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NONRESPONSIVENESS OF IMMATURE B LYMPHOCYTES TO ANTI-IMMUNOGLOBULIN IS REVERSED BY PRONASE

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SUMMARY: Splenic B cells are induced to proliferate upon culture with antibody having specificity for surface membrane immunoglobulins. Cells treated with pronase, washed and then cultured with antibody, exhibited a >5-fold enhancement of DNA synthesis whereas pronase treatment, per se, was not mitogenic. The pronase effect exhibited specificity in that the induction of proliferation with either lipopolysaccharide or dextran sulfate was not enhanced by prior enzyme treatment. Cells from mice at two weeks of age which essentially do not show a proliferative response to antibody become responsive subsequent to pronase treatment. These results are interpreted to suggest a possible growth regulatory role for the pronase sensitive surface membrane component.

Antibodies with specificity for surface membrane immunoglobulins trigger proliferation of B lymphocytes. Nevertheless, splenocyte populations from young mice (about 2 weeks old) as well as from adult X-chromosome linked immunodeficient mice essentially do not exhibit a proliferative response to these same anti-immunoglobulin preparations (1,2). However, the fact that lymphocytes from immunodeficient mice show a substantial proliferative response to anti-immunoglobulin covalently linked to Sepharose (3) establishes that the entities necessary for triggering proliferation in such mice are in place on the surface membrane. To assess whether or not enzymatic digestion of surface membrane materials could alter the responsiveness or unresponsiveness of cells to anti-immunoglobulin, we treated splenic lymphocytes with pronase. In this report, we present evidence demonstrating that nonresponsive cells from immature mice become responsive subsequent to pronase treatment.

MATERIALS AND METHODS

Animals. Female mice (C3H/HeN) at 2-3 and 6-8 weeks of age were obtained from the NIH animal care center.
Reagents. Pronase, dextran sulfate and HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) were purchased from Sigma Chemical Co., St Louis, MO.

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Lipopolysaccharide derived from Salmonella abortus equi was supplied by Difco Laboratories, Detroit, MI. Antibody with specificity for mouse IgM (μ chain specific) either affinity purified or approximately 40 percent pure and prepared in goat was obtained from Cappel Laboratories, Cochranville, PA. Medium 199 was purchased from Grand Island Biological Co., Grand Island, NY. Fetal calf serum was supplied by M. A. Bioproducts, Walkersville, MD. [^{3}H]-Thymidine (6.9 Ci/mmole) was a product of New England Nuclear Corp., Boston, MA.

Cell isolation and culture. Single cell suspensions of spleen were isolated (4) in medium 199 buffered with HEPES to 0.02 M at pH 7.5 and supplemented with penicillin to 35 μg/ml. Splenocytes (5 x 10^5/0.2 ml) were cultured in triplicate for 48 hours in 96 well flat bottom plates (Costar 3596) with goat anti-mouse IgM, lipopolysaccharide or dextran sulfate as indicated. Cultures were pulsed for the last 24 hours by adding 1.0 μCi of [^{3}H]-thymidine. The cells were harvested on filter paper using a cell harvester (Skatron, Flow Laboratories, Rockville, MD) and the radioactivity assessed in a liquid scintillation counter.

Pronase treatment. Ten million splenocytes were suspended in 1.0 ml of HEPES buffered medium 199 with and without 1.5 mg of pronase. The cells were incubated for 1.0 hour at 37°C, washed twice at room temperature with medium 199 containing 10 percent fetal calf serum and finally resuspended in medium 199 supplemented with fetal calf serum to 10 percent and β-mercaptoethanol to 50 μM.

RESULTS AND DISCUSSION

The evidence that lymphocytes from immature mice (2 weeks old) acquire responsiveness to anti-immunoglobulin subsequent to a brief treatment with pronase prior to cell culture is shown in Table 1. Although cells from young adult mice (6-8 weeks old) were responsive in part, there was a marked enhance-

Table 1
Pronase treatment of splenocytes from two week old mice alleviates their proliferative unresponsiveness toward anti-immunoglobulin

Age	Prior cell treatment	[^{3}H]-Thymidine incorporation without anti-immunoglobulin	with anti-immunoglobulin
weeks		cpm	
2	None	9,860 ± 1,080	9,120 ± 600
2	Pronase	11,820 ± 510	113,390 ± 9,330
6-8	None	7,840 ± 560	25,380 ± 540
6-8	Pronase	7,570 ± 800	98,490 ± 1,140

Cells were incubated without or with pronase then washed, cultured and processed as described in Materials and Methods. Where indicated, 200 μg of the $F(ab')_2$ moiety of goat IgG having specificity for mouse IgM was added per culture well. The results are expressed as the mean of triplicate samples ± SEM.

ment in responsiveness after pronase treatment. It is noteworthy that the extent of the proliferative response of pronase treated cells derived from 2 week old and from 6-8 week old animals was quite similar indicating that the stimulation in two week old mice was not a marginal effect.

Cells incubated briefly with pronase exhibited increased ^{3}H -thymidine incorporation only when anti-immunoglobulin was present during the cell culture period (Fig. 1). That pronase was not present and or functional during the culture period as a result of a possible carry-over from the initial incubation was demonstrated by experiments showing that pronase treatment at 3°C rather than at 37°C was without effect on proliferation. In addition, some of our observations are consistent with the view that pronase functions as a consequence of its proteolytic properties. Thus, if aprotinin, a protease inhibitor, or an excess of ovalbumin was included in the pronase incubation mixture there was no enhancement of thymidine incorporation. In this context it is worthy of emphasis that lymphocytes treated with pronase lose their surface membrane immunoglobulin which then reappears after removal of the pronase and upon cell culture (5).

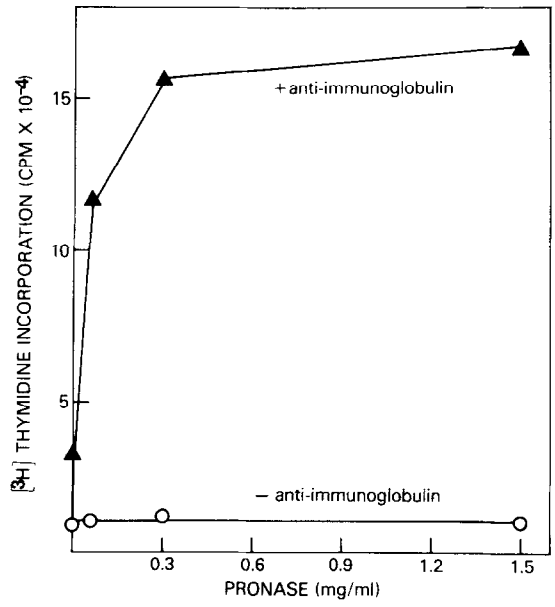

Figure 1. Effect of varying the concentration of pronase. The cells were incubated without or with the indicated concentrations of pronase, washed and then cultured and processed as described in Materials and Methods.

Because the cell populations employed are heterogeneous, it is possible that pronase treatment affects cells in the mixture other than B cells and thereby indirectly converts unresponsive to responsive B cells. To test this possibility, untreated cells were cultured with cells treated briefly in sequence with pronase and then with mitomycin C so as to irreversibly inhibit DNA synthesis by the pronase treated cells. This cell mixture yielded less than 15 percent of the enhanced proliferation that resulted from pronase treatment (data not shown). These results are consistent with the view that the effect of pronase is a result of its action directly on B cells.

The specificity of the effect of pronase is shown in Table 2. While there was a marked proliferative response in the case of anti-immunoglobulin there was no effect of pronase on the response by two other B cell reactive mitogenic agents i.e., dextran sulfate and lipopolysaccharide. This specificity may be a reflection of the fact that lipopolysaccharide and dextran sulfate represent compounds possessed of multiple repeating units whereas anti-immunoglobulin is not a repetitive unit except that it has a valence of 2. Therefore, it is possible that lipopolysaccharide and dextran sulfate do not induce enhanced DNA synthesis subsequent to pronase treatment because the inherent structure of

Table 2

Effect of pronase treatment of cells on the subsequent proliferative response to anti-immunoglobulin, lipopolysaccharide and dextran sulfate

Prior cell treatment	[^{3}H]-Thymidine incorporation using anti-immunoglobulin	lipopolysaccharide	dextran sulfate
		cpm	
None	18,420 ± 290	94,350 ± 1,100	32,760 ± 1,390
Pronase	119,190 ± 4,750	92,680 ± 3,060	33,710 ± 1,670

Cells were incubated without or with pronase then washed, cultured and processed as described in Materials and Methods. Anti-immunoglobulin, lipopolysaccharide and dextran sulfate were used at a final concentration of 200, 50 and 20 µg/culture well, respectively. The results are expressed as the mean of triplicate samples ± SEM in the presence of mitogen corrected for radioactivity incorporated without mitogen. The cpm incorporated in the absence of mitogen were 7,770 ± 270 for untreated cells and 4,360 ± 270 for pronase treated cells.

these mitogens permits them to circumvent the pronase sensitive sites in untreated cells. Alternatively, the pronase sensitive sites may be peculiar to only those sites involved in the triggering mechanism for anti-immunoglobulin and not for lipopolysaccharide or dextran sulfate.

From the findings herein it is intriguing to speculate that the conversion to responsiveness of pronase treated B lymphocytes from immature mice is a consequence of the removal of regulatory entities at the level of the surface membrane which control proliferation. In adult animals such immature cells might exist along with anti-immunoglobulin responsive mature cells which no longer possess the pronase sensitive sites. In this sense the putative regulatory function would be to preclude the proliferation of immature cells. Regardless of its function, it would be of some interest to determine the nature and characteristics of the relevant pronase sensitive sites.

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