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THE SERUM AMYLOID A STIMULATING FACTOR (SAASF) IN THE HAMSTER

By

P. R. HOL, F. W. J. J. SNEL, M. DRAAIJER and E. GRUYS

Department of Veterinary Pathology, University of Utrecht, Utrecht, The Netherlands

INTRODUCTION

Serum amyloid A (SAA) is the precursor of protein AA, the major constituent of amyloid A fibrils (secondary amyloid). Induction of AA-amyloidosis in man and other vertebrates requires chronic inflammatory lesions or repeated injections with tissue-destroying and inflammation-inducing substances. In the hamster, casein (Gruys, Timmermans and van Ederen, 1979; Hol, van Ederen, Snel, Langeveld, Veerkamp and Gruys, 1985a) or casein supplemented with lipopolysaccharide (casein–LPS) (Hol, van Andel, van Ederen, Draaijer and Gruys, 1985b; Hol, Snel, Niewold and Gruys, 1986; Schultz and Pitha, 1985) appeared to be useful as amyloid A and SAA inducers.

The SAA-response has been described as an acute phase phenomenon resulting from hepatic stimulation by a peripherally released factor called SAA-stimulating factor (SAASF) (Selinger, McAdam, Kaplan, Sipe, Rosenstreich and Vogel, 1980a) or SAA-inducer (Sipe, Vogel, Ryan, McAdam and Rosenstreich, 1979). SAASF was first detected in the early acute-phase serum (latent phase serum) of LPS-injected mice, being a factor inducing SAA production in recipient LPS-non-responder mice (Sipe *et al.*, 1979). Similarly, SAASF was detected in the supernate of LPS-stimulated murine peritoneal macrophages (Sipe *et al.*, 1979; Selinger, McAdam, Kaplan, Sipe, Vogel and Rosenstreich, 1980b). Macrophage-derived preparations of human and murine lymphocyte activating factor (LAF) and rabbit endogenous pyrogen (EP) also showed SAASF-activity when injected in mice (Sztein, Vogel, Sipe, Murphy, Mizel, Oppenheim and Rosenstreich, 1981). SAASF, LAF and leucocytic pyrogen (LP) were not separable during purification of LP from human macrophages (McAdam, Li, Knowles, Foss, Dinarello, Rosenwasser, Selinger, Kaplan and Goodman, 1982). Hence it is thought that SAASF is part of a closely related family of monokines (LP, EP, LAF and leucocyte endogenous mediator (LEM)) which are classified as Interleukin-1 (IL-1) (Dinarello, 1984). The in vivo production of SAASF is ascribed to activated macrophages in the inflamed area. However, evidence has been presented that polymorphonuclear leukocytes (PMNs), especially those in acute inflammatory exudates, secrete IL-1-like factors (Goto, Nakamura, Goto and Yoshinaga, 1984; Rodrick, Lamster, Sonis, Pender, Kolodkin, Fitzgerald and Wilson, 1982; Yoshinaga, Nishime, Nakamura and Goto, 1980). EP has been detected in granules of PMNs (Harris, 1982).

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To study whether the SAA-response in the hamsters mentioned above was mediated by a factor resembling SAASF-LAF-IL-1 in mice, early acute phase blood plasma, supernates and lysates of PMN-rich acute peritoneal exudate cells and chronic peritoneal exudate macrophages were tested in vivo for SAASF activity. Moreover, the chronic peritoneal exudate macrophages were examined for LAF activity. The supernates of the latter cells appeared not to contain SAASF, whereas LAF-activity was detected.

MATERIALS AND METHODS

Hamsters

Young adult male Golden hamsters (*Mesocricetus auratus*) were used in all experiments.

Plasma SAA Concentrations After One Subcutaneous Injection with Casein–LPS

Thirty-six hamsters were injected subcutaneously with 2 ml 5 per cent casein (Hammersten, Merck, Darmstadt, FRG) in 0·3 M sodium bicarbonate (pH 7·5) supplemented with 20 μg per ml lipopolysaccharide (LPS: *E. coli* 0127: B8, Difco Laboratories, Detroit, MI, U.S.A.) (casein–LPS). Casein–LPS in our laboratory is routinely used for induction of AA-amyloidosis in hamsters (Hol *et al.*, 1985b). Hamsters were killed by decapitation after ether anaesthesia, 2·5, 4·0, 5·5, 7·5, 9·5 and 24 h later. Blood was collected in heparinized tubes. Plasma was separated by centrifugation at 3000 g for 15 min at 4°C. Plasma samples were used for measurements of SAA-concentrations as described elsewhere (Hol *et al.*, 1985a). Control values were obtained from 6 untreated hamsters.

Preparation and Stimulation of PMN-rich Acute Peritoneal Exudate Cells (APEC)

Three groups of 4 hamsters received intraperitoneally one injection of either 1 ml casein–LPS, 1 ml RPMI-1640 medium (Flow Laboratories Ltd., Irvine, Ayrshire, U.K.) containing 10^8 latex beads (0·8 μm, Dow Diagnostics, Indianapolis, IN, U.S.A.) or 1 ml Freund's incomplete adjuvant (FIA). Four hours later, cells were obtained by washing the peritoneal cavity twice with 15 ml PBS containing 0·5mM EDTA, 100 U per ml penicillin, 100 μg per ml streptomycin and 2 mM glutamine (PBSE). Penicillin, streptomycin and glutamine were added to all culture buffers. After centrifugation (10 min at 500 g at 4°C), cells were twice washed with PBSE and then once with RPMI containing 0·2 U per ml heparin. The cells from each group of hamsters were pooled and suspended in 3 ml RPMI. Small samples of these suspensions were used for counting and differential staining (cytospins; Shandon cytofuge, Shandon Southern Instruments Inc., Sewickley, Pa). Cell suspensions were supplemented with 3×10^8 latex beads and incubated for 2 h at 37°C in 5 per cent CO_2, 95 per cent air and 90 to 95 per cent humidity. After incubation the suspensions were centrifuged for 10 min at 500 g. The pellets were resuspended in 3 ml RPMI and the cells were lysed by freezing and thawing. Supernates and lysates were passed through 0·22 μm filters (Millex G. V., Millipore Corp., Cambridge, MA, U.S.A.) and stored at −20°C.

Preparation and Stimulation of Chronic Peritoneal Exudate Macrophages (CPEM)

Twelve hamsters were infected intraperitoneally with 0·1 ml BCG (42×10^6 colony-forming units Bacillus Calmette Guerin per ml). The animals were rechallenged with 0·2 ml BCG 7 weeks later. After 5 days, peritoneal exudate cells were harvested by peritoneal lavage with PBSE. Cells were twice washed with PBSE and once with RPMI–1640 medium containing 0·5 mM EDTA (RPMI-E) and suspended

to a density of 5×10^6 cells per ml in RPMI containing 5 per cent heat inactivated (30 min at 56°C) foetal calf serum (Flow) (complete RPMI). Adherent cells were obtained by incubation of 5 ml aliquots ($2{\cdot}5 \times 10^7$ cells) in 60 mm Petri dishes (Corning Glassworks, Corning, N.Y.) for 3 h at 37°C in an atmosphere of 5 per cent CO_2, 95 per cent air and 90 to 95 per cent humidity. The non-adherent cells were removed by washing with incomplete RPMI. The adherent cells were >98 per cent macrophages. To each Petri dish, 5 ml RPMI containing 10^8 latex beads per ml was added. The supernates were collected after 24 h incubation. Five ml PBS was added to the remaining cells which were then lysed by freezing and thawing. All solutions were passed through 0·22 μm filters and stored at −20°C.

SAASF-assay

Hamsters were injected intraperitoneally with 1 ml of the following preparations: (i) blood plasma obtained 2·5, 4·0, 5·5, 7·5 and 9·5 h after one subcutaneous casein-LPS injection; (ii) supernates and lysates of APEC; (iii) supernates and lysates of CPEM. Blood was collected after 15 h. SAA concentrations in the blood plasma samples were measured by the ELISA method as described previously (Hol *et al.*, 1985a).

LAF-assay

The LAF activities in the supernates of latex stimulated CPEM were measured as the ability of these samples to augment the Concanavalin A (Con A)-induced incorporation of ^{3}H-thymidine (^{3}H-TdR) by hamster mononuclear spleen cell cultures. From the spleens of two hamsters, two single cell suspensions were prepared by forcing spleen fragments through nylon sieves with a pore diameter of 220 μm into 50 ml PBSE at 4°C. After centrifugation for 8 min at 500 *g*, the cells were suspended in 15 ml PBSE and centrifuged on Ficoll-Pague (Pharmacia Fine Chemicals Inc., Uppsala, Sweden). The mononuclear leukocyte layer was recovered, twice washed and resuspended to 2×10^6 cells per ml in RPMI containing 4 mM l-glutamine and 20 per cent heat-inactivated normal hamster serum. Triplicate cultures were performed in 96-well flat bottom microtitre plates (Greiner u. Söhne GmbH, Nürtingen, BRD) at various Con A concentrations and various dilutions of the test samples. Each well contained 100 μl cell suspension, 5 μl Con A (final well concentration: 0, 0·5, 1·25, 2·5 and 12·5 μg per ml; Sigma Chemical Co., St Louis, MO, U.S.A.) and 95 μl control medium or test sample (final dilution 1 in 2 to 1 in 5 and 1 in 10). Plates were incubated at 37°C in 5 per cent CO_2, 95 per cent air and 90 to 95 per cent humidity. After 64 h, 25 μl 16 μm ^{3}H-TdR (1,0 Ci per mmol; Radiochemical Centre, Amersham, U.K.) was added and the cells were isolated with a Skatron multiple cell culture harvester (Flow) 6 h later. The cells, collected on glass fibre filters, were dried (3 h at 60°C) and denaturated with a mixture of ether and ethanol (1 to 1 v/v). After drying (15 min at 60°C), 2 ml Xylofluor (Lumac B.V., Schaesberg, The Netherlands) was added and the incorporated ^{3}H-TdR measured in a liquid scintillation counter (Nuclear Chicago Isocap 300).

RESULTS

SAA Response of Hamsters to Early Acute Phase Blood Plasma

SAA concentrations in the blood plasma of hamsters showed a small increase 5·5 h after subcutaneous injection with casein–LPS (Fig. 1). SAA concentrations then increased rapidly to 150 ± 11 μg per ml at 24 h (data not shown in Fig. 1). Plasma samples obtained 2·5 and 4·0 h after casein–LPS injection did not induce an SAA response when injected intraperitoneally in

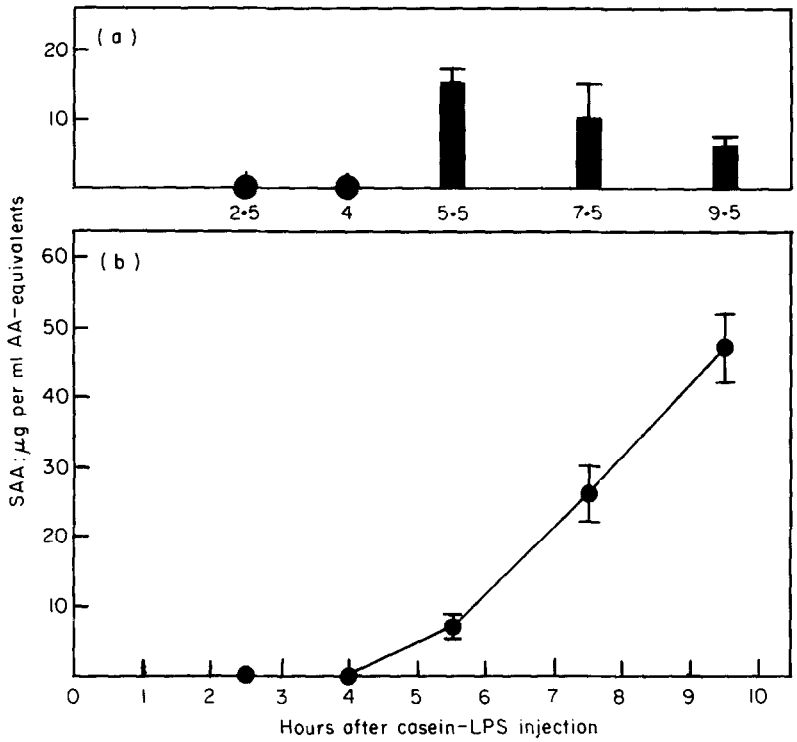

Fig. 1. SAA concentrations in blood plasma of hamsters: (a) after one subcutaneous injection with casein–LPS. Each point represents the mean ± S.E.M. of data from 6 animals. (b) 15 h after an intraperitoneal injection with 1 ml blood plasma obtained from hamsters 2·5, 4, 5·5, 7·5 and 9·5 h after subcutaneous injection with casein–LPS (shown in a). Each bar represents the mean ± S.E.M. of data from 5 animals.

recipient hamsters, whereas the 5·5, 7·5 and 9·5 h plasma samples did (Fig. 1). The highest SAA-stimulating activity was present in the 5·5 h early acute phase plasma.

SAASF in Acute and Chronic Peritoneal Exudate Cells

APEC were collected 4 h after intraperitoneal injections with either casein–LPS, latex or FIA. The differential white cell counts of the exudates are given in Table 1. CPEM (>98 per cent macrophages) were obtained by removing non-adherent cells from BCG-elicited chronic peritoneal exudate cells (>90 per cent macrophages). APEC were stimulated in vitro for 2 h with latex and CPEM for 24 h. After incubation with latex, both APEC and CPEM had extensively phagocytosed latex. In the case of casein-LPS elicited APEC, however, considerable cell death occurred. Supernates and lysates of APEC elicited by latex, FIA and casein–LPS all induced SAA synthesis in hamsters when injected intraperitoneally (Table 2). The responses to the supernates of the latex and FIA, recruited cells, however, were only marginal. Supernates and lysates of CPEM did not induce an SAA response measured 15 h after intraperitoneal injection.

TABLE 1.
DIFFERENTIAL WHITE CELL COUNT OF ACUTE (4 H) PERITONEAL EXUDATES ELICITED WITH DIFFERENT STIMULANTS

Stimulant	*Polymorphonuclear leukocytes*	*Macrophages*	*Basophilic leukocytes*
Cas-LPS	81 ± 3	17 ± 3	2 ± 1
Latex	86 ± 2	11 ± 2	3 ± 1
FIA	89 ± 2	7 ± 3	4 ± 1

Abbreviations are given in the text.

TABLE 2.
SAA-RESPONSE (μG PER ML AA-EQUIVALENTS) OF HAMSTERS 15 H AFTER INTRAPERITONEAL INJECTION WITH SUPERNATES AND LYSATES OF LATEX TREATED CULTURES OF APEC AND CPEM

Cell culture	*Elicited with*	*Cell count*	*SAA-response*†	
			*Supernate**	*Lysate**
APEC	Cas-LPS	13·2	18·9 ± 2·0	25·0 ± 9·0
	Latex	46·6	3·4 ± 1·3	10·6 ± 3·8
	FIA	26·3	2·5 ± 0·7	8·9 ± 5·1
CPEM	BCG	20·0	0 0	0 0

*Each experiment involved 4 hamsters receiving supernates or lysates of hamster cells (number of cells $\times 10^{-7}$ per dose is given under cell count)
†Mean ± S.E.M.
See text for abbreviations.

LAF in Supernates of Chronic Peritoneal Exudate Macrophages

Unstimulated spleen cell cultures showed only low values of ^{3}H-TdR incorporation (20 to 40 cpm). A small increase was noted after stimulation with 0·5 μg per ml Con A (160 to 2000 cpm). Con A concentrations higher than 0·5 μg per ml were markedly mitogenic (20 000 cpm; Fig. 2). Addition of the supernatant of latex stimulated CPEM caused a significant increase in ^{3}H-TdR incorporation at 0·5 μg per ml Con A. This did not occur in the absence of Con A, indicating the presence of LAF. Dilution of the supernate decreased the ^{3}H-TdR incorporation by the spleen cell cultures. In 1 in 10 diluted CPEM-supernates, LAF activity, however, was still obvious.

DISCUSSION

In mice, the SAA response has been described as starting 2 to 3 h after an intraperitoneal injection of LPS. Aliquots of 0·2 ml of serum, obtained 2·5 h after administration of LPS, stimulated SAA synthesis when injected intraperitoneally in LPS-non-responder mice (Sipe *et al.*, 1979). Similar results were obtained in our hamster experiments, in which SAASF was detected in blood plasma 5·5 h after a subcutaneous injection with casein–LPS. It is not likely that our recipient hamsters have reacted to small quantities of LPS in the blood plasma of the casein–LPS injected donors since 2·5 and 4·0 h plasma samples gave negative results. The SAASF-activity in the early acute phase

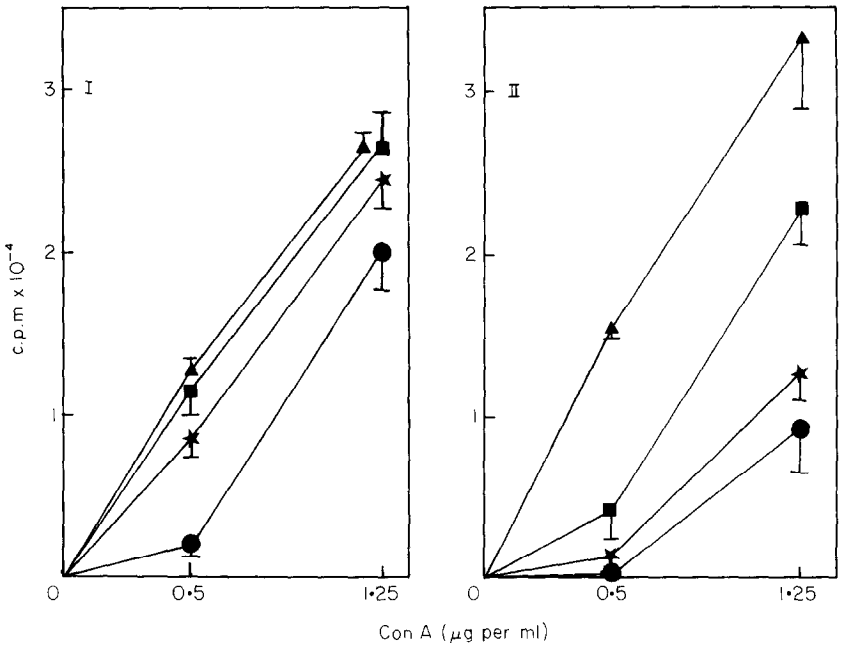

Fig. 2. Influence of supernate of CPEM on response of normal hamster splenocytes (culture I and culture II) to various Con A concentrations. Results are expressed in net counts. Vertical bars indicate the S.E.M. for triplicate cultures. Control: ●, Supernate diluted 2×: ▲, 5×: ■ and 10×: ★.

plasma decreased considerably between 5·5 and 9·5 h. It is likely that SAASF was released only during a short period after injection with casein–LPS. In mice receiving a single intraperitoneal casein injection, only the 2 to 4 h inflammatory exudate revealed LAF (Goto *et al.*, 1984) and early infiltrating PMNs were shown to be responsible for this in vivo production of LAF. A rapid clearance of rabbit LEM from the circulation of the rat has been described (Kampschmidt and Upchurch, 1980). SAASF is possibly destroyed in the acute phase blood plasma by high proteolytic enzyme activities associated with inflammation (Hol *et al.*, 1985a).

Lysates of PMN-rich APEC, whether elicited with casein–LPS, latex or FIA, stimulated SAA-synthesis in hamsters, indicating intracellular SAASF. Extracellular SAASF was detected only in the case of casein–LPS-recruited APEC. This could be due to cell death in this culture as well as to a selective release. The release of IL-1-like factors by PMN-rich murine APEC was shown to depend on the stimulants used to recruit the cells (Rodrick *et al.*, 1982). Murine CPEM have been described as releasing SAASF (IL–1) on stimulation with LPS (Sipe *et al.*, 1979; Selinger *et al.*, 1980b; Sztein *et al.*, 1981). The supernates of 2×10^6 cells induced increased SAA-concentrations (10 to 100 μg per ml) when injected intraperitoneally in LPS-non-responder mice. In the present study we used BCG-elicited CPEM since in vivo infection with BCG has been described as enhancing in vitro the LPS-induced production of IL–1 by peritoneal exudate macrophages and in vivo the SAA response to LPS

(Sipe, Vogel, Sztein, Skinner and Cohen, 1982). CPEM were stimulated with latex which can be easily removed from the culture medium. Latex has been reported to stimulate LAF secretion better than LPS (Gery, Davies, Derr, Krett and Barranger, 1981). In the supernate of hamster CPEM, LAF activity was detected. However, the supernate of 2×10^7 cells did not induce SAA synthesis in recipient hamsters.

These results indicate that, in the hamster, SAASF and LAF(IL-1) are functionally different substances. Separated activities within the group of so called IL-1 factors (EP-LP-LEM-LAF-SAASF (Dinarello, 1984)) have been reported by a few authors; a rabbit and human LAF without EP-activity (Damais, Riveau, Parant, Gerota and Chedid, 1982), a goat LP without effect on plasma zinc and iron (van Miert, van Duin, Verheijden, Schotman and Nieuwenhuis, 1984) and a rabbit EP which does not induce an acute phase fibrinogen response (Kampschmidt, Upchurch and Worthington III, 1983). With respect to the latter, it is interesting that this EP was produced by CPEC (>90 per cent macrophages), whereas EP produced by APEC (3 to 6 per cent macrophages and 92 to 96 per cent granulocytes) induced both a febrile and a fibrinogen response. These findings are in good agreement with our results concerning SAASF in the hamster i.e. the production of LAF but not SAASF by CPEM and the production of SAASF by PMN-rich APEC. Whether the SAASF production by APEC has to be attributed to the PMNs, to the 7 to 17 per cent macrophages, or to the contribution of both, is not clear. However, PMNs are likely candidates since in mice they appeared prior to the macrophages and in a greater number in the subcutaneous tissues of the casein-injected site (Miura, Takahashi and Shirasawa, 1986) and early infiltrating PMNs have been shown to secrete LAF in the casein-inflamed peritoneal cavity (Goto *et al.*, 1984).

SUMMARY

The acute phase SAA response was studied in hamsters. An SAA-stimulating factor (SAASF) was detected in the early acute phase blood plasma of hamsters which were subcutaneously injected with casein–LPS. The latter is routinely used in our laboratory for amyloid induction in hamsters. Acute (4 h) inflammatory exudates (>80 per cent polymorphonuclear leukocytes) were produced by intraperitoneal injection with either casein–LPS, latex or Freund's incomplete adjuvant. Chronic inflammatory exudate macrophages (>98 per cent) were elicited by intraperitoneal injection with Bacillus Calmette Guérin (BCG). Cells were stimulated in vitro with latex. SAASF was detected in the supernates and lysates of the acute exudate cells but not in those of the chronic peritoneal exudate macrophages. Lymphocyte activating factor (LAF), however, was evidently present in the latter samples, indicating that SAASF and LAF (IL-1) are functionally different substances in hamsters.

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