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Differential Expression of mRNA Coding for the Alpha-2-macroglobulin Family and the LRP Receptor System in C57BL/6J and C3H/HeJ Male Mice

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ABSTRACT. **Expression of mouse A2M (MAM), murinoglobulin (MUG), the A2M receptor or LDL-Receptor related protein (A2MR/LRP) and the Receptor Associated Protein (RAP) were measured by northern blotting of mRNA isolated from liver, heart and peritoneal macrophages from C3H/HeJ and C57BL/6J (B6) mice. Marked differences between males of the two mouse strains were observed for MAM and MUG mRNA levels in liver, which were reflected in plasma levels of both proteinase inhibitors, as confirmed by immune-electrophoresis. C3H/HeJ mice had higher levels of the MAM and MUG mRNA and their corresponding plasma proteins than B6 mice. B6 mice expressed higher levels of LRP mRNA relative to C3H/HeJ mice but had lower levels of RAP mRNA. LRP receptor activity, assayed by fluoresceinated-A2M binding, was higher in B6 cells. The present data contribute to the knowledge of genetic background characteristics among male mouse of these two strains, which can take part in many biological events such as lipid metabolism, inflammation and immune response to different infectious agents.**

Key words: Alpha-2-macroglobulin/LRP/murinoglobulin/genetic background/C57BL/6J/C3H/HeJ

Differences in genetic background among mouse strains contribute to their resistance or susceptibility to inflammatory agents. C57BL/6J (B6, haplotype H-2B) and C3H/HeJ mice (haplotype H-2K) respond differently to a large range of inflammatory stimuli, such as killed BCG administration (Allen *et al*., 1977), infection by the intracellular parasite *Trypanosoma* (Trishmann and Bloom, 1982), or by bacteria such as *Chlamydia* (Darville *et al*., 1997) and by *Mycoplasmas* (Cartner *et al*., 1998). The general pattern is that of a higher resistance of B6 as compared to C3H/HeJ mice, but there are always exceptions to this rule (Allen *et al*., 1977, Soeiro *et al*., 2000). Differences in cytokine response (Starobinas *et al*., 1991, Silva *et al*., 1991) and T helper 1/T helper 2 (Th1/Th2) balance (Hondowicz *et al*., 1997) were ascribed as the basis for such differences, but components of the innate immune response were not completely investigated. Other differences, which were not yet directly correlated to their resistance to inflammatory response, were also reported between B6 and C3H/HeJ mice, such as normal ECG pattern (Postan *et al*., 1987) or different susceptibility to cadmium-induced toxicity (Shimada *et al*., 1997).

Proteinases and their inhibitors control several important biological events such as tissue injury repair, blood coagulation and fibrinolysis. The alpha-macroglobulin superfamily (AM) is composed of broad-spectrum plasma inhibitors entrapping and sterically inhibiting proteinases from all known classes (Chu and Pizzo, 1994). Different species of the AM family are widely distributed throughout the animal kingdom. The tetrameric protein murine alpha-2-macroglobulin (MAM) and the monomeric protein murinoglobulin (MUG) represent AM species in mouse plasma. Differences in serum Alpha-2-macroglobulin (A2M) levels were observed among mouse strains (Tunstall *et al*., 1975), and it

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Abbreviations: MAM, mouse A2M; MUG, murinoglobulin; A2MR/LRP, A2M receptor or LDL-Receptor related protein; RAP, Receptor Associated Protein; B6, C57BL/6J mice; AM, alpha-macroglobulin superfamily.

was also reported that A2M plasma levels are differently modulated after C3H/HeJ and B6 mice infection with *T. cruzi* (Luz *et al*., 1994). A2M has been implicated as mediator of the innate immune defense protecting against pathogens and some act as positive acute phase proteins (Chu and Pizzo, 1994, Kataranovski *et al*., 1999). A2M behaves as a carrier of cytokines and growth factors and might regulate their availability and composition in the extracellular space (Feige *et al*., 1996). A2M also may trigger early-acquired immune responses by coupling to antigens or by the entrapment process itself, resulting in enhanced antigen presentation for T cell expansion and antibody production (Chu and Pizzo, 1994).

Reaction with proteinases causes A2M to undergo a conformational change leading to the exposure of a previously buried receptor-recognition site (Chu and Pizzo, 1994). This change leads to the rapid clearance of these complexes through the A2M receptor (Van Leuven *et al*., 1979), identified as the LDL-receptor-related protein (LRP), a member of the LDL-receptor superfamily (for review see Strickland *et al*., 1994). LRP is a large glycoprotein composed of two non-covalently bound sub-units (515 and 85 kDa), with an amazingly wide spectrum of seemingly unrelated ligands (Strickland *et al*., 1994). The 40 kDa human protein that co-precipitates or co-purifies with LRP was named the receptor associated protein (RAP) (Herz *et al*., 1991), previously known in mouse as heparin binding protein (HBP-44) (Furokawa *et al*., 1990). The role of RAP is to function as an intracellular chaperone needed for proper folding, early processing and transport to the cell surface (Strickland *et al*., 1994).

Recently we observed by flow cytometry assays that LRP was overexpressed in peritoneal macrophages in C57BL/6J mice as compared to C3H/HeJ mice (Coutinho *et al*., 1998). We have now measured by northern blotting the tissue mRNA levels coding for MAM, MUG, their receptor LRP, and RAP, the associated protein. In addition, we measured by immune-electrophoresis the MAM and the MUG plasma levels in C3H/HeJ and B6 male mice. The data showed that important quantitative differences exist in mRNA levels between male mouse of these two strains.

Materials and Methods

Animals

C3H/HeJ and C57BL/6J (B6) pathogen-free male mice aged 7–8 weeks old were obtained from the animal facilities of CECAL/FIOCRUZ, Rio de Janeiro, Brazil. Mice were housed five per cage and kept in a conventional room, maintained at 20 to 25°C under a 12/12 hr light/dark cycle. Sterilized water and feed were provided *ad libitum*, and the animals were allowed to equilibrate for 7–15 days before starting the experiments. After euthanasia by cervical dislocation, the organs were removed and weighed on an analytical balance. Three age-matched individual animals were evaluated in each different assay (a total of 12 animals per strain). In every assay, we removed the heart, liver, and peritoneal cells and collected the plasma as described below. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals, resolution 242/99.

Alpha-2-macroglobulin

Human plasma alpha-2-macroglobulin (A2M) was prepared from pooled citrate-treated plasma using Zn^{2+} affinity chromatography as previously reported (Van Leuven *et al*., 1979). A2M-trypsin complexes (P-A2M) were prepared and labeled with fluorescence (P-A2M-FITC) as described (Coutinho *et al*., 1998).

Macrophages and P-A2M-binding

Macrophages were harvested from the peritoneal cavity of the mice and 10^4 cells were plated into 24-well tissue culture plates. After 24 hr of cultivation in DMEM supplemented with 10% fetal calf serum, the cell culture was processed for P-A2M-FITC labeling as previously described (Coutinho *et al*., 1998). The binding specificity was assayed by adding 5mM EDTA instead of $CaCl_2$ to the incubation medium or, by the presence of 100-fold excess of unlabeled P-A2M. To enable the visualization of nuclei the cultures were labeled for 3 min with 4,6-diamino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA) diluted 1:1000.

cDNA probes

cDNA probes for LRP, RAP, MAM and MUG were used either as restriction fragments or polymerase chain reaction-amplified fragments, as described previously (Van Leuven *et al*., 1992, Lorent *et al*., 1994). The mouse GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) cDNA was used as internal control (Piechaczyk *et al*., 1984). The probes were labeled to high specific activity by hexanucleotide-mediated incorporation of [α-^{32}P] dCTP as previously described (Lorent *et al*., 1994).

RNA extraction and Northern blotting

The procedures were essentially as described before (Lorent *et al*., 1994, Van Leuven *et al*., 1987). Adult male mice were euthanized and peritoneal macrophage; heart and liver samples were isolated, immediately frozen in liquid nitrogen and stored at –70°C before RNA extraction and northern blotting. No differences were recorded among organ weights from C3H/HeJ and B6. Comparison between C3H/HeJ and B6 mice focused on liver as it is the relevant organ involved in plasma AM synthesis/clearance, and on heart and peritoneal macrophages as their LRP expression has been studied previously (Coutinho *et al*., 1998, Lorent *et al*., 1994). Hybridization of the membrane was performed sequentially with all the tested probes as described previously (Lorent *et al*., 1994). Briefly, the filters were incubated at 42°C for 7 hr in 5×SSPE ($NaCl/P_i$/EDTA, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 150 mM sodium chloride) 5×Denhardts solution [100×contains 2% each of poly (vinylpyrrolidone), bovine serum albumin and Ficoll 400], 0.5 % SDS, 50 % deionized formamide, 100 μg/ml

denatured salmon sperm DNA, 0.05 % heparin. The filters were then hybridized at 42°C overnight in the previous solution supplemented with 10% dextran sulfate and 2×10^6 cpm/ml of the appropriate radiolabelled probe. The blots were rinsed 3 times with 0.3×SSPE/ 0.5 % SDS and washed with the same solution for 1 hr at 60°C (for MAM and MUG) or 65°C (A2MR/LRP and GAPDH). Stripping procedures were conducted by washing the hybridized membrane with 1.2 M Tris buffer SDS 10% for 3 times at 80°C. Autoradiography was done by exposure to Hyperfilm MP (Amersham, UK) using intensifying screens at –70°C for 2 hr (for MAM, MUG and GAPDH) up to 7 days (for LRP and RAP). Exposed autoradiographs were then quantified densitometrically and the specific probes values normalized for GAPDH mRNA. The results were obtained and averaged from at least four independent experiments. Afterwards, an index of variation (IV) was applied to evaluate the ratio among the obtained values between the two mice strains (B6/C3H/HeJ).

Rocket Immune-electrophoresis

Heparinized blood from C3H/HeJ and B6 adult male mice were collected from the animal tails and rocket immunoelectrophoresis was done as described previously (Van Leuven *et al*., 1992). Briefly 0.8% agarose gels containing 2.5% (v/v) of rabbit antiserum against MUG or MAM were prepared and 2.5 μL of a 1:4 dilution of the different plasma samples were applied to sequential wells, before running the gels in horizontal electrophoresis apparatus at 100 V for 20 hr. Anti-MAM and anti-MUG sera were obtained by immunization of rabbits with the isolated proteins (Van Leuven *et al*., 1987).

Statistical analysis

All values are expressed as the means±standard error. Student's *t*-test was applied to ascertain the statistical significance of the observed differences. Probability (P) values of <0.05 were considered significant.

Results

Northern blotting of RNA isolated from heart and liver showed that MAM, MUG, LRP and RAP cDNA probes specifically identified their corresponding mRNA species (Fig. 1A,1B). Directly comparable signals were obtained by consecutive hybridization of the northern blots (Lorent *et al*., 1995). The characteristic bands of 5 kb for both MAM and MUG were exclusively detected in liver as described before (Lorent *et al*., 1995), while the 15 kb LRP mRNA and the 3.6 kb and 1.8 kb RAP mRNA were detected in liver and in heart (Lorent *et al*., 1994, 1995).

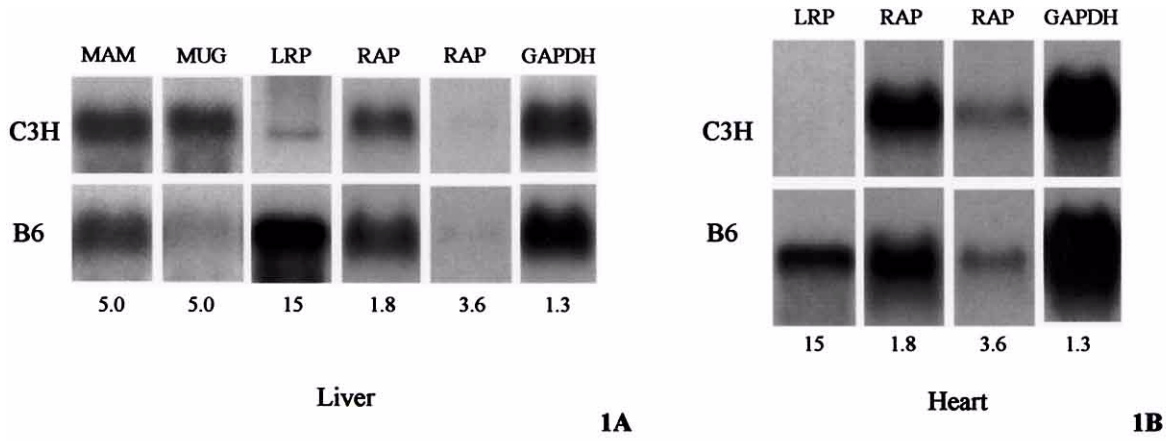
In liver, the 5-kb MUG mRNA was about 4-fold higher in C3H/HeJ mice than in B6 ($p<0.01$), while expression of MAM mRNA was about 2-fold higher in liver extracts of the C3H/HeJ mice ($p<0.09$) (Fig. 1A, 1C). Immunoelectrophoresis of plasma proteins further demonstrated the higher levels of plasma MAM and MUG in CH3/HeJ mice (Fig. 2A). There were significant differences: C3H/HeJ mice had about 37% and 45% more plasma MAM and MUG, respectively, than B6 ($p<0.045$ and 0.01, respectively) (Fig. 2B).

A representative northern blot (Fig. 1) demonstrates that liver and heart from B6 mice contained much higher levels of LRP mRNA than C3H/HeJ mice, in which LRP mRNA was barely detectable in heart (Fig. 1A, B). Densitometric analysis confirmed the significant difference (Fig. 1D): LRP mRNA in heart and liver of B6 mice was about 15-fold and 6-fold higher than in C3H/HeJ mice, respectively ($p<0.01$ and 0.05). RAP mRNA in C3H/HeJ heart and liver samples was about 2-fold higher than in B6, but only the 1.8 kb isoform in heart was significantly higher (Fig. 1E, 1F) ($p<0.03$).

To examine other cell types for a different expression of LRP mRNA observed in C3H/HeJ and B6 we analyzed macrophages, one of the best-studied cells for LRP activity. We probed A2MR/LRP activity with a physiologically ligand: the fast form of A2M, fluorescently labeled. In both mouse strains, the ligand was bound to the macrophage plasma membrane as previously described (Coutinho *et al*., 1998). P-A2M-FITC binding to the surface of peritoneal macrophages harvested from B6 mice displayed higher fluorescent intensity (Fig. 3A) relative to cells obtained from C3H/HeJ mice (Fig. 3D). Binding in the presence of EDTA or a 100-fold molar excess of unlabeled P-A2M was consistently negative (data not shown), as reported previously (Coutinho *et al*., 1998). As predicted from P-A2M binding, northern blotting confirmed that LRP mRNA expression in B6 macrophages (Fig. 3C) was 11-fold compared to C3H/HeJ cells (Fig. 3F).

Discussion

The findings of the present study denote compelling differences in the expression of the two members of the A2M-family in mice, i.e. MAM, MUG and their receptor, LRP, in B6 and C3H/HeJ male mouse strains. These differences are important extensions of some reported differences among these two mouse strains (Tunstall *et al*., 1975, Coutinho *et al*., 1998). The mRNA coding for MAM and MUG was expressed to higher levels in the liver of C3H/HeJ mice relative to B6 mice. Moreover, we demonstrated that these higher mRNA levels were also translated into much higher levels of the corresponding plasma proteins. On the other hand, B6 mice had higher levels of mRNA coding for LRP, which might be taken as indicative of a more efficient clearance and scavenging of the diverse ligands mediated by this receptor. Likewise, peritoneal macrophages from B6 displayed a higher LRP activity revealed by specific and prominent binding of fluoresceinated A2M complexes. Our present results validate previous data obtained by flow cytometry, showing higher functional expression of LRP in macrophages from B6 as compared to C3H/HeJ mice (Coutinho *et al*., 1998). Up to now, no differences were re-



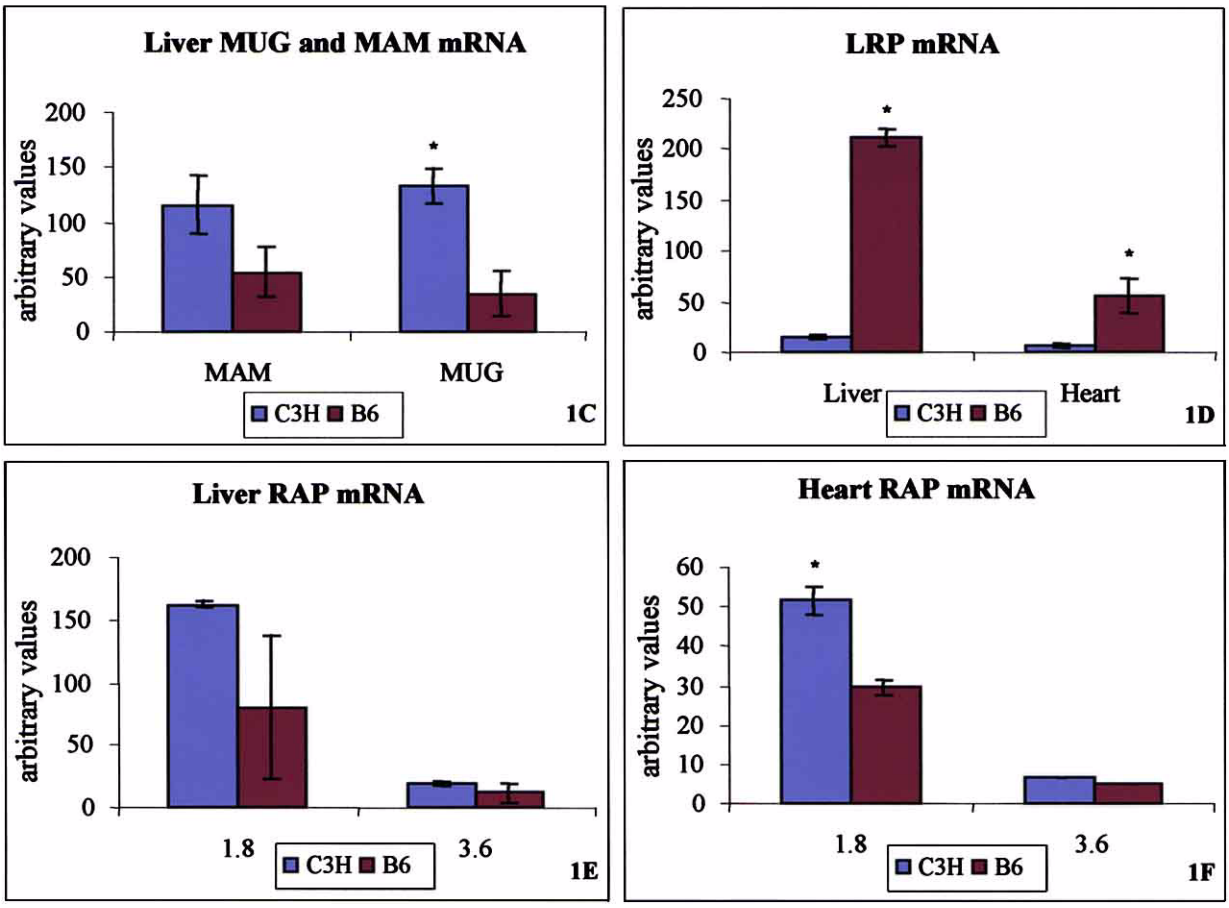


Fig. 1. A representative Northern blotting of RNA extracted from liver (A) and heart (B) of C3H/HeJ and B6 male mice. The mRNA size is indicated in kilobases. The same membrane was sequentially hybridized with cDNA probes specific for MAM, MUG, LRP, RAP and their values normalized with the GAPDH signal. The results (C-F) represent the mean of at least four independent assays. Statistical analysis: Student's *t*-test ($p<0.05$).

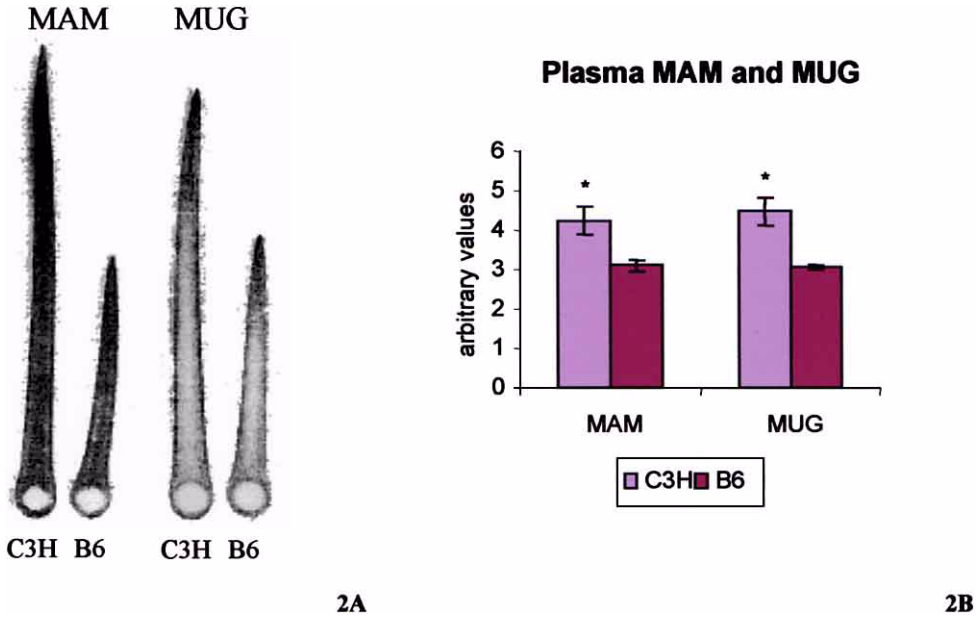


Fig. 2. (A) Immunoelectrophoresis of plasma samples of male mice from the C3H/He and B6 strains. The antiserum was isolated from rabbits immunized with the purified proteins by standard procedure (Piechaczyk *et al.*, 1984). (B) Immunoeletrophoretic quantification of MUG and MAM in the plasma of C3H/HeJ and B6 mice. The ordinate displays arbitrary units to reflect only the relative mean levels obtained in three different assays. * Student's *t*-test: statistically significance ($p<0.05$)

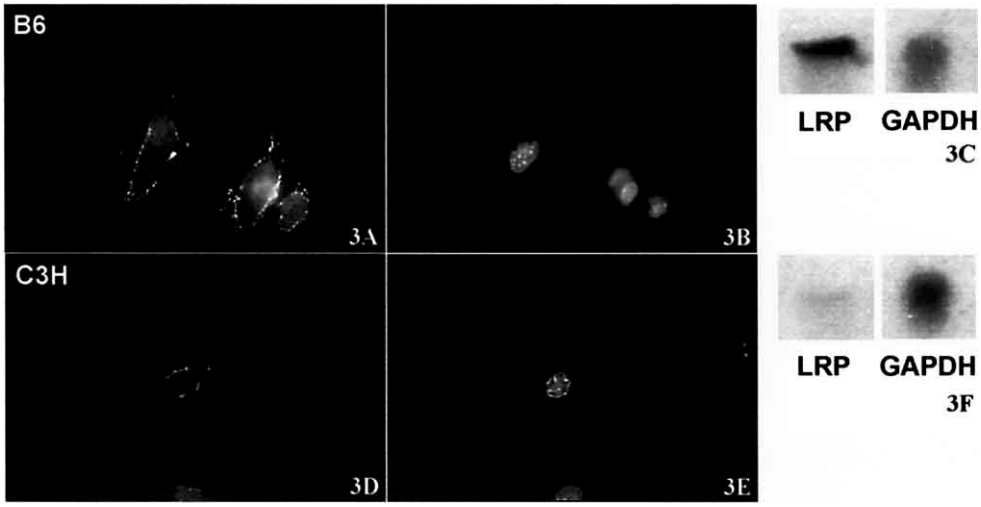


Fig. 3. LRP activity assessed by P-A2M-FITC binding to peritoneal macrophages obtained from both B6 (A) and C3H/HeJ (D) mice. Positive labeling was detected exclusively on the cell surface (A, D). Note that brightness is greater in the cells collected from B6 (A) compared to C3H/HeJ mice (D). The nuclei of the same cells were observed by DNA staining with DAPI (B and E). Northern blotting of total macrophages RNA collected from B6 (C) and C3H/HeJ (F) were sequentially hybridized with cDNA probes specific for LRP and GAPDH, respectively Bars, 100μm.

ported concerning mRNA synthesis for MAM, MUG and LRP among adult animals of different strains under similar physiological conditions. Variations in MAM expression were associated with embryonic development, acute phase inflammation, human congenital antithrombin deficiency and neoplastic conditions (Lorent *et al*., 1994, Kataranovski *et al*., 1999, Tripodi *et al*., 2000, Chu and Pizzo, 1994). In addition, aberrant forms and decreased stability of alpha-2-macroglobulin have been noticed in multiple sclerosis patients (Gunnarsson *et al*., 2000 a, b). In the same way, induction of LRP expression was associated with differentiation and maturation of human monocytes into macrophages (Watanabe *et al*., 1994), with some neoplastic transformations (Strickland *et al*., 1994), and with aging in rat (Field

and Gibbons, 2000).

Since A2M may play a role in innate immunity, we suggest that the differences reported are a genetic background characteristic that could reflect differences in the susceptibility to inflammatory agents. B6 but not C3H/HeJ mice respond to killed BCG administration by marked enlargement of the spleen and lung (Allen *et al*., 1977). The authors showed that the inflammatory response does not seem to be controlled by genes within the major histocompatibility complex but that it was associated with the B6 background.

Recently, we showed that during *T. cruzi* infection (with Y strain) higher A2M plasma levels were measured in C3H/HeJ relative to B6 male mice (Soeiro *et al*., 2000). We supposed that the lower tissue *T. cruzi* parasitism found in infected C3H/HeJ male mouse could denote an inhibitory effect of A2M since *in vitro* studies revealed that the addition of A2M to the interaction medium impaired the parasite invasion (Araújo-Jorge *et al*., 1986). In fact, analysis of *T. cruzi* infection in these experimental models must take into account the differences in the steady state of AM and A2MR/LRP between the uninfected male mice that we presently reported. Both mouse strains responded to *T. cruzi* infection with higher liver MAM and MUG mRNA expression but displayed different LRP mRNA regulation (Soeiro *et al*., 2000) which can reflect differences in the nature and activity of modulating molecules produced during this acute infection. Considering that A2M acts as cytokine and growth factor carrier and/or inhibitor or protector (Chu and Pizzo, 1994, Coutinho *et al*., 1998), it might regulate the levels of important cytokines, such as TNFα (tumor necrosis factorα) and TGFβ (transforming growth factorβ) in inflammatory microenvironments and contribute to differences in the resistance of these mice strains to different inflammatory agents (Starobinas *et al*., 1991, Silva *et al*., 1991).

The multi-functionality of the LRP and its ubiquitous expression raises questions about its role *in vivo* and argues against one single specific function. The knockout of the LRP gene impaired normal mouse development resulting in death of LRP-deficient mouse embryos before 14 days of gestation (Herz *et al*., 1992). Among the various physiologic functions of the LRP family we stress its importance in cholesterol homeostasis, clearance of activated alpha 2-macroglobulin and apolipoprotein E-enriched lipoproteins, and in Alzheimer's disease development (reviewed in Gliemann, 1998). In addition, LRP helps regulating urokinase receptor expression on the cell surface via ligand-mediated internalization followed by return of the naked urokinase receptor to the cell surface (reviewed in Hussain *et al*., 1999, Hussain, 2001). Recently, it has been demonstrated that the binding of a synthetic heparin-mimicking polyaromatic anionic compound (RG-13577) to LRP inhibits the proliferative response of vascular smooth muscle cells to growth promoting factors (Benezra *et al*., 2001). Besides, LRP and the G(i) class of GTPases are involved in neurotoxic mechanisms triggered by ApoE4 to activate a cell type-specific apoptogenic program (Hashimoto *et al*., 2000).

The *in vivo* function of RAP and the significance of its interaction to LDL receptor family are still controversial. *In vitro* and *in vivo* studies have demonstrated that exogenous RAP is a very efficient inhibitor of the binding of nearly all ligands to LRP (Herz *et al*., 1991). However, *in situ* hybridization showed a prominent diversity in cellular distribution of the mRNA coding for RAP and LRP (Lorent *et al*., 1994). Our present data showed that the 1.8 and 3.6 kb mRNA species, derived from alternatively spliced or alternatively polyadenylated primary RAP transcripts, were present at nearly constant levels, except for the 1.8 kb transcript found in heart samples of C3H/HeJ mice, a variation which remains to be investigated.

In conclusion, we provide experimental evidence for quantitative differences in the expression of mRNA coding for A2M, murinoglobulin and LRP in liver, heart and macrophages of C3H/HeJ and B6 male mouse strains. The potential relevance for their function as proteinase inhibitors and for the efficiency of clearance and endocytosis of these and other proteinase-inhibitor complexes, as well as for lipid metabolism and for resistance to parasitic infection, deserves further investigation.

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