Journal of Biological Response Modifiers
7:498–512 © 1988 Raven Press, Ltd., New York

Immunoregulatory Effects of Catrix

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Summary: The immunoregulatory effect of Catrix on in vitro and and in vivo antibody production was examined in mice. Catrix, an acidic mucopolysaccharide complex, contains glycosaminoglycans including chondroitin sulfate. Catrix-S, a soluble derivative, was found to enhance T-dependent and T-independent antibody responses in vivo in a dose-dependent manner, with 100 mg intraperitoneally or 10 mg intravenously being optimal. Lower doses were found to be less effective or inhibitory. In vitro, the enhancing activity of Catrix-S on proliferative response was additive with that of dextran sulfate and lipopolysaccharide but not with chondroitin sulfate C. This immunoaugmenting activity appears to be related to the chondroitin sulfate component of Catrix-S, because both have similar effects on in vivo and in vitro antibody responses and because chondroitinase ABC inactivates activity. The inhibitory activity of Catrix-S could be separated from its stimulatory effects by ammonium sulfate precipitation or by fractionation according to molecular weight. The immunoaugmenting effect was present in the 0–30% saturated ammonium sulfate precipitate and in the 5–10,000-m.w. and 30–100,000-m.w. fractions. The ability of Catrix-S to enhance antibody responses in nude as well as in normal mice, and antibody responses to T-independent as well as to T-dependent antigens, indicates that its activity is due in part to a direct effect on B cells and/or to an indirect effect mediated by macrophages. **Key Words:** Catrix-S—Glycosaminoglycan—Immunoregulatory effect—Chondroitin sulfate.

Catrix is an acidic mucopolysaccharide complex derived from bovine trachea cartilage (1). Although it contains some protein, including denatured collagen, some of its major nonprotein constituents are glycosaminoglycans, including chondroitin sulfate. Catrix has been reported to be of value in the treatment of autoimmune diseases and to have antitumor activity in vivo (2) and antimitotic activity in tissue culture (3). In view of our previous observations (4) that chondroitin sulfate A and C can enhance antibody production in mice and induce murine B-cell proliferation in vitro, it was of interest to determine whether Catrix could be shown to share some of these adjuvant properties.

In the present report we demonstrate that Catrix has immunoregulatory activity

Received November 9, 1987; accepted April 21, 1988.
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in vivo, which is similar to that of chondroitin sulfate, and that fractions of Catrix-S are capable of stimulating murine B-cell proliferation in vitro. However, as noted above, Catrix differs from chondroitin sulfates in its antimitotic effects (3), and also in the breadth of its immunoregulatory effects.

MATERIALS AND METHODS

Animals

BALB/c, $CB6F_1$, and athymic nu/nu BALB/c mice purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). SJL/J and A/J were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). The following mouse strains were used in order to assess possible differences in responsiveness: (SJL/J × BALB.B) F_1, (SJL/J × BALB/c) F_1, (SJL/J × A.TH) F_1, (SJL/J × A.TL) F_1, and B10.9R mice. These strains were bred in the Berg Institute of NYU Medical Center.

Antigens and Adjuvants

Sheep erythrocytes (SRBC; Colorado Serum Co., Denver, CO, U.S.A.) were washed, counted, and intraperitoneally injected at a dose of 1 or 2×10^7 cells per mouse or injected intravenously (i.v.) at a dose of 1 or 2×10^6 cells per mouse. Trinitrophenylated (TNP) derivatives of hemocyanin (KLH) and of B. abortus (BA) (U.S. Department of Agriculture, Ames, IA, U.S.A.) were prepared by the method of Little and Eisen (5) as described previously (6). Doses of 10, 20, or 100 μg TNP-KLH or 40–80 μg TNP-BA were injected i.v. TNP-Ficoll (TNP-F) was prepared as described (7), and doses of 0.2 μg or 10 μg TNP-F were injected intravenously.

1-Chloro-2,4,6-trinitrobenzene (TNCB) (King's Laboratory Inc., Blythwood, SC, U.S.A.) was dissolved in a mixture of 4 parts acetone and 1 part olive oil just prior to use. Chondroitin sulfate type C (ChS.C) (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in saline, and 5 mg was injected intraperitoneally. Lipopolysaccharide-B from *E. coli* (Difco Lab., Detroit, MI, U.S.A.) was used in culture at a final concentration of 5–10 μg/ml. Dextran sulfate (m.w. 500,000) (Sigma) was used at 10–20 μg/ml. ChS.C was used in vitro at 2 mg/ml.

Enzymes

Both chondroitinase ABC and collagenase (Sigma) were dissolved in ice-cold phospate-buffered saline (PBS), pH 7.2, just prior to use. Preincubation of Catrix fractions and ChS.C with enzymes was performed at 37°C for 24 h. The mixture was then dialyzed in Spectrapor membrane tubing (6,000–8,000 m.w. cut off) overnight. The sample was filter sterilized prior to testing.

Assay for Plaque-Forming Cells (PFC)

Single-cell suspensions were prepared from individual spleens and brachial lymph nodes of mice that had been immunized 4 or 5 days previously. The cells

were resuspended in Hanks' balanced salt solution and washed. Cells producing antibody to the immunizing antigen (PFC) were enumerated using a slide modification (8) of the Jerne and Nordin technique (9). Results were expressed as geometric means of PFC per spleen or lymph node. Enumeration of anti-TNP PFC was performed with TNP-SRBC prepared according to Rittenberg and Pratt (10) using goat anti-μ in the agar and rabbit antimouse Ig in the complement for development of indirect PFC (11).

Contact Sensitivity

One-tenth milliliter of 0.1% TNCB was applied to the shaved dorsal neck of mice. Some mice were immediately injected i.p. with Catrix. Five days following sensitization the mice were painted with 0.02 ml of 1.0% TNCB on the ear, as described (12). Ear thickness was measured prior to and 24 h after application of TNCB using an engineer's micrometer (Mitutoyo Manufacturing Co. Ltd., Japan).

Assay of Proliferative Response

A single-cell suspension of normal spleen or lymph nodes was prepared, washed, and counted. Cultures were performed in flat-bottom, 96-well tissue-cultures plates from Becton Dickinson Co. (Oxnard, CA, U.S.A.) in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS; GIBCO) and 5×10^{-5} *M* 2-mercaptoethanol (Eastman Kodak, Rochester, NY, U.S.A.). Cells were cultured at 2×10^5 per 0.2 ml medium. The cultures were incubated at 37°C with 5% CO_2 for 72 h and pulsed with [^{3}H] thymidine (New England Nuclear, Boston, MA, U.S.A.; 0.5 μCi/well, 2 Ci/mmol) 8 h before harvest of cultures of glass fiber filters (Whatman, Hillsboro, OR, U.S.A.) with MASH II automated cell harvestor (MA Bioproducts, Walkersville, MD, U.S.A.). Filters from five replicate cultures were counted in a Packard Tri Carb Scintillation Counter (Packard Instrument Co., Downers Grove, IL, U.S.A.) using toluene-based scintillation fluid containing 2,5-diphenyloxazol and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Research Products International, Elk Grove Village, IL, U.S.A.).

Antibody Production In Vitro

In vitro immunizations were performed according to a modification of the method of Mishell and Dutton (13) using spleen cells from normal mice. Cultures were prepared in Falcon #3001 35 mm Petri dishes containing 1×10^7 spleen cells, 50 μl of 0.5% SRBC in a total volume of 1 ml Iscove's medium supplemented with 10% FCS fetal calf serum, antibiotics, and 5×10^{-5} *M* 2-mercaptoethanol. After 4 days of culture in a rocking chamber, cells were harvested and the number of anti-SRBC PFC was determined as described above.

Catrix

Various lots of Catrix-S (LE-1, LE-9, and LE-15) were used as indicated. Catrix powder, Catrix-S, and a cold aqueous extract of Catrix powder (AE) were prepared as previously described (1).

The lyophilyzed Catrix-S (LE-15) was dissolved in distilled water to a concentration of 50–100 mg/ml and injected intraperitoneally (i.p.) or i.v. into mice in the stated doses. For in vitro assay, Catrix-S was further diluted in Dulbecco's PBS (Gibco, Grand Island, NY, U.S.A.), dialyzed, and added to cultures to the stated concentrations. Catrix-S was dialyzed in Spectrapore membrane tubing #1 overnight against 200 volumes of PBS at a pH of 7.2 with one change of the dialysate. Catrix-S LE-9 and various molecular weight fractions of LE-9 at 5 mg/ml were examined for endotoxin content by the Limulus Amebocyte Lysate Test Method (Associates of Cape Cod Inc., Woods Hole, MA, U.S.A.); they contained ≤1 ng endotoxin per 5 mg.

Molecular weight fractions were prepared by Amicon filtration from LE-9 and from AE. Quantitation of fractions was on lyophilyzed aliquots of each fraction. The distribution over the fractions for LE-9 was >300 Kd, 47%; 100–300 Kd, 26.8%; 30–100 Kd, 3.6%; 10–30 Kd, 9.5%; 5–10 Kd, 7.2%; 1–5 Kd, 4.0%; 0.5–1 Kd, 1.5%; and <0.5 Kd, 0.4% (total recovery 92.7%). Distribution of fractions for AE was >300 Kd, 92.5%; 100–300 Kd, 2.4%; 30–100 Kd, 1.6%; 10–30 Kd, 1.2%; 5–10 Kd, 1.3%; 1–5 Kd, 0.5%; 0.5–1 Kd, 0.5%; <0.5 Kd, 0.05% (total recovery was 90.1%).

Ammonium sulfate precipitations of LE-9 were performed with a total recovery of 68%: for 0–30% $(NH_4)_2SO_4$ the yield was 30.5%; 30–40%, 29.9%; 40–60%, 10.3%; and 60–80%, 4.3%. The supernatant from 100% $(NH_4)_2SO_4$ contained 22.8%.

Statistical evaluations were performed by the Student's *t* test.

RESULTS

Effect of Catrix on the Antibody Response to SRBC In Vivo

The results in Table 1 and Fig. 1 show that two different Catrix-S preparations, LE-1 and LE-9, both enhanced the plaque-forming cell response to SRBC. Both IgM and IgG antibody production were enhanced, but as expected, the IgG component remained a relatively small percentage of the total response on day 4 after a primary injection of antigen. The response was enhanced by i.p.-injected Catrix-S, both after i.v. (Fig. 1A) and after i.p. (Fig. 1B) injection of antigen, suggesting that the effect was *not* due to an interaction between antigen and Catrix-S at the

TABLE 1. *Effect of Catrix-S on 19S and 7S antibody production in mice*[a]

					Geometric mean ± SE of PFC/spleen[c]			
Antigen	Dose	Route	Catrix-S	Dose Route[b]	IgM		IgG	
SRBC	2×10^6	i.v.	Saline	i.p.	3,900 ± 1.7	$p < 0.01$	380 ± 2.1	$p = 0.01$
SRBC	2×10^6	i.v.	LE-1	20 mg i.p.	52,300 ± 1.3		8,800 ± 1.7	
SRBC	1×10^7	i.p.	Saline	i.p.	3,200 ± 1.3	$p < 0.002$	210 ± 1.8	$p < 0.01$
SRBC	1×10^7	i.p.	LE-1	20 mg i.p.	22,600 ± 1.1		6,800 ± 1.3	

[a] (SJL × BALB/c) F_1 mice, n = 4–5.
[b] Catrix-S was injected i.p. at the same time as antigen i.v. or i.p.
[c] Plaque-forming cell assay done on day 4 after antigen injection.

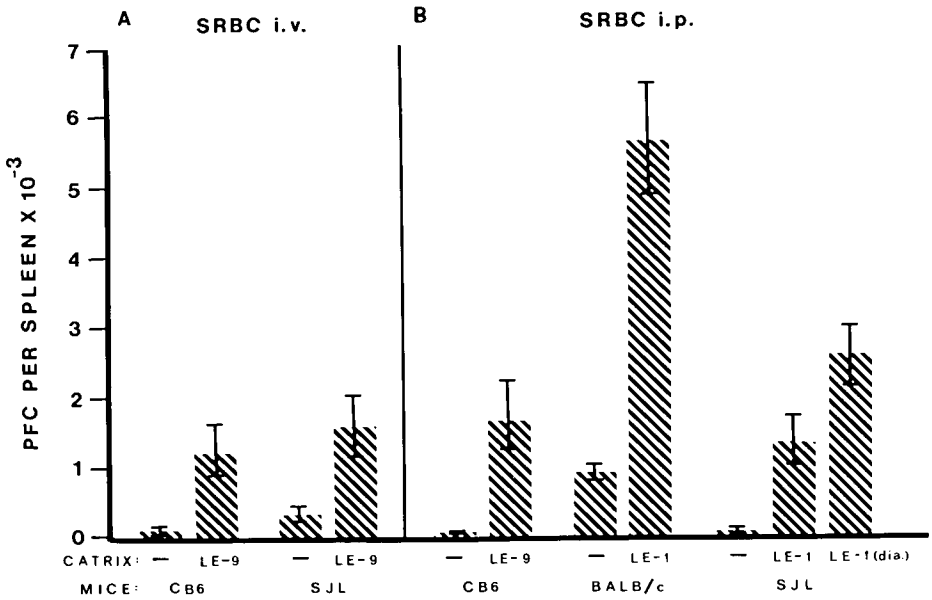

FIG. 1. Augmenting effect of Catrix-S on antibody production to sheep erythrocytes (SRBC) in various mouse strains. Mice received an i.p injection of Catrix-S (20 mg of regular or dialyzed (dia.) LE-1 or 100 mg LE-9) after an i.v. injection of 1–2 × 10^6 SRBC (A) or after an i.p. injection of 10^7 SRBC (B). Anti-SRBC plaque-forming cells per spleen were enumerated on day 4 and expressed as geometric means $\overset{\times}{\div}$ SE (n = 5–10). Effect of Catrix-S in all cases is statistically significant ($p < 0.0001$–<0.001).

site of antigen injection. In addition, the experiment with SJL mice in Fig. 1B shows that the factor responsible for the effect of LE-1 was not dialyzable. The augmenting effect of Catrix-S could be demonstrated in several different mouse strains, including BALB/c, SJL, and F_1 hybrids of these strains, B10.9R (not shown) and outbred Swiss mice (not shown). When 100 mg LE-9 was injected without antigen, the background level of anti-SRBC PFC/spleen measured 4 days later was not higher than in uninjected mice with a geometric mean of 150 $\overset{\times}{\div}$ 1.3.

The effect of different doses of Catrix-S preparations was next examined. In SJL × BALB.B mice (Fig. 2A) 20 mg LE-9 decreased whereas 100 mg enhanced the response to SRBC, both after i.p. and after i.v. injection of the antigen. In contrast, in SJL × A.TL mice there was enhancement by both 20 and 100 mg doses, whereas 2 mg had less or no effect (not shown). In the experiment shown in Fig. 2B, 2 mg LE-1 injected i.p. caused no enhancement (response 107% of control) whereas 2 mg LE-9 i.p. caused a 3-fold enhancement. In the experiment shown in Fig. 2C, the effect of i.v.-injected Catrix-S was examined. Injection of 2 mg LE-9 i.v. caused a decrease to 35% of the control response (not shown) as contrasted with the enhancement caused by 10 mg LE-9 i.v. (Fig. 2C).

The effect of repeated doses of Catrix-S on antibody production is presented in Table 2. It was found that the effect of a single injection at the time of antigen administration was as effective as the third of a series of three injections, as long as it was given at the same time as antigen. If the antigen injection was postponed

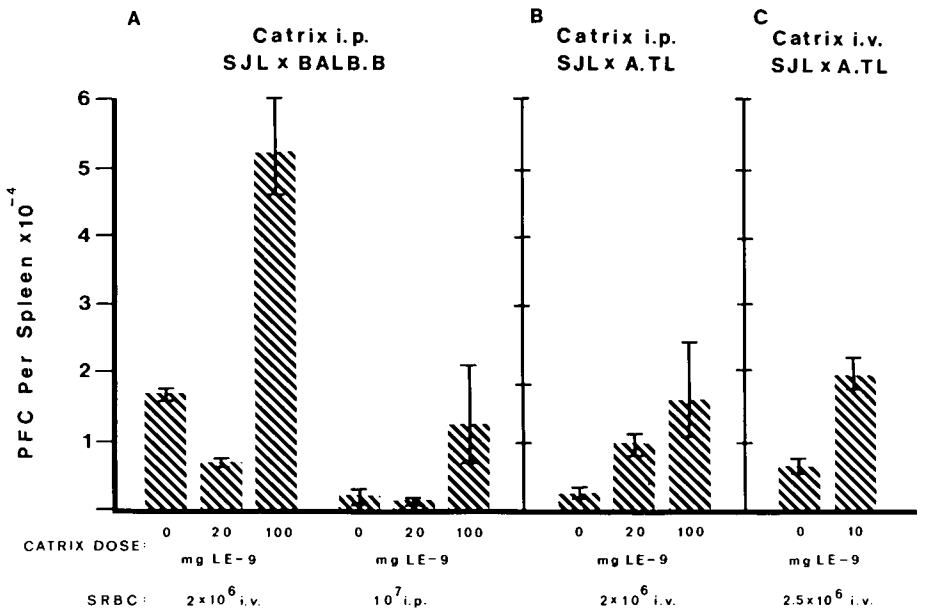

FIG. 2. Comparison of the effect of different doses of i.v.-versus i.p.-injected Catrix-S (LE-9) on antibody production to i.v. or i.p.-injected sheep erythrocytes (SRBC). Anti-SRBC PFC per spleen were determined on day 4 (n = 5). Only the effects of 100 mg i.p. or of 10 mg i.v. are statistically significant.

until 1 week after the last Catrix injection there was no longer any significant effect on the response to 2×10^6 SRBC i.v. Additional studies were performed with intragastrically administered Catrix powder. No effect on the immune response was detected even after repeated administration of up to 2,000 mg Catrix powder or of 100 mg LE-9 or AE intragastrically, regardless of the mouse strain or route of antigen administration (data not shown). There was also no synergistic or antagonistic effect on antibody production when intragastric administration was combined with i.p. injection of Catrix-S (data not shown).

TABLE 2. *Effect of multiple doses of Catrix-S on antibody production in mice*[a]

Catrix-S injections[b]	PFC/Spleen Geometric mean $\overset{\times}{\div}$ SE[c] (n)	p vs control
None	1,500 $\overset{\times}{\div}$ 1.4 (13)	
1 injection simultaneously with antigen	26,600 $\overset{\times}{\div}$ 1.1 (12)	$p < 0.0001$
3 weekly injections; last simultaneously with antigen	22,500 $\overset{\times}{\div}$ 1.6 (5)	$p = 0.01$
4 weekly injections; last 1 week before antigen	2,900 $\overset{\times}{\div}$ 1.3 (4)	NS

[a] (SJL × BALB/c)F_1 and (SJL × BALB.B)F_1 mice were injected with 2×10^6 SRBC i.v. (day 0).
[b] Each Catrix-S injection was 100 mg LE-9, i.p.
[c] PFC assay was performed on day 4 after antigen injection. Statistical evaluations were performed by the Student's *t* test.

Effect of Catrix on the Antibody Response to TNP-Conjugates

Both LE-1 (20 mg) and LE-9 (100 mg) Catrix-S preparations enhanced responses to TNP-KLH, TNP-BA, TNP-F (Table 3). This suggests that the effect of Catrix-S was either mediated by a direct influence on B cells or by an indirect effect mediated by macrophages, as both TNP-BA and TNP-F are T-independent antigens (14,15). In addition, the results in experiment 5 (Table 3) show that the response of athymic mice to TNP-F was also significantly enhanced.

The augmenting effect on the response to TNP-F was still detected when LE-9 was given 2 days after antigen (expt. 5, Table 3). In this respect it resembled the effect of chondroitin sulfate C, which was previously shown to cause a greater increase in the response to TNP-F when given 2 days after rather than on the same day as antigen (4).

In contrast to the high dose of LE-1, a low dose (0.2 mg) caused inhibition of the response to TNP-KLH (expt. 1, Table 3) in SJL × BALB/c mice, similar to what was seen with the response to SRBC (Fig. 2A).

Effect of Catrix on the Antibody Response In Vitro

Dialyzed LE-1 caused a 3–4-fold increase in the primary response of BALB/c spleen cells to SRBC in culture. The effect was dose dependent, optimal at 0.4 mg/ml, and no longer detected at 0.2 mg/ml. A concentration >10 mg/ml was clearly inhibitory (data not shown).

The augmenting effect on antibody production was not due to induction of lymphokine production. Catrix-S did not affect thymocyte proliferation in vitro, either with or without added Con A, and at 10–20 mg/ml it did not induce detectable IL-2 production by spleen cells (≤2 U IL-2/ml). It also failed to affect IL-2 assays when added at 1, 5, or 25 mg/ml together with IL-2 to the IL-2-dependent CTLL assay T cell line. The IL-2 units measured in the presence of Catrix-S were

TABLE 3. *Effect of Catrix on antibody formation to TNP-conjugates expressed as percentages of control PFC responses*

Expt. no.	Antigen			Adjuvant[a]			Strain	Compared with control response[b]	
	Dose	Prep	Route	Dose	Prep	Day		% of Control	p
1	100 μg	TNP-KLH	i.v.	20 mg	LE-1	Day 0	SJL × BALB/c	373 (IgM) 243 (IgG)	$p < 0.02$ $p < 0.02$
	100 μg	TNP-KLH	i.v.	0.2 mg	LE-1	Day 0	SJL × BALB/c	47 (IgM) 5 (IgG)	NS $p < 0.04$
2	20 μg	TNP-KLH	i.v.	100 mg	LE-9	Day 0	SJL × BALB/c	157	$p = 0.05$
3	10 μg	TNP-KLH	i.v.	100 mg	LE-9	Day 0	BALB/c	372	$p < 0.002$
4	80 μg	TNP-BA	i.p.	20 mg	LE-1	Day 0	SJL × BALB.B	241	$p < 0.02$
2	40 μg	TNP-BA	i.p.	100 mg	LE-9	Day 0	SJL × BALB/c	139	$p < 0.05$
5	1 μg	TNP-F	i.v.	100 mg	LE-9	Day 0	BALB/c	159	$p = 0.07$
	1 μg	TNP-F	i.v.	100 mg	LE-9	Day 0	Athymic BALB/c	232	$p < 0.05$
6	0.2 μg	TNP-F	i.v.	100 mg	LE-9	Day 2	BALB/c	239	$p < 0.01$
	0.2 μg	TNP-F	i.v.	5 mg	ChS.C	Day 2	BALB/c	205	$p < 0.01$

[a] Catrix-S (LE-1 or LE-9) or chondroitin sulfate C (ChS.C) were always injected i.p.

[b] Control responses ranged from 12–24,000 PFC/spleen for TNP-Ficoll, from 5–25,500 PFC/spleen for TNP-KLH, and from 6–12,000 PFC/spleen for TNP-BA.

115, 107, and 99% of control values, respectively. Catrix-S slightly inhibited IL-2 production by spleen cells in response to Con A; IL-2 titers attained at 24 h after initiation of culture were 76% of controls at 20 mg Catrix-S/ml and 90% of controls at 10 mg Catrix-S/ml. Serum taken from mice 24 h after injection of Catrix-S did not contain detectable interferon activity (assayed as in reference 16).

Effect of Catrix on Delayed Hypersensitivity

Catrix-S (100 mg LE-9) was injected i.p. at the same time as the first application of trinitrochlorobenzene (TNCB) to the nape of the neck. Ear swelling to a challenge with TNCB was measured 5 days later. The controls exhibited mean increments of ear thickness of 22.3 ± 2.8 and 12.9 ± 2.0 mm × 10^{-2} (n = 5) at 24 and 48 h after challenge. The Catrix-S-injected mice showed ear thickness increments of 24.0 ± 2.4 and 17.2 ± 2.3 mm × 10^{-2} (n = 5) respectively, which were not significantly different from the control values.

Effect of Catrix and Catrix Fractions on Spleen Cell Proliferation In Vitro

Several Catrix-S and Catrix preparations including LE-1, LE-9, and AE were tested for their ability to induce proliferation in spleen cells and to influence the response of spleen cells to the slightly suboptimal concentration of 5 μg/ml LPS (Fig. 3A and B). Undialyzed AE was universally strongly inhibitory (not shown), but after dialysis some preparations of Catrix-S were much less inhibitory and even stimulated a highly significant degree of proliferation, particularly LE-9 used in concentrations of 10 mg/ml or lower. The most stimulatory preparations also slightly enhanced the response of spleen cells to LPS (Fig. 3B). In terms of total

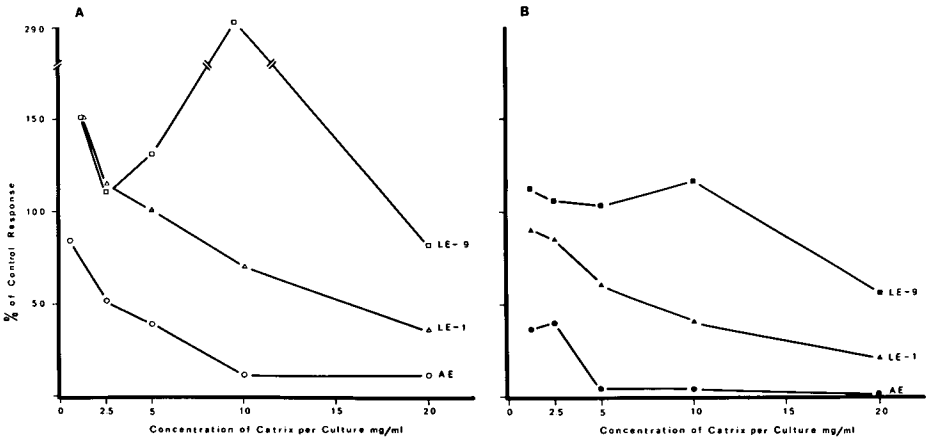

FIG. 3. Proliferative responses of BALB/c mouse spleen cells to varying concentrations of Catrix-S without (A) or with (B) addition of a slightly suboptimal stimulating concentration of *E. coli* lipopolysaccharide (LPS, 5 μg/ml). Results are expressed as the percentages of the cpm of ^{3}H-thymidine incorporated by control cells cultured for 3 days, with or without LPS, respectively: 100% responses − LPS ranged from 1,010 ± 253 to 4,610 ± 283 cpm and 100% responses + LPS ranged from 33,306 ± 1,206 to 72,845 ± 394 cpm. All values are means of five determinations. The results shown are a composite of three experiments.

increments in cpm incorporated, the increases in the absence and presence of LPS induced by Catrix-S were similar, although expressed as percentages of control responses they were much higher in the absence of LPS. When Catrix-S preparations made in the usual manner were compared for activity with AE, the latter, even after dialysis, was very inhibitory both in the absence and presence of LPS (Fig. 3). An exposure of only 3 h in vitro to 20 mg/ml AE reduced the subsequent ability of the (washed) cells to respond to LPS by 39%, whereas a 3-hour preexposure to LE-1 (20 mg/ml) had no significant effect on subsequent responses (data not shown). Moreover, cell viability after incubation with AE was reduced as compared with control cells or LE-1-incubated cells.

In view of the observation that dialyzed Catrix-S preparations such as LE-9 enhanced ^{3}H-thymidine incorporation in spleen cells cultured with Catrix-S alone, fractionation procedures were applied to Catrix-S in an attempt to separate the inhibitory from stimulatory moieties in the preparation. LE-9 was chosen for these studies because it appeared to be the most stimulatory of six preparations tested. LE-9 was fractionated according to molecular size and solubility in different concentrations of $(NH_4)_2SO_4$.

The results in Fig. 4A show that not all the fractions induced equal proliferation. The highest stimulatory activity in LE-9 was found in fractions of 5–10,000 and

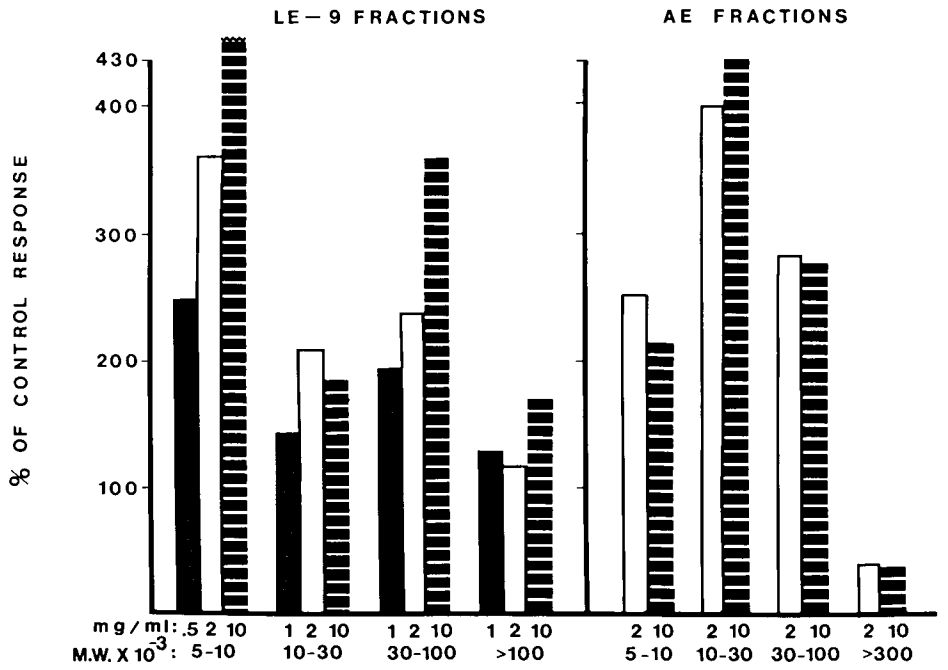

FIG. 4. Proliferative responses of BALB/c mouse spleen cells to different concentrations (mg/ml) of molecular weight (m.w.) fractions of Catrix-S preparation LE-9 or aqueous extract (AE). Results are expressed as percentages of cpm ^{3}H-thymidine incorporated by cells cultured without additives and determined (n = 5 determinations per point) on day 3 of culture. 100% values were 672 ± 71 and 1,810 ± 120 cpm. All samples between 5,000 and 100,000 m.w. stimulated thymidine uptake to levels significantly above the 100% values ($p \leq 0.002$).

30–100,000 molecular weight (m.w.), both of which also costimulated with LPS. For comparison, AE was also fractionated and tested. The inhibitory activity was present in the fraction containing molecules >300,000 in m.w., whereas peak stimulation was obtained with the 10–30,000 and 30–100,000 m.w. fractions (Fig. 4B).

Fractionation by $(NH_4)_2SO_4$ precipitation also resulted in a separation of the most stimulatory fraction (Fig. 5A). In these experiments 30% $(NH_4)_2SO_4$ precipitated all of the stimulatory activity, whereas at the same concentration none of the fractions precipitated by higher concentrations stimulated. Fractions precipitated between 60 and 80% and between 80 and 100% saturated $(NH_4)_2SO_4$ inhibited proliferation by 75 and 96%, respectively, in the absence of LPS and by 62 and 99% in the presence of LPS (not in Figure). The effect on spleen cells from an athymic mouse was also determined with similar results (Fig. 5B), suggesting that the proliferation induced by Catrix-S is in B cells.

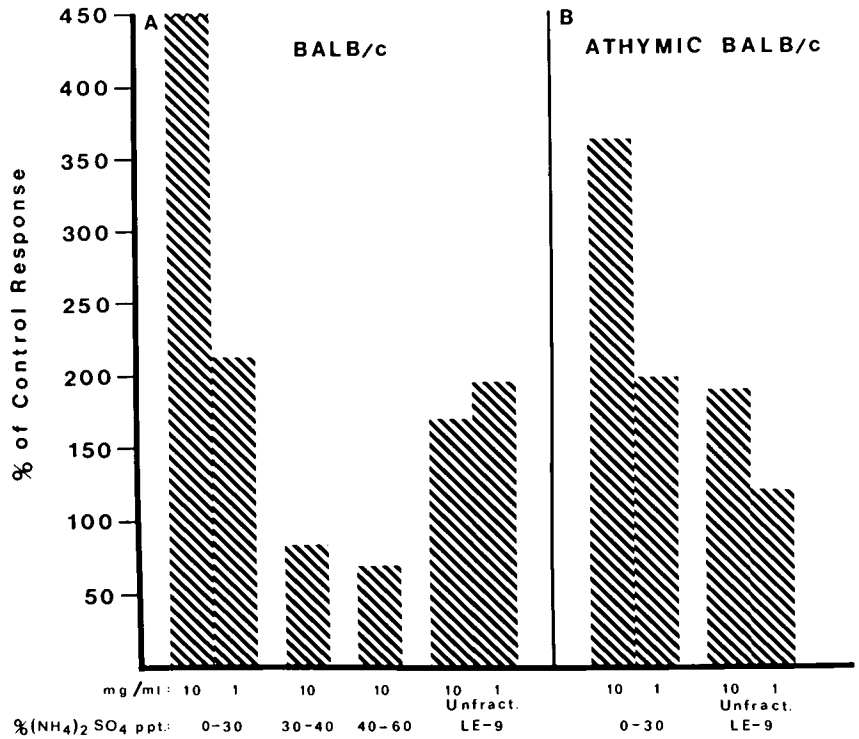

FIG. 5. Proliferative responses of spleen cells from euthymic (A) and athymic nu/nu (B) BALB/c spleen cells to $(NH_4)_2SO_4$ precipitation fractions of Catrix-S (LE-9). The concentrations (mg/ml) of fractions examined and the percentages of saturated $(NH_4)_2SO_4$ used to prepare the fractions are indicated in the figure. Results are expressed as percentages of cpm ^{3}H-thymidine incorporated by cells cultured without additives, as determined on day 3 of culture (n = 5). 100% values were 4,453 ± 243 cpm (A) and 1,330 ± 91 cpm (B). Significance: 0–30% $(NH_4)_2SO_4$ ppt. fraction versus control, $p = 0.0003$ (A) and $p = 0.004$ (B); comparing unfractionated LE-9 with the 0–30% ppt. fraction $p < 0.005$ at 10 mg/ml (A and B) and at 1 mg/ml (B).

For comparison, these fractions were also analyzed for effect on the immune response in vivo. The fraction precipitated by 0–30% saturated $(NH_4)_2SO_4$ enhanced antibody production, like unfractionated LE-9, but none of the other fractions has a significant effect (Fig. 6).

Because the stimulation by the 30% saturated $(NH_4)_2SO_4$ precipitated fraction was *much* higher than that by unfractionated Catrix, the costimulatory activity of this Catrix fraction with other B-cell stimuli was investigated (Fig. 7). This Figure is representative of two or three experiments for each combination of agents examined. The results suggest that the Catrix fraction has an additive effect on B-cell stimulation induced by LPS or by dextran sulfate, but not significantly on the proliferation induced by chondroitin sulfate C.

The various m.w. fractions and unfractionated LE-9 contained ≤1 ng endotoxin per 5 mg as tested in the Limulus Amebocyte Lysis test. At this level of contamination none of the proliferative responses induced by Catrix-S fractions could be ascribed to endotoxin, as detectable proliferation was not induced in murine spleen cells at concentrations of a standard endotoxin preparation of ≤100 ng/ml. In addition, additive stimulation obtained when both LPS and Catrix-S were used

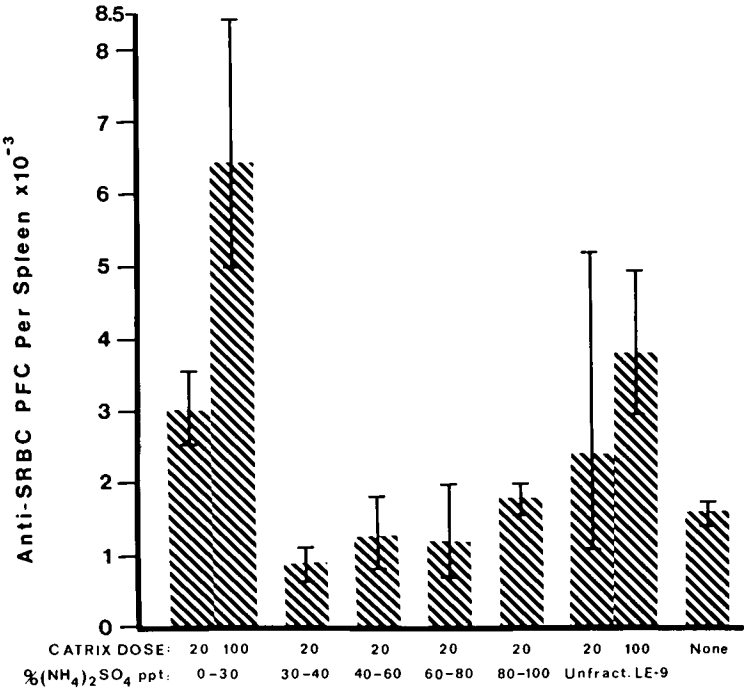

FIG. 6. Effect of $(NH_4)_2SO_4$ fractionated Catrix-S (LE-9) on the antibody response to sheep erythrocytes (SRBC). Doses (mg) injected of unfractionated LE-9 or of fractions precipitated by different percentages of saturated $(NH_4)_2SO_4$ are indicated in the figure. Responses are expressed as geometric means of anti-SRBC PFC/spleen $\overset{\times}{\div}$ SE (n = 5) determined on day 4. Mice (None) injected with SRBC (2×10^6, i.v.) alone showed 1,560 $\overset{\times}{\div}$ 1.1 PFC/spleen. Significance: 0–30% $(NH_4)_2SO_4$ ppt. fraction versus control (None) $p < 0.05$ for both 20 and 100 mg doses.

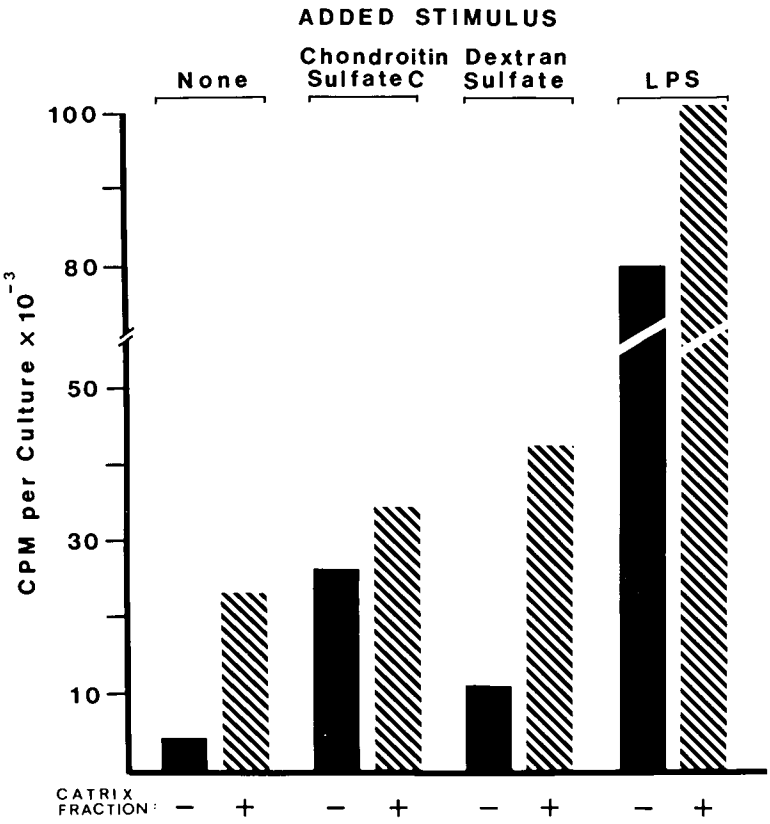

FIG. 7. Costimulatory activity of 30% saturated $(NH_4)_2SO_4$ precipitated fraction of Catrix-S (LE-9) with other B-cell stimuli. The final concentration of the LE-9 fraction in the medium was 10 mg/ml, of dextran sulfate, 10 μg/ml, of chondroitin sulfate, 2 mg/ml, and of LPS, 10 μg/ml. All values represent mean cpm ^{3}H-thymidine incorporated on day 3 of culture (n = 5). The addition of the LE-9 fraction caused a highly significant increase in proliferation without further stimulus ($p < 0.001$), with dextran sulfate present ($p < 0.0001$) and with LPS present ($p = 0.0015$).

together strongly suggested that the stimulatory moiety in Catrix-S was not endotoxin.

Enzyme Sensitivity of Proliferation-Inducing Fraction in Catrix

In order to further characterize the fraction in Catrix that might be stimulating splenic B cells, the sensitivity of the stimulatory molecules to digestion with chondroitinase and collagenase was determined. In previous studies it was shown that the B-cell proliferation-inducing activity of chondroitin sulfate C is much decreased after preincubation with chondroitinase ABC. The results in Table 4 show that this enzyme nearly abolishes the stimulatory activity of two Catrix fractions with high stimulatory activity, the low mw fraction, as well as the fraction precipitated by 30% saturated $(NH_4)_2SO_4$. Pretreatment with collagenase has

TABLE 4. *Effect of enzyme pretreatment on ability of Catrix-S fractions to stimulate spleen cell proliferation*

Catrix fraction used as substrate	Enzyme Treatment	% of Control response remaining[a]	
		Expt. 1	Expt. 2
30% $(NH_4)_2SO_4$ precipitate	Chondroitinase ABC 1 U/100 mg	19	17
	collagenase	104	47
1,000–10,000 MW injection	Chondroitinase ABC 1 U/100 mg	15	ND
	collagenase	55	ND
Chondroitin sulfate C	Chondroitinase ABC 1 U/2 mg	ND	35
	collagenase	ND	86

Catrix fractions were tested at 10 mg/ml; chondroitin sulfate C at 2 mg/ml.
[a] Control cpm ranged from 6,900 to 25,400 cpm (=100%).

much less effect. For comparison, a simultaneously treated chondroitin sulfate C preparation was also tested. The activity of 2 mg condroitin sulfate was greatly reduced by incubation with the same amount of enzyme (1 U) for the same incubation time at 37°C that abolished activity of 100 mg of Catrix.

DISCUSSION

The results presented show that Catrix-S preparations enhance antibody production to a variety of antigens, including T-dependent and T-independent antigens, and regardless of the route of antigen administration (i.v. or i.p.) or of the strains of mice used. The effect of Catrix-S is dose dependent. The most significant enhancement is obtained with 100 mg Catrix-S i.p. or 10 mg i.v., whereas lower doses are either less effective or can even be inhibitory. The enhancement of the response to TNP-F is also seen in athymic mice, suggesting that the effect is not mediated via T cells.

The mechanism by which Catrix-S enhances antibody production appears to be by a direct stimulation of B cells as Catrix-S, and in particular certain Catrix-S fractions, prepared by 30% $(NH_4)_2SO_4$ precipitation or size fractionation, induce proliferation of both normal BALB/c and athymic BALB/c spleen cells in vitro. The observation that certain fractions of Catrix are inhibitory to spleen cell proliferation and, in particular, to LPS-induced B-cell proliferation may also explain why at lower concentrations unfractionated Catrix-S can inhibit antibody responses in vivo. The outcome of an effect of Catrix-S in vivo may thus depend on the ratio of inhibitory and stimulatory components in the Catrix-S.

The substance in Catrix-S that induces the immunomodulatory effects appears to be related to chondroitin sulfate. In the first place, the effect in vivo shows similar properties to those previously shown for chondroitin sulfates C and A. Like chondroitin sulfate, less is needed to enhance antibody production when Catrix-S is injected i.v. than when it is injected i.p. In addition, the immunoaugmenting effect obtained when it is injected 2 days after TNP-F is greater than when it is injected simultaneously with TNP-F. This has also been observed for LPS (17) and chondroitin sulfate C (4) and may, therefore, be a general property of agents that directly activate B cells. In vitro, the effect of Catrix-S is additive

with that of dextran sulfate and of LPS, but not with chondroitin sulfate C. Similar stimulation in vitro was previously described for chondroitin sulfate C which also causes additive effects with dextran sulfate and with LPS. Lastly, the effect of Catrix-S appears sensitive to digestion with chondroitinase ABC, an enzyme with specificity for chondroitin sulfates and related glycosaminoglycans. Thus, this cartilage-derived material, rich in proteoglycans containing chondroitin sulfate and related glycosaminoglycans, apparently stimulates B cells in vivo and in vitro by virtue of its chondroitin sulfate-like content.

Further work is needed to determine whether any of the clinically beneficiary effects described for Catrix and Catrix-S are related to its immunoaugmenting properties. The complete absence of any effect on antibody production after oral administration of cold water extract, soluble Catrix-S, or Catrix powder may be helpful in determining which activity is clinically relevant, as both oral (Catrix) and parenteral administration (Catrix-S) forms are used in patients.

It should be noted that the in vitro stimulation of B cells by Catrix fractions required dialysis. The nature of the inhibitory, low molecular weight fractions that are lost by dialysis has not been examined here, but in vitro inhibition of tumor cell growth by low molecular weight substances in Catrix has been observed in other studies (18). It is also of interest that aqueous extracts of cartilage appear to contain strongly inhibitory high m.w. material that is either absent or much less prominent in autoclaved extracts (i.e., Catrix-S such as LE-9). The molecular size of the B-cell stimulatory material in aqueous extract also appears somewhat larger than in LE-9. Thus, autoclaving may reduce the size of the active molecules and destroy the large m.w. inhibitory material present in aqueous extract.

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