphocytes with anti-Ig induced an increase of the activity of the NHP-protein kinases in nuclei. In order to confirm this possibility, in vitro phosphorylation was carried out with the nuclei isolated from anti-Ig-stimulated cells. Nuclei were isolated from cells which had been incubated with 25 μg/ml of anti-Ig for 4 h at 37°C. 100 million nuclei in 1 ml of the reaction buffer were incubated with 20 μmol of γ-^{32}P-ATP for 15 min at 37°C. After stopping the reaction by the addition of cold ethanol, differential extractions of nuclear proteins were performed and the incorporation of ^{32}P into each fraction of nuclear proteins was measured. As a control experiment, nuclei isolated from the cells which had been cultured in the absence of anti-Ig were used. As shown in Fig. 1, a significant increase of ^{32}P incorporation into NHP fraction was observed in the nuclei isolated from anti-Ig-stimulated cells. The increase of ^{32}P incorporation was more than two times as much as that observed in control nuclei. On the other hand, no significant increase or decrease of the phosphorylation of 0.14 M NaCl-soluble fraction or histones was observed. The same experiment was repeated three times and in every experiment about a twofold increase of ^{32}P incorporation in NHP was observed without any concomitant increase or decrease of ^{32}P incorporation in histones or 0.14 M NaCl-soluble fraction. The result suggested that an activity of NHP-specific protein kinase was induced in the nuclei isolated from anti-Ig-stimulated cells.

Induction of an Increased Phosphorylation of NHP in Nonstimulated Nuclei with Cell-Free Extracts from Anti-Ig-Stimulated Cells. The above result suggested strongly that there might be some cytoplasmic factors in anti-Ig-stimulated cells which were responsible for the induction of an activity of NHP-protein kinase in nuclei. In order to study this possibility, the following experiment was performed. After 2 h incubation of lymphocytes with anti-Ig, cells were disrupted and the cell-free extract which was free from membranes and nuclei was obtained by ultracentrifugation. Independently, intact nuclei were isolated from nonstimulated lymphocytes. Nonstimulated nuclei were incubated with the cell-free extract from anti-Ig-stimulated cells. After 2 h incubation at

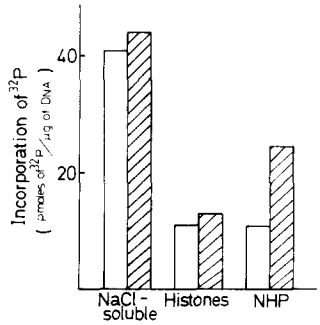

FIG. 1. In vitro phosphorylation with nuclei from anti-Ig-stimulated cells. Cells were cultured with 25 μg/ml of anti-Ig for 4 h and nuclei were isolated. After in vitro phosphorylation with γ-^{32}P-ATP, nuclear proteins were separated and incorporation of ^{32}P into each nuclear fraction was measured. Hatched columns show an incorporation of ^{32}P into nuclear proteins isolated from anti-Ig-stimulated nuclei and open columns show a ^{32}P incorporation in nonstimulated nuclei.

37°C, the nuclei were washed to remove the cell-free extract and in vitro phosphorylation was carried out with these nuclei. As a control experiment, nonstimulated nuclei were incubated with the cell-free extract obtained from nonstimulated cells which had been cultured in the absence of anti-Ig. The incorporation of ^{32}P into each nuclear protein fraction is shown in Table I. A significant increase of ^{32}P incorporation into NHP was observed in the nuclei which had been incubated with the cell-free extract from anti-Ig-stimulated cells. The same experiment was repeated four times and the average increase of ^{32}P incorporation into NHP fraction was about two times as much as that observed in the control experiment (Table I). Again, no significant increase or decrease of ^{32}P incorporation into NaCl-soluble fraction or histones was observed. The result was exactly the same as that observed in the nuclei isolated from anti-Ig-stimulated cells and suggested that the cell-free extract obtained from anti-Ig-stimulated cells contained the factor which induced the activation of NHP-protein kinases in isolated nuclei from nonstimulated cells.

To exclude the possibility that the observed increase of ^{32}P incorporation into NHP might be due to a decrease in the nuclear ATP pool because of the reduced ATP pool in cell-free extract from anti-Ig-stimulated cell, a large excess of cold ATP (3 mM) was added to the incubation mixture of cell-free extract and normal nuclei. After 2 h incubation at 37°C, nuclei were washed to remove cell-free extract and cold ATP and in vitro phosphorylation of nuclei was performed. As shown in Table II, the increased incorporation of ^{32}P into NHP of the nuclei which had been incubated with cell-free extract from anti-Ig-stimulated cells was not significantly reduced by the addition of a large excess of cold ATP, indicating that the increased incorporation of ^{32}P into NHP of "activated nuclei" was not due to decrease of the ATP pool in cell-free extract of anti-Ig-stimulated cells. Another possibility which had to be considered was that the active substance in cell-free extract might be NHP-protein kinase itself. In order to study this possibility, the following experiment was undertaken, in which the activity of protein kinase in cell-free extract was measured with NHP isolated from nuclei as a substrate. NHP or histones which had been extracted from 100

TABLE I

In Vitro Phosphorylation with Nonstimulated Nuclei which had been Incubated with the Cell-Free Extracts of Anti-Ig-Stimulated Cells

Exp.	Nuclei incubated with the extracts from*:	Incorporation of ^{32}P into‡:			NHP stimulation§
		0.14 M NaCl	Histone	NHP	
		pmol of $^{32}P/\mu g$ of DNA			%
1	Nonstimulated cells	28.9	17.0	28.9	
	Anti-Ig-stimulated cells	26.8	15.9	60.0	207
2	Nonstimulated cells	21.2	11.6	12.5	
	Anti-Ig-stimulated cells	28.8	9.7	26.3	208
3	Nonstimulated cells	12.2	8.5	24.5	
	Anti-Ig-stimulated cells	10.0	7.5	41.4	169
4	Nonstimulated cells	28.0	10.0	10.5	
	Anti-Ig-stimulated cells	26.0	11.0	26.0	248

* Lymphocytes were incubated with or without 25 μg/ml of anti-Ig. After 2 h incubation, cells were thoroughly washed and cell-free extracts were isolated.

‡ 100 million normal nuclei were incubated with the cell-free extracts. After 2 h incubation, nuclei were washed and in vitro phosphorylation was performed.

§ Percent of control (%) was calculated as follows: Percent of control (%) = [(^{32}P incorporation in NHP of nuclei incubated with the extract from anti-Ig-stimulated cells)/(^{32}P incorporation in NHP of nuclei incubated with the extract from normal cells)] × 100.

TABLE II

Effect of Exogenously Administered ATP into the Incubation Mixture of Nuclei and Cell-Free Extracts on the Induction of the Phosphorylation Activity in Nuclei

Cell-free extracts from*:	ATP‡	Phosphorylation§,‖ of NHP	NHP stimulation
		pmol of $^{32}P/\mu g$ of DNA	%
Nonstimulated cells	−	32 ± 2.0	
Anti-Ig-stimulated cells		65 ± 2.5	203
Nonstimulated cells	+	25 ± 0.5	
Anti-Ig-stimulated cells		48 ± 2.0	192

* Lymphocytes were cultured with or without 25 μg/ml of anti-Ig for 2 h and cell-free extracts were isolated.

‡ The concentration of cold ATP in the incubation mixture of normal nuclei and cell-free extracts was 3 mM.

§ After the incubation of nuclei with the cell-free extracts for 2 h, nuclei were washed and in vitro phosphorylation of nuclei was performed.

‖ The average of duplicate experiments was shown.

million nonstimulated nuclei were incubated with cell-free extract of anti-Ig-stimulated cells for 15 min at 37°C in the presence of γ-^{32}P-ATP. As shown in Table III, no significant increase of the phosphorylation of isolated NHP was observed when they were incubated with cell-free extract of anti-Ig-stimulated cells. In contrast, a twofold increase of ^{32}P incorporation into NHP was observed when intact nuclei which had been activated with the same cell-free extract

TABLE III
Protein-Kinase Activity in Cell-Free Extracts from Anti-Ig-Stimulated Cells

Cell-free extracts from*:	Phosphorylation of nuclear proteins			
	Histone‡	NHP‡	Intact nuclei§	
			Histone	NHP
	pmol of ^{32}P/μg of DNA			
Nonstimulated cells	89.6	11.9	17.0	28.9
Anti-Ig-stimulated cells	107.2	8.2	15.9	60.0
Control, %	120	69	94	208

* Lymphocytes were incubated with or without 25 μg/ml of anti-Ig for 2 h and the cell-free extracts were isolated.

‡ Histones or NHP were isolated from 1×10^8 of nonstimulated cells and incubated with the cell-free extracts for 15 min in the presence of γ-^{32}P-ATP.

§ Nuclei isolated from 1×10^8 nonstimulated cells were incubated with the cell-free extracts for 2 h. After washing nuclei, in vitro phosphorylation was performed.

were incubated with γ-^{32}P-ATP. All of these results clearly showed that cell-free extract from anti-Ig-stimulated cells contained a factor which was not a NHP-protein kinase itself but could activate a NHP-protein kinase in nuclei.

Kinetics of the Induction of the Cytoplasmic Factor in Anti-Ig-Stimulated Cells and of the Activation of NHP-Protein Kinase in Nuclei. Lymphocytes were stimulated with anti-Ig for various time periods and the activity of cell-free extract to induce an increased phosphorylation of NHP in nonstimulated nuclei was studied. As shown in Fig. 2, the cell-free extract obtained from the cells which had been stimulated with anti-Ig for 2 h showed the maximum activity, indicating that the cytoplasmic factor was maximally induced in cells at 2–4 h after anti-Ig stimulation.

In the experiment shown in Fig. 3, the time-course in which cell-free extract induced the activity of NHP-protein kinase in nuclei was followed. Nonstimulated nuclei were incubated for various time periods with cell-free extract from anti-Ig-stimulated cells and the activity of NHP-protein kinase induced in the nuclei was measured by in vitro phosphorylation. As shown in Fig. 3, the incubation of normal nuclei with cell-free extract of anti-Ig-stimulated cells for 2 h at 37°C was required for the induction of the maximum activity of NHP-protein kinase in nuclei. The incubation of nuclei with cell-free extract for 2 h at 4°C did not induce any significant increase of the phosphorylation activity in nuclei, suggesting the requirement of the energy-dependent active metabolism for the activation of nuclei with cell-free extract.

Some Properties of the Cytoplasmic Factor Responsible for the Activation of Nuclei. In order to determine the nature of the active component in cell-free extract of anti-Ig-stimulated cells, we subjected it to several treatments before incubation with normal nuclei. As shown in Table IV, dialysis of cell-free extract against the reaction buffer for 24 h at 4°C did not reduce the activity of the extract, indicating that the active factor in the cell-free extract was not a low molecular weight substance, such as a divalent cation or a cyclic nucleotide. Heating the extract at 56°C for 30 min did not abolish the activity, whereas heating at 90°C for 30 min reduced the activity to the background level. Diges-

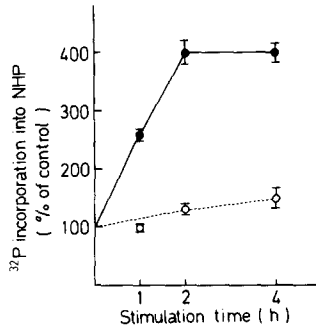

FIG. 2. Kinetics of the induction of the active substance in the cell-free extracts by anti-Ig stimulation. Nonstimulated nuclei were incubated with the cell-free extracts isolated from the cells which had been stimulated with anti-Ig for various time periods (●—●) or with the extracts isolated from the cells which had been cultured without anti-Ig (○—○). After in vitro phosphorylation of "activated nuclei," ^{32}P incorporation into NHP fraction was compared to that observed in normal nuclei.

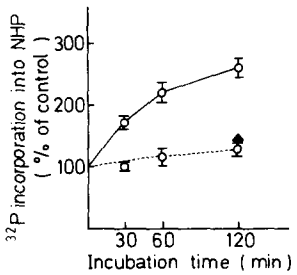

FIG. 3. Kinetics of the induction of the phosphorylation activity in nonstimulated nuclei with the cell-free extracts. Nonstimulated nuclei were incubated with the cell-free extracts from anti-Ig-stimulated cells (○—○) or from nonstimulated cells (○---○). After the incubation for various time periods, in vitro phosphorylation of "activated nuclei" was performed. ^{32}P incorporation into the NHP fraction was compared to that observed in normal nuclei. ^{32}P incorporation observed in nuclei which had been incubated with the cell-free extracts from anti-Ig-stimulated cells at 4°C for 2 h is also shown (▲).

tion of the extract with trypsin completely destroyed its activity, indicating that the active component in the extract is most probably a protein. The results in Table IV show that the inactivation of the extract with trypsin was neither due to the soybean inhibitor used nor to any residual activity of trypsin present during the incubation with nuclei. The active component in the extract was precipitated with ammonium sulfate between 33 and 50% saturation.

Discussion

Our present experiment clearly shows that cell-free extract from anti-Ig-stimulated cells contained an active component which induced an increased phosphorylation of NHP in nonstimulated quiescent nuclei. The result that the extract induced an increased phosphorylation of NHP present in intact nuclei but did not increase the phosphorylation of isolated NHP indicated that the active component in the extract was not a NHP-protein kinase itself. Furthermore, the fact that a 2 h incubation of nuclei with the extract at 37°C had been

TABLE IV
Properties of Cytoplasmic Factor from Anti-Ig-Stimulated Cells

Treatments of the cell-free extracts*	Phosphorylation in NHP (experiment/control)‡
None	2.20 ± 0.70
56°C, 30 min	1.95 ± 0.63
90°C, 30 min	0.97 ± 0.20
Trypsin	0.65 ± 0.02
Trypsin + trypsin inhibitor	1.74 ± 0.18
Dialysis	2.41 ± 0.33
Fractionation with $(NH_4)_2SO_4$	
<33%	1.34 ± 0.18
33–50%	3.10 ± 0.17
>50%	1.27 ± 0.09

* Cell-free extracts were obtained from anti-Ig-stimulated or nonstimulated cells. Several treatments of the cell-free extracts were performed before the incubation with nuclei.

‡ Nuclei were incubated with the treated cell-free extracts and in vitro phosphorylation was performed. ^{32}P incorporation into NHP in nuclei incubated with the extracts of anti-Ig-stimulated cells was compared with that observed in nuclei incubated with the extracts of nonstimulated cells.

required for their activation suggested that an active component in the extract was not a NHP-protein kinase itself but it induced the activity of a NHP-protein kinase in nuclei. In the present study, however, it could not be excluded that the extract exerted its function by inhibiting the activity of NHP-phosphatase in nuclei. In any event, the active component in the extract induced the increased phosphorylation of NHP in nuclei. It may be involved in the signal-transmission responsible for B-cell differentiation, since our previous experiment (21) suggested that the increased phosphorylation of NHP was intimately involved in the differentiation process of B cells. At present, the mechanism with which the extract induced the increased phosphorylation of NHP in nuclei and the nature of the active component in the extract are unknown. It should be noted, however, that the active component was not a small molecular substance, such as a cation or a cyclic nucleotide, but probably a protein, since it was nondialyzable and sensitive to trypsin treatment. The result, however, does not necessarily exclude the possibility that the active substance might be a cyclic AMP-binding protein and it might show the function by augmenting the incorporation of cyclic AMP into nuclei.

The presence of some substances in cytoplasm, which may be concerned in the activation of quiescent nuclei, has been reported by several investigators. Benbow and Ford (26) showed that the cytoplasm from early embryos of *Xenopus laevis* induced DNA synthesis of nuclei isolated from nondividing liver cells. More recently, Jazwinski et al. (27) reported the presence of the cytoplasmic substance in proliferating tumor cell lines which induced DNA synthesis of quiescent nuclei. At present we can not tell whether the active component shown in our present experiment is similar or not with those reported by Benbow and Ford (26) or Jazwinski et al. (27), since it has not yet been examined in our experiment whether the extract of anti-Ig-stimulated cells induced DNA

synthesis in nonstimulated nuclei. However, it would be reasonable to speculate that the active component in anti-Ig-stimulated cells might be concerned in the differentiation of B cells to the stage sensitive to the soluble factor from T cells (8) rather than in the induction of proliferation. Previously, we have shown that stimulation of B lymphocytes with anti-Ig induced acceptor sites for the soluble factor from T cells without any requirement of cellular division (8). More recently, it was shown in our preceding paper that the addition of dibutyryl cyclic AMP along with anti-Ig augmented the increased phosphorylation of NHP as well as the IgG production induced with anti-Ig and soluble factor, whereas the addition of the same concentration of dibutyryl cyclic AMP along with the soluble factor in the second stage of culture inhibited not only the IgG production but also the increased phosphorylation of NHP induced with the soluble factor (21). The same biphasic effect of dibutyryl cyclic AMP on the increased phosphorylation of NHP with anti-Ig or soluble factor and on IgG production suggested strongly that the increased phosphorylation of NHP was intimately involved in the processes of the differentiation and proliferation of B lymphocytes to IgG-producing cells. In the present experiment, exactly the same patterns of the phosphorylation of nuclear proteins were observed in the nuclei isolated from anti-Ig-stimulated cells and the nuclei incubated with cell-free extract from anti-Ig-stimulated cells, i.e., in both populations of nuclei only the phosphorylation of NHP was increased and no significant changes in phosphorylation of histones or 0.14 M NaCl-soluble proteins were observed. The result suggests that the active component in the cell-free extract of anti-Ig-stimulated cells may be concerned in the transduction of the membrane-mediated signals, which are induced with anti-Ig and responsible for the increased phosphorylation of NHP as well as for the induction of B-cell differentiation to the soluble factor-responsive stage.

However, in our present and preceding experiments (21), only the changes in the phosphorylation of total NHP were studied and we have not yet examined what kinds of NHP were phosphorylated by stimulation with anti-Ig. As it is well known, NHP are very heterogenous (18, 23) and Johnson and Hadden (20) have shown that a certain molecule of NHP, whose mol wt was about 52,000 daltons, was phosphorylated by incubation of human lymphocytes with cyclic GMP. It is essential to study which molecule(s) of NHP is phosphorylated after anti-Ig stimulation. If the NHP phosphorylated in the nuclei from anti-Ig-stimulated cells are the same as those found in the nuclei incubated with the cell-free extract, it could be concluded that the active component in cell-free extract is responsible for the signal transduction.

In the present experiment, the maximum activity was found in the cell-free extract from the cells stimulated with anti-Ig for 2 h, showing that the active substance was induced in the cytoplasm within 2 h after anti-Ig stimulation. Our preceding experiment (21), as cited above, suggested the intimate involvement of a cyclic AMP-dependent process in the phosphorylation of NHP and showed that the cyclic AMP-dependent process proceeded 1–2 h after anti-Ig stimulation. Taking all of these results together, it will be supposed that the active component in the extract may be involved in the process modulated by cyclic AMP.

The kinetic study revealed that the active component in the extract signifi-

cantly increased already at 1 h after anti-Ig stimulation. This result suggested that the induction of the active component may be due to the activation of the substance already present in the resting cells rather than due to *de novo* synthesis, although we should examine the effect of inhibitors of protein synthesis to confirm this possibility. In this sense, the mechanism with which the active component was induced may have some similarity to the activation of cytosol receptor for steroid hormones (28), in which cytosol receptor induced the gene expression in target cells by binding with steroid hormones. Work is now in progress to define whether the induction of the active component is related to the functions of microtubules or microfilaments. Very recently, ligand-specific esterase activation was observed by Henson et al. (29) in the secretion process of platelet. If this situation could be applied to lymphocytes, the specific esterase might be activated by anti-Ig and it might be responsible for the activation of the cytoplasmic factor. The studies about how the active substance is induced by anti-Ig stimulation and about the molecular nature of the substance will give useful information for revealing the mechanisms of the signal transduction given through Ig receptors.

Summary

An increased in vitro phosphorylation of nonhistone nuclear proteins (NHP) was observed in the nuclei isolated from rabbit lymphocytes which had been stimulated with anti-Ig for 4 h. No concomitant increase of phosphorylation in histones or 0.14 M NaCl-soluble proteins was observed. The increase of in vitro phosphorylation of NHP was also observed in the nuclei isolated from nonstimulated cells when these nuclei were preincubated for 2 h with cell-free extracts from anti-Ig-stimulated cells. The active substance in cell-free extracts was maximally induced when lymphocytes were stimulated with anti-Ig for 2 h. The induction of an increased phosphorylation of NHP in nonstimulated nuclei with the cell-free extracts was not due to decrease of the adenosine triphosphate pool in the extracts from anti-Ig-stimulated cells. The active substance in cell-free extracts was not NHP-protein kinase itself, but it probably activated NHP-protein kinase in quiescent nuclei. The active substance was nondialyzable and probably protein. It was resistant against heating at 56°C for 30 min, but the activity was completely destroyed by heating at 90°C for 30 min. The active substance may be responsible for the transduction of the membrane-mediated signals given through Ig receptors to nuclei.

We express our great appreciation to Professor Kaoru Onoue, Kyushu University for his helpful discussion and to Miss Kuniko Masuda for her secretarial assistance in preparing the manuscript.

Received for publication 29 March 1977.

References

1. Greaves, M. F. 1976. Cell surface receptors: a biological perspective. *Receptors and Recognition*. 1:1.
2. Warner, N. L. 1974. Membrane immunoglobulins and antigen receptors on B and T lymphocytes. *Adv. Immunol.* **19**:67.
3. Kincade, P. W., A. R. Lawton, D. E. Bockman, and M. D. Cooper. 1970. Suppression

of immunoglobulin G synthesis as a result of antibody-mediated suppression of immunoglobulin M synthesis in chickens. *Proc. Natl. Acad. Sci. U. S. A.* 67:1918.

4. Kishimoto, T., and K. Ishizaka. 1971. Regulation of antibody response *in vitro*. I. Suppression of secondary response by anti-immunoglobulin heavy chains. *J. Immunol.* 107:1657.
5. Andersson, J., W. E. Bullock, and F. Melchers. 1974. Inhibition of mitogenic stimulation of mouse lymphocytes by anti-mouse immunoglobulin antibodies. I. Mode of action. *Eur. J. Immunol.* 4:715.
6. Kearney, J. F., M. D. Cooper, and A. R. Lawton. 1976. B lymphocyte differentiation induced by lipopolysaccharide. III. Suppression of B cell maturation by anti-mouse immunoglobulin antibodies. *J. Immunol.* 116:1664.
7. Kishimoto, T., and K. Ishizaka. 1975. Regulation of antibody response *in vitro*. IX. Induction of secondary anti-hapten antibody response by anti-immunoglobulin and enhancing soluble factor. *J. Immunol.* 114:585.
8. Kishimoto, T., T. Miyake, Y. Nishizawa, T. Watanabe, and Y. Yamamura. 1975. Triggering mechanism of B lymphocytes. I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B cells. *J. Immunol.* 115:1179.
9. Watanabe, T., T. Kishimoto, T. Miyake, Y. Nishizawa, H. Inoue, Y. Takeda, and Y. Yamamura. 1975. Triggering mechanism of B lymphocytes. II. Induction of ornithine decarboxylase in B cells by anti-immunoglobulin and enhancing soluble factor. *J. Immunol.* 115:1185.
10. Taylor, R. B., P. H. Duffus, M. C. Raff, and S. de Petris. 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nat. New Biol.* 233:225.
11. Unanue, E. R., K. A. Ault, and M. J. Karnovsky. 1974. Ligand-induced movement of lymphocyte surface macromolecules. IV. Stimulation of cell motility by anti-Ig and lack of relationship to capping. *J. Exp. Med.* 139:295.
12. Mendelsohn, J., S. A. Skinner, and S. Kornferd. 1971. The rapid induction by phytohemagglutinin of increased α-aminoisobutyric acid uptake by lymphocytes. *J. Clin. Invest.* 50:818.
13. Freedman, M. D., M. C. Raff, and B. Gomperts. 1975. Induction of increased calcium uptake in mouse T-lymphocytes by concanavalin A and its modulation by cyclic nucleotides. *Nature (Lond.).* 255:378.
14. Smith, J. W., A. L. Steiner, W. M. Newberry, Jr., and C. W. Parker. 1971. Cyclic adenosine 3′,5′-monophosphate in human lymphocytes. Alterations after phytohemagglutinin stimulation. *J. Clin. Invest.* 50:432.
15. Hadden, J. E., E. M. Hadden, M. K. Haddox, and N. D. Goldberg. 1972. Guanosine 3′,5′-cyclic monophosphate: a possible intracellular mediator of mitogenic influences in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 69:3024.
16. Edelman, G. M., I. Yahara, and J. L. Wang. 1973. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 70:1442.
17. Becker, E. L., and E. R. Unanue. 1976. The requirement for esterase activation in the anti-immunoglobulin-triggered movement of B lymphocytes. *J. Immunol.* 117:27.
18. Kleinsmith, L. J. 1974. Acidic nuclear phosphoproteins. *In* Acidic Proteins of the Nucleus. I. L. Cameron and J. R. Jeter, Jr., editors. Academic Press, Inc., New York. 103.
19. Johnson, E. M., J. Karn, and V. G. Allfrey. 1974. Early nuclear events in the induction of lymphocyte proliferation by mitogen. *J. Biol. Chem.* 249:4990.
20. Johnson, E. M., and J. W. Hadden. 1975. Phosphorylation of lymphocyte nuclear acidic proteins. Regulation by cyclic nucleotides. *Science (Wash. D. C.).* 187:1198.
21. Kishimoto, T., Y. Nishizawa, H. Kikutani, and Y. Yamamura. 1977. Biphasic effect

of cyclic AMP on IgG production and on the changes of non-histone nuclear proteins induced with anti-immunoglobulin and enhancing soluble factor. *J. Immunol.* 118:in press.

22. Levy, R., S. Levy, S. A. Rosenberg, and R. T. Simpson. 1973. Selective stimulation of non-histone chromatin protein synthesis in lymphoid cells by phytohemagglutinin. *Biochemistry.* 12:224.
23. Teng, C. S., C. T. Teng, and V. G. Allfrey. 1971. Studies of nuclear acidic proteins: evidence for their phosphorylation, tissue specificity, selective binding to deoxyribonucleic acid and stimulatory effects on transcription. *J. Biol. Chem.* 246:3597.
24. Rickwood, D., P. G. Riches, and A. J. McGillivray. 1973. Studies of the *in vitro* phosphorylation of chromatin non-histone proteins in isolated nuclei. *Biochim. Biophys. Acta.* 299:162.
25. Burton, K. 1956. Studies of the conditions and mechanism of the diphenylamine reactions and colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62:315.
26. Benbow, R. M., and C. C. Ford. 1975. Cytoplasmic control of nuclear DNA synthesis during early development of *Xenopus laevis*: a cell free assay. *Proc. Natl. Acad. Sci. U. S. A.* 72:2437.
27. Jazwinski, S. M., J. L. Wang, and G. M. Edelman. 1976. Initiation of replication in chromosomal DNA induced by extracts from proliferating cells. *Proc. Natl. Acad. Sci. U. S. A.* 73:2231.
28. Means, A. R., and B. W. O'Malley. 1974. Oestrogen-induced differentiation of target tissue. *In* Biochemistry of Cell Differentiation. J. Paul, editor. University Park Press, Baltimore, Md. 161.
29. Henson, P. M., D. Gould, and E. L. Becker. 1976. Activation of stimulus-specific serine esterases (proteases) in the initiation of platelet secretion. I. Demonstration with organophosphorus inhibitors. *J. Exp. Med.* 144:1657.