AMYLOID A GENE FAMILY EXPRESSION IN DIFFERENT MOUSE TISSUES

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Serum amyloid A (SAA)[1] is a family of acute phase proteins (1–3), unique among these in being an apoprotein of high density lipoprotein (HDL) (4, 5). In response to a variety of inflammatory conditions, plasma SAA levels are dramatically elevated several hundred to a thousandfold and may reach a concentration of 1 mg/ml (6–8). The various inflammatory stimuli are thought to elevate SAA via a common monocyte-derived circulating mediator (9). IL-1 has been identified as one such mediator (10–16). In the mouse, SAA is encoded by three genes (SAA_1, SAA_2, and SAA_3) and a pseudo gene (17). The liver is a major site of SAA synthesis where messenger RNA for each of the three genes is elevated 500–2,000-fold after LPS injection (18, 19). The SAA_1 and SAA_2 polypeptides are synthesized and secreted by hepatocytes (20–22) and constitute as much as 20% of the HDL apoprotein of mice challenged with LPS (23). The SAA_3 gene is a recent discovery (2), and identification of the polypeptide has not yet been reported.

SAA is perhaps more widely known as the precursor to amyloid A (AA) protein. It is the major component of amyloid fibrils deposited in tissues in the disease amyloidosis associated with chronic inflammation (4, 5, 24–27). Of the three possible SAA gene products, murine AA protein is derived from only SAA_2 (9) by a mechanism that selectively removes it from the circulating pool of both SAA_1 and SAA_2 (22). Though liver is considered to be the major if not the only source of SAA, some reports (28–30) have suggested extrahepatic sites of synthesis, particularly the spleen, and that deposit of AA in amyloidosis is mediated via local synthesis. Using a murine SAA cDNA, we have previously shown (22) that the spleen is not active in synthesizing SAA mRNA during splenic amyloid deposit induced by repeated casein injections in mice. We wished to determine if the three individual members of the SAA gene family in the liver respond to inflammation induced by different inflammatory agents and if any of the SAA genes are expressed in extrahepatic tissues. We found differential hepatic SAA response to these agents; casein elevated hepatic SAA_1 and SAA_2 mRNA while LPS elevated all three. Extrahepatic SAA mRNA was also found, primarily after LPS injection, and several qualitative and quantitative patterns of SAA_1, SAA_2, and SAA_3 mRNA expression are evident among these tissues. It is clear that the SAA gene family does not exhibit a simple all-or-none response

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[1] *Abbreviations used in this paper:* AA, amyloid A; HDL, high density lipoprotein; SAA, serum amyloid A.

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to casein- or LPS-induced inflammation but exhibits a diverse expression of individual members of the family; this varies among tissues and between the two inflammatory agents used.

Materials and Methods

RNA Preparation and Northern Blot Analysis. Male BALB/c mice 2–3 mo of age were given an intraperitoneal injection of LPS (50 μg in 0.5 ml of PBS) or a subcutaneous injection of casein (0.5 ml of a 10% solution). 18 h after injection, animals were killed and we removed the appropriate tissues. Peritoneal macrophages were elicited by injecting 2 ml of sterile thioglycolate intraperitoneally. 2 d later we injected 10 μg of LPS intraperitoneally, and after another day cells were collected by peritoneal lavage, washed in PBS, and plated in petri dishes at 2×10^6 cells/60-mm dish in RPMI medium containing 20% FCS. After 1 h, attached cells were washed and scraped from the plates in PBS and pelleted for RNA extraction. Liver for time course data was obtained from BALB/c mice after LPS injection and CBA mice after casein injection. Total RNA was isolated from fresh tissues or cells after polytron homogenization in 5 M guanidinium isothiocyanate and LiCl precipitation (31) as described elsewhere (32). RNA was denatured and electrophoresed through 1.5% agarose containing 2.2 M formaldehyde (33) and transferred to Gene Screen Plus nylon membrane (New England Nuclear, Boston, MA) (34). Blots were incubated in hybridization solution (35) modified to include salmon sperm DNA (20–40 μg/ml) and polyethylene glycol 6000 (10%). After 3–4 hr blots were hybridized with 1×10^6 cpm/ml of nick-translated ^{32}P-labeled SAA cDNA (36) for 16 hr at 42°C, washed as described previously (14), and exposed to X-ray film (Kodak XAR-5) at −70°C apposed to an intensifying screen (Dupont Cronex lightning plus).

Oligonucleotide Probes. To distinguish between SAA_1, SAA_2, and SAA_3 mRNA, oligonucleotide probes specific for each were synthesized (courtesy of Howard Hughes Medical Institute, DNA Synthesis Laboratory, University of Washington). All three probes were designed from a region in the 3′ untranslated sequences beginning 14 nucleotides after the termination codon where all three genes are divergent (3), as shown in Fig. 2*a*; these are distinct from the oligonucleotides used by Yamamoto et al. (37). Uniqueness of each oligonucleotide for their respective mRNA was checked by searching each of the three SAA mRNA sequences for regions of homology to each of the oligonucleotide sequences (Sequence Homology Search Program, International Biotechnologics Inc., New Haven, CT). Oligonucleotides were end-labeled with T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD) following the manufacturer's directions, except that 10 pmol of oligonucleotide were reacted in a 10-μl reaction with 50 μCi [γ^{32}P] ATP (3,000–4,000 Ci/mmol). Oligonucleotides were separated from unincorporated [γ^{32}P]ATP by chromatography through P-10 resin (Bio-Rad Laboratories, Richmond, CA) in 10 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EDTA, 10 μg/ml yeast tRNA (32). Probe-specific activity of $2–4 \times 10^6$ cpm/pmol was regularly obtained, assuming 100% recovery of oligonucleotide.

Denaturing gel electrophoresis and blotting analysis was performed as described above. Prehybridization was performed in 6× SSC (1× = 105 mM NaCl, 15 mM Na citrate), 5X Denhardt's solution, 0.5% SDS, 100 μg/ml yeast tRNA, and 0.05% NaPP at 45°C for 1–4 h. End-labeled oligonucleotide probes were heated at 65°C for 5 min, diluted to 3–5 ng/ml in hybridization solution (as above except with 0.1% SDS). Blots were hybridized with oligonucleotide for 16 h at 45°C, then washed at room temperature in 6X SSC, 0.1% SDS three times for 20 min followed by one wash at 45°C for 5 min. Autoradiography was then carried out as described above.

The melting temperature (T_m) for SAA_1 and SAA_2 oligonucleotide is 52°C, while that of the SAA_3 oligomer is 54°C (38). For oligomer hybrids <20 nucleotides long, the T_m is reduced 5°C for each mismatched base pair. SAA_1 and SAA_2 probes are mismatched at 4 positions, while the SAA_3 probed is mismatched at 12 and 13 positions compared with complimentary regions of the SAA_1 and SAA_2 probes, respectively. Therefore, hybridization was performed at 45°C, which is 7°C below the T_m of SAA_1 and SAA_2 oligomers

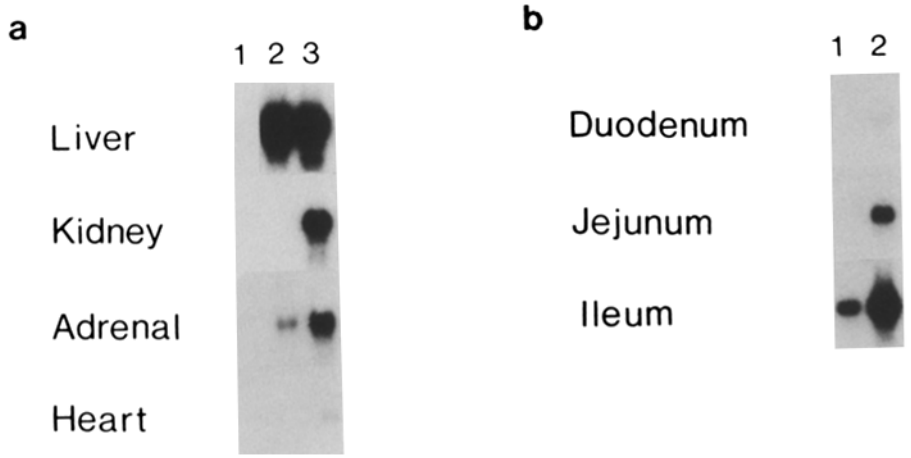

FIGURE 1. Representative Northern blot of SAA mRNA in several mouse tissues. (*a*) 5 μg of total RNA from each tissue, except 0.5 μg of liver RNA, 18 h after injection with casein or LPS, were denatured, electrophoresed, blotted, and hybridized with ^{32}P-labeled SAA cDNA as described under procedures: (*1*) unstimulated; (*2*) casein; (*3*) LPS. A typical autoradiograph is shown. (*b*) As in *a* except the autoradiograph exposure was three times longer, and (*1*) unstimulated; (*2*) LPS.

and only 2°C below the temperature recommended for perfectly matched molecules (38). Under these conditions, each oligonucleotide probe should hybridize specifically to their respective mRNAs. Evidence of the specificity of these probes is seen in Fig. 3, where the pattern of expression is distinctly different among most tissues.

Results

The relative amount of SAA mRNA in 14 murine tissues was examined after casein or LPS injection by hybridization of Northern blots with recombinant mouse SAA_1 cDNA. The resultant pattern of SAA mRNA expression after casein injection is quite different from the pattern after LPS injection (Fig. 1). Casein stimulated ~1,000-fold elevation of hepatic SAA mRNA compared with uninjected controls (Table I). Of the remaining 13 tissues, casein induced SAA mRNA elevation only in the adrenal gland. SAA mRNA was detected in small and large intestine, but at a level equivalent to the unstimulated quantity. In contrast to the limited effect of casein on tissues, LPS elicited SAA mRNA accumulation in all tissues examined (Table I). As with casein injection, liver was stimulated ~1,000-fold after LPS injection. Extrahepatic tissues exhibit a broad range of SAA levels from trace amounts in pancreas and brain to modest amounts in the large intestine, kidney, and lung. A more detailed examination of small intestines revealed a gradient of response to LPS (Fig. 1); only trace amounts could be detected in the duodenum, while the jejunum contained ~0.5% and ileum ~5% of hepatic levels (Table I). Three major regions of the brain were also examined for differential SAA mRNA levels (cerebrum, cerebellum and pons, medulla and stem), and were found to express equal levels of SAA mRNA (data not shown).

We used separate oligonucleotide probes designed from the 3′-untranslated portion of each SAA messenger RNA (Fig. 2*a*) to assess the specific SAA_1, SAA_2,

TABLE I
Relative Levels of Mouse SAA mRNA Induced by LPS or Casein

Tissue	Treatment		
	Control	Casein	LPS
Liver	0.1	100	100
Adrenal	0	0.5	2.0
Large intestine	1.5	1.0	9.0
Small intestine	0.2	0.2	1.0
Duodenum	0	ND	0
Jejunum	0.1	ND	0.5
Ileum	0.5	ND	5.0
Kidney	0	0	5.0
Lung	0	0	2.5
Testes, skeletal muscle, stomach, spleen, heart, pancreas, brain, pituitary	0	0	0.05*–0.5

Total RNA was prepared from individual tissues, except pituitary and adrenal glands which were pooled from 6–10 animals. 5 μg of RNA were examined by Northern blot analysis. Filters were hybridized with ^{32}P-labeled SAA cDNA as in Fig. 1. Each filter contained 5, 0.5, 0.05, and 0.005 μg of 18-h post-LPS total liver RNA as standards. Various exposures of the resulting autoradiographs were analyzed by quantitative scanning densitometry. Absorbances in the linear range of the film exposure were compared with absorbances of the liver RNA standards. Relative values are expressed as percent of that found in 18-h post-LPS liver RNA. The 5′ end of our SAA_1 cDNA ($pSAA_1$) contains a highly conserved region (100%) spanning amino acid residues 32–44 (3, 17) and represents 12% of $pSAA_1$ length. The remaining $pSAA_1$ sequence 3′ to this conserved sequence is ~45% homologous to SAA_3 but >90% homologous to SAA_2 mRNA. Only nick-translated SAA_1 cDNA fragments complementary to the highly conserved region of SAA_3 mRNA will be stable under the hybridization and wash conditions used. Therefore SAA_3 mRNA is detected with ~12% the sensitivity of SAA_1 and SAA_2 mRNA.

* Plasmid $pSAA_1$ could detect mRNA in as little as 2.5 ng of total RNA from mouse liver 18 h after injection of LPS. This represents ~3 SAA_1, 3 SAA_2, and 24 SAA_3 mRNA molecules per hepatocyte, assuming 6,000 molecules of each SAA mRNA per hepatoycte as reported by Lowell (18), and establishes the lower limits of SAA mRNA detection in these experiments.

These data were determined from one set of tissues. RNA from most tissues has been isolated in one more or up to three more experiments with similar results.

or SAA_3 mRNA expression in hepatic and extrahepatic tissues. The time course of liver mRNA expression for SAA_1, SAA_2, and SAA_3 after casein or LPS injections are shown in Fig. 2*b*. SAA_1 and SAA_2 mRNAs exhibit essentially the same time course after a single casein injection; they were only slightly elevated by 4 h (3%), followed by a gradual rise to ~25%s of the maximum level by 9 h. The maximum level was reached by 16 h, where they remained through 29 h. SAA_3 mRNA was minimally elevated by casein injection and reached only 3% of the maximum SAA_1 and SAA_2 levels. The accumulation of SAA_1 and SAA_2 mRNA after LPS injection was much more rapid than after casein injection. They were both near maximal elevation by 4 h (90%) and remained elevated up to 29 h when the levels began to decline (70%). At 48 h, SAA_1 and SAA_2 mRNA were ~15% of the maximum level after 9 and 16 h. The SAA_3 mRNA was expressed at a high level compared with casein injection, but accumulated at a

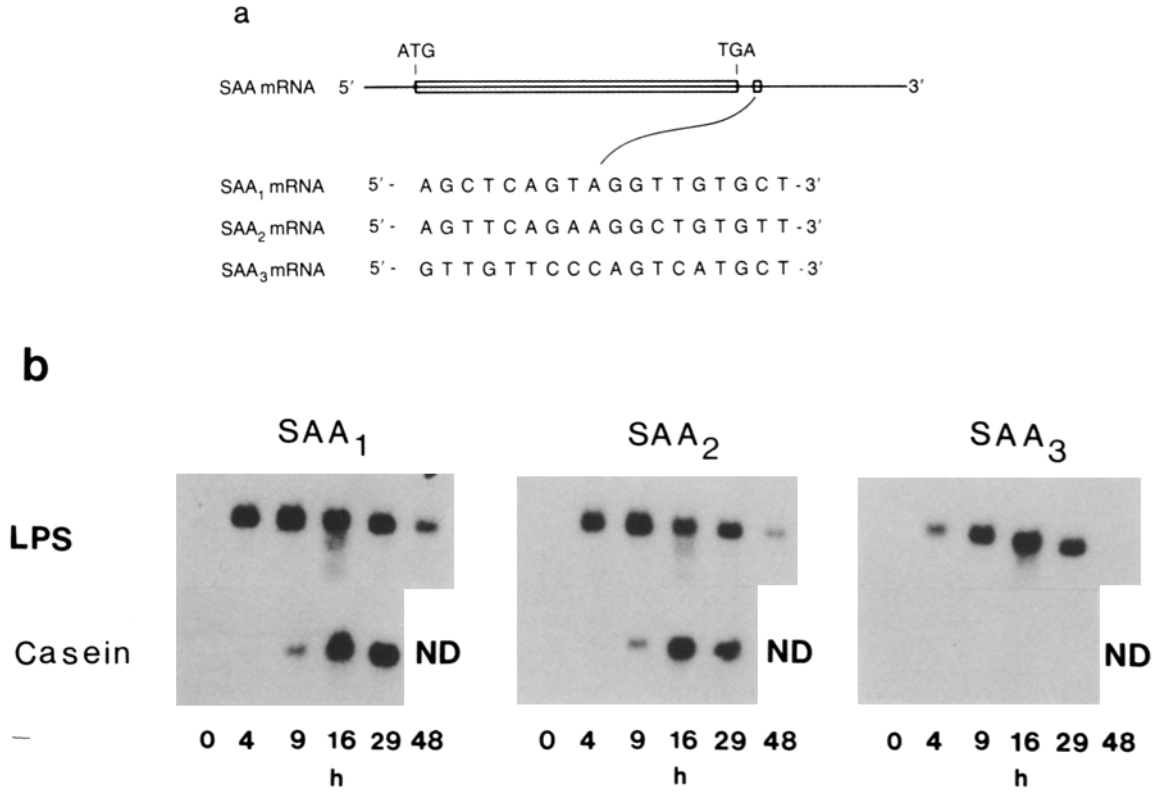

FIGURE 2. Hepatic SAA_1, SAA_2, and SAA_3 mRNA detected with oligonucleotide probes. (*a*) Nucleotide sequences of SAA_1, SAA_2, and SAA_3 message-strand DNA (reference 3) from which complementary oligonucleotide probes were designed. (*b*) Time course of hepatic SAA_1, SAA_2, and SAA_3 mRNA accumulation after LPS or casein injection. Mice received a single injection of LPS or casein and total RNA was isolated at the indicated times from individual livers after LPS injection or from pairs of livers after casein injection. Filters were hybridized with ^{32}P-labeled oligonucleotide probes as described under Materials and Methods. A representative autoradiograph is shown. Quantitative densitometry of several autoradiographs was performed as in Table I, and absorbances were calculated relative to the density of liver RNA 16 h after LPS injection.

slower rate than that of LPS-induced SAA_1 and SAA_2, reading 25% of the maximum level at 4 h, 50% by 9 h, and the maximum equal to SAA_2 by 16 h; disappearance is slightly faster than that of SAA_1 and SAA_2 mRNA so that by 48 h the level is reduced to 3%. The different rate of SAA_1 and SAA_2 mRNA accumulation induced by casein compared with LPS injection was also observed in the time course of SAA accumulation in the circulation determined by RIA in groups of four animals at each point (data not shown).

Analysis of extrahepatic tissue also revealed distinct patterns of SAA_1, SAA_2, and SAA_3 gene expression (Fig. 3). The tissues can be grouped into three patterns (Table II): (*a*) tissues expressing all three genes (liver and kidney), (*b*) tissues expressing SAA_1 and SAA_3 (small and large intestine), and (*c*) tissues that express predominantly or only SAA_3 (adrenal gland, lung, spleen, heart, testis, stomach, skeletal muscle, brain, pituitary gland, pancreas, peritoneal macrophages). Liver and kidney express all three mRNAs with the liver exhibiting

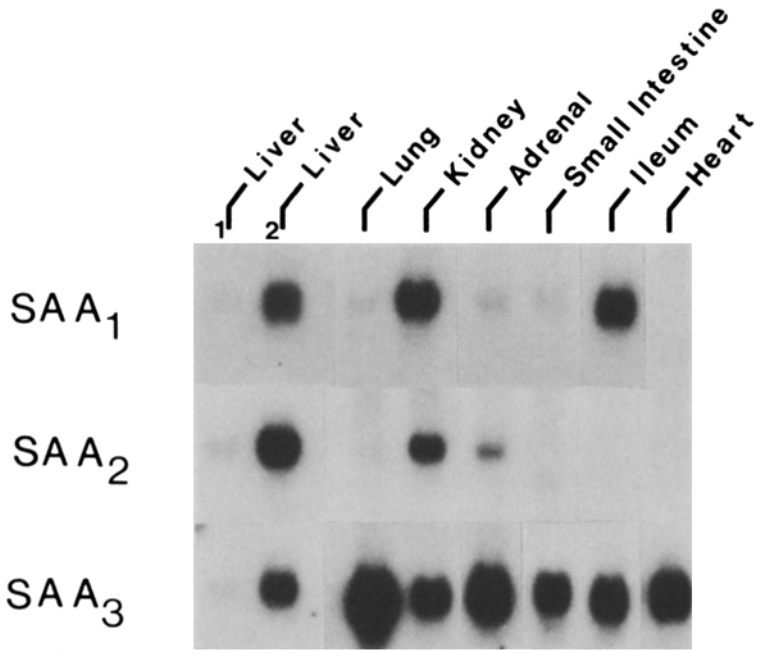

FIGURE 3. Representative Northern blot of SAA_1, SAA_2, and SAA_3 mRNA in several mouse tissues 18 h after LPS injection. 20 μg of total RNA from each tissue, and 0.2 μg (lane *1*) and 2 μg (lane *2*) of liver were analyzed as described in Fig. 2.

nearly equal amounts of each one. In the kidney, all SAA mRNAs were lower than liver and exhibit a distinct fourfold difference between SAA_1 (20%) and SAA_2 (5%) (Table II). SAA_2 was not detected in the small or large intestine, while SAA_1 was low and SAA_3 substantial. Analysis of the duodenum, jejunum, and ileum revealed a different quantitative pattern for SAA_1 and SAA_3 expression (Table II). SAA_3 levels varied by only approximately threefold between them, but SAA_1 was very prominent in the ileum, being 10-fold higher than in jejunum and not detected at all in duodenum. Of the remaining tissues, lung and adrenal gland expressed high levels of SAA_3 (60 and 35%) and low levels of SAA_1 and SAA_2. Low levels of SAA_2 were found in skeletal muscle and stomach while SAA_3 levels were moderate and SAA_1 was not detected. SAA_1 and SAA_2 mRNA was not detected in the remaining tissues, but we found different levels of SAA_3: peritoneal macrophages, 100%; testis, heart, and spleen, 25–35%; and pancreas and brain 1–2% of hepatic levels.

Discussion

The differences in tissue expression of the three SAA mRNAs, the different time course in liver, and the differing tissue response to specific acute-phase stimulants raise a new set of questions about the mode of regulation at the molecular level of this family of genes and the possible functions of their products. A variety of stimuli, including tissue injury and inflammatory agents, are effective in elevating SAA levels. The different stimuli are thought to act at least in part

TABLE II

Relative Levels of SAA_1, SAA_2 and SAA_3 mRNA in Different Tissues after Casein or LPS Injection

Tissue	Treatment					
	Casein			LPS		
	SAA_1	SAA_2	SAA_3	SAA_1	SAA_2	SAA_3
Liver	100	100	2	100‡	100	100
Kidney*				20	5	11
Large intestine*	1	0	0	4.5	0	25
Small intestine*	0.5	0	0	1	0	10
Duodenum				0	0	3
Jejunum				1	0	7
Ileum				15	0	10
Adrenal	0	0	2	1	2	35
Lung*				1	0.5§	60
Spleen*				0	0	25
Heart*				0	0	30
Testis*				0	0	12
Skeletal muscle*				0	1	8
Stomach*				0	0.5	7
Brain,* pituitary,* pancreas*				0	0	1
Macrophages*				0	0	100

20 μg of total RNA from each tissue or 2 μg of macrophage RNA were examined by Northern blot analysis as in Fig. 3. Autoradiograph exposures in the linear response range were examined by quantitative densitometry and absorbances were compared with those of standards constructed with total post-LPS (18-h) liver RNA as described for Table I.

* These tissues do not respond to casein.

‡ Values are relative to total liver RNA 18 hr after LPS injection.

§ Lowest detectable SAA mRNA levels correspond to 30 molecules/heptocyte, assuming 6,000 molecules of each SAA mRNA per hepatocyte (18).

through a common macrophage/monocyte-derived mediator or mediators (14, 16, 21); IL-1 has been identified as a mediator of SAA induction (10–13). Whether monokines besides IL-1 are involved in SAA production is not known. The data reported here indicate that there is more than one factor responsible for the induction of the several SAA genes. The most notable fact is that SAA_3 gene expression is different from SAA_1 and SAA_2. Thus hepatic SAA_3 mRNA is expressed at high levels in response to LPS but is expressed only at a low level after casein administration. Moreover, the majority of extrahepatic tissues respond only to LPS and then mainly with expression of SAA_3 mRNA. Besides IL-1, macrophages and monocytes secrete other monokines, including lymphotoxin, IFN-γ, tumor necrosis factor, and glucocorticoid-antagonizing factor in response to bacterial infection or endotoxin administration (39, 40). Perhaps one or several of these factors is involved in control of SAA_3 mRNA expression.

SAA_3 RNA is expressed in a wide variety of tissues at levels ranging over 100-fold between liver and brain (Table II). This differential behavior raises the following question: are the differentiated cells of these tissues responsible for SAA_3 mRNA expression or is it expressed by a single dispersed cell system present in these tissues? For this role there are several candidate cells: (*a*) endothelial or (*b*) smooth muscle cells of the vasculature, or (*c*) resident tissue

macrophages. We found after LPS stimulation that RNA from peritoneal macrophages contain as much SAA_3 mRNA as that found in liver RNA (Table II); moreover, thioglycollate-elicited macrophages did not express elevated SAA mRNA in response to casein (data not shown), as is the case with all extrahepatic tissue except adrenal gland. Fixed macrophages may be responsible for production of the SAA_3 mRNA in some tissues like lung, but in others, heart and testis for example, this seems unlikely because the macrophage population is low, while SAA_3 levels are quite substantial. It is also of interest that endothelial cells elaborate a variety of inflammatory mediators including IL-1 in response to LPS (41). Could they also express SAA_3? Whether tissue macrophages and other members of the reticuloendothelial system express SAA_3 mRNA in response to LPS and whether they alone are responsible for extrahepatic tissue SAA_3 mRNA remains to be determined.

The small intestine is interesting; the SAA_3 level varied only threefold over its length after LPS injection, while SAA_1 was expressed most dramatically in the ileum (Table II). In addition, SAA_1 mRNA was detected in control ileum and large intestine. Perhaps the presence of gram-negative bacteria and their production of endotoxin in these regions of the intestinal tract cause SAA_1 expression in otherwise unstimulated control animals and this is only moderately enhanced after LPS injection.

A recent report by Ramadori et al. (42) using a cDNA common to all three SAA mRNAs showed accumulation of SAA mRNA in the spleen after LPS injection. The authors suggested local production of SAA as a source of AA protein. However, this is not the case since murine AA protein is derived from only the SAA_2 polypeptide (9) and the spleen expresses only the SAA_3 mRNA (Table II). Furthermore, in a previous report (22) no SAA mRNA was detected in the spleen during casein-induced splenic amyloid accumulation.

Our finding that hepatic SAA_3 mRNA reached a level equivalent to that of SAA_1 and SAA_2 mRNA after LPS injection differs from the data of Yamamoto et al. (37). They reported that SAA_3 mRNA was barely detectable while SAA_1 and SAA_2 mRNA were expressed at high levels. The reason for this discrepancy is not clear. Perhaps this discrepancy is a result of the different sets of oligonucleotide probes used or because Yamamoto et al. analyzed RNA levels 24 h after LPS injection when the SAA_3 mRNA levels were declining.

The widespread pattern of tissues expressing SAA_3 messenger RNA resembles that of apoE mRNA expression (43), while the pattern of SAA_1 and SAA_2 mRNA expression resembles more closely the restricted pattern of A-1 mRNA (44). Differences in tissue expression and gene responses to acute-phase stimuli suggest that SAA_3 may have a different function from that of the SAA_1 and SAA_2. Structural evidence points to SAA_1 and SAA_2 having a selective affinity for certain lipids, as indicated by their association with particular lipoprotein subclasses (20, 23). Is it possible that SAA_3 also has a special affinity for certain lipids? We have not yet found, nor have others reported, evidence of SAA_3 protein in the circulation; perhaps the SAA_3 polypeptide remains cell bound. Of interest is the fact that the putative SAA_3 signal peptide (3) contains a core region of hydrophobic and uncharged residues like other signal peptides (45); however, it contains glutamine at position -1, a very unusual amino acid at this position.

In addition, the putative signal peptide cleavage site has a low processing probability (46). If SAA_3 is not secreted, it could be involved with intracellular lipid and other lipophilic substance transport.

The present study provides information on the variety of tissues capable of synthesizing SAA mRNAs and the differential expression of the three murine SAA genes. Further studies are necessary to: (*a*) identify the cell type(s) responsible for extrahepatic SAA mRNA synthesis, particularly SAA_3; (*b*) determine whether SAA_3 is secreted or remains intracellular as a first step in elucidating its function; and (*c*) elucidate the mechanisms and determine the factors involved in the control of SAA_1, SAA_2, and SAA_3 mRNA expression.

Summary

Serum amyloid A (SAA) is a major acute-phase reactant and apoprotein of high density lipoprotein (HDL). SAA is encoded by a family of three active genes. We examined hepatic expression and searched for extrahepatic expression of the three SAA mRNAs after injection with casein or LPS. Studies using an SAA cDNA, which detects all three SAA mRNAs, revealed that after casein injection liver SAA mRNA was elevated ~1,000-fold. Adrenal gland expressed SAA mRNA at a low level (0.5% of hepatic level), and was the only extrahepatic tissue with elevated SAA mRNA after casein injection. The small intestine, primarily the ileum, and the large intestine of unstimulated control animals contained 5- and 15-fold higher SAA mRNA levels than control liver. LPS also elevated liver SAA mRNA ~1,000-fold. However, in contrast to casein injection, every extrahepatic tissue examined expressed SAA mRNA. Lung and kidney contained 2–5% and large intestine contained nearly 10% of SAA mRNA levels found in liver RNA. SAA mRNA levels were lower in the remaining tissues and ranged from 0.1% in the brain and pancreas to 1.0% in the small intestine, with the ileum containing 50-fold more than the duodenum.

Analysis of liver with SAA_1, SAA_2, and SAA_3 mRNA–specific oligonucleotide probes revealed that SAA_1 and SAA_2 mRNA were elevated ~50-fold higher than SAA_3 mRNA after casein administration. LPS, however, induced all three SAA mRNAs equally. In extrahepatic tissues, SAA_1, SAA_2, and SAA_3 mRNAs were expressed differentially and can be grouped into three general classes: (*a*) tissues expressing all three genes, (*b*) tissues expressing SAA_1 and SAA_3, and (*c*) tissues expressing predominantly or only SAA_3.

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