EXPERIMENTAL AMYLOIDOSIS: THE INDUCER IS A POLYCLONAL B-CELL ACTIVATOR TO WHICH SUSCEPTIBILITY IS UNDER GENETIC CONTROL*

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Deposition of amyloid material in various tissues is an occasional sequelae of various autoimmune disorders, chronic infections, and myeloma in man (1). Histologically similar lesions can be induced in experimental animals by repeated injections of casein (2). As the amyloid deposits in both these instances comprise material derived from gamma globulin molecules (3) it has been suggested that both clinical (1) and experimental amyloidosis (4) invoke hypersensitivity or autoimmune reactions. However, amyloidosis can be induced in neonatally thymectomized mice (5) and animals made immunologically tolerant to casein (5). Therefore, the immunological background to at least casein-induced experimental amyloidosis remains obscure. In this communication we have studied the in vitro effects of casein on spleen cells from amyloid-susceptible and amyloid-resistant mice with reference to polyclonal antibody production and DNA synthesis.

Materials and Methods

Animals. Inbred mice of strains A/Jax, CBA/Jax, and CBA/C57BL from the colonies at the Department of Bacteriology, Life Sciences, U. C. L. A., Los Angeles, Calif., and the Department of Immunobiology, Wallenberglaboratory, Stockholm, Sweden were used. They were kept under similar conditions and were originally purchased from The Jackson Laboratory, Bar Harbor, Maine. The animals were 3- to 4-mo old when used and they were all females.

Mitogens. Lipopolysaccharide (LPS)[1] from *Escherichia coli* 055:B5 was used as a B-cell mitogen and polyclonal activator (PBA) (6). It was produced and handled as described previously (6). Phytohemagglutinin (PHA-P, Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.) was used as a T-cell mitogen. Casein in the form of sodium caseinate was purchased from National Biochemical Co., Chicago, Ill., and dissolved in buffered isotonic saline. It was ultrafiltrated before used in tissue culture experiments.

In Vitro Procedures

TISSUE CULTURE TECHNIQUES. Mouse spleen cells were prepared and kept in culture essentially as described by Mishell and Dutton (7) although at a microscale. Thus, 0.2 ml of the cell suspension at a concentration of 10^7/ml was cultivated in a well on a sealed microplate (Microtest;

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[1] *Abbreviations used in this paper:* LPS, lipopolysaccharide; PBA, polyclonal activator; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin.

Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). All cultures were done under serum-free conditions.

THE AGAR PLAQUE TECHNIQUE. The agar plaque technique of Jerne et al. (8) with modifications (9) was used for detection of single antibody-forming cells. Target cells were NNP-haptenated sheep red blood cells (type II) according to a chemical coupling procedure of Pasanen and Mäkelä (10). Cells from three cultures were pooled. Mean values from two slides of this pool are indicated.

DNA SYNTHESIS. DNA synthesis of cultivated cells was determined by exposing the cells to 2 μCi [^{3}H]thymidine (The Radiochemical Centre, Amersham, England) for 18 h under microculture conditions as above. After this the cells were collected on glass filters, washed several times with water, and the retained radioactivity determined in a liquid scintillation counter (Tri-Carb, Packard Instrument Co., Inc., Downers Grove, Ill.).

In Vivo Procedures. The capacity of sodium caseinate to induce peritoneal exudate was checked by injecting 2 ml of a 10% solution of sodium caseinate intraperitoneally. 3 days later the peritoneal cavity was rinsed with 5 ml of saline and the recovered cells counted in phase-contrast microscopy. Thymus cell-deprived mice were obtained by thymectomizing adult animals which were 2 wk later lethally irradiated and repopulated with 50×10^6 syngeneic fetal liver cells. Their spleens were used 3 wk later.

Results

The Effect of Casein on DNA Synthesis and Polyclonal Antibody Synthesis in Mouse Spleen Cells. Various concentrations of sodium caseinate were added to mouse (CBA/C57BL) spleen cells, and the DNA synthesis (Table I) and polyclonal antibody synthesis (Table II) as revealed by the number formation of direct anti-NNP-producing cells (NNP-PFC) was determined after 2 days. The values were compared with those obtained with cells exposed to LPS and PHA, respectively.

It can be seen that casein was a mitogen (Table I), although weaker than LPS and PHA. Sodium caseinate induced strong polyclonal antibody formations in mouse spleen cells (Table II) as did LPS, but not PHA (Table II). Casein induced a maximal response at a slightly lower concentration than LPS.

The kinetics of induction of polyclonal antibody formation with LPS and sodium caseinate were similar, although peak responses for casein appeared to be somewhat delayed (Table III). The responder cell to sodium caseinate was present in spleen cells from thymectomized lethally irradiated fetal liver reconstituted mice, because such cells responded both with increased DNA and

TABLE I

DNA-Synthetic Response of Mouse (CBA × C57BL) Spleen Cells Exposed for 2 days to Various Doses of PHA, LPS, and Casein

Dose/ml	Substance added		
	PHA	LPS	Sodium caseinate
μg	*mean of three cultures ± 1 SE*		
—	3.460 ± 180	3.460 ± 180	3.460 ± 180
0.1	18.770 ± 1.320	3.250 ± 110	3.810 ± 340
1	59.720 ± 3.150	6.040 ± 1.940	9.130 ± 640
10	44.160 ± 1.240	19.160 ± 1.950	15.460 ± 990
100	9.330 ± 1.770	38.800 ± 2.510	14.600 ± 1.570
500	1.460 ± 1.550	33.590 ± 1.790	9.990 ± 1.320

TABLE II

Number of Anti-NNP Plaque-Forming Cells/10^6 Recovered Cells in CBA × C57BL Mouse Spleen Cells Exposed to Various Doses of PHA, LPS, and Casein, Respectively, for 2 days

Dose/ml	Substance added		
	PHA	LPS	Casein
μg			
—	16	16	16
0.1	12	20	18
1	28	34	38
10	8	182	494
100	0	346	896
500	0	702	540

TABLE III

Number of Anti-NNP Plaque-Forming Cells/10^6 Recovered Cells in Spleen Cells from CBA × C57BL Mice Exposed to 100 μg LPS and Casein, Respectively, for Varying Times

Days in culture	Substance added		
	LPS	Casein	None
1	42	38	14
2	490	522	28
3	525	970	32
4	180	806	10

polyclonal antibody synthesis to casein, whereas they were absolutely nonresponsive to the mitogenic effect of PHA (Table IV).

Genetic Variation in Susceptibility of Mouse Spleen Cells to Sodium Caseinate. Since casein-induced experimental amyloidosis can be readily induced in CBA/J mice, but not in A/J mice (2), spleen cells from these two strains of mice were exposed to casein (Table V) and their polyclonal antibody synthesis was determined (Table V). It can be seen that sodium caseinate was a much stronger inducer of polyclonal antibody synthesis in spleen cells from CBA/J mice than from A/J mice, although the background response in nonstimulated CBA spleen cells was also higher.

The tissue-irritating capacity of sodium caseinate was compared in CBA/J and A/J mice. To this end, the animals were injected intraperitoneally with 2 ml of a 10% solution of sodium caseinate and the number of cells in the peritoneal exudate determined 3 days later (Table VI). The tissue-irritant capacity of sodium caseinate was similar in these strains of mice.

Discussion

Much evidence now suggests (1) that amyloidosis in man is immunological in nature. This is mainly so because of the prominence of immunoglobulin material in many of the amyloid deposits.

TABLE IV

DNA-Synthetic response and Anti-NNP PFC Response of Spleen Cells from Normal (N) and Thymectomized Lethally Irradiated Fetal Liver Reconstituted (T × FL) Mice (CBA × C57BL) Exposed to PHA (1 μg) and Casein (100 μg) for 2 days

Substance added	DNA synthesis		NNP-PFC	
	T × FL	N	T × F	N
None	6.450 ± 920	3.120 ± 470	28	12
PHA	4.920 ± 1.200	87.120 ± 2.770	6	18
Casein	17.040 ± 620	19.180 ± 1.190	748	454

TABLE V

Polyclonal Antibody Synthesis (anti-NNP-PFC) of Spleen cells from Amyloid-Susceptible (CBA/J) and Amyloid-Resistant (A/J) Mice Exposed to 100 μg of Casein for 2 (exp. I) or 3 (exps. II and III) days

Material added	NNP-PFC/10^6 recovered cells in mouse strain					
	A/J			CBA/J		
	Exp. I	II	III	Exp. I	II	III
Casein	80	106	158	655	1,208	906
None	12	8	8	36	54	40

TABLE VI

Number of Cells × 10^6 in the Peritoneal Cavity of A/J and CBA/J Mice 3 days after an i.p. Injection of 2 ml 10% Sodium Caseinate or Saline

Material injected	A/J	CBA/J
	mean ± SE of six mice	
Casein	8.4 ± 1.2	7.6 ± 0.8
Saline	0.80 ± 0.16	0.52 ± 0.08

Experimental amyloidosis in mice and guinea pigs appears to be analogous to casein-induced amyloidosis (2). It is not known why repeated injections of casein into animals result in lesions very similar to those seen in amyloidosis in man. Although immunological factors have been invoked (4), a direct immunological effect has been excluded, because both neonatally thymectomized animals (5) and animals made tolerant to casein (5) will develop casein-induced amyloidosis. Lately, it has been suggested (11) that casein would affect subpopulation of T cells, because lymphoid cells of the preamyloid animal displayed an impaired response to T-cell mitogens. Although not experimentally verified, it was also suggested (11) that casein would in fact negatively influence a suppressor T-cell subpopulation (12).

In this communication we demonstrate that casein has a mitogenic effect on what appears to be B lymphocytes (Tables I and IV) and that it induces strong polyclonal antibody synthesis in mouse spleen cells (Tables II and IV). The selectivity of its triggering capacity in B cells (relatively weak inducer of DNA-synthesis, but strong inducer of polyclonal antibody formation) makes it most similar to purified protein derivative of tuberculin (PPD) as a PBA (13). Our data explain why neonatally thymectomized animals and animals made immunologically tolerant to casein still develop amyloidosis if regularly injected with casein, because the PBA properties of casein would be unaffected by both these regimens.

We also demonstrated that spleen cells from amyloid-susceptible CBA/J mice responded much stronger with polyclonal antibody production to casein than cells from the amyloid-resistant A/J mice (Table V), and that a similar difference could not be observed between these two strains of mice regarding the tissue irritating and chemoattractant effects of sodium caseinate (Table VI). From our data we suggest that a main feature of casein-induced experimental amyloidosis is the capacity of casein to promote the development of polyclonal antibody synthesis in B cells and that susceptibility to this activity of casein is under genetic influence.

Two other agents have also been used as inducers of experimental amyloidosis, i.e., LPS from *E. coli* (14) and Freund's adjuvant (15). It is not surprising that both these substances contain material which is strongly mitogenic to mouse B lymphocytes (13).

In several instances (1) where amyloidosis is seen in man there is also a possibility of a sustained exposure to molecules with PBA properties, i.e., in chronic gram-negative infections (LPS) and in tuberculosis (PPD). In other cases, a malignant monoclonal hypergammaglobulinemia (multiple myeloma) may result in increased tissue deposition of gamma globulin in the amyloid form. However, as amyloidosis is also seen in agammaglobulinemic individuals (1), it cannot be due to a simple hyperproduction of gamma globulin of polyclonal or monoclonal origin which eventually leads to amyloidosis.

As well as there are genetic differences in the susceptibility to PBA in mice (Table V and reference 16) there may be similar differences in man. In addition there may be genetic variations in the catabolism of proteins and in the ability of the reticuloendothelial system to deposit gamma globulin in the amyloid form. I also believe that different classes of gamma globulin may have different affinities for tissue deposition. Thus, I wish to explore whether there is any selectivity with regard to class or physical properties of the immunoglobulin molecules induced to secretion by casein.

Summary

Experimental amyloidosis in mice can be induced by repeated injections of casein. It has now been demonstrated that casein induces strong polyclonal antibody synthesis in mouse B spleen lymphocytes. This effect is much more pronounced in spleen cells from amyloid-susceptible mice (CBA/J) than amyloid-resistant mice (A/J). It is suggested that amyloidosis can be due in some

instances to a constant exposure for molecules which induce polyclonal B-cell activation.

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