Immunology 1991 **73** 58–63

Suppression of experimental allergic encephalomyelitis by α_2-macroglobulin

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Accepted for publication 28 December 1990

SUMMARY

Lewis rats sensitized with guinea-pig spinal cord in Freund's complete adjuvant developed an acute-phase protein response. This was characterized by a marked increase in plasma α_2-macroglobulin (α_2 M) levels which, however, declined towards normal values before the onset of clinical signs of experimental allergic encephalomyelitis (EAE). In contrast, levels of two other acute-phase proteins, fibrinogen and caeruloplasmin, remained variably elevated over the entire study period. Recovery from EAE coincided with an increase in α_2 M levels. Infusion of purified α_2 M effectively protected the rats against clinical EAE and this was associated with a restimulation of the acute-phase response. The protected rats were shown to be sensitized to myelin basic protein and to have comparable mononuclear infiltration of the central nervous system with the diseased animals. It is postulated that the infusion of α_2 M leads to the inhibition of the effector pathways of the delayed type hypersensitivity response.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease process induced in susceptible rat strains by the injection of spinal cord tissue emulsified in Freund's complete adjuvant. It is usually a non-relapsing disease with complete recovery being the normal outcome.[1] The injection of Freund's complete adjuvant into rats is known to produce a chronic, multi-organ, adjuvant disease.[2] This is accompanied by an acute-phase protein response.[3] In view of a report describing the inhibition of the clinical signs of EAE by the injection of a variety of proteinase inhibitors,[4] we were interested in the role of the endogenous proteinase inhibitor and major acute-phase protein of rats, α_2-macroglobulin (α_2 M), in this disease process. α_2 M irreversibly binds neutral endopeptidases of all four classes: serine, metallo, thiol and carboxy peptidases.[5] The Lewis rat, the animal of choice for induction of EAE, is a high responder with respect to α_2 M. In response to an intense inflammatory challenge α_2 M levels in plasma rise from microgram levels to as high as 15 mg/ml.[6]

The aim of this study was to document the acute-phase response following the induction of EAE in Lewis rats and, in particular, to relate plasma levels of α_2 M to the onset and severity of disease as well as to the recovery phase.

Abbreviations: α_2 M, α_2-macroglobulin; Cp, caeruloplasmin; EAE, experimental allergic encephalomyelitis; Fb, fibrinogen.

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MATERIALS AND METHODS

Induction of EAE

Animals. All animals were bred at the Animal Breeding Establishment at the John Curtin School of Medical Research (JCSMR), Australian National University. Female Lewis/JC rats were bred from pairs obtained from the Commonwealth Scientific and Industrial Research Organisation, Brisbane, Australia, in 1972. Due to contamination in Brisbane, the Lewis strain rats bred at the JCSMR carry the AA allele; most other Lewis rats are aa. This strain, now known as Lewis/JC, is fully susceptible to EAE. They were used at 8–10 weeks of age and weighed approximately 120–150 g.

Induction of EAE. Spinal cords were excised from adult Hartley guinea-pigs, meninges and vasculature removed, and a 20% W/W suspension prepared by homogenization in 0·9% saline using a Mulsijet syringe (Mulsijet, Elmhurst, IL), and aliquots were stored at −70°. For use, the homogenate was emulsified in an equal volume of Freund's incomplete adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia) to which had been added 4 mg/ml *Mycobacterium butyricum* (Difco, Detroit, MI). This inoculum is designated GPSC/FCA. Rats were injected with 0·2 ml, divided between the hind footpads. For control groups, bovine serum albumin (Sigma, St Louis, MO) was substituted for GPSC.

Clinical assessment of EAE. Rats were weighed daily, and assessed for clinical signs using a scale of 0–5, as follows: 0, asymptomatic; 1, weight loss, flaccid tail; 2, ataxia, slow righting reflex; 3, paraparesis, incontinence; 4, paraplegia or

quadriplegia, no voluntary limb movement; 5, moribund or death.

Histology. Animals were anaesthetized with Avertin, and perfused via the left ventricle with saline, followed by fixation/perfusion with formol saline. The brain and spinal cord were then removed into formol saline and embedded in paraffin. Mid-sagittal sections were cut at 6μ thickness, and stained with haematoxylin and eosin to demonstrate cellular infiltrates in lesions or with toluidine blue to show areas of demyelination.

Purification of α_2 M

Male Sprague–Dawley rats, between 150 and 200 days old, were injected subcutaneously with commercial grade mineral turpentine (0·50 ml in each of four sites on shaved flanks) to elicit an acute-phase response. After 48 hr the rats were anaesthetized under ether and injected through the tail vein with 1000 Kallikrein inhibitory units (KIU) aprotinin (Sigma) in 0·5 ml 0·85% NaCl, before being bled from the heart into tubes on ice containing trisodium citrate at a final concentration of 0·01 M.

The blood was centrifuged at 1000 ***g***, 15 min, 4° and the plasma retained. Platelets were removed by high speed centrifugation (23,500 ***g***, 20 min, 4°). To the plasma was added one volume of water and 0·8 volumes of 1% Rivanol (Sigma). The Rivanol/plasma preparation was centrifuged at 160 ***g***, 20 seconds at 4°, the supernatant discarded and 30 ml 0·1 M acetate buffer (pH 4·5 containing 0·02% sodium azide and 50 KIU/ml aprotinin) added to the pellet. The tube was then shaken gently until the precipitate had dissolved (30–40 min). To clarify, the solution was centrifuged at 10,000 ***g***, 10 min, 4°. The supernatant was dialysed at 4° over 48 hr against 100 times its volume of 0·01 M phosphate buffer, pH 7·25, containing 0·15 M NaCl and 0·02% sodium azide. The fibrinogen which precipitated during dialysis was removed by centrifugation at 4000 ***g***, 10 min, 4°. The supernatant was applied to CM Affigel Blue (Bio-Rad, Richmond, CA) consisting of 100 ml CM Affigel Blue in a sintered glass funnel. The α-globulin fraction was eluted with the above phosphate buffer and used in the first infusion experiment.

To purify α_2 M, the α-globulin fraction was concentrated, applied to a 2·6 × 100 cm column of Sepharose CL6B (Pharmacia, Uppsala, Sweden), and the first peak containing the α_2-macroglobulin was concentrated and applied to a 1·6 × 60 cm column of DEAE Sephacel (Pharmacia). α_2 M was separated from α_1-macroglobulin by using a gradient of 0–0·25 M NaCl in 0·05 M Tris-HCl buffer, pH 8·0. The first peak eluted contained α_2 M. The preparation was applied to a SDS–polyacrylamide gel and showed one major band at 185,000 MW under reducing conditions of 5% 2-mercaptoethanol in SDS disaggregation buffer. Immunoelectrophoresis showed that only one detectable protein was present when tested against a rabbit antiserum against rat 'acute-phase' plasma proteins, and only one precipitin arc was present when the preparation was electrophoresed against concanavalin A. The α_2 M was further characterized by isoelectric focusing and shown to be in the free (unbound) form.[5] The protein was stored at 4° as a sterile solution in pyrogen-free Dulbecco's phosphate-buffered saline (DPB).

Measurement of caeruloplasmin, fibrinogen, α_2 M and corticosterone

Rats were anaesthetized under ether and blood obtained by cardiac puncture within 2 min of handling. Caeruloplasmin was measured by its oxidase activity[7] and fibrinogen by a turbidometric assay.[8] α_2 M was quantified by rocket immunoelectrophoresis against goat anti-human α_2 M antiserum (Cappel Laboratories, West Chester, PA; 150 μl per 12 ml of 1% agarose M per glass plate measuring 9·2 cm × 8·2 cm);[9] 5 μl plasma or serum was placed in each well, electrophoresis carried out for 16 hr at 6V/cm in 0·025 M sodium barbitone, pH 8·6, and the plates then dried and stained with 0·5% Coomassie blue G250. Corticosterone was measured by a fluorimetric assay.[10]

Preparation of rat albumin

Albumin was eluted from the CM Affigel Blue column during the α_2 M purification with 1·4 M NaCl in the phosphate running buffer following elution of the α-globulins. The albumin preparation was characterized by immunoelectrophoresis.

Preparation of trypsin-bound α_2 M

To obtain an equimolar ratio of the components, 10 mg α_2 M was incubated with 250 μg trypsin (bovine; Sigma Type III) at 37°, 20 min, then placed on ice immediately. Isoelectric focusing showed this preparation to have a higher pI than the untreated α_2 M. To test for any remaining trypsin activity, 0·3 mg trypsin-bound α_2 M was incubated with 10 mg azocoll, a general protease substrate (according to the Calbiochem Technical Bulletin), in a total volume of 1·0 ml for 60 min at 37°. The absorbance of the supernatant was measured at 520 nm against a standard curve of trypsin and the bound form demonstrated trypsin activity of < 300 ng/ml.

Protein infusion

Solutions of proteins in DPB were administered via an osmotic pump (type 2ML1 from Alza Corporation, Palo Alto, CA), which was implanted subcutaneously on the back. This allowed a uniform rate of infusion over 7 days. In all experiments, EAE controls inoculated with GPSC/FCA were sham operated. At the end of the treatment, the pumps were removed and checked to make sure they had emptied completely and the surrounding tissues were inspected for signs of infection.

Macrophage procoagulant assay (MCPA)

Procoagulant lymphokine activity was measured as described previously.[11] The MCPA is based on the finding that specifically sensitized T cells recognize antigen presented on appropriate stimulator cells, and react by producing lymphokine(s) which, in turn, causes the production of procoagulant on the surface of macrophages. It is the procoagulant which is measured by the capacity to reduce the clotting time of normal plasma. The test antigen, myelin basic protein, was purified from guinea-pig spinal cord by the method of Diebler, Martensen & Kies.[12] The control antigen, ovalbumin, was obtained from Sigma.

Statistical analysis

Data were analysed by Student's *t*-test.

RESULTS

Effect of α_2 M infusion on development of EAE

The ability of GPSC/FCA to induce the signs of EAE is shown in the typical experiment summarized in Table 1. The clinical

Table 1. Clinical scores for Lewis rats injected with guinea-pig spinal cord emulsified in Freund's complete adjuvant (GPSC/FCA) with no infusion and with the infusion of either α_2-macroglobulin or rat albumin from Days 8 to 15 at 0·5 mg protein/rat/day

	Clinical scores for each rat in group		
	Infusion		
Days after sensitization	GPSC/FCA*	GPSC/FCA + α_2 M†	GPSC/FCA + rat albumin‡
0–11	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0
12	3 2 2 2 0	0 0 0 0 0	0 0 0 0 0
13	3 4 2 2 0	2 1 0 0 0	2 2 0 0 0
14	4 4 3 3 3	3 2 0 0 0	3 3 3 0 0
15	4 4 3 2 2	2 0 0 0 0	4 4 3 3 0
16	4 4 3 3 2	2 1 0 0 0	4 3 3 2 2
17	4 3 2 1 0	4 0 0 0 0	4 4 3 2 0
18	3 2 1 0 0	4 0 0 0 0	3 2 1 0 0
19	2 1 1 0 0	3 0 0 0 0	1 1 1 0 0
20	2 1 1 0 0	2 0 0 0 0	2 1 1 1 0
21	2 1 1 0 0	2 1 0 0 0	2 2 1 0 0
23	0 0 0 0 0	0 0 0 0 0	2 1 0 0 0
24–27	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0

Assessment of EAE: 0, asymptomatic; 1, weight loss, flaccid tail; 2, ataxia, slow righting reflex; 3, paraparesis, incontinence; 4, paraplegia or quadriplegia, no voluntary limb movements; 5, moribund or death.

Statistical analysis of data from Table 1:

	$\bar{x}$	n	SEM	P
1. * GPSC/FCA	8·250	12	1·548	1 versus 2 = < 0·01
2. † GPSC/FCA + α_2 M	3·100	10	0·315	2 versus 3 = < 0·01
3. ‡ GPSC/FCA + albumin	7·182	11	1·31	1 versus 3 = NS

where $\bar{x}$ is derived from the aggregate daily score for each group.

scores for each of five rats are given in descending order and the scores in a particular column are not necessarily those for the same animal. The results show that signs became evident after 12 days, were most predominant after 14–16 days and then gradually decreased until all the animals were asymptomatic after 23 days. In a control experiment in which eight animals were injected with BSA/FCA none displayed signs of EAE.

Infusion of the α-globulin fraction (containing 1 mg α_2 M/rat/day) from Days 8 to 15 effectively ablated the clinical signs and led to the testing of purified α_2 M to determine whether it was the protective component. At an infusion rate of 1 mg/rat/day from Days 8 to 15, protection was near complete, with the maximum clinical scores amongst the group of five rats being 1 (weight loss, and/or flacid tail) for one animal on Day 13 and three on Day 16. This finding was reproduced over three experiments (GPSC/FCA mean = 5·43 ± sem 1·42 versus α_2 M infused mean = 1·27 ± sem 0·92, P = 0·02 and where the mean is derived from the aggregate daily scores for each group). Reduction of the dose to 0·5 mg/rat/day from Days 8 to 15 gave the results shown in Table 1. The effect was less marked than at 1 mg/rat/day but a reduction is evident in the number of animals displaying signs and in the severity of the disease. In contrast rat albumin 0·5 mg/day had no such effect (Table 1).

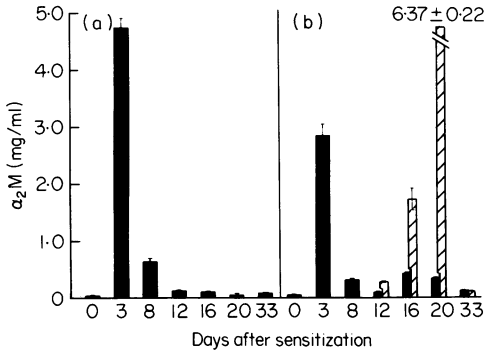

Figure 1. (a) α_2 M response in Lewis rats injected with bovine serum albumin emulsified in Freund's complete adjuvant (BSA/FCA) (■); and (b) for Lewis rats injected with guinea-pig spinal cord emulsified in Freund's complete adjuvant (GPSC/FCA) with (diagonal stripes) and without (■) infusion of α_2 M at 1 mg/rat/day from Days 8 to 15. Three rats were bled for each time point. Data show mean values ± SD.

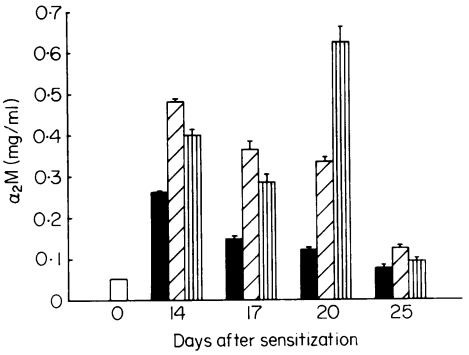

Figure 2. α_2 M response in Lewis rats injected with guinea-pig spinal cord emulsified in Freund's complete adjuvant with no infusion (solid bars), and with the infusion of either α_2 M (diagonal stripes) or rat albumin at 0·5 mg/rat/day (vertical stripes) from Days 8 to 15. Four rats were bled for each time point. Data show mean values ± SD.

Measurement of plasma α_2 M

In parallel with the above clinical studies, plasma samples were examined for changes in concentration of α_2 M. As shown in Fig. 1, the control animals injected with BSA/FCA displayed the expected profile for α_2 M, with a rapid rise and decline. In contrast those injected with GPSC/FCA showed a secondary peak after 16–20 days (Fig. 1) (control value 0·038 ± 0·003 (SD) versus Day 16 value 0·432 ± 0·023 mg/ml), which was reproduced in three experiments. This secondary peak was markedly enhanced in the animals infused with 1 mg α_2 M/day from Days 8 to 15; with the maximum of approximately 6 mg α_2 M/ml plasma being far in excess of the amount of α_2 M infused, namely 7 mg.

Further studies on the secondary peak were carried out on the plasma from rats infused with 0·5 mg α_2 M/day and whose clinical signs are recorded in Table 1. The results (Fig. 2) show a significant increase in α_2 M concentration in the rats infused with α_2 M; the values are considerably less than those induced with 1 mg α_2 M/day but are still in excess of the amount infused. Rat albumin also led to an increase in plasma α_2 M concentration

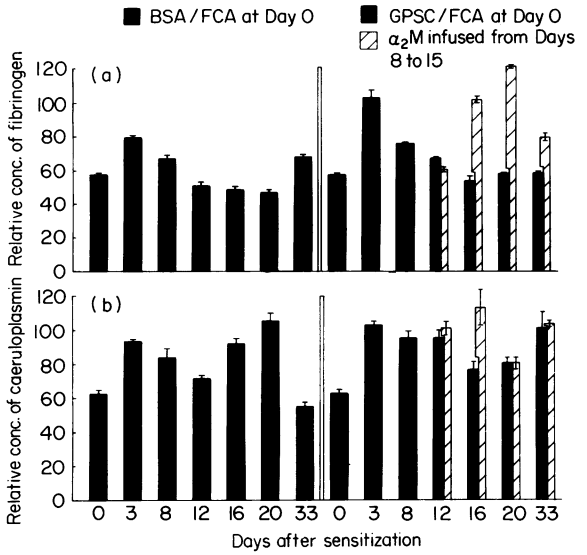

Figure 3. (a) Fibrinogen response in Lewis rats injected with bovine serum albumin emulsified in Freund's complete adjuvant (BSA/FCA); and for Lewis rats injected with guinea-pig spinal cord emulsified in Freund's complete adjuvant (GPSC//FCA) with (diagonal stripes) and without infusion of α_2 M at 1 mg/rat/day from Days 8 to 15. Three rats were bled for each time point. Data show mean values ± SD. (b) Caeruloplasmin response in Lewis rats injected with bovine serum albumin emulsified in Freund's complete adjuvant (BSA/FCA); and for Lewis rats injected with guinea-pig spinal cord emulsified in Freund's complete adjuvant (GPSC/FCA) with (diagonal stripes) and without infusion of α_2 M at 1 mg/rat/day from Days 8 to 15. Three rats were bled for each time point. Data show mean values ± SD.

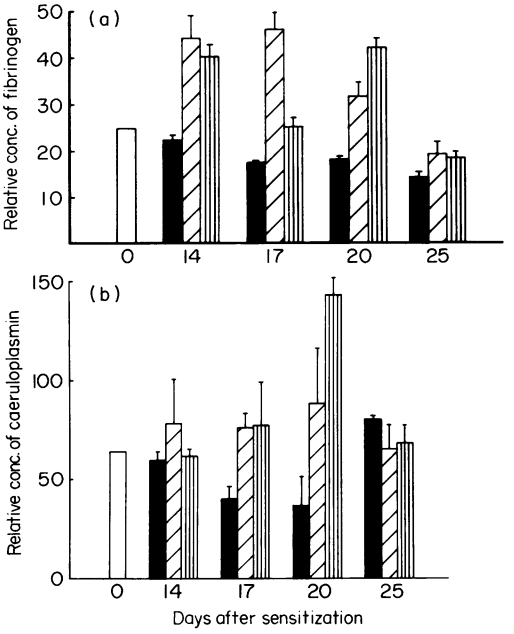

Figure 4. (a) Fibrinogen response in Lewis rats injected with guinea-pig spinal cord emulsified in Freund's complete adjuvant with no infusion (solid bars), and with the infusion of either α_2 M at 0·5 mg/rat/day (diagonal stripes) or rat albumin 0·5 mg/rat/day (vertical stripes) from Days 8 to 15. Four rats were bled for each time point. Data show mean values ± SD. (b) Caeruloplasmin response in Lewis rats injected with guinea pig spinal cord emulsified in Freund's complete adjuvant with no infusion (solid bars), and with the infusion of either α_2 M at 0·5 mg/rat/day (diagonal stripes) or rat albumin 0·5 mg/rat/day (vertical stripes) from Days 8 to 15. Four rats were bled for each time point. Data show mean values ± SD.

(Fig. 2), although no reduction of clinical signs was observed (Table 1).

To determine whether α_2 M, either free or enzyme-bound, would stimulate α_2 M production, increasing amounts of α_2 M (up to 1·5 mg) were injected subcutaneously into rats. While injections of pyrogen-free phosphate-buffered saline (carrier buffer) caused a 10-fold increase in plasma α_2 M, injections of free or enzyme-bound α_2 M did not increase the response when the contribution of the injected material was allowed for.

Measurement of other acute-phase proteins

Plasma samples were also analysed for Fb and Cp. Infusion of 1 mg α_2 M/day resulted in the enhancement of the concentration of Fb from Days 16 to 33 (Fig. 3a) and of Cp at Day 16 (Fig. 3b). Infusion of 0·5 mg α_2 M/day or 0·5 mg albumin/day also led to an increase in the concentration of both Fb (Fig. 4a) and Cp (Fig. 4b).

The direct subcutaneous injection of free or enzyme-bound α_2 M (see above) in graded doses up to 1·5 mg did not enhance the serum concentration of Cp above that produced by injection of carrier buffer alone.

Histological and immunological studies

An examination was undertaken of the intensity and pattern of leucocyte infiltration in the spinal cord of animals with clinical signs of EAE and those protected with α_2 M (1 mg/day). In both cases there was extensive infiltration with perivascular accumu-

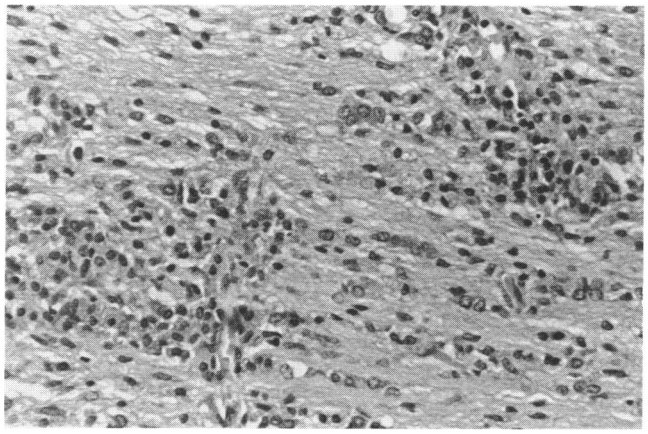

Figure 5. H&E stained section of a spinal cord from a clinically disease-free, α_2 M-infused rat 16 days after sensitization with GPSC/FCA. Showing extensive mononuclear infiltration; ×245.

lation of mononuclear leucocytes, as shown by the example in Fig. 5.

At Day 20 following challenge for EAE, popliteal lymph nodes were obtained from both diseased animals and those protected by infusion with α_2 M (1 mg/day). Cultured lymph node cells were challenged with myelin basic protein and the supernatants tested for procoagulant lymphokine activity. In each case there was a mean reduction in clotting time of 25% compared with 3·5% for cells challenged with the control antigen, ovalbumin.

DISCUSSION

The injection of Freund's complete adjuvant is an effective stimulus of the acute-phase response. The prolonged elevation of caeruloplasmin levels in particular and also fibrinogen is in keeping with the information relating to chronic adjuvant disease.[2] α_2 M behaved differently from caeruloplasmin or fibrinogen. In relation to this finding there is now good evidence for the primary role of the cytokine interleukin-6 in stimulating the production of the acute-phase proteins by hepatocytes.[13] For α_2 M there is, however, additional regulation by glucocorticoids.[14] Levels of corticosterone, the major glucocorticoid of rats,[10] were followed in a group of animals that were challenged for EAE but not infused (data not shown). Serum levels of this hormone remained at control values (mean 20 μg/100 ml) up to Day 10 following challenge but then increased and remained elevated (range 30–45 μg/100 ml) up to Day 60 when the experiment was terminated. The time-course of the clinical signs and the steroid response in the Lewis/JC rats used in this study differ from that reported for conventional Lewis rats.[15] These authors reported that steroid levels were elevated by Day 10 and subsided with the clinical signs on Day 16 to reach normal values by Day 21. Recently it has been demonstrated that rat strains vary in the vigour of the steroid response in relation to EAE and that this difference is not linked to the major histocompatibility complex.[16] There is a consensus, however, that steroids play a key role in mediating the remission of clinical disease. In relation to this, the infusion of α_2 M could not protect adrenalectomized Lewis/JC rats from a fulminating EAE (N. Hunter, unpublished observations). While there is an absolute requirement for corticosterone to enable the synthesis of α_2 M in the rat, it should be noted that in the present study high levels of α_2 M were attained at Day 3 after challenge, when serum steroid levels are within the normal range. It is possible, however, that high levels of steroid in the recovery phase could facilitate α_2 M synthesis.

In relation to the observed boosting of the acute-phase proteins in infused animals, the injection of α_2 M in the free or proteinase-bound form into normal animals did not result in an increased acute-phase response over that produced by injection of buffer alone. The response of animals with an ongoing inflammatory process could, however, differ from normal animals. The major difference between the group of rats infused with a low dose of α_2 M and albumin was the higher level of α_2 M at Day 14 and Day 17 in the α_2 M-infused animals. It could be that a minimum threshold of α_2 M is necessary for protection. This is supported by the drop in the level of protection in this group. Another possibility is that there is a special characteristic of the α_2 M preparation. In this regard it is of interest to note a report[17] that an α_2 M preparation with high levels of esterolytic activity, and therefore probably largely in the proteinase-bound form, effectively reduced oedema induced by carrageenan injection,[18] whereas a preparation shown to be predominantly in the 'free' form was ineffective.[17] Proteinase-bound form α_2 M, however, was not detected in the preparation used in the present study. An alternative explanation is the possibility of isomeric forms of α_2 M with differing biological properties. Isomers have been reported for human α_2 M[19,20] and we observed an apparent heterogeneity in the elution of α_2 M from the Cibacron Blue affinity gel.

On a molar basis, α_2 M was between five and six orders of magnitude more effective in protecting rats from EAE than were low molecular weight proteinase inhibitors.[4] This suggests that the proteinase binding properties of α_2 M are not the only basis for protection. This should be considered, however, in the context of the broad spectrum of inhibitory activities for α_2 M compared with other proteinase inhibitors so that α_2 M would bind a variety of proteinases which could contribute to clinical disease.

There are a number of possible steps where α_2 M could intercede in the inflammatory reaction, including suppression of the immune response, inhibition of proteinase secretion, inhibition of oxygen radical formation and binding of proteinases. In addition to its role in proteinase capture, α_2 M has other properties which are relevant to a role in EAE. α_2M in either the free or proteinase-bound form is reported to modulate the secretion of proteinases by macrophages.[21] Also, the enzyme-bound form of α_2 M is reported to inhibit the release of superoxide anion from stimulated macrophages.[22] In addition, α_2 M has been shown to bind myelin basic protein.[23] This could lead to masking of antigenic sites on the basic protein or to inhibition of proteolytic attack. In relation to this, guinea-pigs were protected against EAE by mixing heterologous serum with myelin basic protein before challenge in adjuvant.[24] Also, rats were reportedly protected by premixing myelin basic protein with caeruloplasmin, transferrin or α_2 M.[25] The protective mechanism was thought to relate to the masking of antigenicity.

More recent studies have emphasized the role of the electrophoretically fast form of α_2 M in the regulation of the immune response. Thus α_2 M–trypsin complexes in which the activity of the trypsin was blocked by the low molecular weight proteinase inhibitor aprotinin were effective in antagonizing the induction by interferon-gamma of class II histocompatibility proteins on human and murine macrophages.[26] In contrast, aprotinin blocked the capacity of α_2 M–trypsin complexes to inhibit T-cell proliferation induced by antigen-pulsed monocytes.[27] The immunosuppressive action of enzymatically active trypsin entrapped within α_2 M was related to the capacity of the proteinase to degrade interleukin-2.[28] The data obtained in this present study of EAE suggest, however, that α_2 M does not prevent lymphocyte sensitization or migration into the central nervous system. If α_2 M is the protective agent then it seems that it works by blocking a later stage in the effector pathway of cell-mediated immunity.

The rat, like other rodents, but unlike man, has two major macroglobulin proteinase inhibitors.[29] In this respect the rat provides a good model for studying the biological properties of α_2 M because this protein is a major acute-phase protein.[30] Further studies are in progress to determine if α_2 M is a key protein in the regulation of the disease process.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Health and Medical Research Council of Australia.

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