

## Session II. Allergy and Intercellular Signal Transmission Mechanism

### Positive and negative signals transduced through surface immunoglobulins in human B cells

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*Cross-linking of surface IgM and surface IgD by anti-IgM antibodies and anti-IgD antibodies, respectively, showed different effects on the growth of normal human peripheral blood B cells and the human B lymphoma cell line, B104. Only cross-linking of surface IgM transduced signals that inhibited cell division of peripheral blood B cells and B104 cells at the G<sub>2</sub>/M interphase. In B104 cells, the inhibition of cell division was followed by rapid B104 cell death. The negative signals were inhibited by cyclosporin A and FK-506 at lower concentrations than those that inhibited proliferation of the B cells. Anti-IgM antibody-induced B104 cell death was dependent on Ca<sup>2+</sup> influx and macromolecular synthesis. B104 cells treated with anti-IgM antibodies showed neither DNA fragmentation or morphology of apoptosis but showed DNA single-strand breaks and morphology of necrosis. Nicotinamide inhibited anti-IgM antibody-induced B104 cell death and the involvement of poly(adenosine diphosphate-ribosylation) was suggested in the process of the B104 cell death. With regard to the intracellular mechanisms responsible for the different signals, however, no qualitative difference was detected in putative signal transducers, including tyrosine phosphorylated protein, phosphatidyl inositol turnover, Ca<sup>2+</sup> influx, activation of protein kinase C, and messenger ribonucleic acid expression of c-fos and Egr-1 when surface IgM and surface IgD were crosslinked. Further investigations of the mechanisms responsible for the different signals transduced through surface IgM and surface IgD will provide better understanding of immunodeficiencies and autoimmune diseases. (J ALLERGY CLIN IMMUNOL 1994;94:612-9.)*

**Key words:** B cell, signaling, antigen receptor, tyrosine kinase, apoptosis

Signals caused by antigen stimulation are the initial specific triggers for B-cell activation, inactivation, or deletion followed by their clonal expansion, anergy, or deletion, respectively. The positive and negative signals have been considered to play important roles in preparing enough numbers of immunocompetent B cells and self-tolerance, respectively. The two different signals

#### Abbreviations

|        |                                   |
|--------|-----------------------------------|
| ADP:   | Adenosine diphosphate             |
| ATP:   | Adenosine triphosphate            |
| IFN:   | Interferon                        |
| IL-4:  | Interleukin-4                     |
| mRNA:  | Messenger ribonucleic acid        |
| NAD:   | Nicotinamide-adenine dinucleotide |
| PBL-B: | Peripheral blood B cells          |
| PKC:   | Protein kinase C                  |
| sIg:   | Surface immunoglobulins           |

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Supported by a grant from the Ministry of Health and Welfare of Japan and by a grant from Sandoz Co., Ltd.

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0091-6749/94 \$3.00 + 0 1/0/57121

are both fundamentally transduced through surface immunoglobulins (sIg), probably in association with other co-signaling pathways, under certain circumstances, in vivo. Recently the signal transducing mechanisms of sIg have been consid-

erably examined, and it has been shown that sIg are associated with a set of molecules that include the *mb-1* and B29 gene products, Ig- $\alpha$  and Ig- $\beta$ , respectively,<sup>1-3</sup> and with several tyrosine kinases, including Lyn, which are activated by sIg cross-linking.<sup>4-8</sup> The covalently linked MB-1 and B29 heterodimers are noncovalently associated with sIg and are phosphorylated in tyrosine residue by sIg cross-linking.<sup>9</sup> However, the mechanisms underlying the positive and negative signaling have not yet been made clear. We examined the effects of signals induced by cross-linking of sIgM or sIgD by anti-IgM or anti-IgD antibodies, respectively, on cell growth of normal human peripheral B cells (PBL-B) and a sIgM- and sIgD-positive human B lymphoma cell line, B104. We also examined the changes in putative signal transducing molecules induced by cross-linking of sIgM and sIgD.

## MATERIAL AND METHODS

### Cells

Normal heparinized human peripheral venous blood was obtained from healthy volunteers. PBL-B were isolated and purified as described previously.<sup>10</sup> The human B lymphoma cell line, B104, which coexpresses sIgM and sIgD, has been described previously.<sup>11,12</sup> The B104 cells were maintained at  $2 \times 10^5$  to  $2 \times 10^6$  cell/ml in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum,  $5 \times 10^{-5}$  mol/L 2-mercaptoethanol, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin.

### Reagents

The following mouse hybridomas were purchased from American-type Culture Collection (Rockville, Md.): HB57 (DA4.4 anti-human IgM antibody), HB138 (anti-human IgM antibody), and HB70 (anti-human IgD antibody). The monoclonal antibodies were purified from ascitic fluids with ammonium sulfate and diethylaminoethyl column chromatography. Cyclosporin A and FK-506 were provided by Sandoz Co., Ltd., (Basel, Switzerland) and by Fujisawa Pharmaceutical Co. (Osaka, Japan), respectively. 3-Methoxybenzamide was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise specified.

### Analysis of effects of reagents on cell growth

Exponentially growing B104 cells were seeded on flat-bottomed 96-well culture plates in triplicate with or without the reagents and were cultured in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37° C for the planned periods. Viable cells were determined by trypan blue dye exclusion.

### Extraction of intracellular nucleotides

To extract nicotinamide-adenine dinucleotide (NAD) and adenosine triphosphate (ATP),  $3 \times 10^6$  viable cells, after various treatments, were washed with cold phosphate buffered saline and incubated in 0.5 mol/L perchloric acid for 15 minutes at 0° C. After centrifugation at  $1500 \times g$  for 10 minutes, the supernatant was neutralized with 0.33 mol/L potassium phosphate buffer, pH 7.5, and 1 mol/L potassium hydroxide. After centrifugation, the clear supernatant was kept at -20° C until assayed for NAD and ATP.

### Measurement of NAD and ATP

The amount of NAD in the extract fluid was measured by an enzyme cycling assay as previously described.<sup>13,14</sup> The cycling assay was initiated at 37° C in a dark place by the addition of 1  $\mu$ g alcohol dehydrogenase (Sigma Chemical Co., St. Louis, Mo.), and the reaction was terminated by the addition of 0.5 ml of 12 mmol/L iodoacetate. The optical density of reduced methyl-thiazolyltetrazolium (Sigma) was measured at 570 nm. ATP was quantified by high-performance liquid chromatography on a Partisil SAX 10 anion-exchange column (Whatman, Inc., Clifton, N.J.) eluted with 0.5 mol/L potassium chloride/0.25 mol/L monopotassium phosphate, pH 3.4, at 1.5 ml/minute.

### Measurement of poly(adenosine diphosphate [ADP]-ribose) synthesis

Poly(ADP-ribose) synthesis was measured as described previously.<sup>15,16</sup> Briefly,  $4 \times 10^6$  cells, after various treatments, were first permeabilized by a hypotonic buffer (10 mmol/L Tris-hydrochloride pH 7.8, 1 mmol/L ethylenediaminetetraacetic acid, 4 mmol/L magnesium chloride, and 30 mmol/L 2-mercaptoethanol) for 30 minutes at 0° C. After centrifugation, the cells were then resuspended in 50  $\mu$ l of the hypotonic buffer and 25  $\mu$ l of a reaction mixture consisting of 100 mmol/L Tris-hydrochloride pH 7.8, 120 mmol/L magnesium chloride, and 1.25  $\mu$ Ci (adenine 2, 8, -<sup>3</sup>H)-NAD (NEN Research Products, Boston, Mass.). After incubation at 30° C for 45 minutes, the reaction was terminated by an excess of ice cold 20% trichloroacetic acid. The trichloroacetic acid-insoluble fraction was collected on a GF/C filter (Whatman, Inc.), and radioactivity was measured in a liquid scintillation counter.

### Statistical analysis

Statistical analysis was performed with the Student *t* test.

## RESULTS

### Signals transduced via sIgM and sIgD have different effects on cell division of normal mature B cells

When normal human PBL-Bs were stimulated with anti-IgM antibodies or anti-IgD antibodies, they showed DNA synthesis on day 3 almost at

the same degree. Interleukin-4 (IL-4) enhanced the DNA synthesis in both cases. The results show that cross-linking of sIgM and sIgD have the same effect on DNA synthesis of PBL-B cells.<sup>10, 17</sup> On the contrary, when cross-linking of sIg was examined in terms of the effect on cell division, anti-IgM antibodies and anti-IgD antibodies showed quite different effects. When PBL-Bs were stimulated with anti-IgD antibodies, a considerable number of M phase cells were detected after 3 days of culture. The number of M phase cells induced by anti-IgD antibodies was increased in the presence of IL-4. However, when PBL-Bs were stimulated with anti-IgM antibodies in the presence or absence of IL-4, the numbers of M phase cells were significantly lower than those induced by anti-IgD antibodies with or without IL-4. Moreover, anti-IgM antibodies decreased the number of M phase cells induced by anti-IgD antibodies and IL-4 without any decrease either in DNA synthesis or the total number of cells at S and G<sub>2</sub>/M phases of the cell cycle. *Staphylococcus aureus* Cowan I (SAC) also induced DNA synthesis and M phase cells of PBL-B, and anti-IgM antibodies inhibited the induction of M phase cells without inhibition of the DNA synthesis and the total number of cells at S and G<sub>2</sub>/M phases. On the contrary, anti-IgD antibodies did not inhibit but enhanced the induction of M phase cells by SAC. The results showed that cross-linking of sIgM and sIgD induced quite different signals in B cells; that is, although both anti-IgM antibodies and anti-IgD antibodies induced DNA synthesis, signals via sIgM but not sIgD act during the late stage of the cell cycle and inhibit the cell cycle transition from G<sub>2</sub> to M phase.<sup>10, 17</sup>

Positive signals on cell growth transduced via sIg cross-linking or by SAC have been shown to be inhibited by cyclosporin A and FK-506, both of which are inhibitors of calmodulin-dependent phosphatase, calcineulin.<sup>18</sup> In our system, cyclosporin A and FK-506 at concentrations of or higher than 10 nmol/L and 1 nmol/L, respectively, inhibited DNA synthesis and induction of M phase cells by SAC, anti-IgM antibodies plus IL-4 or anti-IgD antibodies plus IL-4. However, cyclosporin A and FK-506, when used at lower concentrations (10<sup>-4</sup> to 1 nmol/L and 10<sup>-5</sup> to 10<sup>-2</sup> nmol/L, respectively), which did not inhibit the DNA synthesis and the cell division of PBL-B, inhibited the anti-IgM antibody-induced negative signal on cell division; they increased the numbers of M phase cells in the PBL-B cultured with SAC plus anti-IgM antibodies or with anti-IgD anti-

bodies and IL-4 plus anti-IgM antibodies. The concentrations of cyclosporin A and FK-506 did not increase the number of M phase cells in PBL-B cultured with anti-IgD antibodies plus IL-4, but they increased the M phase cell numbers in the culture with anti-IgM antibodies plus IL-4.<sup>17</sup> The results show that cross-linking of sIgM transduces both positive and negative signals for cell division through a calcineulin-dependent pathway and that the negative signals are preferentially inhibited by cyclosporin A and FK-506 at lower concentrations. Interestingly, the lower concentrations of cyclosporin A and FK-506 also increased the number of M phase cells induced by SAC. The results are compatible with former results, which show that SAC may show the effects partly through sIgM cross-linking.

Whether PBL-B, whose division was blocked at the G<sub>2</sub>/M interphase by anti-IgM antibodies, were going to die was somewhat unclear because relatively small populations of PBL-B could be induced to reach G<sub>2</sub>/M phases by SAC or anti-IgD antibodies plus IL-4, although viable cell number analyses suggested that those cells died in a short period.

#### **Cross-linking of sIgM but not that of sIgD induced death of a human B lymphoma cell line, B104**

Although examinations that use normal B cells should be better for understanding normal immune responses, such examinations sometimes may not show clear results because PBL-B contain cells at different maturation or activation stages. The fact that only a limited proportion of PBL-B can be induced to enter the M phase by SAC or anti-IgD antibodies plus IL-4 is an example. From this point of view, lymphoma or leukemia B cell lines are very useful because their maturation is frozen at a certain stage of cell differentiation and represent a single cell population. We therefore examined the effects of cross-linking of sIgM and sIgD by using a human B lymphoma cell line, B104, and found that signals transduced via sIgM and sIgD also induced different phenomena in B104 cells.

B104 cells were established from PBL-Bs of a patient with malignant B cell lymphoma in the leukemic stage. B104 cells express large amounts of sIgM and small amounts of sIgD, showing a relatively immature phenotype of mature B cells; tests for Epstein-Barr virus nuclear antigens have negative results.<sup>11</sup> Cross-linking of sIgM or sIgD by anti-IgM antibodies or anti-IgD antibodies, respec-

tively, induced the increase in concentrations of intracellular free  $\text{Ca}^{2+}$  ion at almost the same degree.<sup>11</sup> Both anti-IgM antibodies and anti-IgD antibodies induced activation of protein kinase C (PKC), phosphatidyl inositol turnover, *c-fos* and *Egr-1* messenger ribonucleic acid (mRNA) expression in B104 cells, although anti-IgM antibodies were more effective.<sup>19</sup> The difference was presumably because of the smaller amount of surface expression of sIgD than that of sIgM in B104 cells because in PBL-B, which express sIgD as many as or more than sIgM, cross-linking of sIgM and sIgD by anti-IgM antibodies and anti-IgD antibodies, respectively, induced the same levels of PKC activation, phosphatidyl inositol turnover, and *c-fos* and *Egr-1* mRNA expression. These results showed that both sIgM and sIgD on B104 cells have signal transducing ability with some quantitative differences.

Cross-linking of sIgM by anti-IgM antibodies, but not of sIgD by anti-IgD antibodies, induced death of B104 cells within 12 to 24 hours after culture.<sup>11, 12</sup> Anti-IgD antibodies did not influence the effect of anti-IgM antibodies when added at different times of the culture. Cell cycle analyses revealed that anti-IgM antibodies inhibited cell cycle transition from  $G_2$  to M phase, as similarly seen in the anti-IgM antibody-treated PBL-B. The anti-IgM antibody-induced B104 cell death was cancelled by depletion of  $\text{Ca}^{2+}$  ion from the culture medium or by actinomycin D or cycloheximide, showing cell death is dependent on the influx of extracellular  $\text{Ca}^{2+}$  ion and macromolecular synthesis.<sup>12</sup> Cyclosporin A and FK-506 completely inhibited anti-IgM antibody-induced B104 cell death,<sup>12</sup> the same as shown above in PBL-B.<sup>17</sup>

The B104 cell death induced by anti-IgM antibodies may be inhibited by costimulation with cytokines. In the cytokines tested, interferon (IFN)- $\alpha$  and - $\beta$ , when added within 24 to 48 hours after stimulation by anti-IgM antibodies, cancelled anti-IgM antibody-induced inhibition of cell cycle transition from  $G_2$  to M phase and saved B104 cells from death.<sup>11</sup> IL-1, -2, -3, -4, -5, and -6, TNF- $\alpha$  and IFN- $\gamma$  were not effective. IFN- $\alpha$  and - $\beta$  also inhibited the above mentioned negative signals induced by anti-IgM antibodies in PBL-B.<sup>10</sup>

#### **Anti-IgM antibody-induced B104 cell death is not compatible with classic apoptosis**

The results mentioned above showed that anti-IgM antibody-induced B104 cell death is an active process that needs new macromolecule synthesis. It has been shown that cross-linking of CD3 and T

cell receptor complexes induces apoptotic death of T cells, which is characterized by apoptotic body formation and DNA fragmentation. On the contrary, anti-IgM antibody-treated B104 cells did not show either morphology of apoptosis or DNA fragmentation. Instead, the anti-IgM antibody-treated B104 cells showed a morphology of necrosis, swelling of the cytoplasmic apparatus with a relatively intact nuclear membrane.<sup>12</sup> Although neither DNA fragmentation nor small-sized DNA was detected, the DNA unwinding assay, which can detect both DNA single and double-strand breaks, revealed many DNA strand breaks in anti-IgM antibody-treated B14 cells. The DNA strand breaks were thus considered as DNA single-strand breaks (nick). The nick was detected before massive cell death became apparent and mitomycin C and cyclosporin A, both of which inhibited anti-IgM antibody-induced B104 cell death, inhibited nick formation. Because cells with massive DNA strand breaks have been considered unable to enter the M phase of the cell cycle, the interruption of cell cycle transition at the  $G_2$ /M interphase seen in anti-IgM antibody-treated B104 cells is compatible with the result of DNA strand breaks. It is also suggested that nick plays a role in anti-IgM antibody-induced B104 cell death.

#### **Possible involvement of poly(ADP-ribosylation) in anti-IgM antibody-induced B104 cell death**

A possible explanation for the contribution of nick to anti-IgM antibody-induced B104 cell death is that massive single-strand breaks may induce alterations and degradative changes of DNA molecules.<sup>20, 21</sup> However, the existence of nick does not necessarily result in cell death because it may be repaired by activation of poly-(ADP-ribose) polymerase. It has been suggested that excessive activation of poly(ADP-ribose) polymerase as a result of massive DNA strand breaks may cause depletion of NAD and ATP, thus resulting in cell death because of lack of energy sources.<sup>22, 23</sup> Because DNA single-strand breaks existed in anti-IgM antibody-treated B104 cells before their death became apparent, and because their morphology represented a necrosis-like feature, we supposed that activation of poly-(ADP-ribose) polymerase plays a role in anti-IgM antibody-induced B104 cell death. In fact, NAD and ATP pools in anti-IgM antibody-treated B104 cells were significantly decreased in a time-dependent manner before cell death could be detected

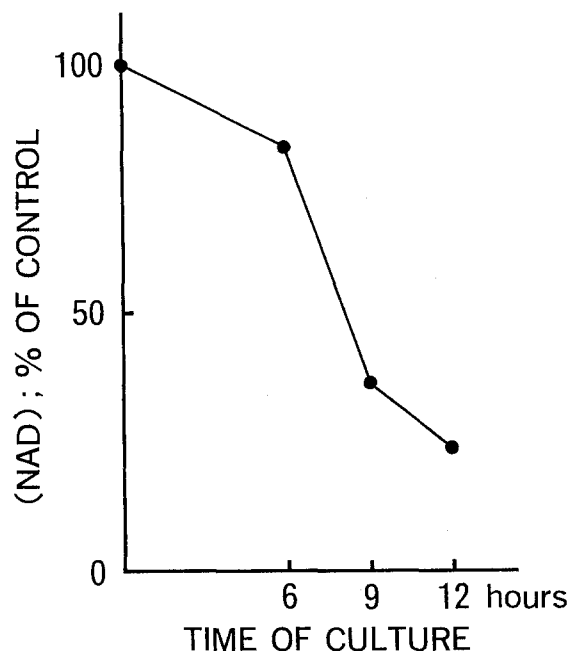


**FIG. 1.** Changes in NAD pools induced by stimulation with anti-IgM antibodies. B104 cells were stimulated with 1  $\mu$ g/ml of monoclonal anti-IgM antibody (DA4.4), and intracellular NAD pools were determined after indicated culture periods.

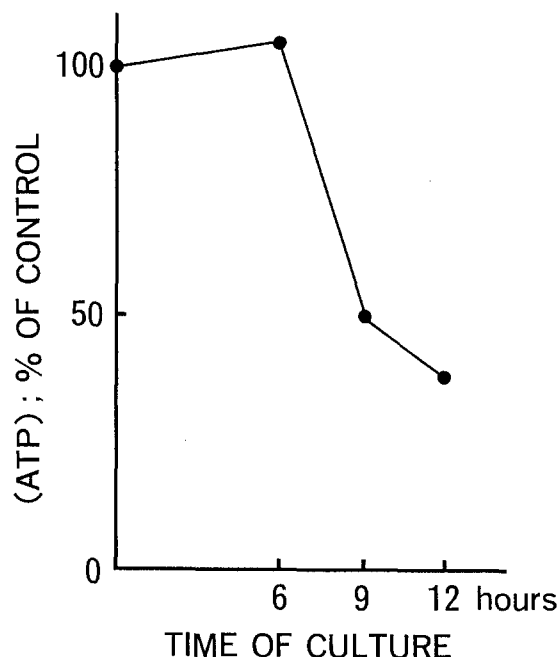


**FIG. 2.** Changes in ATP pools induced by stimulation with anti-IgM antibodies. B104 cells were stimulated with 1  $\mu$ g/ml of anti-IgM antibody DA4.4, and intracellular ATP pools were determined after indicated culture periods.

(Figs. 1 and 2). Increased transfer of ADP-ribose moieties to proteins was seen simultaneously (Fig. 3). Moreover, nicotinamide, a poly(ADP-ribosyl)ation inhibitor and a source of NAD, and 3-methoxybenzamide, another poly(ADP-ribosyl)ation inhibitor, inhibited anti-IgM antibody-induced B104 cell death in a dose-dependent manner (Fig. 4). These results collectively suggested the involvement of poly(ADP-ribosyl)ation in anti-IgM antibody-induced B104 cell death. However, it should be noted that B104 cells showed necrotic changes as early as 6 hours after stimulation with anti-IgM antibodies, by which time neither NAD nor ATP showed a significant drop. The incremental transfer of ADP-ribose moieties to proteins was not as large as expected. Therefore it is possible that other lethal mechanisms may also be involved in the overall cell death program. A possibility that the changes in NAD and ATP levels are secondary phenomena according to cell death has not yet been completely ruled out.

On the other hand, it has been shown that crosslinking of sIgM on a murine B cell line, WEHI 231, by anti-IgM antibodies causes WEHI 231 cell death by apoptosis after DNA fragmentation.<sup>24</sup> This observation, when combined with

our results, showed that signals transduced via sIgM can induce the morphology of apoptosis and necrosis in B cells. It is likely that the existence or lack of apoptosis after DNA fragmentation induced by cross-linking of sIgM may be dependent on the endonuclease activity of cells.

#### Protein tyrosine phosphorylation by cross-linking of sIgM and sIgD

sIg have been shown to be noncovalently associated with the products of two B cell-specific genes, *mb-1* and *B29*. These products are considered to play an important role not only in surface expression of Ig but also in signal transduction through sIg in association with tyrosine kinase(s), including Lyn, Fyn, and Syk. The tyrosine kinases are activated by sIg cross-linking and phosphorylate the tyrosine residues of a set of proteins including MB-1 and B29. A 75 kd protein (HS1), which has a proline-rich motif and a DNA-binding domain, has been shown as one of the major proteins that are tyrosine phosphorylated by sIgM cross-linking<sup>25</sup> and thus are considered to play an important role in sIg-mediated signal transduction. We examined the possible participation of MB-1, B29, and HS1 in the different signal transducing abilities of sIgM and sIgD.

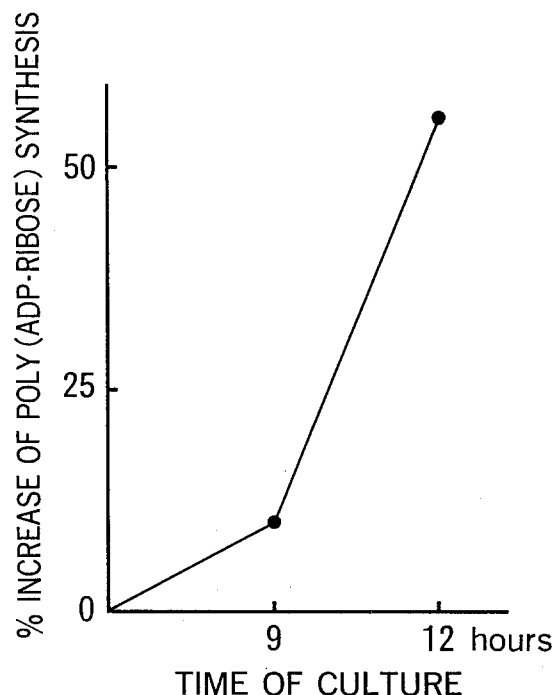


**FIG. 3.** Enhancement of poly(ADP-ribose) synthesis by stimulation with anti-IgM antibodies. B104 cells were stimulated with 1  $\mu$ g/ml of anti-IgM antibody DA4.4 and poly(ADP-ribosyl)ation was determined after indicated culture periods.

Cross-linking of either sIgM or sIgD induced tyrosine phosphorylation of a set of the same proteins including MB-1 and B29 in almost the same manner, although molecular weights of MB-1 and B29 associated with IgM were different from those associated with IgD.<sup>26, 27</sup> The difference was because of the difference in *N*-glycosylation of these molecules. HS1, which was neither tyrosine phosphorylated nor coimmunoprecipitated with sIg complexes without stimulation, was tyrosine phosphorylated and coimmunoprecipitated with the antigen receptor complexes after cross-linking of sIgM or sIgD. After cross-linking of sIgM or sIgD, HS1 was considered to associate with Lyn, as previously reported.<sup>25</sup> From these points of view in combination with the above mentioned results of  $Ca^{2+}$  influx, PKC activation, and expression of *c-fos* and *Egr-1* mRNA, no qualitative difference in signal transduction mechanisms of sIgM and sIgD was detected.

## DISCUSSION

Cross-linking of sIgM and sIgD shows different effects on B cell proliferation, and only sIgM cross-linking transduces negative signals that suppress the cell cycle transition from  $G_2$  to M phase

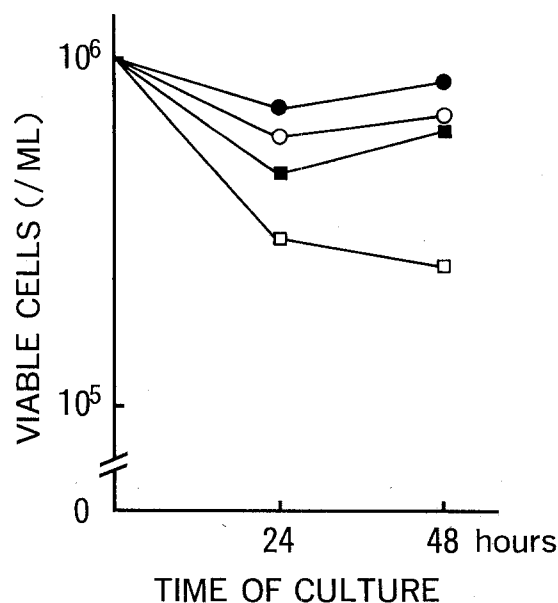


**FIG. 4.** Protective effects of nicotinamide and 3-methoxybenzamide on anti-IgM antibody-induced B104 cell death. B104 cells were cultured with 1  $\mu$ g/ml of anti-IgM antibody DA4.4 in absence (open square) or presence of 5 mmol/L nicotinamide (closed square), 10 mmol/L nicotinamide (closed circle), or 2.5 mmol/L 3-methoxybenzamide (open circle). Viable cell numbers were determined after indicated culture periods.

in normal human PBL-B and a human B lymphoma cell line, B104. The cell cycle arrest at the  $G_2/M$  interphase in B104 cells seems to be, at least in part, because of nick induced by sIgM cross-linking. The negative signals most likely use the same intracellular signal transducing pathway as the positive signals, at least in terms of the involvement of calcineurin. Cross-linking of both sIgM and sIgD induced tyrosine phosphorylation of the same proteins, including MB-1, B29, and HS1, phosphatidyl inositol turnover,  $Ca^{2+}$  influx, PKC activation, and *c-fos* and *Egr-1* mRNA expression. It has been proposed that lack of PKC and *Egr-1* mRNA activation is responsible for the negative signaling through sIgM.<sup>28, 29</sup> Our data show that this is not the case in B104 cells and in normal PBL-B and suggest that presence or absence of PKC activation is not directly involved in the different signaling. However, when B104 cells were pretreated with a PKC activator, phorbol 12-myristate 13-acetate (PMA), stimulation with anti-IgM antibodies did not induce enough  $Ca^{2+}$  influx, and anti-IgM antibody-induced B104 cell death was not seen.<sup>30</sup> Calcium ionophore induced B104 cell death; PMA inhibited the cell death. The influence of PKC activation on sIg-trans-

duced negative signals thus needs further examination.

Regarding the differences between sIgM-associated and sIgD-associated molecules and between intracellular events induced by sIgM cross-linking and sIgD cross-linking, sIgM-associated MB-1 and B29 have a different *N*-glycosylation pattern from sIgD-associated MB-1 and B29.<sup>26, 27</sup> The signal transducing ability of sIgM and sIgD may depend not only on their intracellular moieties but also on their extracellular ones.<sup>31</sup> Thus it is possible that the difference in the *N*-glycosylation of sIgM and sIgD is responsible for the different signaling. On the other hand, the quantity but not quality of phosphatidyl inositol turnover, PKC activation, and expression of *c-fos* and *Egr-1* mRNA was also different when sIgM or sIgD were cross-linked.<sup>19</sup> The quantitative difference may also play a role in the different signaling; relatively weak stimuli may induce positive signaling whereas strong stimuli may induce negative signaling. Whether the difference in signals transduced through sIgM and sIgD is a result of the qualitative difference in their specific signal transducing apparatuses is still unclear and needs further examination.

In a murine B cell line, WEHI 231, it has been shown that stimulation with anti-IgM antibodies causes apoptotic cell death. Our data show anti-IgM antibody-induced B cell death does not necessarily represent apoptosis. It is noteworthy that anti-*Fas* antibodies, which have been shown to induce apoptosis in various types of cells, induced B104 cell death; however, the dying B104 cells did not show the morphology of apoptosis but showed that of necrosis.<sup>32</sup> Poly(ADP-ribosyl)ation, which was suggested to be involved in the process of anti-IgM antibody-induced B104 cell death, was also suggested to play a role in anti-*Fas* antibody-induced B104 cell death whereas both poly(ADP-ribosyl)ation and apoptosis after endonuclease activation were suggested to be involved in the process of anti-*Fas* antibody-induced U937 cell death.<sup>32</sup>

It is unlikely that all B cells that received antigen stimulation through sIgM *in vivo* die as seen in B104 cells. Therefore mechanisms that cancel the sIgM-transduced negative signals may exist. In fact, it has been shown recently that sIgM-transduced negative signals are canceled by cosignals through CD40.<sup>33, 34</sup> In B104 cells, stimulation with anti-CD40 antibodies did not cancel anti-IgM antibody-induced B104 cell death, although B104 cells express considerable numbers

of CD40 molecules (unpublished observation). B104 cells are thus useful for examining the CD40-mediated signals and the interaction of signals through sIgM and CD40.

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