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Immunomodulatory Effect of Recombinant Human Superoxide Dismutase (SOD) on Human B Lymphocyte Function *in Vitro*

KEIKO MORIKAWA AND SHIGERU MORIKAWA*

*Departments of Internal Medicine and *Pathology, Shimane Medical University, Enya-cho 89-1, Izumo, Shimane, Japan*

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Recent investigations demonstrate that B lymphocytes possess an oxygen-generating system which is similar to the phagocytic NADPH-oxidase system. Reduction of nitroblue tetrazolium by stimulated tonsillar B cells is inhibited by superoxide dismutase (SOD). However, the biological significance of the superoxide-generating property of B cells remains to be explored. In this study, we examined the immunomodulatory effect of a recombinant human SOD (rh-SOD) on the activation of human B lymphocytes *in vitro.* A supplement of rh-SOD in the B cell culture increased the proliferation of unstimulated B cells in the presence of SAC, but not of SAC-preactivated B cells in the presence of cytokines such as IL-2 or IL-4. In addition, rh-SOD enhanced the immunoglobulin generation by B cells at the terminal stage of differentiation. Inactivation of the enzymatic activity of SOD by treatment with anti-SOD antibody abrogated the enhancing effects. These data suggest that the superoxide—generating system in B cells may be involved in the cellular activation process.

INTRODUCTION

The antimicrobial action of the phagocytes is intimately connected with their potential to perform one-electron reduction of molecular oxygen (O_2) to superoxide (O_2^-), from which other species can be derived, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), or hydrochloride (−OCL) (1). Recent investigations demonstrate that B lymphocytes from various sources (2–8) have an ability to generate superoxide, despite the fact that they are nonphagocytic cells. As stimulation with phorbol ester (7) or anti-immunoglobulin (Ig) (2, 5) can induce superoxide generation in B cells, it is likely that B lymphocytes can provide a locally restricted antigen-activated source of reactive oxygen species during an immune response. Although the NADPH-dependent oxidase system in B cells is similar to the phagocytic NADPH-oxidase system in the reduction of nitroblue tetrazolium (NBT), cytochrome b-245 antigen, and RNA for the β-chain of cytochrome b-245, the capacity of B cells for generating superoxide is rather low in comparison with that of phagocytic cells. Interestingly, this oxygen-generating system is specific for B lymphocytes, and T lymphocytes are unable to produce superoxide (2). Actually, radical oxygen intermediates (O_2^-, H_2O_2, and $OH\cdot$) can interfere with the mitogenesis of peripheral blood mononuclear cells (9–12). In addition, it has been reported that generation of radical oxygen intermediates can induce tyrosin phosphorylation in lymphocytes (13).

Small quantities of oxygen free radicals are constantly generated in a number of normal biochemical processes. To cope with physiological amounts of superoxide, the cells possess an enzyme, superoxide dismutase (SOD), that catalyzes the dismutation of this radical to hydrogen peroxide. Three forms of SOD with distinctive distributions are characterized by their metal requirement. CuZnSOD is found mainly in the cytosol of eukaryotes, FeSOD is found in prokaryotes, and MnSOD is found both in prokaryotes and in mitochondria of eukaryotes (14). SODs are important initial components in the cellular defense against oxygen toxicity since O_2^- can react with H_2O_2 to generate singlet oxygen and hydroxy radicals, which are even more reactive and cytotoxic than O_2 or H_2O_2. Besides the intracellular presence of SOD, the enzyme is found in human serum in high concentrations (15). It protects capillary endothelial cells against the damaging effects of superoxide (16). According to a recent study, high autoantibody titres against MnSOD are detectable in sera of patients with acute Epstein–Barr virus (EBV) infection, but not in patients with other acute viral infections or in healthy persons (17). The anti-MnSOD autoantibodies were capable of blocking the function of MnSOD (17). EBV is not only capable of infecting and immortalizing B cells, but is also capable of activating resting B cells. Acute EBV infection becomes apparent

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with the clinical picture of infectious mononucleosis (IM). EBV-transformed B cells are competent in generating superoxide in the immune responses (3–8). Previous reports describe that SOD can enhance the growth of human myeloid progenitor cells (18) and murine phagocytic mononuclear cells (19). However, the effect of SOD on B lymphocytes remains to be elucidated.

In the present study, we examined the effect of SOD on B cell activation by using a newly purified recombinant human SOD preparation (rh-SOD) (20). The rh-SOD could enhance the proliferation of B lymphocytes in the presence of a stimulator capable of superoxide-generating activity. In addition, it increased the immunoglobulin generation of terminally differentiated B cells at the transcriptional level.

MATERIALS AND METHODS

Cell Preparation

Human B lymphocytes were obtained from tonsillar samples by tonsillectomies from juvenile patients with chronic tonsillitis as previously described (21). Briefly, tonsils were dispersed into single-cell suspensions and mononuclear cells were isolated on Ficoll–Hypaque density gradients. Monocytes and natural killer cells were depleted by incubating the cells with 5 m*M* L-leucine methyl ester (Sigma, St. Louis, MO) in serum-free medium. T cells were removed by rosetting twice with 2-aminoethylisothiouranium bromide (Sigma)-treated sheep erythrocytes. Nonrosetted cells (E−) were further purified by isolating B cells from the 40/50, 50/55, 55/65% interfaces of a discontinuous Percoll density gradient (Pharmacia, Uppsala, Sweden). The isolated cell population contained 90–95% surface Ig+ cells, and $CD3^+$(T), $CD13^+$ (granulocyte), and $CD14^+$ (monocyte) cells were less than 1%, as determined by flow cytometry.

Reagents

Rh-SOD (sp act 4700 IU/mg) (20) was donated from Nippon Kayaku Company (Tokyo, Japan). The enzymatic activity of the preparation was assayed according to the cytochrome c reduction test. Although the enzyme differs from naturally occurring SOD in human red blood cells only in that its N-terminal amino acid is not acetylated, it has no significant differences in physicochemical and spectrophotometric properties from the native SOD. The drug was dissolved in phosphate-buffered saline (PBS) and further diluted in medium before being used. *Staphylococcus aureus* Cowan I (SAC) and PMA were obtained from Calbiochem–Behring (La Jolla, CA) and Sigma, respectively. Recombinant human interleukin-2 (IL-2) was a generous gift from Shionogi Pharmaceutical Company (Osaka, Japan). Recombinant human interleukin-4 (IL-4) was purchased from Genzyme (Boston, MA). Affinity-purified anti-human SOD polyclonal antibody from sheep IgG fraction was obtained from the Binding Site Ltd. (Birmingham, UK).

Measurement of Lymphocyte Proliferation

Triplicate lymphocyte cultures were incubated in 96-well, flat-bottomed microplates. The culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA), streptomycin (100 μg/ml), penicillin (100 U/ml), and 2-mercaptoethanol (0.005 m*M*). One hundred thousand B cells were cultured in 200 μl of medium in the presence or absence of stimulators for 3 days at 37°C in 5% CO_2. The cells were pulsed with 1 μCi of [^{3}H]thymidine per cell during the last 18 hr of culture and were harvested with a multiple-cell harvester.

Detection of Immunoglobulin (Ig) Production

Ig production was estimated in a T-cell-independent system as described elsewhere (21). Briefly, high-density B cells were prestimulated with SAC for 48 hr, and then viable cells were collected by Ficoll–Hypaque density gradient. These B cells were recultured with rh-SOD and/or IL-2 for an additional 7 days at a cell density of 2×10^5 in 200 μl. The amount of Ig secreted in the culture was measured by enzyme-linked immunosorbent assay (ELISA).

Antibody Neutralization of SOD

Neutralization of rh-SOD activity with affinity-purified anti-human SOD antibody was performed as described elsewhere (20). Briefly, grading dilutions of anti-human SOD antibody were mixed with an equal volume of rh-SOD and incubated at 4°C for 1 hr before examination for the effect on B cells.

Northern Blot Analysis

Total RNA was isolated by the guanidine thiocyanate procedure and 20 μg of the RNA was electrophoresed through a 1.2% agarose gel and transfered to nylon membranes. Northern blot analysis with randomly primed ^{32}P-labeled DNA probe was performed as described previously (21). Plasmid containing the appropriate human cDNA probe was isolated by alkaline lysis and subjected to Sepharose CL-6B chromatography. An appropriate DNA insert was isolated from the plasmid by restriction endonuclease digestion and a fragment from plasmid pGEM containing human germline heavy-chain constant-region C1-C3. Autoradiographic signals were quantitated by densitometric scanning of

TABLE 1

Effect of rh-SOD on the Proliferation of SAC-Preactivated B Cells in the Presence or Absence of Costimulators

SOD (μg/ml)	None	IL-2	IL-4
0	5.44 ± 0.10	23.81 ± 0.37	16.21 ± 0.39
6	5.36 ± 0.06	22.22 ± 0.32	15.94 ± 0.46
30	5.50 ± 0.03	23.80 ± 0.52	16.31 ± 0.40
150	5.43 ± 0.04	22.46 ± 0.12	16.17 ± 0.05
750	5.25 ± 0.10	22.52 ± 0.24	15.65 ± 0.26

Note. High-density B cells from tonsillar samples were prestimulated with SAC for 3 days and then washed and recultured for an additional 4 days. IL-2 or IL-4 was added at the initiation of the secondary cultures. The proliferative response was estimated by [^{3}H]thymidine incorporation. The data show are the mean (cpm × 10^{-3}) ± SE of triplicate cultures.

Fuji photographs (Fuji photo film Co. Ltd., Kanagawa, Japan) of Northern blots taken under a LKB Ultrascan XL enhanced laser densitometer (Pharmacia).

Detection of Ig-Containing Cells

Ig-containing cells were detected by direct immunofluorescence. Cytocentrifuge preparations of each sample were made and cells were fixed in 95% ethanol and 5% glacial acetic acid at −20°C. After washing, cells were stained with fluorescein-conjugated goat-antihuman Ig. The percentage of brightly stained Ig-containing cells was determined using a fluorescein microscope. At least 400 cells were counted on each slide. The viable cell number did not differ significantly between samples.

RESULTS

Effect of rh-SOD on the Proliferative Response of B Lymphocytes

The effect of rh-SOD was investigated on the proliferative response of B lymphocytes *in vitro.* Percoll-fractionated low-, intermediate-, and high-density B cell populations were incubated in the culture medium supplemented with different concentrations of rh-SOD (6 to 750 μg/ml) for 3 days. The proliferative response of each density of B cells in the presence of SAC was increased in the culture supplemented with rh-SOD in a concentration-dependent manner (Fig. 1). Next, we examined the effect of rh-SOD on the activated B cells. *In vitro* preactivated B cells with SAC showed significant levels of spontaneous DNA synthesis in the subsequent 3-day cultures. Addition of IL-4 or IL-2 further enhanced it. Supplement of rh-SOD in the cultures did not show a positive effect on B cell proliferation (Table 1). Human B lymphocytes (2) can release oxygen radicals when stimulated with B cell mitogen. However, stimulation by IL-2 and IL-4 are ineffective for inducing superoxide production (10). The positive and negative results observed in our study appeared to correspond with the superoxide-generating activity of B cells to different stimuli.

Effect of Anti-SOD Antibody in rh-SOD-Induced B Cell Proliferation

To identify whether the enhancing effect of rh-SOD on the B cell proliferation was specifically induced by the action of rh-SOD, the rh-SOD (150 μg/ml) was treated with grading dilutions of affinity-purified anti-SOD polyclonal antibody for neutralization and they were employed for the experiment. Neutralized rh-SOD decreased their enhancing action on the B cell proliferation in proportion to the levels of dilution as shown in Fig. 2. The ratio of anti-SOD antibody to rh-SOD appeared to be optimal at 1:25 in our study. The result indicates that the increase of DNA synthesis in B cells is specifically induced by the enzymatic activity of rh-SOD.

Effect of rh-SOD on Ig Generation by B Lymphocytes

B cells were stimulated with SAC for 48 to 72 hr, then washed thoroughly and recultured in the pres-

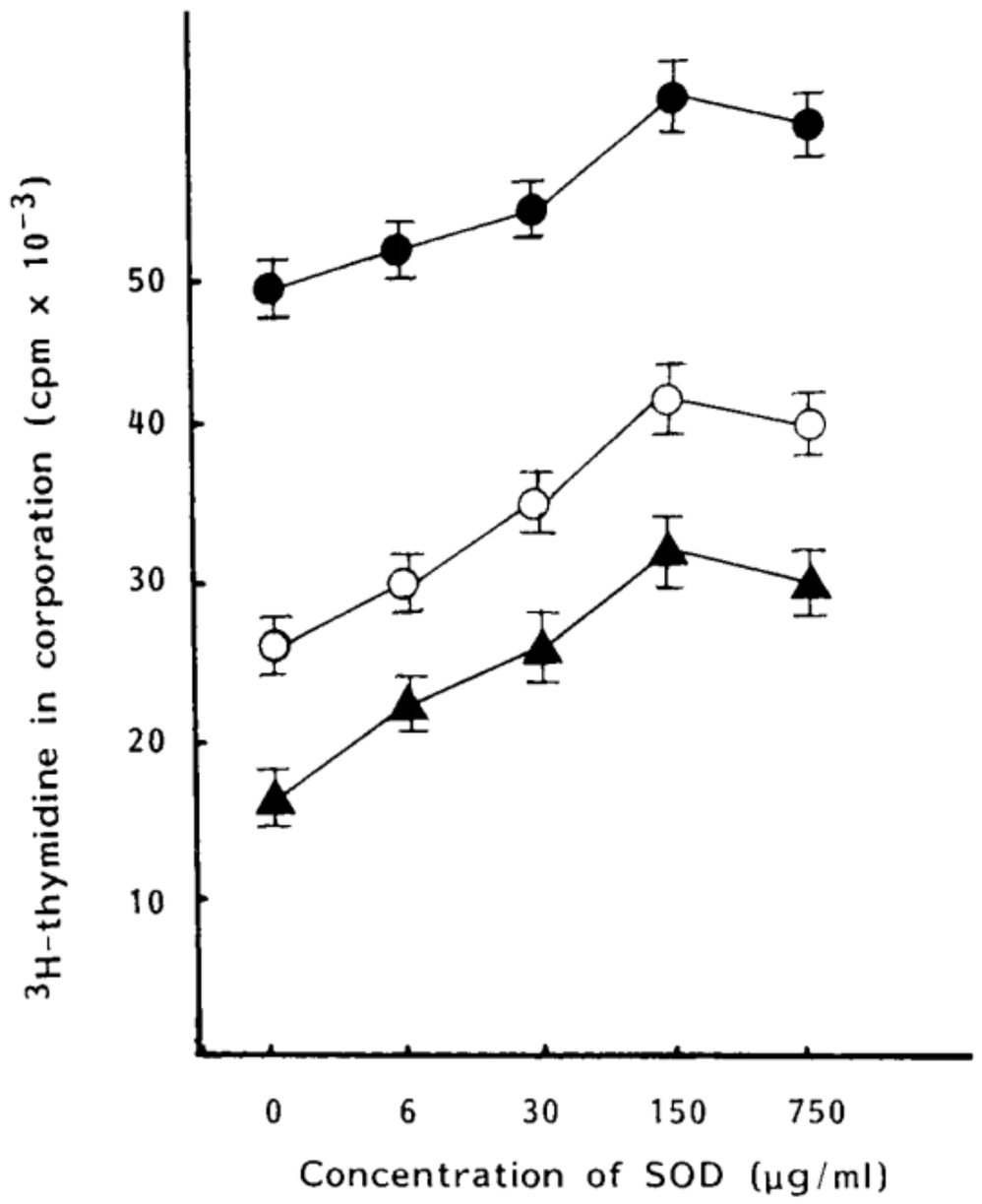

FIG. 1. Tonsillar B cells were fractionated by Percoll buoyant density gradient as described under Materials and Methods. Each density of B cells (40/50% interface, ▲; 50/55% interface, ○; 55/65% interface, ●) was cultured in triplicate for 3 days in the presence of SAC and various concentrations of rh-SOD were added. The cells were pulsed with [^{3}H]thymidine during the last 18 hr of culture. Values of [^{3}H]thymidine incorporation of SAC-unstimulated cells were less than 1000 cpm in the presence or absence of rh-SOD.

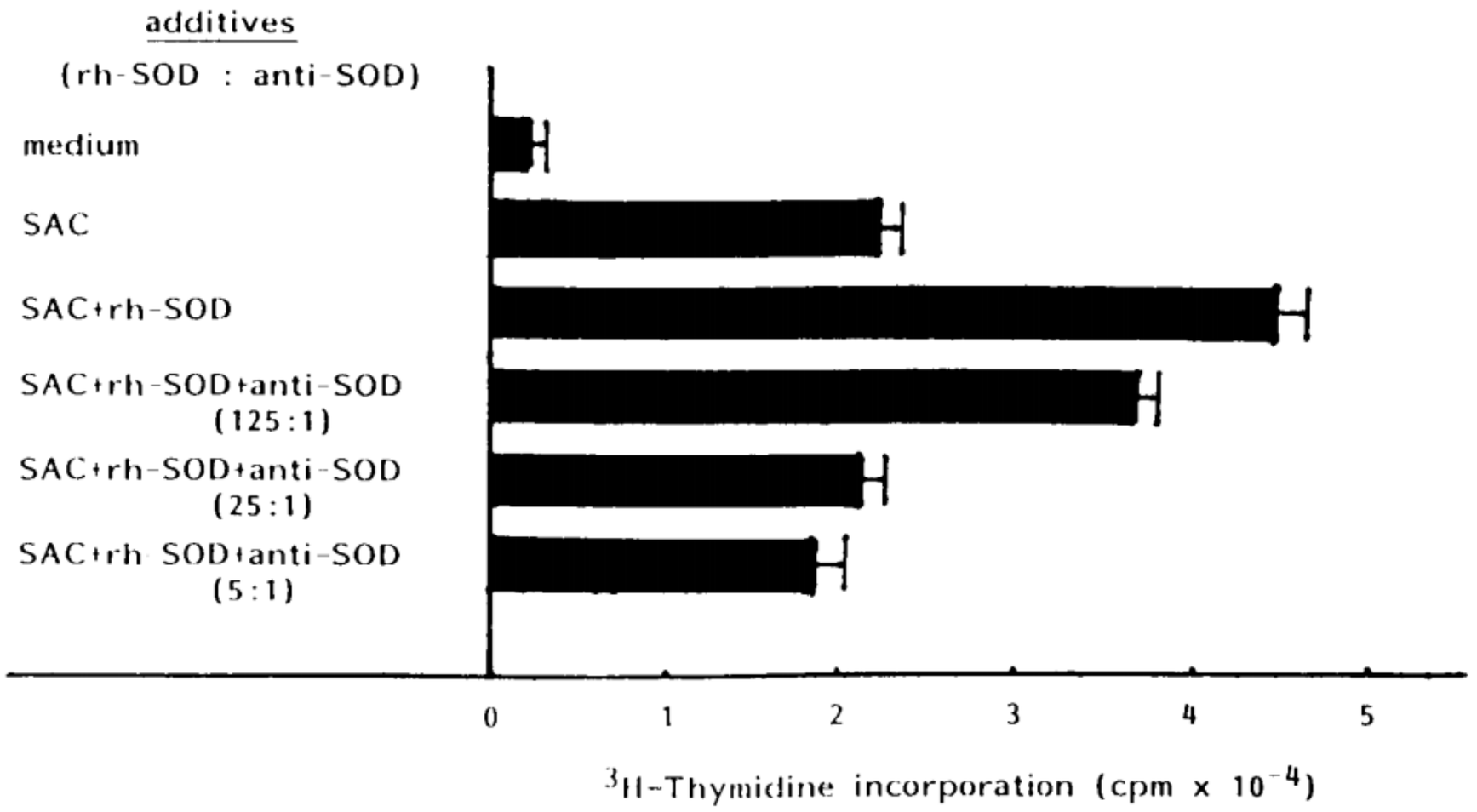

FIG. 2. Inhibitory effect of anti-SOD antibody on the rh-SOD induced proliferative responses. Tonsillar B cells were cultured for 3 days in the presence of SAC (1:10^5 v/v), rh-SOD, or neutralized rh-SOD with anti-SOD antibody. The proliferative response of B cells were estimated by [^{3}H]thymidine incorporation as described in the legend to Fig. 1.

ence of rh-IL-2 for another 7 days. The rh-IL-2 induced significant Ig production (IgM and IgG) in the SAC-prestimulated B cells. The addition of rh-SOD to the cultures at concentrations of 6 to 750 μg/ml resulted in the increase of both isotypes of Ig secretion, as shown in Fig. 3.

Time Course Analysis of the Action of rh-SOD on Ig Production

The rh-SOD (150 μg/ml) was added at different times in order to examine when rh-SOD exerted its effect on Ig production by B cells. The amount of Ig in the culture supernatant was measured after 7 days of culture (Table 2). When rh-SOD was added at the initiation of the 7-day culture, Ig secretion was increased. The effect of rh-SOD was rather prominent when added 4 days after the initiation. Rh-SOD was still effective even when added at the last day of the culture in this system. The data suggest that SOD acts on the B cells in the terminal stage of differentiation.

Frequency of Cytoplasmic Ig-Positive (cIg$^+$) Cells in the Presence of rh-SOD

The effect of rh-SOD on the frequency of cIg$^+$ cells was examined in parallel with the kinetic study. The preparation of cIg$^+$ cells was performed on the last day of a 7-day culture and analyzed by direct immunofluorescence. The results shown in Table 2 indicated that the effect of SOD was directed toward the B cells with antibody-producing capacity.

Abrogation of rh-SOD-Induced Ig Generation by Anti-SOD Antibody

It was investigated whether the anti-SOD antibody could block the effect of rh-SOD on Ig generation. Neu-

FIG. 3. Effect of rh-SOD on Ig generation by T-cell-independent system. SAC-preactivated B cells were cultured in the presence of IL-2 for 7 days. Amounts of IgG and IgM secreted in the culture supernatants were determined by ELISA. The results are expressed as the mean of triplicate culture supernatants and represent one of five separate experiments.

TABLE 2

Kinetics of the Effect of SOD on Ig Generation by B Cells

Additives	Days of treatment	Ig secretion (ng/ml) IgM	Ig secretion (ng/ml) IgG	cIg^+ cells (%)
Il-2	7	568	1135	10.2
Il-2/SOD	7	1135	2390	9.8
	5	1120	2400	11.2
	3	1159	2540	10.1
	1	1235	2602	11.5

Note. Tonsillar B cells were prestimulated with SAC for 2 days and the recultured for an additional 7 days in the presence of rh-IL-2. SOD was added to each culture at the time indicated.

tralization of rh-SOD with anti-SOD antibody at the ratio of 1:25 (anti-SOD antibody:rh-SOD) was prepared by the same procedure described above. The data showed that anti-SOD antibody was capable of abrogating the promoting effect of rh-SOD on Ig generation (Table 3). Therefore, the increase of Ig generation appeared to be a specific event induced by rh-SOD.

Increase of the μmRNA Level in rh-SOD-Treated B Cells

To examine at what level of Ig generation SOD exerts its action, we examined the μmRNA contents in B cells treated with IL-2 alone, or in combination with rh-SOD. Total cellular RNA was analyzed by using randomly primed ^{32}P-labeled DNA probes as described previously (21). The relative amounts of specific transcripts in B cells were determined by densitometric scanning. RNA extraction was performed 96 hr after stimulation, because only the secretory form of μmRNA (2.4-kb band) would be expressed after 4 days of culture

TABLE 3

Inhibitory Effect of Anti-SOD Antibody on rh-SOD-Induced Ig Response

IL-2	rh-SOD	Anti-SOD	IgG
–	–	–	308
–	+	–	337
–	–	+	289
+	–	–	1235
+	+	–	2230
+	+	+	1120
–	–	+	1159

Note. SAC-activated B cells (2×10^5 cells/well) were cultured for 7 days in the presence or absence of IL-2 (400 U/ml), rh-SOD (75 μg/ml), or neutralized rh-SOD antibody (×25 dilution). Amounts of Ig secreted in the culture supernatants were determined by ELISA. The values represent the mean of triplicate cultures from one of two different experiments.

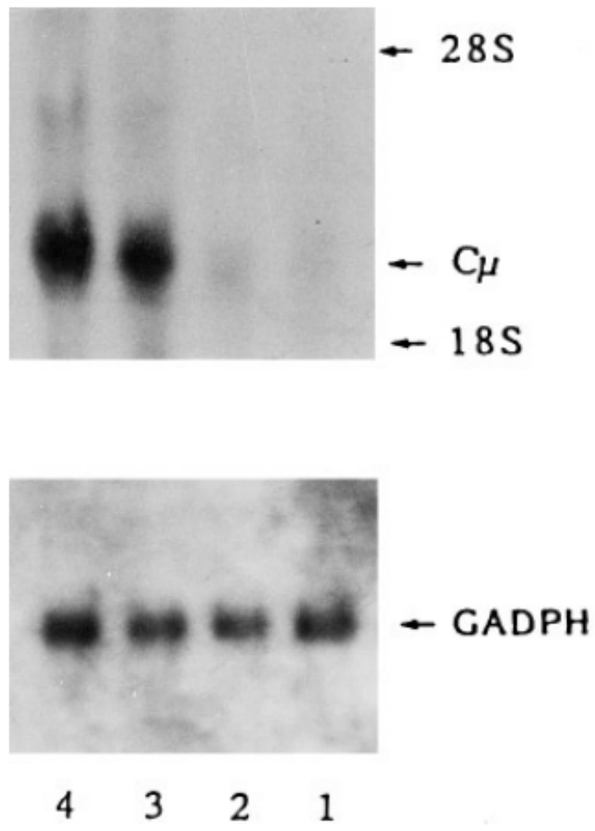

FIG. 4. RNA transfer blot analysis of mRNA levels isolated from SAC-activated B cells after 4 days of culture in the presence or absence of stimulators. Twenty micrograms of total RNA per lane was subjected to 1.2% agarose gel electrophoresis. Lane 1, medium alone; 2, rh-SOD (150 μg/ml) alone; 3, IL-2 (400 U/ml); 4, IL-2/rh-SOD.

in an Ig production system as described (22). As shown in Fig. 4, the cells treated with IL-2 alone showed the 2.4-kb band of the secretory form of μmRNA. In contrast, the amount of μmRNA was greater in rh-SOD/IL-2-treated cells, although some variations in the efficacy exist between individuals, as shown in Table 4. These findings indicate the increase of Ig production at the level of mRNA coding for IgM.

DISCUSSION

We have shown that the proliferation and differentiation of human B lymphocytes increased in the presence of SOD in culture. The effect of SOD on B cell proliferation was observed only when B cells were stimulated with agents which can induce superoxide-generating activity on B cells. Therefore, the enhancing effect of rh-SOD on B cell proliferation seems to be connected with its enzymatic activity as a scavenger of superoxide. In addition, Ig production of activated B cells increased with addition of SOD in a dose-dependent fashion. The effect of SOD was directed toward the B lymphocytes of terminal differen-

TABLE 4

Relative Amounts of μmRNA Isolated from SAC-Activated B Blasts

Culture condition	Exp.	Medium	SOD	IL-2	SOD + IL-2
Signal Intensity	1	0.040	0.048	0.272	0.577
	2	0.053	0.061	0.213	0.509

tiation stages. The enhancement was abrogated with treatment of anti-SOD antibody. As the process of resting B lymphocytes maturing into Ig-secreting cells consists of distinctive stages of proliferation and differentiation, the data presented here indicate that SOD may act independently on the different stages of B cells in proliferation and differentiation.

The mechanism underlying the relationship between CuZnSOD activity and B cell stimulation is not directly addressed by these experiments. Augmentation of B cell activation by SOD suggests either that superoxide is inhibitory to the activation or alternatively that the levels of H_2O_2 generated by SOD are stimulatory. H_2O_2 passes through the membrane and can act with cytoplasmic molecules such as DNA. It is thought that the growth enhancement of human bone marrow cells and murine phagocytic mononuclear cells by SOD might be mediated by the generation of H_2O_2 from O_2^-.(18, 19) H_2O_2 has an inhibitable effect on the proliferative response of T lymphocytes (9–12) although T lymphocytes do not have a superoxide-generating system (2). Indeed, SOD has no effect on mitogen-induced T cell proliferation (10, and our observation). H_2O_2 can be reduced to OH·, which reacts with various cellular components. Based on the ability of a variety of compounds, all of which share the property of scavenging OH·, it is possible that OH radicals are mediators for the transduction of mitogenic signals (10).

Some of the hydrogen radical scavengers are inhibitors on lymphocyte mitogenesis, but are potent inducers of cellular differentiation (23, 24). The induction of cell differentiation by these agents is related to their ability to scavenge free radicals.

The need for a high concentration of rh-SOD to enhance B cell activation observed in this experiment is also seen in the experiments of the myeloid progenitor cells (18). This suggests that cellular penetration and intracellular localization of exogenous SOD to detoxify cytoplasmic free radicals are less efficient (25). The effect of rh-SOD may be also explained by extracellular free-radical scavenging. Oxygen radicals generated in B cells might travel through the cell wall. The possibility that superoxide radicals can cross membranes has been demonstrated *in vitro* and *in vivo* (26, 27).

In this study, we investigated the effect of CuZnSOD on B cell activation. Although enzymatic activity of three SODs in the dismutation of O_2^- is not different, synthesis of CuZnSOD and FeSOD is constitutive whereas that of MnSOD is inducible (28). If MnSOD activity is abrogated by anti-MnSOD autoantibodies as observed in IM (17), it is likely to substitute CuZnSOD and FeSOD for MnSOD. The target cells of EBV infection have been considered to be exclusively B lymphocytes, which leads to the production of antibodies of various specificities, including antibody against MnSOD. On the other hand, EBV-transformed B cells are capable of generating oxygen species in response to the antigen stimulation. These facts suggest that the oxygen-generating property of B lymphocytes is connected with the B-cell-activation process. SOD activation in connection with B cell stimulation has been reported in patients with viral hepatitis (29).

Although the physiological role of SOD is primarily the protection of cells from the toxicity of O_2^-, our finding may indicate an accessory role of SOD.

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