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Immunosuppressive Activity of Bovine Seminal Ribonuclease and its Mode of Action.

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Abstract

Two preparations of dimeric BS RNase – native and recombinant proteins caused identical immunosuppressive effects on MLC-stimulated human lymphocytes. The monomers of RNase A and BS RNase were ten times less active. The inhibitory effect on MLC-stimmulation was followed by 90 % inhibition of cell-mediated lympholysis (CML) caused by BS RNase (10 μg/ml). This effect indicated that BS RNase suppressed the recognition phase of the cytotoxic reaction, resulting in inhibition of generation of cytotoxic effector cells. BS RNase exerted a similar effect on generation of cytotoxic LAK cells. Cytotoxic activity of LAK cells or CTLs against K562 target cells was abrogated only when BS RNase was added at the beginning of the sensitizing phase, but the cytotoxicity of effector cells in the destruction phase was not influenced. The effect of RNase A on the generation of cytotoxic cells was much less pronounced. To get more information about the site of action, the effect of BS RNase on early lymphocyte stimulation by PHA was investigated by using fluorescein cell probes. BS RNase (100 μg/ml) prevented a shift in fluorescein emission occurring within one hour of activation using fluorescein diacetate as a marker for changes in the cytoplasmic matrix. On the contrary, it did not block the shift in fluorescence emission when tested with diphenylhexatrien as a marker for changes in membrane fluidity. Furthermore the effect of BS RNase on expression of membrane antigens expressed on activated human lymphocytes was estimated. BS RNase significantly inhibited the expression of CD25, CD38 and CD71 antigens on PHA-, Con A- and MLC-stimulated human T and B lymphocytes. No substantial change in expression of these antigens was observed on IL-2-stimulated cells, but DNA synthesis was totally abrogated. These results indicate that the mode of action of BS RNase on activated T and B lymphocytes is based mainly on the suppressed expression of receptors for interleukin-2-α-chain and transferrin.

Introduction

In the 1970's and early 1980's we reported that BS RNase is a potent antispermatogenic agent (1) having both immunosuppressive (2, 3) and cytostatic activities (4, 5). Because of these extraordinary biological properties BS RNase has been ranked among the ribonucleases with a special biological action denoted as RISBASes (6). BS RNase has an eighty percent identity in amino acid sequence with bovine pancreatic ribonuclease (RNase A) which is a prototype of the superfamily of the secretory ribonucleases. Unlike BS RNase, RNase A exerts none of these special biological activities. The special biological properties of BS RNase were accounted to its dimeric form, since this structure is unique among the vertebrate RNase superfamily (6). Recently the DNA sequence of the gene encoding BS RNase was determined and recombinant preparations of BS RNase were synthetized (7, 8). We have found that the biological activities of this recombinant molecule (aspermatogenicity, antitumor, embryotoxic, and immunosuppressive effects) are identical with those of the native BS RNase preparation (9). These results confirmed our previous suggestion that BS RNase isolated from bovine seminal plasma alone is responsible for the biological activity without contribution of supposed impurities. The main interest of our laboratory has focussed on the immunosuppressive activity of BS RNase isolated from bovine seminal vesicles (1). BS RNase, at the concentration of 10 μg/ml, strongly inhibited the mitogen and antigen stimulation of human lymphocytes in culture (5, 10). *In vivo* studies showed that this substance suppressed regional GVH reaction and caused significant prolongation of skin graft survial in mice (3). In contrast, BS RNase did not show any adverse effects on hemopoietic cells in human bone marrow cultures (10).

The present study provides additional data on the immunomodulating effect of BS RNase and provides some information on its mode of action. In some expriments the effects of recombinant BS RNase and bovine pancreatic RNase A were compared. To obtain more data about the site of action we have followed the effect of BS RNase on early lymphocyte stimulation by PHA using fluorescein cell probes and we also tested the effect of BS RNase on expression of various surface antigens following mitogen and antigen stimulation of human T and B lymphocytes.

Materials and Methods

Bovine seminal ribonuclease (BS RNase) was isolated from bull seminal fluid by precipitation with acetic acid and ammonium sulphate and subjected to CM Sephadex C-50 and Sephadex G-100 column chromatography. The purity of BS RNase was determined by disc electrophoresis using an acrylamide gel, starch-gel electrophoresis and immunoelectrophoresis in agar gel by peak homogeneity at the last step of separation and by ultracentrifugation pattern (1). The monomeric and dimeric recombinant preparations of BS RNase were produced in E. coli strain BL21(DE3) and partially purified as described (7, 19). Bovine pancreatic ribonuclease (RNase A) was obtained from Boehringer GmbH,

Germany. Phytohemagglutinin (PHA) was obtained from Wellcome Diagnostics, England. Concanavalin A (Con A) was produced by Pharmazia, Uppsala, Sweden. Interleukin-2 (IL-2), a lyophilized lectin-free preparation was purchased from Biotest Co., Germany. Monoclonal antibodies (mAb) against human lymphocyte surface antigens were commercially purchased from Dako a/s, Denmark, or Immunotech s/a, France, as follows: FITC-conjugated anti-CD71 (transferrin receptor, clone Ber T/9, Dako), FITC conjugated anti-CD25/IL-2R alfa-chain, clone ACT-1, Dako) and FITC-conjugated anti-CD38 (clone T16, Immunotech) were used in the direct immunofluorescence (DI) assay, while anti-CD25 (clone ACT-1, Dako) was used in the indirect immunofluorescence (II) assay. Anti-CD45/FITC (clone J.33, Immunotech) was used as leukogate control, while anti-CD3/PE (clone UCHT1, Immunotech) and anti-CD19/PE (clone J4.119, Immunotech) were used for selective gating of T and B lymphocyte populations, respectively. Mouse anti-human IgG1/FITC and IgG1/PE (Immunotech), mouse anti-human IgG1/FITC and rabbit anti-mouse $F(ab)_2$/FITC (Dako) were used as negative isotopic controls and second immunofluorescence reagents, respectively. All cytometric studies were done on a Coulter Epics-XL flow calibrated cytometer with EPICS check fluorespheres (Coulter Corp., Hialeah, Florida, USA).

Preparation of lymphocytes

Lymphocytes used in the following experiments were isolated from heparinized peripheral blood of normal persons on Ficoll-Paque solution gradients (10). Lymphocytes withdrawn from the interphase of a gradient were centrifuged, washed twice with PBS solution and then resuspended in PBS or RPMI 1640 medium supplemented with 20 % of heat-inactivated mixed human AB serum and antibiotics.

Stimulation of human lymphocytes

Lymphocyte cultures were set up in triplicates in microtiter cultivation plates (type FB produced by A/S NUNC, Denmark). Briefly, 10^5 lymphocytes/well were cultured for three days with 5 µg/ml of PHA or 5 µg/ml of Con A and for 5 days with 50 U/ml of IL-2 in a total volume of 0.2 ml of RPMI 1640 medium at 37 °C in a humidified atmosphere of CO_2 (5 % v/v). In the two-way directed MLC reaction, 10^5 lymphocytes from each of two unrelated donors were mixed and cultured under the same conditions for 6 days. Four hours before the termination of culture, each sample was pulsed with 24 kBq of (6-^{3}H)-thymidine (Inst. for Resarch, Development and Application of Radioisotopes, Prague, specific activity 980 GBq/mmol) and then harvested in a Scatron cell harvester. The incorporated radioactivity into the newly synthetized DNA was measured in a beta-scintillation counter (Beckman). The mean values of triplicates expressed as counts per minute (cpm) were calculated. Suppression of DNA synthesis caused by RNases was expressed as the percentage of control cpm values.

Cell-mediated lympholysis (CML)

CML assay was performed according to the modified KRISTENSEN procedure (11). The effector cells were prepared in a one-way directed MLC of donor lymphocytes and recipient cells (irradiated by 20 Gray) mixed 1:1 in a microtiter plate (U-type). Target cells were prepared by 6-day incubation of donor lymphocytes which were stimulated beginning on the fourth day with PHA. These targets were pulsed with ^{51}Cr (produced by Du Pont, NEN Division, Germany) for 1 hour. In the second (destruction) phase, the cytolytic activity of effector cells was tested. A mixture of effector and target cells (40:1 or 20:1) was incubated for 4 hours. Then 100 µl of supernatant was carefully withdrawn and the released radioactivity was measured in a gamma counter. All cultivations were done in CO_2

(5 % v/v) humidified atmosphere at 37 °C. Cytolytic activity of effector cells was expressed as a percent of cytotoxicity according the formula ER-SR/MR-SR × 100. Experimental release (ER) represents the radioactivity released from cultures containing target and effector cells, spontaneous release (SR) is radioactivity released spontaneously in absence of effector cells, and maximum release (MR) represents the release of radioactivity after destruction of target cells by detergent.

Cytotoxic activity of LAK cells was estimated and calculated under the same conditions as mentioned in the CML assay. The effector cells were prepared by 5-day incubation of normal human lymphocytes with IL-2 (50 U/ml) and their lytic activity was tested against K562 cells as targets at an E:T ratio 20:1.

Assay of fluorescence polarization in activated lymphocytes

The lymphocyte suspension in PBS (4×10^6/ml) was incubated for 30–60 minutes under various conditions:

A. control cells in PBS,
B. cells with a standard solution of PHA (50 µl/ml),
C. cells with BS RNase (100 µg/ml),
D. cells with PHA (50 µl) + BS RNase (100 µg/ml).

The events occurring in cytoplasm were followed by fluorochromasia with fluoresceindiacetate (FDA) which is actively transported into cytoplasm where it is hydrolysed into a luminescent product-fluorescein (12). Briefly, 0.5 ml of lymphocyte suspension was mixed with 2 ml of FDA solution in PBS (5×10^{-7} M/l) and quickly transferred into the spectrofluorometric cuvette at a temperature of 25–26 °C. The intensity of luminescent emission was immediately measured following 10 minutes at the excitation wave length of 470 nm and emission wave length of 510 nm. The events occurring on the cell membrane were detected by 1,6-diphenyl-1,3,5-hexatriene (DPH). DPH is one of the most efficient fluidity probes available for the hydrocarbon region of lipids whose fluorescence quantum yields related to environment polarization. Changes in polarization emission, occurring during cell stimulation, can be interpreted as changes in cell membrane fluidity (13). Briefly, 1 ml of lymphocyte suspension was mixed with 1 ml of DPH solution in PBS (2×10^{-6} M/l). Changes in membrane fluidity were slower than those in the cytoplasm, thus the measurement was carried on for 45 minutes at an excitation wave length of 365 nm and emission wave length of 425 nm.

Cytometric studies

Human lymphocytes isolated on Ficoll-Paque solution gradient from heparinized blood of normal individuals and suspended in RPMI 1640 medium (10^6/ml) were activated by PHA, Con A, IL-2 or in MLC reaction as described above. These lymphocyte suspensions were washed twice and resuspended in PBS to the final concentration of 10^6/ml. Briefly, 10 µl of individual mAb was added to 100 µl of lymphocyte suspension and incubated in the dark for 15 min at 20 °C. After two washes with RPMI medium, the cell pellet was resuspended with PBS to the original concentration for FACS analysis. In the indirect immunofluorescence assay, 10 µl of rabbit anti-mouse $F(ab)_2$ FITC was added to the cell pellet followed by 15-min incubation in the dark. After two washes in PBS, cells were examined for fluorescence in Coulter EPICS-XL flow cytometer. FS, SSC, log.FL1, log.FL2 data for 10,000 events in the acquisition gate were saved in list mode data. Final analysis was performed using Coulter analysis software, version 1.5.

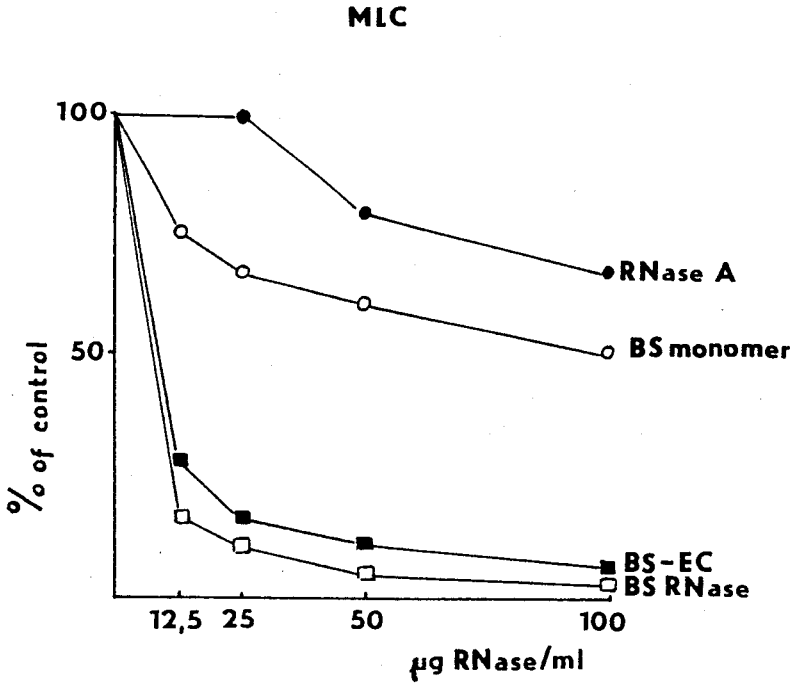

Figure 1. Effect of BS RNase from bovine seminal plasma (□), BS RNase from E. coli (■), BS RNase monomer (○) and RNase A (●) on the proliferation of MLC-stimulated human lymphocytes in 6-day culture. Each point is the mean of 3 replicates from 3 experiments and it is reported as a per cent of the control, which was cultivated without ribonuclease. The actual values for ^{3}H-thymidine incorporation in MLC-stimulated lymphocytes ranged from 42,000 to 78,000 cpm/well.

Results

Effect of RNases on MLC-stimulated lymphocytes

Previously we reported that dimeric BS RNase prepared from bovine seminal vesicles displayed a remarkable immunosuppressive activity both *in vitro* and *in vivo* (2, 3, 5, 9). Here we have examined the effect of this preparation on MLC-stimulated human lymphocytes and compared it with the effect of two recombinant preparations (BS RNase dimer and monomer) and monomeric RNase A. As shown in Figure 1, both dimeric RNases exerted strong inhibitory effects on lymphocyte proliferation, whereas the activity of both monomers was not pronounced.

The effector cells generated in one-way directed MLC reaction became highly cytotoxic against the stimulating lymphocyte population. This reaction, designated as cell-mediated lympholysis (CML), was abrogated by BS RNase at the concentration of 20 µg/ml (Fig. 2). The inhibitory effect occurred only in case when BS RNase was added at the beginning of MLC re-

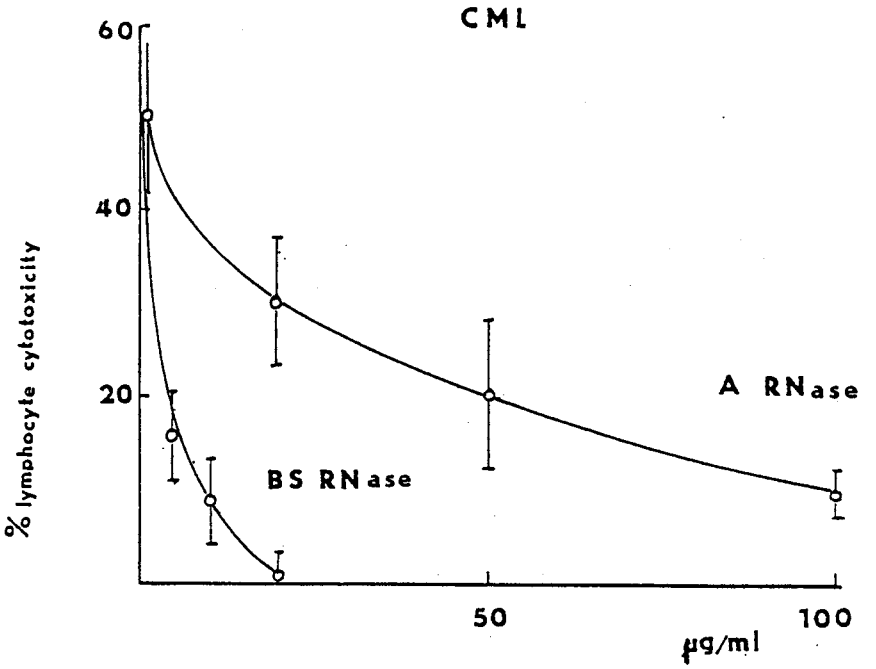

Figure 2. Inhibitory effect of BS RNase and RNase A on cytolytic activity of effector cells in CML reaction. Each point represents the mean of 3 replicates ± SD from 3 experiments.

action, which resulted in reduced production of cytotoxic effector cells. The lytic activity of effector cells was not suppressed by BS RNase added prior to the lytic phase (Table 1). These experiments indicated that BS RNase sup-

Table 1. Effect of BS RNase on the sensitizing or destructive phase of CML reaction.

Effector cells (MLC)	Specific combination Targets	Lysis %	Control combination Targets	Lysis %
AB_x	B	50.7	A	5.4
AB_x + BS RNase	B	12.2	A	3.5
AB_x	B + BS RNase	46.0	A + BS RNase	5.3
CD_x	D	38.9	C	8.2
CD_x + BS RNase	D	3.8	C	5.7
CD_x	D + BS RNase	40.8	C + BS RNase	4.0

A combination of lymphocytes from 4 normal unrelated persons (A, B, C, D). Stimulating lymphocytes were irradiated with 20 Gy using cobalt source (x). BS RNase (10 µg/ml) was added either to the sensitizing phase (MLC) or to the destructive (lytic) phase of CML reaction. Target cells were 3-day old PHA blasts derived from the stimulator donor lymphocytes.

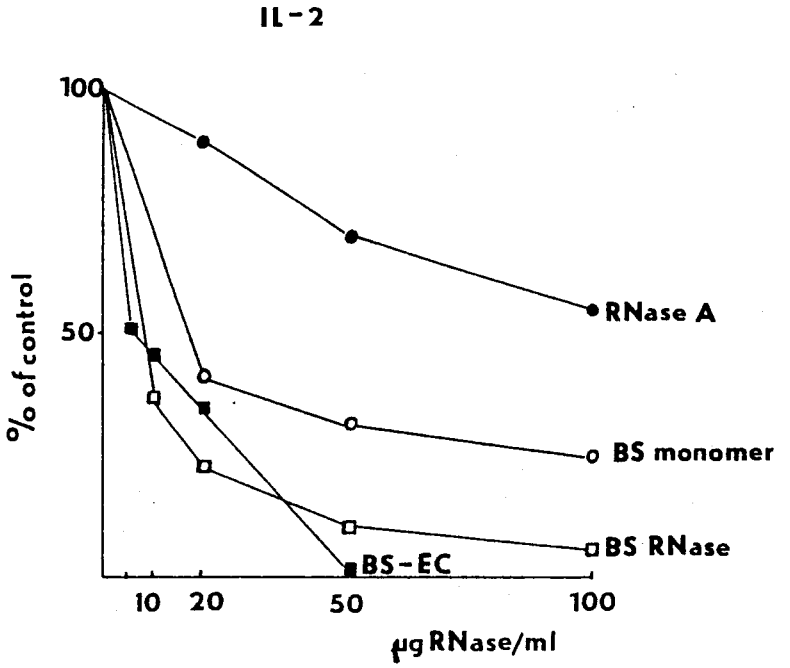

Figure 3. Effect of BS RNase from bovine seminal plasma (□), BS RNase from E. coli (■), BS RNase monomer (○) and RNase A (●) on the proliferation of IL-2-stimulated human lymphocytes in a 5-day culture. Each point is the mean of 3 replicates from 3 experiments and is reported as a percent of the control, which was cultivated without ribonuclease. The actual values for ^{3}H-thymidine incorporation into IL-2-stimulated lymphocytes ranged from 32,000 to 59,000 cpm/well.

presses only the recognition phase resulting in reduced production of cytotoxic cells. It is well known that T cells require IL-2 for their proliferation and that in presence of IL-2 very active killer cells can be generated from cultured lymphocytes (14). Our tested RNase preparations blocked the stimulating effect of IL-2 on lymphocyte proliferation similar to the MLC blocking. Both dimeric BS RNases used at a concentration of 50 µg/ml suppressed lymphocyte proliferation by 90 % and 100 %, respectively, whereas the inhibition by monomeric preparation (M) and RNase A was 70 % and 30 % (Fig. 3). Cytolytic activity of LAK cells, generated after a 5-day incubation of lymphocytes with IL-2 and tested against K562 target cells, was abrogated by BS RNase (20 µg/ml) when added at the beginning of the culture (Figure 4). The effect of RNase A was again much less pronounced. Adding of BS RNase to the generated LAK cells did not influence their lytic activity even when five-fold higher doses of BS RNase were used (Table 2).

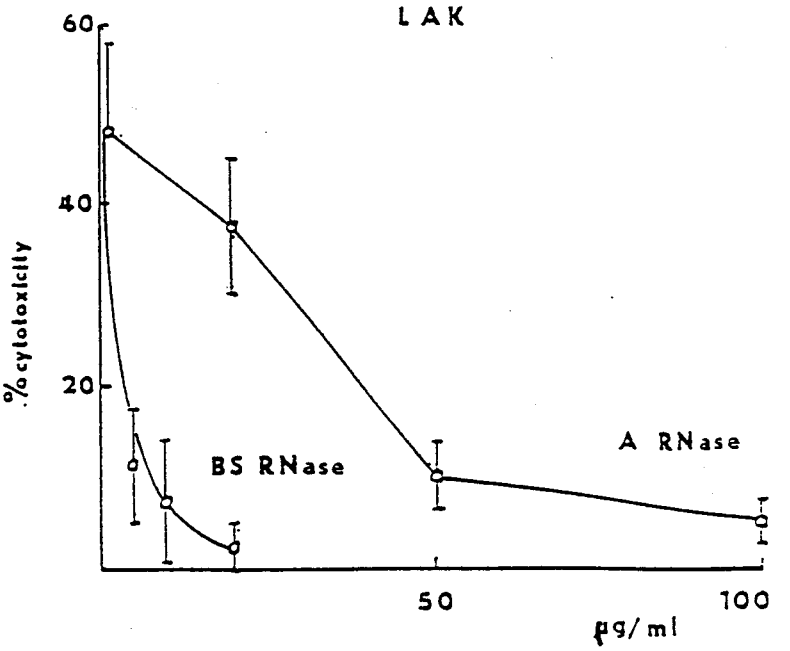

Figure 4. Inhibitory effect of BS RNase and RNase A on generation of lymphokine-activated killers (LAK cells). Cytotoxic activity of LAK cells was estimated against K562 cells as targets at a ratio of E:T 20:1 in a 6-day culture. Each point represents the mean of 3 replicates ± SD from 3 experiments.

Effect of BS RNase on fluorescence polarization in activated lymphocytes

To obtain better knowledge about the site of action of BS RNase we have used the method of fluorescence polarization with two fluorescein cell probes. The events occurring in cytoplasm were followed with fluorescein-

Table 2. Effect of BS RNase on the sensitizing or destructive phase of LAK cell cytotoxicity generation.

At the beginning of the experiment	After a 5-day incubation	Cytotoxicity %
Il-2	–	79.1
Il-2 + BS $RNase_{50}$	–	13.2
Il-2	BS $RNase_{50}$	81.5
Il-2	BS $RNase_{250}$	78.2

Interleukin-2 (IL-2 50 U/ml) was added to human lymphocyte culture at the beginning of the experiment. BS RNase (50 or 250 μg/ml) was added either at the beginning (sensitizing phase) or after 5-day incubation 2 h before the destructive phase with ^{51}Cr-labelled K 562 cells as targets. Effector:target ratio was 20:1.

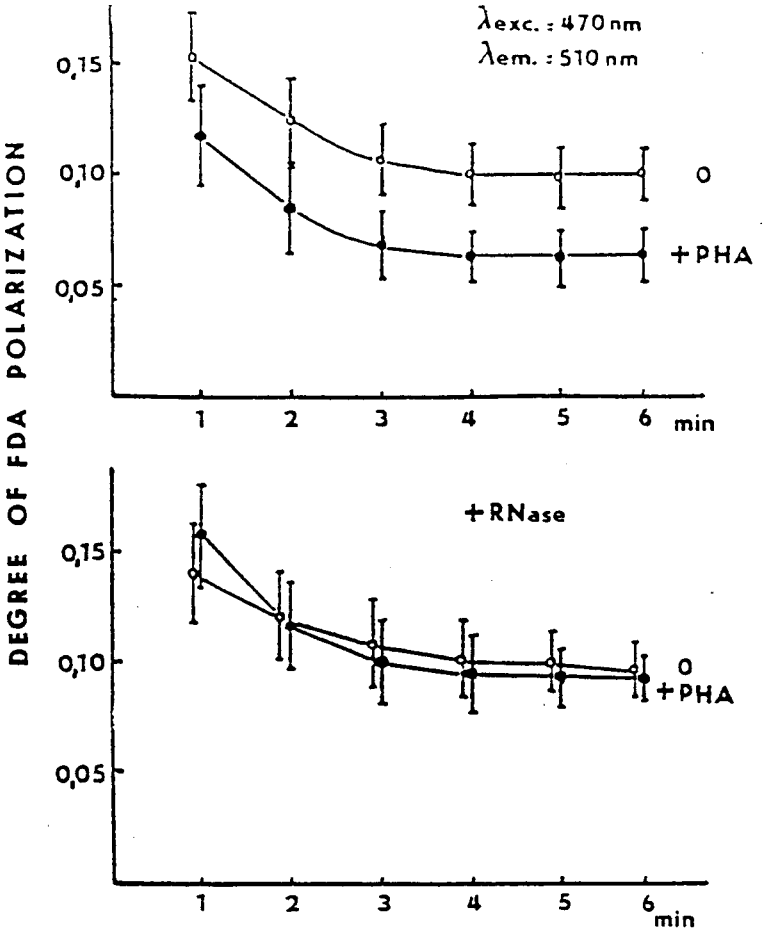

Figure 5. Effect of BS RNase (100 μg/ml) on the shift in fluorescence emission was measured after 1-h preincubation of normal human lymphocytes with PHA. Fluorescein diacetate (FDA) was used as a marker for changes occurring in cytoplasmic matrix.

diacetate (FDA) and the changes in membrane fluidity were detected by diphenylhexatrien (DPH). The degree of polarization of intracellular fluorescein decreases when lymphocyte in steady state (G_0 or G_1) enter into the cell cycle (15). PHA added to the lymphocyte culture 60 min before measuring caused the shift in luminescence emission comparable to the culture of non-stimulated lymphocytes. The course of fluorescein polarization and its decrease after 60 min preincubation with PHA is demonstrated in the upper part of Figure 5. The lower part of Figure 5 shows the effect of BS RNase added to PHA or administered alone. The data of this experiment shown in Figure 5 demonstrate clearly that BS RNase prevents a shift in fluorescence emission occurring within one hour of activation as tested with fluorescein

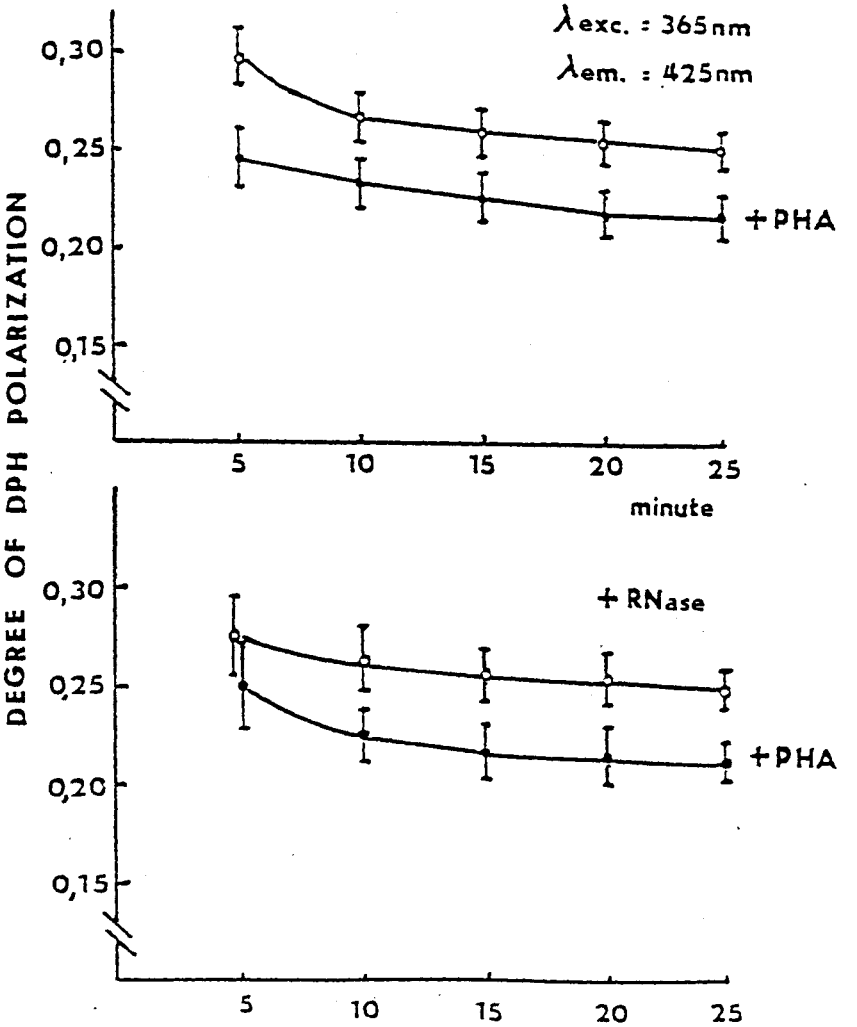

Figure 6. Effect of BS RNase (100 μg/ml) on the shift in fluorescence emission was measured after 1-h preincubation of normal human lymphocytes with PHA. 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a marker for changes occurring in membrane fluidity.

diacetate (FDA) as a marker for changes in the cytoplasmic matrix. In contrast, BS RNase did not inhibit the shift in fluorescence emission when tested with DPH, as a marker for changes in membrane fluidity (Fig. 6).

Table 3. Effect of BS RNase on the expression of surface antigens on activated human lymphocytes.

Stimulator	BS RNase 100 µg/ml	% of positive cells CD25	CD38	CD71	CD45
PHA	–	55	45	27	98
	+	13	31	11	98
Con A	–	83	48	68	98
	+	32	26	22	98
MLC	–	22	40	10	N.D.
	+	7	26	4	N.D.
IL-2	–	17	39	17	N.D.
	+	14	33	12	N.D.

Peripheral human lymphocytes isolated on Ficol-Paque solution were cultured in RPMI 1640 medium at 37 °C in a humidified atmosphere of 5 % CO_2 with PHA (25 µg/ml), Con A (25 µg/ml) and IL-2 (50 U/ml) for 3 and 5 days, respectively; MLC-stimulated lymphocytes were cultured under the same conditions for 6 days. All examinations were done without or with BS RNase (100 µg/ml) added at the beginning of cultivation. Primarily labelled mAb anti-CD25, anti-CD38, anti-CD71 or CD45 (10 µl/ml) were added for 15 min and after two washes with PBS cytofluorometric assay was performed. The values are the mean from two experiments. In each sample 10,000 cells were analyzed.

Effect of BS RNase on expression of membrane antigens CD25, CD38 and CD71

Human lymphocytes stimulated by various mitogens undergo blastic transformation followed by expression of some membrane antigen. Previously we have found that synthesis of new DNA in mitogenic or antigenic stimulated human lymphocytes was very potently inhibited by BS RNase (3, 10). Here we tested BS RNase effects on expression of membrane antigens CD25, CD38 and CD71 expressed on PHA-, Con A-, IL-2- or MLC-stimulated human lymphocytes. MAb anti-CD45 was used as leukogate control. As shown in Table 3, BS RNase potently inhibits expression of all three activation antigens on PHA- and Con A-stimulated lymphocytes. The effects on expression of these surface antigens in MLC-stimulated lymphocytes were less pronounced but inhibition also occurred. In contrast, no substantial change in expression of the tested surface antigens was caused by BS RNase on IL-2-stimulated lymphocytes, while DNA synthesis in these cells was abrogated (see Fig. 3). Furthermore selective gating of T ($CD3^+$) and B ($CD19^+$) lymphocytes demonstrated strong suppressive effect of BS RNase simultaneously on both T and B lymphoid cell subpopulations, without any significant difference. The effect of RNase A on activation antigens was not pronounced (Table 4). These results indicate that the mode of action of BS RNase on T and B lymphocytes is based primarily on an inhibitory effect on the expression of surface antigens for IL-2-α-chain and transferrin receptors.

Table 4. Effect of BS RNase and RNase A on the expression of surface antigens on activated T and B lymphocytes.

RNase 100 μg/ml		% of positive T cells ($CD3^+$) CD25	CD38	CD71	% of positive B cells ($CD19^+$) CD25	CD38	CD71
BS RNase	–	85.8	72.1	55.8	86.5	68.4	72.1
	+	22.4	17.5	12.1	30.2	9.5	6.4
RNase	–	87	88	82	39	34	21
	+	88	89	80	30	34	37

Human lymphocytes (10^6/ml) were activated with Con A (25 μg/ml) and cultured in RPMI 1640 medium without or with BS RNase or RNase A (100 μg/ml), respectively, for 3 days. Then mAb (10 μl/10^6 cells) were added for 15 min and after two washes with PBS cytofluorometric assay was performed. T and B lymphocytes were selectively gated using anti-CD3 and anti-CD19 mAbs and the expression of activation antigens was analyzed individually on the basis of double-stain labelling.

Discussion

The immunosuppressive properties of mammalian seminal plasma has been reported by many authors (16–18). Immunosuppressive activity of human seminal plasma and its clinical significance was also described in a review article (19). Bovine seminal ribonuclease is one of the numerous components present in seminal fluid which are supposed to be responsible for immunosuppressive activity. But its occurrence in bull seminal plasma is quite unique since pig and human seminal plasma do not have detectable amounts. Its position is also unique among the other members of the superfamily of secretory ribonucleases. It is the only one which exerts biological properties including aspermatogenic, antitumor, embryotoxic and immunosuppressive activites and it is the only one which occurs in dimeric form (6). Recently, determination of the DNA sequence of BS RNase gene enabled the preparation of recombinant BS RNase molecules and a variety of mutants (7, 8, 9, 24). The recombinant preparations showed identical biological activities to the native preparation from bull seminal plasma. The lower activity of some recombinant preparations may depend on the ratio of two forms i.e. biologically more active M × M and a less active M = M form which create an equilibrium under various conditions (9).

Some authors reported that the dimeric form is essential for biological activities of RNases (9, 20, 21). This is probably true for the antitumor or immunosuppressive effects of RNase A or BS RNase, but our recent immunosuppression studies with angiogenin (22) and some BS RNase mutants showed that monomeric ribonucleases also can exert a remarkable immunosuppressive or aspermatogenic effects (unpublished data). In contrast, dimeric BS RNase, in which His119 was changed to an aspartic acid, lost both the catalytic and the biologic activities (24). This finding correlates with other authors who also reported that the catalytic activity is essential for the

special biological activities of RNases (21, 23). Another member of the RNase A superfamily, onconase, is a monomeric RNase which also express a remarkable antitumor activity and is now in the course of clinical trials for cancer therapy (23). The recent experiments provided with onconase and various RNase mutants in combination with retinoic acid resulted in an immense increase of RNase cytotoxicity. The authors suggested that RNases reach the cytosol through a disrupted Golgi apparatus more efficiently (23). However, we did not see any potentiation of BS RNase immunosuppressive activity in combination with all-trans retinoic acid (unpublished data).

Our previous *in vitro* and *in vivo* studies (3, 5, 10) showed that BS RNase is a potent immunosuppressive agent. The present results supported those studies and also provide an explanation for some BS RNase modes of action. The generation of cytotoxic T lymphocytes in a CML reaction and the generation of cytotoxic LAK cells was abrogated by BS RNase (10 μg/ml) when it was added at the beginning of lymphocyte culture. The generated cytotoxic activity could not be suppressed by 20–30fold higher concentrations of BS RNase. These results correspond with the impotency of BS RNase for affecting NK and ADCC cytotoxicity that we reported previously (10). They indicate that BS RNase suppresses the recognition phase of the cytotoxic reaction resulting in reduced production of cytotoxic effector cells, but it does not influence the lytic reaction occurring in the destructive phase, even when at much higher concentrations.

RNase as an enzyme of nucleic acid metabolism may likely attack RNA's generated during mitogenic or antigenic stimuli of cell proliferation, including newly synthetized messenger RNA. We have found that DNA synthesis in PHA- or IL-2-stimulated lymphocytes was suppressed even when BS RNase was added to the culture 48 h after the mitogen (5). In contrast, we never saw any inhibitory or masking effects on the cell membrane. BS RNase (100 μg/ml) did not inhibit formation of SRBC rosettes with T and/or B cells from a normal donor or a patient with chronic lymphatic leukemia (25) nor did it affect binding of monoclonal antibodies to various differentiation membrane antigens or polycloncal antibodies binding to MHC antigens in pigs (unpublished data). Therefore we used the method of fluorescence polarization with fluorescein cell probes to test events occurring on the membrane and in the cytoplasm. This technique confirmed our previous finding that BS RNase enters the cell within a very short time (30–60 minutes) and acts inside the cell.

On the basis of our experiments with IL-2 we suggest that BS RNase can also influence the production of IL-2 or the expression of IL-2 receptors by activated lymphocytes. The experiments monitoring the expression of CD25, CD38 and CD71 antigens on mitogen- and antigen-activated T and B lymphocytes have shown that BS RNase inhibited significantly the expression of all three activation antigens, whereas the inhibition by RNase A did not occur. The inhibitory effect on the expression of the IL-2-α-chain receptor (CD25) correlates with the findings of TAMBURINI (21) who demonstrated a drastic inhibition of expression of IL-2-α chain receptor on OKT3-

stimulated lymphocytes whereas the secretion of IL-2 by T cells was not affected. However, other findings of ours differ from TAMBURINI's in two aspects. First, we have seen inhibition of PHA- or IL-2-induced lymphocyte proliferation still after a 24 or 48 h delay of BS RNase administration to the cell culture and second, adding of BS RNase monoclonal antibody with BS RNase did not abrogate the immunosuppressive activity (unpublished data). A similar picture, showing an unchanged aspermatogenic activity of BS RNase in the presence of high titer of BS RNase polyclonal antibody as detected in mice, was also reported (26).

Our previous data (3, 5, 10) and the results provided here indicate that both bovine seminal RNases, prepared from seminal fluid or from E. coli by a recombinant technique, represent potent immunosuppressive agents interferring with various immune reactions. BS RNase enters the target cell within an hour and abrogates mitogen- or antigen-induced immune response of human T and B cells and simultaneously blocks generation of cytotoxic T cells. Finally our cytometric studies demonstrate that the inhibition of antigen- and mitogen-induced T and B lymphocyte proliferation is based mainly on a suppressed expression of the IL-2-α-chain and transferrin receptors.

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