Brief Definitive Report

SUPPRESSION OF IN VITRO ANTIBODY RESPONSE BY A SERUM FACTOR (SAA) IN EXPERIMENTALLY INDUCED AMYLOIDOSIS*

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Secondary amyloidosis typically occurs in chronic disease states in which there is presumed to be persistent antigenic stimulation. Therefore, it has long been hypothesized that secondary amyloidosis might be the result of an abnormality in antibody production. The finding that amyloid fibril proteins in primary amyloidosis are composed mainly of fragments of immunoglobulin light chains strengthened the argument that immunoglobulin abnormalities were involved in amyloidogenesis (1). However, the deposits in secondary amyloidosis have been shown to be largely composed of a small molecular weight protein (8,400 daltons) which bears no relationship to any known immunoglobulin molecule (2, 3). This protein (AA) is felt to be derived from a serum alpha globulin (SAA) (mol wt approx. 100,000) which has been shown to be elevated in amyloidosis and probably is present in small amounts in all normal sera (4, 5). The function of this protein (SAA) and its role in amyloidogenesis are the subject of this report. The availability of an experimental model of amyloidosis in the mouse which appears to be analogous to human secondary amyloidosis (6) has allowed us to demonstrate that SAA suppresses antibody formation.

Materials and Methods

Mouse Sera. 8-wk old female CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) were made amyloidotic by 21 daily injections of 0.5 ml of 10% casein as previously described (6). The animals were bled 3 days after the last casein injection. Normal 10- to 12-wk old female CBA/J mice were bled for control serum.

Preparation of Antiserum. Amyloid fibrils, isolated from the spleens of amyloidotic CBA/J mice as previously described (7), were solubilized in 4 molar guanidine and fractionated on Sephadex G100. The major retarded fraction (protein AA) was isolated and used to immunize New Zealand white rabbits by serial injections in complete Freund's adjuvant.

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Spleen Cell Cultures. The Mishell-Dutton culture system was used for antibody stimulation in vitro (8). 10- to 12-wk old CBA/J mice were immunized with 0.1 cc of a 10% suspension of sheep red blood cells (SRBC) (Colorado Serum Company, Denver, Colo.). 3 days later the mice were sacrificed and sterile spleen cell suspensions made in Eagle's minimal essential media (MEM) which had been supplemented with 10% fetal bovine serum (Reheis Chemical Company, Chicago, Ill.). 1 ml of cell suspension (1.5×10^7 cells) was placed in a 30 mm petri dish with varying amounts of either normal mouse serum or amyloid mouse serum and 50 μl of 1% SRBC as antigen. On the fourth day the cells were harvested and SRBC antibody-forming cells determined using the Jerne hemolytic plaque assay. Each experimental and control group consisted of 6–10 separate cultures. Viability testing was done using trypan blue dye exclusion. To measure indirect plaque-forming cells (PFC) two sets of agar plates were prepared for each sample. In one set direct PFC were developed with guinea pig complement. The second set was incubated with rabbit antimouse IgG and complement. Indirect or 7S PFC were calculated as the difference between total PFC and direct PFC.

Amyloid serum was absorbed by incubating with an equal volume of rabbit anti-AA serum for 48 h and centrifuging at 18,000 *g* for 1 h. The data were analysed statistically using the Student's *t* test, and are expressed as geometric mean ± standard error. For clarity some data are expressed as response index (ratio of PFC/culture of experimental to PFC/culture of controls).

Results

Initial experiments were done to determine if serum from amyloidotic mice would suppress antibody formation in vitro. 50 μl of amyloidotic serum were added to each experimental culture (5% vol/vol) while equal volumes of normal mouse serum (NMS) were added to cultures as controls. The amyloidotic serum caused significant suppression of direct (19S) PFC in three separate experiments (Table I). Since the spleen cell donors were primed before culture, it is possible that some 7S PFC were produced which were not suppressed. Therefore the experiment was repeated using an indirect (7S) PFC assay. Again 50 μl of amyloidotic serum suppressed 90% of the control response (259 PFC for NMS versus 26 PFC for amyloid serum $P < 0.025$). Percent of viable cells after 4 days in culture as determined by trypan blue dye exclusion was the same in the cultures incubated with amyloidotic serum as in control cultures (60%). This excludes a nonspecific cytotoxic effect on cultured lymphocytes as the cause of suppression.

The suppression of antibody response to SRBC was proportional to the amount of amyloidotic serum added to the culture and ranged from 99.8% suppression at a concentration of 10% serum down to 69% at 1% serum (Table II). Significant suppression was achieved with as little as 1% serum in the culture.

To determine if the suppressive factor was serum A protein (SAA), specific rabbit antiserum to murine amyloid protein AA was used to absorb the amyloid serum. This antiserum, which was produced to the tissue protein AA, showed a line of identity between AA and amyloid mouse serum by double diffusion in

TABLE I
Inhibition of In Vitro Antibody Response by Amyloid Serum

Exp.	Normal mouse serum (5%)	Amyloid mouse serum (5%)	Inhibition	*P*
	PFC/culture ± SE	*PFC/culture ± SE*	%	
1	2,456 ± 35	37 ± 13	98	<0.001
2	1,135 ± 321	48 ± 18	96	<0.025
3	4,366 ± 551	832 ± 83	81	<0.005

TABLE II
Dose-Response Effect of Amyloid Serum on Antibody Response to SRBC

	Concn.	Direct PFC/ culture ± SE	Inhibition	*P*
	%		%	
Normal mouse serum	5	6,431 ± 602		
Amyloid mouse serum	0.5	3,108 ± 375	52	<0.1
	1.0	1,974 ± 139	69	<0.005
	2.0	720 ± 150	89	<0.001
	5.0	326 ± 48	95	<0.005
	10.0	15 ± 6	99.8	<0.001

agar. The amyloid serum was also absorbed with normal rabbit serum as a control. The results (Fig. 1) show that amyloid mouse serum (AMS) suppressed PFC response to 20% of normal mouse serum controls ($P < 0.05$); and after absorption with normal rabbit serum (NRS), the degree of suppression was still highly significant ($P < 0.005$). Absorption of the AMS with anti-AA reversed the suppression ($P < 0.005$ comparing PFC response of absorbed and unabsorbed

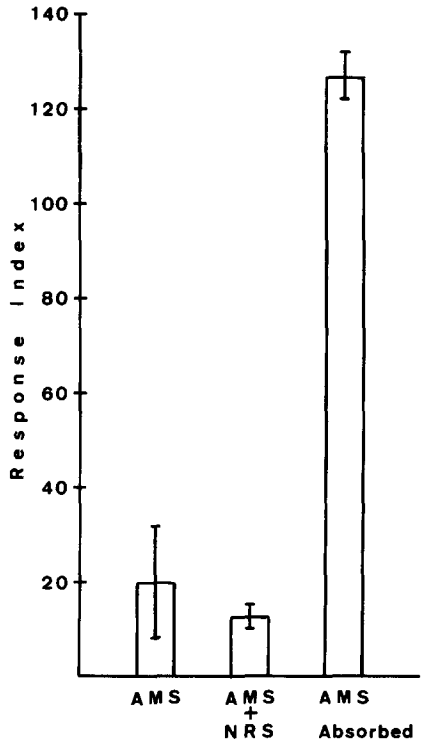

FIG. 1. Reversal of amyloid serum suppression of PFC by absorption with rabbit antiamyloid AA. Results expressed as response index (ratio of PFC/culture in experimental to PFC/culture in normal mouse serum controls). Amyloid serum suppresses significantly ($P < 0.05$). After absorption with normal rabbit serum, amyloid serum still suppresses ($P < 0.005$). Absorption with rabbit anti-AA reverses suppression of amyloid serum.

AMS). Rabbit anti-AA when added to control cultures alone had no stimulatory effect upon PFC response to SRBC.

Since normal mouse serum has previously been shown to have a suppressive effect on antibody formation (9), four separate batches of normal CBA/J sera were added to cultures and compared to cultures having only fetal calf serum. We found that 5% normal mouse serum suppressed between 30% and 50% of the normal SRBC response in three out of four batches of serum. Since we have demonstrated trace amounts of SAA in normal mouse serum by radioimmunoassay,[1] it appeared possible that SAA could account for the suppressive effect of normal mouse serum. Therefore, we absorbed normal mouse serum with specific anti-AA and added the absorbed serum to spleen cell cultures (Fig. 2). Absorption with anti-AA removed the suppressive effect of normal mouse serum. Control cultures containing both normal rabbit serum (5%) and NMS (5%) did not show any reversal of PFC suppression.

Discussion

Casein-induced amyloidosis in the mouse and guinea pig appears to be analogous to human secondary amyloidosis (7, 10). All have as a major component of the fibril deposits a small molecular weight protein (AA). The entire sequence of 76 amino acids is known for the human protein (3). The guinea pig-derived protein has been sequenced to 25 residues and is homologous to the human AA except for a 5 amino acid peptide at the amino terminus.

In all three species there is a circulating alpha globulin which immunologically cross reacts with antiserum to the autologous tissue protein AA but in each case is species specific. In the human this alpha globulin (SAA) which is believed to be a precursor of the fibril protein has a mol wt of approximately 100,000 (3, 5). It is present in small amounts in all normal sera and is markedly elevated in secondary amyloidosis, cancer, and many acute and chronic inflammatory diseases (4, 5, 11). Sera from amyloidotic mice have elevated levels of SAA as shown by Ouchterlony analysis and radioimmunoassay (12, footnote 1). Normal CBA/J serum has low levels of SAA by radioimmunoassay. No specific functional activity of this circulating protein has been previously described.

The present studies clearly show that serum from amyloidotic mice markedly suppresses in vitro antibody response to SRBC. This suppression is reversed by absorbing the serum with rabbit antiserum specific for murine amyloid protein AA but is not reversed by normal rabbit serum. Thus the suppressive factor in amyloidotic serum would appear to be SAA. This is the first demonstration of an immune suppressive function for amyloid protein SAA.

Veit and Michael have previously shown that mouse serum suppresses the primary immune response to SRBC (9, 13). This suppression was greater in serum from immunized mice. The possibitity exists that this suppression is related to SAA, and is supported by the demonstration that anti-AA reverses the suppression of normal mouse serum in this study.

The mechanisms involved in immune suppression by SAA are not clear. Antibody response to SRBC is thymus-dependent and, therefore, suppression

[1] Benson, M.D. Manuscript in preparation.

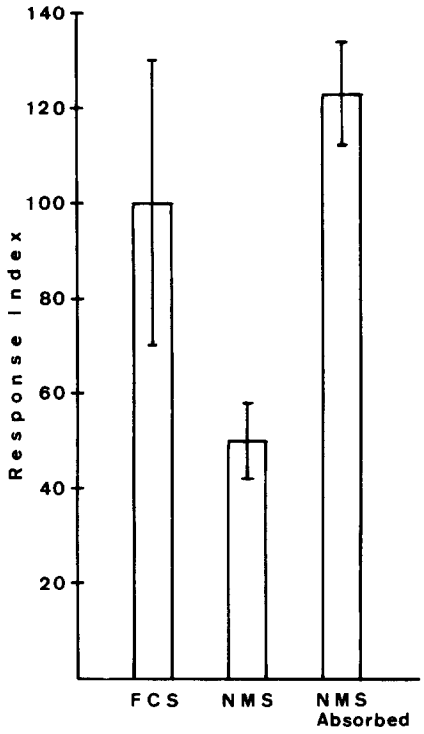

FIG. 2. Reversal of NMS suppression of PFC by absorption with antiamyloid AA. Results expressed as response index (ratio of PFC/culture of experimental to PFC/culture in fetal calf serum).

could be either affected upon thymus or bone marrow-derived cells. If SAA is the same factor that has been studied by Veit and Michael, it would appear from their data that it suppresses T cells and not B cells directly. Even so, the SAA could function by direct action upon the antigen-sensitized cells or through suppressor cells. It would appear that the SAA is a nonspecific inhibitor of antibody response. Whether it regulates other processes of the immune system, such a cell-mediated immunity, has not yet been determined.

Within the past few years a large amount of data have been accumulated which suggest abnormalities in immune functions in casein-treated animals (14, 15). Whether any of these findings are related to SAA is not yet known. The present studies tend to suggest that SAA is a normal hormonal factor which is elevated in response to antigenic stimulation. Therefore, there may be no abnormality in immune regulation but just the normal control of antibody response. In secondary amyloidosis the abnormality may be only the inability of the body to deal with the sustained high levels of SAA caused by chronic antigenic stimulation.

Summary

Serum from CBA/J mice made amyloidotic by chronic casein injections has been shown to suppress in vitro antibody response to SRBC. Similar suppression

was also found with normal mouse serum but to a much lesser degree. This suppressive activity of both amyloidotic serum and normal serum was removed by absorption of the sera with antiserum to protein AA, the major constituent of casein-induced (secondary) amyloid fibrils. This antiserum to the amyloid fibril protein AA (mol wt 8,400 daltons) detects an immunologically cross-reacting serum alpha globulin (SAA) (mol wt approx. 100,000). It is postulated that the serum factor (SAA) is a regulator of antibody response and may be present in elevated amounts as the result of chronic antigenic stimulation.

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