DETECTION OF A MEDIATOR DERIVED FROM ENDOTOXIN-STIMULATED MACROPHAGES THAT INDUCES THE ACUTE PHASE SERUM AMYLOID A RESPONSE IN MICE

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Amyloid A fibrils accumulate in human tissues during amyloidosis secondary to diseases characterized by recurrent inflammation, and in tissues of experimental animals that have been subjected to repeated or chronic inflammatory stimulation (1–3). Antibodies to the amyloid A fibril protein (AA)[1] cross-react with a protein in serum which is called serum amyloid A (SAA) (4, 5). SAA has an apparent mol wt of 160,000–180,000 (6, 7) and has the same density as high density lipoproteins, 1.15 g/cc in man (8) and 1.13 g/cc in mouse (J. Sipe, unpublished observations). SAA is converted to a species of 12,000–13,000 mol wt upon gel filtration in dissociating agents (6–8), and there is considerable homology between the amino acid sequences of this denatured SAA and that of the 8,000- to 10,000-mol wt AA polypeptide which is solubilized from amyloid A fibrils (2, 9). SAA concentrations are elevated in chronic inflammatory and neoplastic diseases (10, 11), and it has been documented in man (12) and mouse (13) that SAA is an acute phase protein whose concentration in serum is highly increased after an inflammatory stimulus.

Despite study, the mechanism by which SAA is produced remains unknown. SAA has been found in fibroblasts and connective tissues (14), and in vitro SAA synthesis by cultured polymorphonuclear leucocytes has been observed (15). During an acute phase response to inflammation, SAA synthesis (16) occurs in hepatocytes (17), as does synthesis of the quintessential acute phase reactant, C-reactive protein (CRP) (18). In a previous study (19), we demonstrated that lipopolysaccharide (LPS)-sensitive lymphocytes and macrophages were necessary for LPS-induced SAA production, indicating a primary role for these cells in the acute phase SAA response. LPS has been shown to induce production of a mediator, glucocorticoid antagonizing factor (GAF), that acts on hepatocytes (20). We have therefore investigated whether a similar type of mediator might be involved in SAA production.

C3H/HeJ mice do not produce a significant SAA response to phenol extracted *Escherichia coli* K235 LPS, in contrast to C3H/HeN mice which exhibit a several hundred-fold increase in SAA concentration at the peak of the acute phase response to endotoxin (13). We have therefore utilized this strain pair to investigate the

[1] *Abbreviations used in this paper:* AA, antibodies to the amyloid A fibril protein; CRP, C-reactive protein; GAF, glucocorticoid antagonizing factor; LPS, lipopolysaccharide; SAA, serum amyloid A.

possibility of an SAA inducer and have found that macrophages make an SAA inducer in response to LPS. These findings demonstrate the mechanism by which LPS produces increased SAA concentrations and lay a logical framework for explaining how chronic inflammation may result in amyloidosis.

Materials and Methods

Mice. Female C3H/HeN mice, aged 6–10 wk, were obtained from the Division of Research Resources, National Institutes of Health, Bethesda, Md. Female and male C3H/HeJ mice, aged 4–8 wk, were obtained from The Jackson Laboratory, Bar Harbor, Maine. No significant sex differences have been observed in the acute phase response of C3H or other mice to endotoxin although males tend to fight and elevated SAA concentrations have been observed in these injured mice (K. McAdam and J. Sipe, unpublished results). Serum containing SAA inducer was obtained by bleeding C3H/HeN mice from the orbital plexus between 2 and 2.5 h after the i.p. administration of K235 LPS (1 μg in 0.2 ml of pyrogen-free saline). Control serum was obtained from untreated or saline-injected animals as specified.

Adoptive transfer of bone marrow cells was carried out with C3H/HeJ or C3H/HeN bone marrow cells prepared from 12-wk-old mice by the method of Michalek and co-workers (Suzanne Michalek, personal communication). The method consists of grinding femurs and tibias in RPMI-1640 medium, filtering the suspended cells through gauze to remove small pieces of bone, and suspending the cells at a concentration of 10^8/ml for injection. Chimeric mice were tested 5 wk after adoptive transfer for their ability to produce SAA inducer.

Reagents. Endotoxin was prepared from *E. coli* K235 by the phenol-water extraction procedure of McIntire et al. (21). The dose response curve for SAA induction in C3H/HeN and C3H/HeJ mice was similar to that reported earlier (13). With the present preparation of LPS, <5 μg/ml SAA was induced by 5 μg LPS after 18 h in C3H/HeJ mice, whereas ~1,000 μg/ml SAA was induced in C3H/HeN mice. Sterile thioglycollate broth was obtained from the National Institutes of Health Media Unit. RPMI-1640 medium (Grand Island Biological Company, Grand Island, N. Y.) was supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and 15 mM Hepes buffer.

Production of SAA Inducer by LPS-Treated Cell Cultures. Mice were injected with 3 ml of sterile thioglycollate i.p. and 6 d later the induced peritoneal exudate cells were collected by peritoneal lavage with pyrogen free saline (Abbott Diagnostics, Diagnostic Products, Chicago, Ill.). These cells were routinely found to be >90% macrophages by differential staining of cytospin preparations (Shandown Southern Products Limited, Runcorn, Cheshire, England). Adherent cells were obtained by incubation of 2×10^6 peritoneal exudate cells in serum-free RPMI in TC-24 culture dishes (Costar, Data Packaging, Cambridge, Mass.) at 37°C for 3 h after which the nonadherent cells were removed by washing. Adherent cell monolayers contained >98% macrophages. In some experiments, the nonadherent cells were not removed. Spleen cell preparations were prepared by teasing the spleens and resuspending the washed cells to 2×10^6 spleen cells/ml in serum-free RPMI. Cells were incubated with or without 10 μg/ml LPS.

SAA Measurements. SAA concentration was estimated in serum obtained by bleeding animals from the orbital plexus at various times after injection of serum from LPS-treated C3H/HeN or C3H/HeJ mice, or medium from LPS-treated cell cultures. A 10-μl aliquot of serum to be tested for SAA was added to 500 μl of 10% formic acid and incubated for 24 h at 56°C. Either the entire dilution, or aliquots of it, was lyophilized and resuspended in casein barbital buffer, and the SAA concentration was estimated according to the capacity of the sample to compete with ^{125}I-AA binding by immobilized anti-AA antibodies (22). Each dilution of sample was assayed in duplicate on at least two separate occasions. At least 10 samples were employed to normalize the data for immunoassays carried out on separate occasions. The SAA concentration for individual animals was averaged for the multiple determinations, and the arithmetic mean and SEM determined. The SEM is indicated after each mean concentration of SAA. Occasionally, as is indicated in the legends, a single value was discarded if its value was greater than the mean plus 1.5 times the SD of the mean.

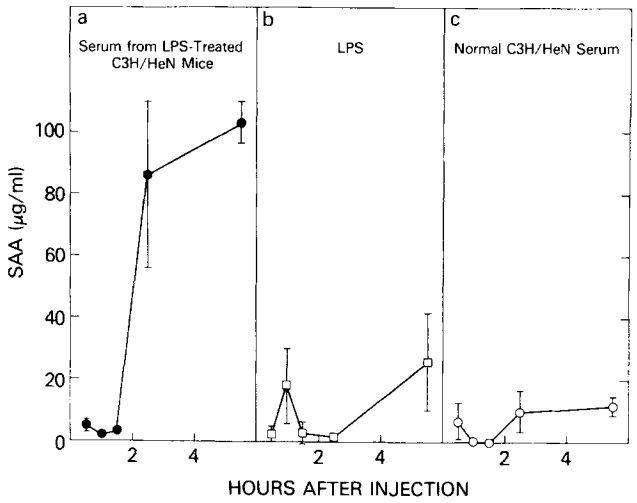

FIG. 1. Appearance of SAA in C3H/HeJ mice. (*a*) 0.2 ml of serum from C3H/HeN mice 2.5 h after i.p. injection of 1 μg of LPS was administered i.p. to C3H/HeJ mice. The SAA concentration of the injected serum was 10 μg/ml. (*b*) LPS, 1 μg in 0.2 ml pyrogen free saline was administered to C3H/HeJ mice. (*c*) 0.2 ml of serum from untreated C3H/HeN mice was administered to C3H/HeJ mice. The SAA concentration of the administered serum was <1 μg/ml. Male C3H/HeJ mice, 6 wk old, were employed. Each point represents the arithmetic mean of the SAA determination for serum of three individual animals. The horizontal bars represent the mean ± SEM. Two values were discarded: 1-LPS 2.5 h and 1-LPS 1.5 h.

Results

Detection of SAA Inducer in Serum of LPS-Treated Mice. The process of SAA induction in C3H/HeN mice by LPS has been characterized previously (13, 16). Initially there is a 2–3 h latent period before the SAA concentration begins to rise; then there is a period of synthesis during which the SAA concentration increases exponentially to almost 1,000 μg/ml by 16–24 h after treatment with 5 μg or more LPS; and lastly there is a deinduction period during which the SAA concentration returns to preinduction levels by 48 h after LPS treatment. C3H/HeJ mice show a diminished SAA response to all forms of endotoxin and in particular to K235 phenol-extracted LPS. At the peak of the acute phase response, 5 μg of K235 LPS elicits <5 μg/ml SAA in C3H/HeJ mice as compared with 1,000 μg/ml in C3H/HeN mice (13).

In the present study it was found that serum from C3H/HeN mice obtained 2.5 h after administration of LPS, which is at the end of the latent period before the normally observed exponential rise in SAA concentration, was capable of stimulating SAA synthesis by C3H/HeJ mice. This data is shown in Fig. 1. The SAA concentration in C3H/HeJ sera was observed to increase between 1.5 and 2.5 h after i.p. injection of 0.2 ml of serum from C3H/HeN mice treated with 1 μg of LPS, and rose to ~100 μg/ml by 5.5 h (Fig. 1a). The same quantity of LPS did not stimulate significant quantities of SAA in C3H/HeJ mice (Fig. 1b) although it did elicit an SAA concentration of 185 μg/ml in C3H/HeN mice 5.5 h after administration (data not shown). Serum from untreated C3H/HeN mice did not stimulate SAA in C3H/HeJ mice (Fig. 1c), nor did serum from C3H/HeJ mice obtained 2.5 h after the i.p. injection of 1 μg of LPS (data not shown).

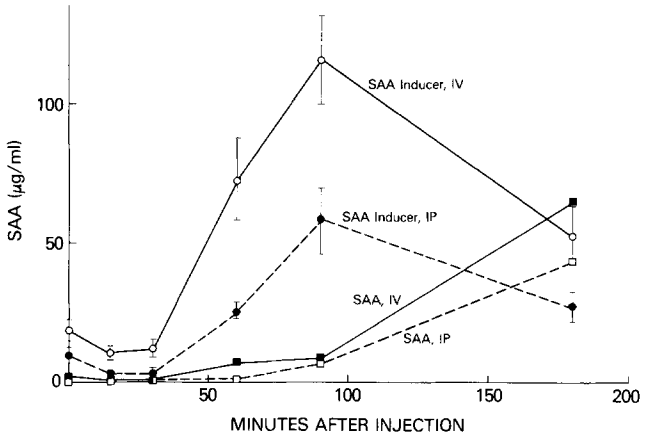

FIG. 2. Time course of appearance of SAA mediator in serum of C3H/HeN mice after either i.p. or i.v. injection of 1 μg of LPS. The mediator was induced in seven groups of eight C3H/HeN female mice, 6 wk old. Each of the mice was injected with 1 μg of LPS in 0.5 ml of pyrogen free saline and bled 0, 15, 30, 60, 90, or 180 min later. The seventh group was used as a control. To determine the presence of SAA inducer, 0.2 ml of pooled serum from each of the groups was administered i.p. to each of eight female C3H/HeJ mice, 6 wk old. Each point represents the arithmetic mean of the eight individual sera ± SEM, except for the 0-min i.p. group for which one high value was discarded.

SAA inducer is present in serum of C3H/HeN mice at maximal concentrations by ~90 min after administration of 1 μg LPS, and has begun to diminish by the end of the latent period of SAA induction, at the beginning of the exponential rise in SAA concentration (Fig. 2). The time course is similar for induction of SAA inducer whether the i.v. or i.p. route of administration of LPS is employed. Injection of LPS intravenously leads to the appearance of higher concentrations of both SAA inducer and SAA (Fig 2) as compared with the i.p. route. This may be due to a dose response effect resulting from effectively higher concentrations of LPS and LPS-derived SAA inducer in the blood stream when the i.v. route is employed. However, it has been experimentally convenient to employ the i.p. route for the preparation of large quantities of SAA inducer.

The time course for induction of SAA by serum SAA inducer is much different than the profile typically obtained for the acute phase SAA response to LPS (Fig. 3). When SAA is induced in C3H/HeJ mice with 0.2 ml of latent period serum (SAA < 10 μg/ml) from C3H/HeN mice treated with 1 μg of LPS, the SAA concentration is 30–90 μg/ml (Figs. 1 and 3) by 4 h after administration, and this is ~50% of the maximum concentration observed (Fig. 3). In contrast, the acute phase SAA response of C3H/HeN mice to LPS itself is marked by a 1,000-fold increase in SAA concentration which has reached only 5–10% of its maximum value at 4 h (16). This is consistent with the premise that SAA is not induced in C3H/HeJ mice by passively transferred LPS. Furthermore, the data shown in Figs. 3 and 4 indicate that 0.2 ml of serum from C3H/HeN mice given 1 μg of LPS stimulated up to 100-fold greater SAA concentrations in C3H/HeJ mice than did 1 μg of LPS itself.

The dose dependence of SAA induction by serum SAA inducer is shown in Fig. 4. The minimum vol of serum that can be feasibly employed appears to be 0.2 ml. Note

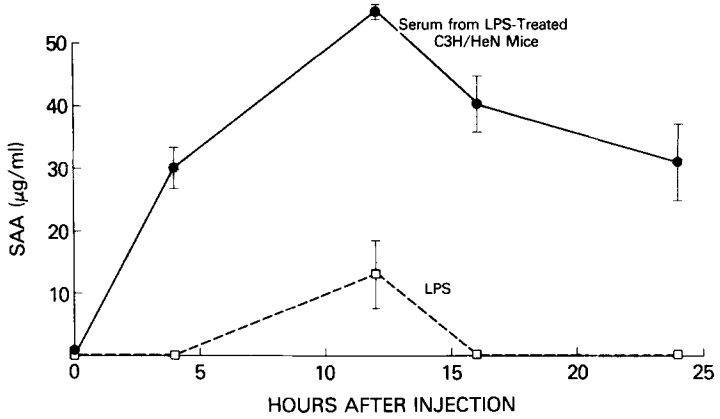

FIG. 3. Time course of induction of SAA in C3H/HeJ mice by latent period serum from C3H/HeN mice and by LPS. Female C3H/HeJ mice 6 wk old were employed. 0.2 ml of either serum from C3H/HeN mice treated with 1 μg of LPS or 1 μg of LPS in pyrogen-free saline were injected into each C3H/HeJ mouse i.p. Six mice were bled for each time point indicated. Each point represents the mean ± SE, for all six mice except for the 4- and the 16-h LPS points for which one high value each was discarded.

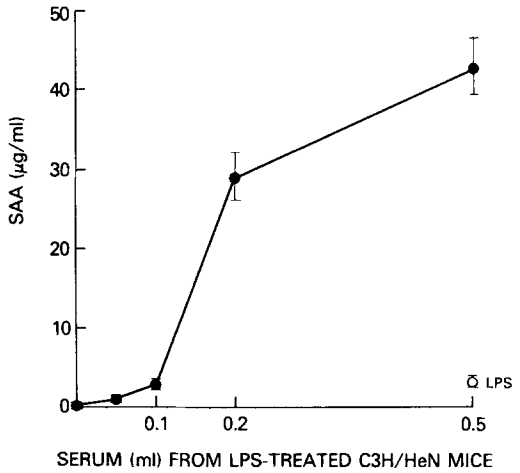

FIG. 4. Dose dependence of SAA induction by serum inducer. 25 C3H/HeN mice were given 1 μg of K235 LPS i.p. and bled 2.5 h later. The serum was collected from the pooled blood and immediately administered i.p. to six C3H/HeJ mice (12 wk old) for each specified volume. The SAA concentration for the pool was subsequently determined to be 10 μg/ml.

that 0.5 ml of serum was >100-fold more effective than LPS in stimulating SAA, when volume factors are taken into account.

Evidence for the Production of SAA Inducer by Macrophages. The resistance of C3H/HeJ mice to mount an acute phase SAA response to LPS appears to be caused by a lymphoid cell defect in the ability to respond to LPS by formation of the SAA inducer. Evidence for this was obtained using chimeric animals (Table I). Pooled serum from 10 C3H/HeJ mice to which had been adoptively transferred bone marrow cells from C3H/HeN mice, induced a mean SAA concentration of 11 μg/ml, whereas

TABLE I

Production of SAA Inducer by LPS-treated Chimeric Mice

Mice	LPS	SAA inducer
		μg/ml SAA
C3H/HeN BM → C3H/HeJx	+	11 ± 2
C3H/HeJ BM → C3H/HeJx	+	3 ± 1
C3H/HeN	+	30 ± 6
C3H/HeN	−	<1

0.2 ml pooled serum from 10 mice in each of the four groups above was administered i.p. to C3H/HeJ female mice (5 wk old). The arithmetic mean and its SE was obtained from all 7 animals in group 1, for 7 of 9 animals for group 2, for 9 of 10 animals in group 3, and for all 9 animals in group 4. The SAA concentration of the administered serum was <2 μg/ml in all cases.

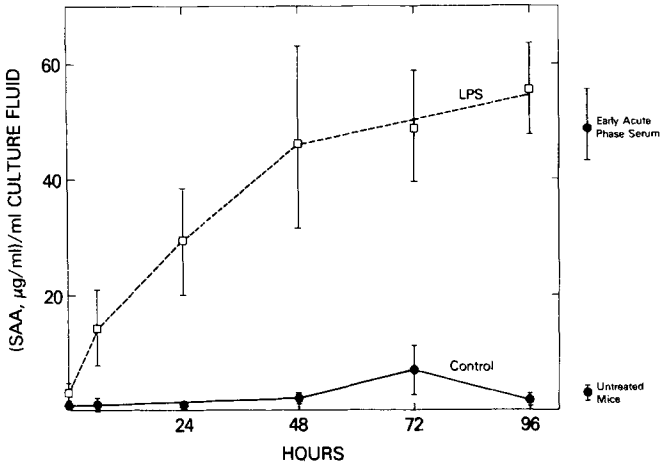

FIG. 5. Induction of SAA in C3H/HeJ mice by LPS-treated peritoneal exudate cells from C3H/HeN mice. 1 ml of SAA-free culture medium was injected i.p. to each of six 6-wk-old females which were bled 18 h later. Each value shown is the arithmetic mean with its SE in brackets. The amount of SAA induced by 0.2 ml serum from C3H/HeN mice 2.5 h after LPS and by 0.2 ml serum from untreated mice is indicated to the right of the graph.

pooled serum from 10 C3H/HeJ mice reconstituted with C3H/HeJ bone marrow cells induced a mean SAA concentration of 3 μg/ml. These values are statistically significant with $P < 0.01$. In the same experiment, latent period serum from LPS-injected normal nonreconstituted C3H/HeN mice induced 30 μg/ml SAA in C3H/HeJ mice.

Further evidence for the derivation of SAA inducer from bone marrow-derived cells comes from the data shown in Fig. 5. Thioglycollate-induced peritoneal macrophages were incubated for varying lengths of time with 10 μg/ml LPS, and the culture medium was harvested, filter sterilized, and injected i.p. into C3H/HeJ mice. About 30% of the maximal amount of SAA inducer was detected in the culture medium by 6 h, indicating that the in vitro SAA inducer is potentially the same one which is acting in vivo. Furthermore, it can be seen that 1 ml of the culture medium from 2×10^6 peritoneal cells is as effective as 0.2 ml of serum from C3H/HeN mice given 1 μg of LPS.

TABLE II

Effect of Extensive Washing on Ability of Peritoneal Exudate Cells to Produce SAA Inducer In Vitro in Response to Endotoxin

Cell population	SAA inducer*			
	Control	*n*	LPS (10 μg/ml)	*n*
	μg/ml SAA		*μg/ml SAA*	
C3H/HeN PEC (nonadherent cells present)	8.2 ± 1.8	(7/8)	79.2 ± 14.8	(8/8)
C3H/HeN ADH (extensively washed to remove nonadherent cells)	10.5 ± 3.4	(6/8)	84.1 ± 14.8	(7/7)

* Culture medium was obtained as described in Materials and Methods. 1 ml was injected into seven or eight animals. The mean and its SE were computed from the number of animals indicated in the numerator.

TABLE III

Comparison of Ability of Endotoxin to Stimulate SAA Inducer in Various Cell Populations

Cell population	SAA	
	Control	LPS (10 μg/ml)
	μg/ml	
C3H/HeN PEC	0.9 ± 0.2	44.3 ± 1.7
C3H/HeN spleen cells	5.6 ± 1.6	5.0 ± 2.1
C3H/HeJ PEC	2.8 ± 0.7	6.7 ± 1.8

Culture medium was obtained as described in Materials and Methods. 1 ml was injected into six animals, and the mean and its SE were computed for all six animals in each case.

The macrophage origin of LPS-derived SAA inducer is further supported by the data shown in Table II. Washed, adherent peritoneal exudate cells were as effective in producing SAA inducer in response to LPS as was the entire cell population. In contrast, LPS-stimulated spleen cells from C3H/HeN mice did not make SAA inducer, nor did LPS-stimulated macrophages from C3H/HeJ mice (Table III).

Discussion

We have shown that the endotoxin-induced acute phase SAA response involves two distinct populations of cells. We have detected a factor that appears in serum during the initial or "latent" part of the acute phase SAA response to endotoxin which can stimulate SAA synthesis in mice that are resistant to the acute phase stimulus itself. The idea, put forth by Rosenstreich and McAdam (19), that a lymphoid cell may be responsible for initiating the SAA response to endotoxin is supported by the evidence presented herein that chimeric C3H/HeJ mice reconstituted with C3H/HeN bone marrow cells are capable of producing the SAA inducing factor in response to LPS. It appears likely that the SAA inducer arises from the interaction of LPS with macrophages, in view of the observation that SAA inducer can be obtained in vitro by endotoxin stimulation of thioglycollate-induced C3H/HeN macrophages but not from a population of LPS-treated C3H/HeN spleen cells that contains <10% mac-

rophages. SAA inducer begins to appear in culture medium within the first 6 h of LPS treatment; thus, it is possible that the in vitro and in vivo inducers are the same. Preliminary results (J. Sipe, unpublished observations) indicate that the serum SAA inducer is a trypsin-sensitive macromolecule, the activity of which is markedly reduced by incubation of latent period serum at 37°C.

The possibility that the SAA inducing factors observed both in vivo and in vitro might arise artifactually by transfer of a precursor of SAA would seem to be ruled out by the fact that the SAA concentrations of both latent period serum and culture medium were minimal. As formic acid denaturation is always employed for the antigenic assay of SAA, it seems unlikely that nonimmunoreactive AA determinants are being transferred.

At present, little can be said about the chemical nature of SAA inducer. Our present understanding of the origin of acute phase reactants (23) would suggest that SAA inducer arises from macrophage injury by endotoxin and that SAA inducer subsequently stimulates the SAA synthesizing cell. It is tacitly assumed in this concept that the inducer is a cell product rather than a product of the inflammatory stimulus itself. This seems reasonable, in view of the wide range of agents which can induce acute phase reactants such as SAA and CRP and in view of the fact that localized inflammation can bring about a strong SAA response (12; A. Weinblatt and J. Sipe, unpublished observations). However, it may be that C3H/HeN macrophages alter K235 (ph) LPS so that it is now stimulatory for C3H/HeJ mice. Such a mechanism has been postulated for induction of B-cell mitogenesis in C3H/HeJ mice by LPS (24). The previously mentioned lability of SAA inducer in serum would seem to rule out this possibility.

The identification of an endotoxin-induced mediator of SAA biosynthesis raises many interesting questions about its role in the synthesis of other acute phase reactants and inducible liver proteins: Does the SAA inducing factor elicit other acute phase reactants such as CRP (12) and do the numerous inflammatory agents which stimulate an SAA response act via a common mediator or by different ones? Biochemical studies are currently in progress to isolate and characterize the endotoxin-produced mediator of SAA induction from LPS-stimulated macrophage culture supernates. The in vitro induction of SAA by an inflammatory agent, such as endotoxin, has not yet been described; clearly, the chemical characterization of SAA inducer will be facilitated by an in vitro assay.

The existence of this newly recognized mediator of the inflammatory response also raises many interesting questions as to its role in amyloidogenesis. It may be that the same mediator acting on different cell populations induces AA polypeptides of different amino acid sequence; or that various mediators, which can induce different AA polypeptides, are produced according to the nature of the inflammatory agents. Perhaps an individual mediator of SAA induction may have a preferential affinity for the hepatocyte, but when present in high concentrations due to prolonged inflammation it may stimulate other tissues, such as spleen, to synthesize amyloid A protein (3, 25, 26). Thus, the chemistry and concentration of the mediator may be important in the pathogenesis of amyloidosis as well as factors which have already been recognized, such as microheterogeneity of the AA polypeptide (25) and proteolytic processing of the AA polypeptide (27).

The required intervention of an LPS-sensitive lymphocyte or macrophage in the

production of SAA suggests a plausible mechanism by which chronic inflammation induces amyloidosis. The isolation and characterization of this SAA inducer may greatly facilitate studies on the pathogenesis of amyloidosis.

Finally it will be interesting to learn whether the SAA inducer is newly synthesized in response to LPS or is released by macrophages involved in the processing of LPS, and to learn if any other biological activities reside in the SAA inducer. SAA inducer appears to be distinct from interferon because interferon was not detectable in latent period serum, and because mouse interferon did not induce significant amounts of SAA (J. Sipe, unpublished observations). Future work will determine what relationship SAA inducer has to other mediators of endotoxicity, such as tumor necrosis factor and GAF.

Summary

The mechanism by which LPS stimulates an acute phase serum amyloid A (SAA) response in C3H mice has been studied. A factor (SAA inducer) appears in the blood of C3H/HeN (lipopolysaccharide [LPS]-sensitive) mice ~1 h after administration of LPS, which, when passively administered, can induce C3H/HeJ mice to produce SAA although they are resistant to the LPS itself. SAA inducer has been detected in the culture medium of LPS treated C3H/HeN macrophages but not spleen cells. Thus, two stages in the induction of the acute phase SAA response are now recognized: a latent period of 2–3 h during which the SAA concentration remains at baseline values and in which SAA inducer appears, and the period of synthesis of SAA which lasts for ~24 h past induction. It is proposed that a macrophage response to LPS is responsible for production of the serum mediator which induces SAA synthesis.

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