FETUIN, AN INHIBITOR OF LYMPHOCYTE TRANSFORMATION

The Interaction of Fetuin with Phytomitogens and a Possible Role for Fetuin in Fetal Development

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The phytohemagglutinins (PHAP)[1] derived from *Phaseolus vulgaris* consist of a family of five isomitogens made up of varying proportions of two similar, but nonidentical subunits designated "L" and "R" (1). L-PHAP is a potent mitogen and leukoagglutinin which contains four L subunits, and which lacks hemagglutinating and red blood cell (RBC) binding properties (2, 3). H-PHAP is a mixture of three isomitogenic proteins of increasingly positive charge (4), the subunit structure of which may be represented as 2L-2R, 1L-3R, and 4R, respectively (1, 5). These three isomitogens are also capable of binding to lymphocytes, and they can evoke a lymphocyte mitogenic response in proportion to their L-subunit content (4). In contrast to L-PHAP, however, they can bind to and agglutinate RBC by virtue of their R-subunit affinity for glycoprotein structures present on the RBC membrane (1–3, 5, 6). Furthermore, H-PHAP can bind to and precipitate with a variety of serum glycoproteins which possess glycopeptide structures resembling those found on RBC, whereas L-PHAL lacks this ability (4, 7). Presumably, the ability to interact with serum glycoproteins is bestowed upon H-PHAP molecules by virtue of their R-subunit binding sites (1).

One serum glycoprotein, fetuin, possesses oligosaccharide subunits similar in structure to those present on red cells which form the attachment site for H-PHAP (6). Both fetuin and its glycopeptide can inhibit H-PHAP-induced hemagglutination (6, 8). Fetuin is also capable of reversing the binding of H-PHAP to lymphocyte membranes and of inhibiting the ability of H-PHAP to stimulate lymphocyte amino acid uptake and DNA synthesis (9). This report examines the ability of H- and L-PHAP to bind to or precipitate with fetuin, as well as the ability of fetuin to inhibit lymphocyte transformation by these and other mitogenic phytoproteins.

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[1] *Abbreviations used in this paper:* ATS, antithymocyte antiserum; BSA, bovine serum albumin; CON-A, concanavalin A; IRA, immunoregulatory α-globulin; MLC, mixed lymphocyte culture; PBS, 0.15 M NaCl, 0.01 M PO_4, pH 7.0; PHAP, phytohemagglutinin; PWM, pokeweed mitogen; RBC, red blood cell.

Materials and Methods

The isolation of H- and L-PHAP, and the procedure used for their radioiodination have been described previously (2, 5). Pokeweed mitogen (PWM) and concanavalin A (CON-A) (10) were employed as mitogens in certain experiments. Antithymocyte antiserum (ATS) was prepared in New Zealand white rabbits by five or six successive weekly i.v. injections of saline suspension of 10^8 human fetal thymocytes derived from 16–20-wk-old aborted fetuses. 1 wk after the last injection, the rabbits were bled; the isolated serum was heat inactivated at 56°C for 30 min and stored frozen at −20°C. Detailed descriptions of the methods used for isolating and culturing lymphocytes and determining their incorporation of [2-^{14}C]thymidine into DNA have been provided (2, 5). 4-ml replicate cultures containing 1.5–2 × 10^6 lymphocytes, free of RBC or platelet contamination, in RPMI-1640 supplemented with L-glutamine, antibiotics, and 12.5% human AB serum (in the case of ATS, heat-inactivated AB serum was employed) or autologous plasma were utilized in most experiments. In certain experiments, noted in the text, a 1-ml culture system was employed.

Peripheral blood mononuclear cells for use in one-way mixed lymphocyte cultures (MLC) were isolated by a Ficoll-Hypaque technique (9). 4-ml triplicate cultures containing 2 × 10^6 responding cells and 1.0 × 10^6 mitomycin-treated stimulating cells were incubated for 5 days before the addition of [2-^{14}C]thymidine and harvesting 16 h later (10).

Fetuin (Spiro method), purchased from Grand Island Biological Co. (Grand Island, N. Y.) (cat. no. 9185), was dissolved in water at a concentration of 50 mg/ml and dialyzed vs. 0.15 M NaCl before use. A maximum of 10 mg of fetuin in 200 μl of 0.15 M NaCl was added to individual lymphocyte cultures just before the addition of mitogen (maximum final fetuin concentration, 2.5 mg/ml). Fetuin was radioiodinated with ^{125}I by the iodine monochloride method (11). The final preparation had a specific activity of approximately 1,000 cpm/μg, of which 95% was precipitable by 50% $(NH_4)_2SO_4$.

The effect of fetuin on lymphocyte survival and viability was examined by incubating lymphocytes in the presence of 2.5 mg/ml fetuin under conditions identical to those used for mitogen-induced transformation. After 88 h incubation, cell counts were performed, and the proportion of remaining cells which were viable was determined by the trypan-blue exclusion technique.

The uptake of ^{125}I-labeled fetuin by lymphocytes was studied in both short- and long-term experiments. Mononuclear cells, free of platelets and RBC, were isolated by the Ficoll-Hypaque method, and half of the cells so obtained were passed over a nylon column to deplete the monocyte population. 5 million cells from each cell isolate were suspended in 1.2 ml tissue culture medium devoid of protein save for 2.5 mg/ml [^{125}I]fetuin and incubated at 37°C for 30 min. The glass tubes (including controls lacking any cells, to correct for nonspecific adsorbtion) were washed five times with 4 ml medium. After the addition of 2 ml medium, all tubes were monitored for residual radioactivity. To examine the interaction between fetuin and human lymphocytes over longer time periods, 4 million lymphocytes were cultured under conditions identical to those used to study mitogen-induced lymphocyte transformation. All culture tubes contained 2.25 mg/ml cold fetuin together with 0.25 mg/ml [^{125}I]fetuin, and some received 5 μg/ml L-PHAP at zero time; cell-free control tubes were also included. At 1, 18, 42, and 66 h appropriate tubes were removed from incubation, and after five 4-ml washes, the culture tubes were subjected to scintillation counting.

The ability of fetuin to precipitate with H- and L-PHAP in agar gel was determined by the Ouchterlony double diffusion technique (4). Formation of soluble complexes between the PHAP mitogens and fetuin was examined by gel diffusion chromatography (12). Approximately 50 μg of [^{125}I]H- or L-PHAP were mixed with 20 mg fetuin, or with 20 mg crystalline bovine serum albumin (BSA) in a total volume of 0.8 ml 0.15 M NaCl, 0.01 M PO_4, pH 7.0 (PBS). The mixture, which contained 10% sucrose, was entered into a 1.5 × 100-cm column of Biogel A 0.5 M, 200-400 mesh (Bio-Rad Laboratories, Richmond, Calif.) and eluted by upward-flow chromatography in 3.0-ml fractions at a flow rate of 7–8 ml/h. Chromatography was carried out at room temperature, and 0.01% sodium azide in PBS was used as the eluting buffer. [^{125}I]H-PHAP, [^{125}I]L-PHAP, BSA, and fetuin were chromatographed separately on several occasions. In addition, a mixture of 50 μg [^{125}I]L-PHAP and 10 mg unlabeled L-PHAP was studied by this technique. The void volume of the column was determined by means of a dextran blue marker, and the column was further calibrated with an IgG myeloma protein (mol wt 155,000) and horse heart cytochrome *c* (mol wt 12,400). Elution fractions were monitored for ^{125}I in a gamma counter (model 1095, Nuclear-Chicago Corp., Des Plaines, Ill.),

and for protein, cytochrome *c*, or dextran blue, by absorbancy at 280, 407, or 674 nm, respectively, as required.

Results

H-PHAP was capable of precipitating with fetuin in agar gel, whereas L-PHAP lacked this ability when tested against a wide range of fetuin concentrations (Fig. 1). Within 3-1/2 h, precipitin lines formed between H-PHAP and fetuin at the higher concentrations employed, but these lines had disappeared after a further 17 h of incubation. This phenomenon, reminiscent of the dissolution of antigen-antibody aggregates in the presence of excess antigen, suggested the possibility of the formation of soluble complexes between fetuin and H-PHAP. We therefore undertook a gel chromatographic analysis of the reaction between

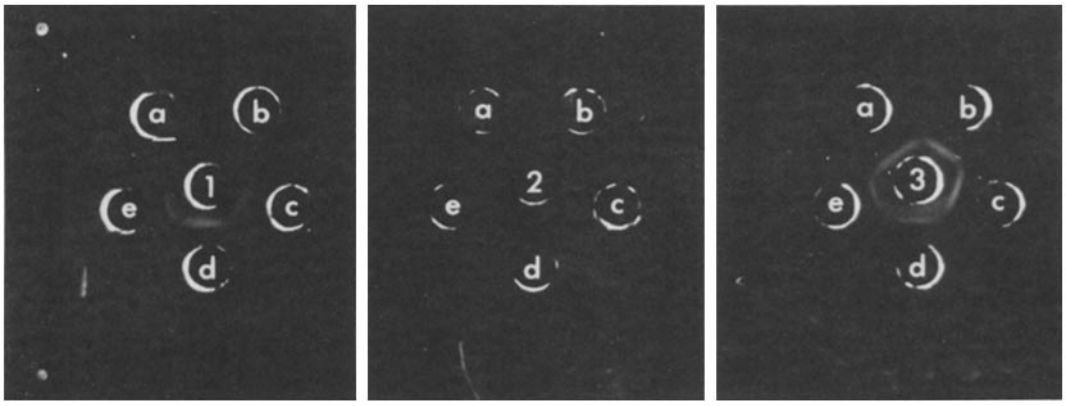

Fig. 1. Ouchterlony analysis of the interaction between fetuin and the PHAP mitogens. The PHAP mitogens were placed in the center wells at a concentration of 1.6 mg/ml. The peripheral wells contained fetuin at the following concentrations (mg/ml); (*a*) 50; (*b*) 25; (*c*) 12.5; (*d*) 6.3; (*e*) 3.1. (1) H-PHAP, 20.5 h incubation; (2) L-PHAP, 20.5 h incubation; (3) H-PHAP, 3.5 h incubation.

[^{125}I]H- and L-PHAP and a 400-fold excess (wt/wt) of fetuin. The results of these studies are shown in Fig. 2. Both [^{125}I]H-PHAP and [^{125}I]L-PHAP emerged from the column with a peak at fraction 25, indicating an apparent mol wt of approximately 110,000.[2] Their behavior was not altered by cochromatography with dextran blue or 20 mg BSA or, in the case of L-PHAP, with a 200-fold excess of unlabeled mitogen. Fetuin (not shown) displayed anomalous behavior on gel chromatography (15) and also emerged with an apparent mol wt of 110,000 at tube 25. No alteration in the pattern of fetuin elution could be seen in the presence of 1/400 its weight of either PHAP mitogen. The behavior of H-PHAP was strikingly altered in the presence of fetuin, however, and over 70% of the radioactively labeled material recovered was shifted to an apparent mol wt of 350,000 or greater (tubes 16–18). Thus, a minimum estimate of the composition

[2] Gel diffusion chromatography has previously been noted to underestimate the true mol wt (136,000) of PHAP isomitogens (2, 13, 14).

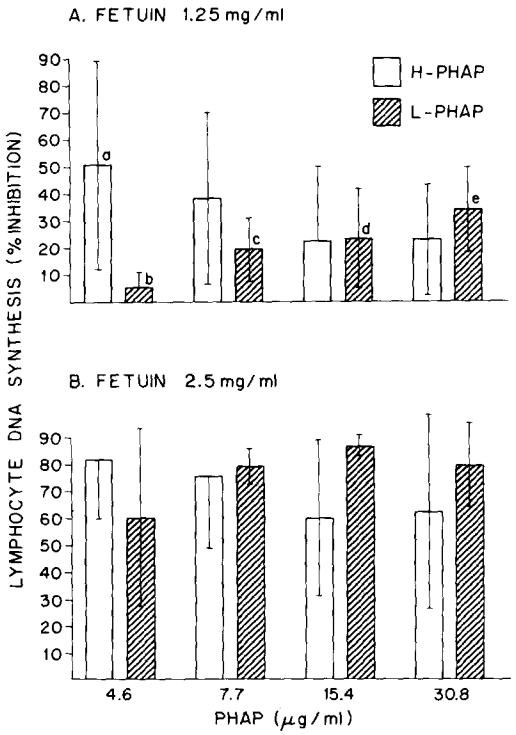

Fig. 2. The behavior of [^{125}I]L- and H-PHAP (50 μg) when chromatographed on Biogel A 0.5 M, either alone or in the presence of a 400-fold excess (wt/wt) of fetuin. V_0, void volume.

of soluble complexes formed between fetuin and H-PHAP would suggest a 2:1 fetuin:H-PHAP molecular ratio; the majority of these complexes were probably larger. Only 5% of the radiolabeled H-PHAP emerged in its normal position, indicating that >90% H-PHAP was involved in soluble complex formation with fetuin. These estimates are made reasonable by the observation that the total amounts of H-PHAP recovered from the column (fractions 1–40) in the presence of, or absence of fetuin were similar, namely, 86.5% and 82±7%, respectively.

The behavior of [^{125}I]L-PHAP in the presence of a 400-fold excess of fetuin was quite different. Approximately 7% of the recovered L-PHAP was shifted to a position consonant with a mol wt of ≧290,000 (tubes 16–19). The major elution peak emerged at tube 24, a position which is equivalent to a mol wt of approximately 125,000–130,000, indicating that the interaction between L-PHAP and fetuin was very much weaker than that between H-PHAP and fetuin, and was insufficient to sustain even a 1:1 molecular association between the two proteins. Again, the total recovery of L-PHAP in tubes 1–40 was not altered whether or not fetuin was present (74.5±5 without vs. 78% with fetuin). Thus H-PHAP is indeed capable of forming soluble complexes with fetuin, whereas the affinity of L-PHAP for fetuin is very much lower and any molecular complexes which form dissociate rapidly.

We have been able to confirm previous observations concerning the ability of

fetuin to inhibit H-PHAP-induced lymphocyte transformation (9) (Table I). Despite L-PHAP's much lower affinity for fetuin, the ability of L-PHAP to transform lymphocytes was also markedly inhibited by fetuin at a concentration of 2.5 mg/ml, and to a notable, but significantly less profound degree than for H-PHAP, at a concentration of 1.25 mg/ml. In agreement with observations by Mendelsohn et al. (9), we have found that fetuin is not nonspecifically toxic to lymphocytes at these concentrations since the amount of DNA synthesis in unstimulated control lymphocytes was not depressed by fetuin, and since the ability of fetuin to depress lymphocyte DNA synthesis in a single lymphocyte preparation at a given fetuin concentration varied with different mitogens (Tables I and II). In addition, long-term incubation of human lymphocytes in the presence of 2.5 mg/ml fetuin does not significantly reduce either the number of

TABLE I

The Ability of Fetuin to Inhibit L- and H-PHAP-Induced Lymphocyte Transformation

Exp.	% Inhibition			
	2.5 mg/ml fetuin		1.25 mg/ml fetuin	
	L-PHAP*	H-PHAP*	L-PHAP*	H-PHAP*
1	64	98.8	7	73.2
2	78.9	98.5	45.7	76.2
3	97.7	99.7	49.6	95.5
4	90.6	97.2	26.4	65.5
5	94.6	100	21.4	77.4
Mean	85.2	98.9	30	77.6
SD	±13.8	±1.1	±17.7	±11.1
P values‡	$P < 0.1 > 0.05$		$P < 0.001$	

In each experiment a single preparation of lymphocytes was used in 4-ml cultures. The greater inhibition of lymphocyte transformation by fetuin at 2.5 vs. 1.25 mg/ml for both L- and H-PHAP is highly significant (*P* values < 0.001 and < 0.01, respectively).

* Used at a concentration of 4 μg/ml.

‡ Comparison between L- and H-PHAP at each fetuin concentration.

cells remaining in culture nor their viability as measured by trypan-blue exclusion (Table III).

We have also confirmed the capacity of increasing doses of H-PHAP to cause a partial reversal of the inhibitory effects of fetuin on H-PHAP-stimulated lymphocyte transformation (9). When increasing doses of L-PHAP were employed in a similar fashion, however, a paradoxical effect was noted. Increments in L-PHAP concentration not only failed to overcome the inhibitory effects of fetuin, but led as well to an increase in its inhibitory action (Table IV). On repeated examinations, this result was most striking at a fetuin concentration of 1.25 mg/ml, where the increase in fetuin inhibition at higher L-PHAP doses achieved a high level of statistical significance (Fig. 3).

Although we have not made any detailed studies of the physical interaction between CON-A or PWM and fetuin, we have demonstrated that the presence of

TABLE II

A Comparison of the Ability of Fetuin to Inhibit Lymphocyte Transformation Induced by a Variety of Phytomitogens

Mitogen (dose)	Fetuin	[2-^{14}C]thymidine incorporation (mean ± SD)	DNA synthesis inhibition	*P* values
	mg/ml	*cpm*	%	
L-PHAP (4 μg/ml)	0	2,845 ± 95	—	—
	1.25	2,236 ± 88	21.4	<.01*
	2.5	154 ± 190	94.6	<.01*
H-PHAP (4 μg/ml)	0	2,237 ± 261	—	—
	1.25	505 ± 133	77.4	<0.01*
	2.5	0	100	—
PWM (0.5 μg/ml)	0	1,987 ± 39	—	—
	1.25	1,542 ± 98	22.4	<0.01*
	2.5	339 ± 162	83	<0.01*
CON-A (12.5 μg/ml)	0	1,329 ± 320	—	—
	1.25	486 ± 180	63.5	<0.02*
	2.5	31 ± 54	98	<0.01*

A single lymphocyte preparation was employed throughout in 4-ml cultures.
* Compared with the amount of DNA synthesis observed in the absence of fetuin.

TABLE III

The Effect of Varying Fetuin Concentrations on Lymphocyte Survival (% of Starting Cell Numbers) and Lymphocyte Viability (Trypan-Blue Exclusion), after 88-h Culture in the Absence of Mitogens

Exp.	Fetuin concentration (mg/ml)							
	0		0.63		1.25		2.5	
	Surviving cells	Viable	Surviving cells	Viable	Surviving cells	Viable	Surviving cells	Viable
	%	%	%	%	%	%	%	%
1	29	92	41.9	89	54.8	91.5	29.7	66
2	100	91.5	100	96.5	100	92	100	94.5
3	71	97.5	48.4	91	51.6	66	64.5	46
4	42.9	86	46.8	86.5	40.7	81.5	44.5	80
5	71.3	90	57.8	90	49.6	86.5	48.7	77
Mean±SD	62.8 ± 27.7	91.4 ± 4.1	59.0 ± 23.6	90.6 ± 3.7	59.3 ± 23.3	83.5 ± 10.7	57.5 ± 26.8	72.7 ± 18.1

There is no significant difference in either parameter between control and fetuin-exposed lymphocytes at any fetuin concentration.

fetuin can also inhibit the ability of lymphocytes to synthesize DNA when these mitogens are employed (Table II). In addition, fetuin proved to be a potent inhibitor of ATS-induced lymphocyte transformation (Table V) as well as of the lymphocyte DNA-synthetic response in the one-way MLC (Table VI).

Attempts to demonstrate irreversible association between lymphocytes and

TABLE IV

The Effect of Increasing Mitogen Dose on the Bility of Fetuin to Inhibit L- and H-PHAP-Induced Lymphocyte Transformation

Mitogen	Dose	Fetuin	[2-^{14}C]thymidine incorporation (mean ± SD)	DNA synthesis inhibition
	mg/ml		*cpm*	%
H-PHAP	4.6	—	22,057 ± 167	—
		1.25	6,935 ± 1,050	68.6
		2.5	462 ± 75	97.9
	7.7	—	21,890 ± 737	—
		1.25	12,189 ± 24	44.3
		2.5	693 ± 266	96.8
	15.4	—	22,803 ± 4,224	—
		1.25	19,293 ± 392	15.4
		2.5	1,328 ± 229	94.2
	30.8	—	19,914 ± 1,027	—
		1.25	18,101 ± 943	9.1
		2.5	1,490 ± 202	92.5
L-PHAP	4.6	—	4,397 ± 839	—
		1.25	5,053 ± 1,300	0
		2.5	1,415 ± 874	67.8
	7.7	—	4,914 ± 311	—
		1.25	3,833 ± 231	22
		2.5	905 ± 182	81.6
	15.4	—	5,252 ± 166	—
		1.25	3,388 ± 474	35.5
		2.5	578 ± 200	90.1
	30.8	—	4,833 ± 178	—
		1.25	1,950 ± 466	59.6
		2.5	120 ± 195	97.5

A separate preparation of lymphocytes was employed for each mitogen. In this experiment, a 1-ml culture system containing 1.5–2 × 10^6 lymphocytes was used.

[^{125}I]fetuin were unsuccessful. Fetuin neither binds to, nor is ingested by, lymphocytes to any significant extent, whether the exposure of lymphocytes to fetuin occurs over a short or long period in tissue culture; addition of L-PHAP in optimum mitogenic doses did not alter this result (Table VII).

Discussion

The evidence for a strong physical interaction between fetuin and H-PHAP is persuasive; the two proteins can coprecipitate in agar gel, and the dissolution of

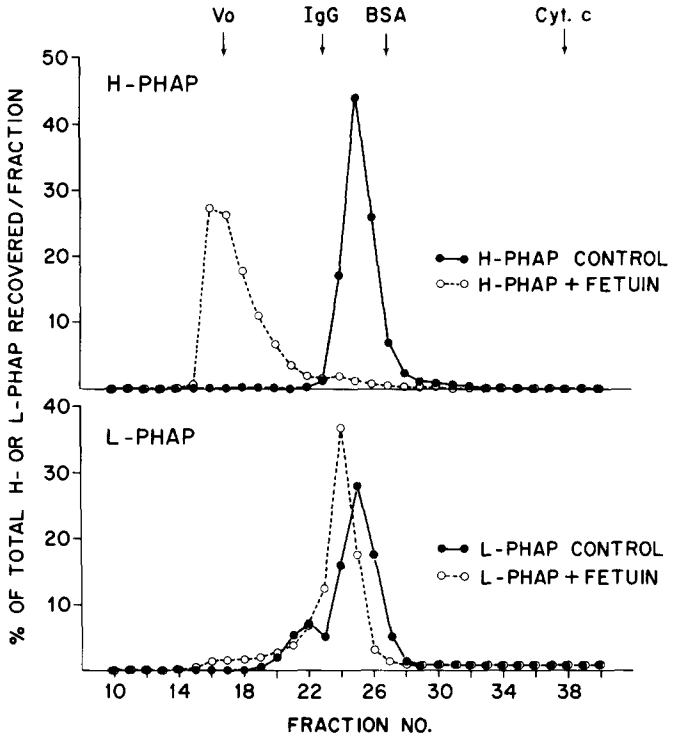

Fig. 3. The effect of increasing mitogen concentrations on the inhibition of PHAP-induced lymphocyte transformation by fetuin. A total of 10 different experiments (five H-PHAP, five L-PHAP) utilizing different lymphocyte preparations in a 1-ml culture system are represented. At a fetuin concentration of 1.25 mg/ml (A) the following statistical comparisons were made: *a* vs. *b*, $P < 0.05$, *b* vs. *c*, $P < 0.05$; *b* vs. *d*, not significant; *b* vs. *e*, $P < 0.01$. Similar statistical comparisons at a fetuin concentration of 2.5 mg/ml (B) revealed no significant differences.

TABLE V

The Inhibition of ATS-Induced Lymphocyte Transformation by Fetuin

ATS dose	Fetuin (mg/ml)	[2-^{14}C]thymidine incorporation (mean ± SD)	DNA synthesis inhibition	*P* values*
μl	*mg/ml*	*cpm*	%	
20	0	22,478 ± 5,343	—	—
	1.25	16,335 ± 4,662	27.3	NS‡
	2.50	607 ± 686	97.3	<0.01
50	0	49,506 ± 3,974	—	—
	1.25	6,087 ± 3,537	87.7	<0.001
	2.50	1,934 ± 1,675	96.1	<0.001

* Compared with the amount of DNA synthesis observed in the absence of fetuin.
‡ NS, not significant.

TABLE VI

The Effect of Fetuin on Lymphocyte Transformation Induced by One-way MLC

Exp.	Fetuin	[2-^{14}C]thymidine incorporation (mean±SD)	DNA synthesis inhibition	*P* values
	mg/ml	*cpm*	%	
1	0	7,781 ± 171	—	—
	1.25	462 ± 510	94.1	<0.001*
	2.5	217 ± 54	97.2	<0.001*
2	0	8,710 ± 313	—	—
	1.25	6,488 ± 852	25.5	<0.02*
	2.5	8 ± 14	99.9	<0.001*
3	0	65,383 ± 5,998	—	—
	1.25	34,074 ± 4,975	47.9	<0.01*
	2.5	13,463 ± 10,420	79.4	<0.01*
4	0	27,350 ± 931	—	—
	1.25	6,329 ± 8,517	76.9	<0.02*
	2.5	9,641 ± 4,273	64.7	<0.01*
Mean±SD of four experiments	1.25		61.1 ± 30.4‡	
	2.5		85.3 ± 16.5‡	

* Compared with the amount of DNA synthesis observed in the absence of fetuin.
‡ The difference between these values is not significant.

the precipitates thus formed in the presence of high fetuin:H-PHAP concentration ratios is reflected in the formation of high-mol-wt soluble complexes between fetuin and H-PHAP in free solution at high fetuin:H-PHAP concentration ratios. The gel diffusion experiments would support the conclusion that, in the presence of a large excess of fetuin, each H-PHAP molecule combines with at least two molecules of fetuin, and that some H-PHAP molecules may in fact combine with more than two fetuin molecules. The L-PHAP molecule, on the other hand, shows little, if any affinity for fetuin; it lacks the ability to form agar gel precipitates with fetuin, and during gel diffusion in the presence of a large fetuin excess only 6–7% of the molecules in L-PHAP are shifted to significantly higher mol wt regions. This figure corresponds closely to an earlier estimate that we have made (8%), on the basis of NH_2-terminal amino acid analysis, of the degree to which our L-PHAP preparations are contaminated by R subunits, presumably in the form of the 3L-1R PHAP isomitogen (1). The low affinity which L-PHAP displays for fetuin is reminiscent of its inability to combine with other serum glycoproteins, and parallels as well its almost total inability to bind to or agglutinate RBC (4). The L subunit of PHAP isomitogens fails to recognize similar glycopeptide structures on fetuin and RBC membranes, which, however, interact strongly with R subunit-containing PHAP molecules (H-PHAP) (6). These observations lend reinforcement to previous evidence which suggests that

TABLE VII

The Interaction of [^{125}I]Fetuin with Lymphocytes

	Cell type	Incubation time	% [^{125}I]fetuin bound	
			Mitogen free	+ 5 μg/ml L-PHAP
		h		
Short-term lymphocyte cultures*	Lymphocytes	0.5	0.03	ND‡
	Lymphocytes (85%) + monocytes (15%)§	0.5	0.05	ND
Long-term lymphocyte cultures‖	Lymphocytes (77%) + monocytes (23%)§	1	0.005	0.007
		18	0.003	0.009
		42	0.002	0.005
		66	0	0.019

* 5×10^6 cells incubated in 1.2 ml RPMI 1640 + 2.5 mg/ml [^{125}I]fetuin (2.41×10^6 cpm).

‡ ND, not done.

§ Determined by latex particle ingestion.

‖ 4×10^6 cells incubated in 4 ml RPMI 1640 + 12.5% AB serum, containing 2.25 mg/ml fetuin and 0.25 mg/ml [^{125}I]fetuin (8.64×10^5 cpm). The values shown are the mean results of two separate experiments.

the receptor for PHAP isomitogens on lymphocytes must be different from that on RBC membranes or serum glycoproteins; that the ability of H-PHAP to bind to and transform lymphocytes in a reflection of their hybrid structure; and that such binding and transformation take place predominantly, if not exclusively, through the L subunits of H-PHAP. Thus, with increasing R:L subunit ratios, the H-PHAP isomitogens become increasingly potent as hemagglutinins, but lose their lymphocyte-transforming activity (4). The 4R H-PHAP isomitogen is almost completely devoid of lymphocyte-transforming ability.[3]

Since fetuin can prevent binding of H-PHAP to lymphocytes, and can even reverse a preexisting association between H-PHAP molecules and the lymphocyte membrane (9), while simultaneously lacking the ability to combine with the L subunits which mediate such binding, it cannot truly be described as a competitive inhibitor of the ability of H-PHAP to attach to lymphocyte membrane receptors. Rather, the presence of large amounts of fetuin would allow for the formation of soluble complexes between fetuin and the H-PHAP molecule, mediated by the strong affinity of the R subunit for the fetuin glycopeptide. The H-PHAP molecules in such complexes may then be unable to bind to lymphocytes through a process of steric hindrance, or possibly through some allosteric conformational change in their L subunit lymphocyte-binding site. The partial removal of previously bound H-PHAP from lymphocyte membranes by the addition of fetuin may simply reflect the fact that the R subunit on hybrid H-PHAP molecules already bound has a higher affinity for the

[3] Kornfeld, S. Personal communication.

fetuin glycopeptide than their L subunit has for the lymphocyte membrane receptor. After their removal through such a process, reattachment of H-PHAP molecules would be prevented by the mechanisms described above. These processes would adequately explain the inhibition of H-PHAP-induced lymphocyte transformation by fetuin and would also be consistent with the observation that the inhibitory effects of fetuin may be partially overcome by increments in the H-PHAP stimulating dose, since the new equilibrium reached in the competition between fetuin-R subunit and lymphocyte-L subunit associations would result in a greater amount of H-PHAP bound to, and stimulating, lymphocytes.

Since CON-A has also been shown to react strongly with a variety of serum glycoproteins (16) the mechanism by which fetuin inhibits its mitogenic action may resemble that described for H-PHAP. While we can say little about the physical interaction between PWM and fetuin, in the case of L-PHAP it is doubtful that the weak interaction it manifests with fetuin would suffice to account for the ability of the latter to inhibit L-PHAP lymphocyte-mitogenic activity. Although the mitogenic activity of L-PHAP at moderate concentrations (4–5 μg/ml) is significantly less inhibited by 1.25 mg/ml fetuin than that of H-PHAP, at a fetuin concentration of 2.5 mg/ml this difference is largely abolished, and cannot be explained by nonspecific toxic effects of fetuin upon the lymphocytes, for reasons which have already been discussed (9). These observations raise the possibility that fetuin has some direct effect upon the lymphocyte itself which is able to suppress its response to mitogenic stimuli. The paradoxical difference between H- and L-PHAP with respect to the ability of increasing mitogen concentrations to overcome this inhibitory effect of fetuin would also suggest that simple mitogen binding cannot account for the inhibitory effects of fetuin upon L-PHAP-induced lymphocyte transformation, since these effects would then be expected to parallel those observed with H-PHAP. It is also pertinent to note that the ability of fetuin to inhibit lymphocyte transformation by the two PHAP mitogens is significantly different only at the lowest mitogen dose, and then only at a fetuin concentration of 1.25 mg/ml. At all other mitogen:fetuin concentration ratios the inhibitory effects of fetuin for L- and H-PHAP are not significantly different. The reversal of fetuin inhibition by increasing H-PHAP concentrations reaches a plateau at 15.4 μg/ml; further increase in the H-PHAP dose does not result in a proportional fall in the inhibitory effects of fetuin. Thus it appears that, in the case of H-PHAP, the inhibitory effects of fetuin may initially depend upon simple competition for mitogen through soluble complex formation; but a second process resembling that seen with L-PHAP may also play a role in preventing transformation. The paradoxical increase in the ability of fetuin to inhibit lymphocyte transformation at higher L-PHAP doses also suggests that the direct inhibitory effect of fetuin on lymphocyte transformation is in some way related to, and dependent upon, the intensity of the mitogenic stimulus.

The experiments with ATS also support the theory that fetuin can directly inhibit the lymphocyte response to mitogens, unless fetuin happened to share an immunologically similar structural determinant with human fetal thymocytes. However, the most compelling evidence for a direct role of fetuin in suppressing

lymphocyte responses to mitogenic stimuli comes from a consideration of its effect upon the one-way MLC, where no soluble mitogenic material with a possible independent affinity for the fetuin molecule is involved. In whatever fashion fetuin inhibits the MLC, it does not seem to involve a firm, irreversible association with either the stimulating or responding cell populations, as our experiments with [^{125}I]fetuin demonstrate.

Fetuin is the bovine representative of a class of proteins, the α-fetoproteins, which are present in fetal sera but lacking in homospecific adult sera, and whose role in fetal life is not well understood. The analogous human α-fetoprotein is present in near-maximal concentrations (approximately 200 mg/100 ml) by the 10th wk of gestation. This high serum level is maintained until approximately 22 weeks' gestation and thereafter falls progressively to <5 mg/100 ml near term (17). One striking property of fetuin is its ability to act as a serum substitute and growth factor for many mammalian cell lines in tissue culture (18). It has also been reported to stimulate the proliferation of hematopoietic stem cells when injected into mice (19). Thus it may play a general role as a supporter of, and stimulus for, the high rate of cell proliferation characteristic of fetal development. In this context, its inhibitory effect upon in vitro lymphocyte proliferation may reflect a second highly specialized function in fetal life essential for successful embryonic development, namely, the development of immunologic tolerance to autologous antigens. Its role in this process may rest upon three aspects related to our in vitro observations: (*a*) Fetuin does not adversely affect resting lymphocyte viability. (*b*) It is able to inhibit the proliferation of lymphocytes in the presence of a mitogenic stimulus. (*c*) Its inhibitory effect upon lymphocyte proliferation is proportional to the intensity of the mitogenic stimulus and might conceivably contribute to cell death should the stimulus be strong enough. These premises would allow for the preservation of the repertoire of specificities required of an intact, universally responsive, postnatal immune system, while at the same time suppressing, and possibly aiding in the elimination of, those lymphocyte clones subjected to sustained and intensive provocation by autoantigens in fetal life. Further experiments are in progress to test the validity of these hypotheses.

The capacity of fetuin for inhibiting the proliferative response of lymphocytes to a variety of mitogenic stimuli is reminiscent of the similar effects of another serum α-protein, the immunoregulatory α_1-globulin (IRA) (20). Even though IRA also possesses the ability to combine with and precipitate H-PHAP, but not L-PHAP, it is able to suppress lymphocyte transformation by both mitogens approximately to the same degree (21). A variety of studies has made clear that the immunosuppressive functions of IRA depend upon some direct, but poorly understood effect which it exerts upon the lymphocyte itself (22, 23), and that it is capable of such diverse actions as the inhibition of the lymphocyte mitogenic response to phytomitogens and antilymphocyte serum, as well as to antigens and to the mixed leukocyte reaction (22). In addition, IRA inhibits antibody formation and skin graft rejection when it is administered to mice before appropriate challenge (24, 25). While we do not understand the precise mechanism by which α-glycoproteins such as fetuin or IRA inhibit lymphocyte responsiveness, the special ability which fetuin possesses to perturb the limiting

membrane of macrophages and to stimulate pinocytosis may offer a clue (26), since the initial step in the immune response, or in the activation of lymphocytes by phytomitogens, depends upon the ability of mitogens or antigen to combine with membrane receptors. Derangement of lymphocyte membrane stability, or preventing access to crucial sites on the lymphocyte cell surface early in the course of mitogen action via a "blindfolding" effect (21, 23), by a substance such as fetuin, may interfere with these initial steps.

Summary

Fetuin, the bovine α-fetoprotein, contains glycopeptide sequences similar to those found on red cells. As a result, it is capable of strong physical interaction with the phytohemagglutinin isomitogens (H-PHAP) which possess two or more R (red cell binding) subunits as part of their tetrameric structures. Fetuin shows little or no interaction with L-PHAP, a phytohemagglutinin made up of four L subunits which also lack red cell affinity. Despite these differences fetuin is able to inhibit both H- and L-PHAP-induced lymphocyte transformation and is also capable of inhibiting the mitogenic effects of pokeweed mitogen, concanavalin A, antithymocyte antiserum, and the one-way mixed lymphocyte culture. In the case of L-PHAP, the inhibitory effect of fetuin is proportional to the intensity of the mitogenic stimulus. The inhibitory effects of fetuin upon lymphocyte transformation may result from perturbation or "blindfolding" of the cell membrane in a manner analogous to other immunosuppressive serum α-globulins. α-Fetoproteins may play an immunoregulatory role during fetal development.

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