Autoantibodies to recombinant lipocortin-1 in rheumatoid arthritis and systemic lupus erythematosus

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SUMMARY Corticosteroids may mediate some of their anti-inflammatory effects via induction of a specific 38 kD protein, lipocortin-1. Autoantibodies to lipocortin-1 were measured by enzyme linked immunosorbent assay (ELISA) in 90 healthy subjects and in 63 patients with rheumatoid arthritis (RA), 36 with systemic lupus erythematosus (SLE), 26 with polymyalgia rheumatica, and 13 with chronic airways disease. Sixteen patients with RA receiving prolonged, high steroid doses (prednisolone >7.5 mg/day) had raised IgM antilipocortin-1 levels, while 19 patients with RA untreated with steroids had normal levels. This association was independent of disease activity. In SLE, raised antilipocortin-1 levels were associated with active disease and were independent of steroid treatment. Antilipocortin-1 antibody levels were not raised in patients with polymyalgia rheumatica and chronic airways disease. Thus steroid treatment alone appears insufficient to induce antilipocortin-1 antibodies, unless an underlying autoimmune state is also present. In RA, antilipocortin-1 antibodies may impair anti-inflammatory actions of steroids and render some patients 'steroid resistant'.

Corticosteroids are potent anti-inflammatory drugs frequently used in the treatment of a wide range of rheumatic and other disorders. Their mode of action is multifactorial but in general requires the induction of specific genes within the cell nucleus and subsequent formation of new proteins. One such steroid induced protein is lipocortin-1,¹ a 38 kD molecule originally identified as lipomodulin² or macrocortin.³ Lipocortin-1 inhibits phospholipase A₂ activity in *in vitro* assays and thus suppresses the generation of phospholipid derived inflammatory mediators, including prostaglandins, thromboxanes, and leucotrienes.⁴ The mechanism by which lipocortin-1 exerts its antiphospholipase A₂ effect is uncertain, as recent data imply an indirect inhibition of enzyme activity where lipocortin competes with enzyme for its phospholipid substrate.⁵ A family of six distinct lipocortin-like proteins has recently been described, with highly conserved sequences. They have

common calcium and phospholipid binding properties with a conserved fourfold repeat structure, suggesting a common functional element, though the exact nature of this is not yet determined.⁶

Although corticosteroids are frequently used in the treatment of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and polymyalgia rheumatica, the clinical observation of high corticosteroid requirements for control of inflammatory disease in some patients has long been recognised.⁷ Furthermore, the 'steroid pseudorheumatism' syndrome in RA may occur with prolonged periods of corticosteroid treatment at doses of prednisolone greater than 7.5 mg/day.⁸ Even with minor reductions of dose, symptoms often worsen and objective synovitis may flare, implying a functional corticosteroid insufficiency in patients receiving prolonged corticosteroid treatment.^{7,8}

Recently, autoantibodies to lipomodulin (lipocortin) have been described in some patients with RA and SLE,⁹ and thus it has been proposed that such autoantibodies may account for apparent

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'steroid resistance' in those patients with RA and SLE requiring high maintenance doses of corticosteroids to control their inflammatory disease. As recombinant lipocortin-1 is now available¹⁰ this has been used in a solid phase enzyme linked immunosorbent assay (ELISA) system to detect auto-antibodies against lipocortin-1 in serum samples from patients with connective tissue diseases and from appropriate controls.

Patients and methods

PATIENTS AND CONTROLS

One hundred and twenty five patients of the Royal National Hospital for Rheumatic Diseases, and Royal United Hospital, Bath, took part in this prospective cross sectional study. Sixty three had classic or definite RA¹¹: of these, 19 had never received corticosteroids (RA-NIL), 28 were receiving prednisolone <7.5 mg/day (RA-LOW), and 16 required prednisolone >7.5 mg/day (RA-HIGH), for a period of >12 months with at least

three unsuccessful attempts to reduce dosage, for control of inflammatory disease (Table 1). Thirty six patients had SLE¹²: 11 had never received corticosteroids (SLE-NIL), 11 were receiving prednisolone <7.5 mg/day (SLE/LOW), and 14 required maintenance prednisolone >7.5 mg/day (SLE-HIGH) for disease control (Table 2). Twenty six patients had a clinical diagnosis of polymyalgia rheumatica¹³; all were taking corticosteroids, nine at prednisolone doses >7.5 mg/day. As controls, serum samples from 67 random healthy blood donors, volunteers and 23 healthy elderly subjects, without major medical illness or corticosteroid treatment, were also analysed. A comparison group of 13 patients with reversible chronic obstructive airways disease requiring maintenance corticosteroid, (10 receiving high corticosteroid doses) were also studied; these last 10 patients fulfilled criteria for 'corticosteroid resistant' asthma (patients whose forced expiratory volume in one second does not increase by more than 15% after a seven day course of at least 20 mg of prednisolone daily).¹⁴

Table 1 Clinical data for the group with rheumatoid arthritis

Variable	RA-NIL* (n=19)	RA-LOW* (n=28)	RA-HIGH* (n=16)
Age (years)†	58(50-66)	68(58-76)	60(50-67)
Sex distribution M:F	4:15	5:23	6:10
Disease duration (years)†	7(2-9)	20(9-25)	19(9-27)
Erosions (n)†	17	26	15
Nodules (n)	4	16	12
Seropositive (n)	17	26	15
Extra-articular disease (n)	5	13	7
Recent rheumatoid factor level (IU/ml)†	188(60-392)	89(7-251)	134(29-422)
Active joint areas†	8 (6-12)	5 (3-8)	6 (2-9)
Immunosuppressive drugs (n)	3	4	8

*RA-NIL=patients who had never received corticosteroids; RA-LOW=patients receiving <7.5 mg/day prednisolone; RA-HIGH=patients receiving >7.5 mg/day prednisolone.

†Values expressed as median (interquartile range) or number (n) of patients.

Table 2 Clinical data for the group with systemic lupus erythematosus

Variable	SLE-NIL* (n=11)	SLE-LOW* (n=11)	SLE-HIGH* (n=14)
Age (years)†	40(30-60)	41(32-62)	44(33-62)
Sex distribution M:F	0:11	2:9	0:14
Disease duration (years)†	6(2-10)	5(2-14)	16(5-19)
ARA‡ criteria	6	4	6
ANA‡ titre†	640(40-640)	160(40-640)	400(40-640)
Anti-dsDNA (U/ml)†	24(14-38)	15(3-22)	30(15-68)
Active disease (n)	6	4	9
Immunosuppressive drugs (n)	0	2	4

*SLE-NIL=patients who had never received corticosteroids; SLE-LOW=patients receiving prednisolone <7.5 mg/day; SLE-HIGH=patients receiving prednisolone >7.5 mg/day.

†Values expressed as median (interquartile range) or number (n) of patients.

‡ARA=American Rheumatism Association; ANA=antinuclear antibody.

Rheumatoid patients were assessed for seropositivity, radiological erosions, presence of rheumatoid nodules, and other extra-articular manifestations. The number of active joints areas (swollen and tender) and duration of early morning stiffness were assessed as clinical markers of disease activity. All patients with RA were receiving stable non-steroidal anti-inflammatory drug treatment with or without analgesic drugs, and most rheumatoid patients receiving maintenance corticosteroids were also taking disease modifying drugs, including immunosuppressives. Patients with SLE were clinically and serologically assessed for evidence of disease activity within the preceding three months, according to a composite score of various parameters: group A criteria included active synovitis, cutaneous disease, serositis, pulmonary disease, deteriorating renal function (active urine sediment, proteinuria >500 mg/24 h, falling creatinine clearance), active cerebral disease without other identifiable cause, myositis (proximal limb weakness and raised creatine kinase enzymes or abnormal electromyography), vasculitis, or fever without other identifiable cause; group B criteria included arthralgia, myalgia, neutropenia $<2.0 \times 10^9/l$, lymphopenia $<1.0 \times 10^9/l$, and hypocomplementaemia. Inactive SLE was defined as none of the above features, whereas active disease was defined as 1 group A criterion or two criteria from group B.

Laboratory investigations included plasma viscosity, C reactive protein, rheumatoid factor assessed by nephelometry (Hyland, normal range 0–40 IU/ml), antinuclear antibodies by immunofluorescence using HEP₂ cells, anti-dsDNA by modified Farr assay (Amersham; normal 0–25 U/ml, anticardiolipin antibody binding levels by ELISA,¹⁵ and immune precipitins to extractable nuclear antigens identified by Ouchterlony immunodiffusion technique or counterimmunoelectrophoresis.

Patients requiring corticosteroids were given the lowest possible maintenance dose to achieve control of the underlying inflammatory disease. The current corticosteroid dose, duration of corticosteroid treatment, and duration of treatment with prednisolone >7.5 mg/day (a semiquantitative assessment of the need for high maintenance corticosteroid treatment) were analysed. Concurrent disease modifying treatment, including immunosuppressive drugs, was also assessed.

RECOMBINANT HUMAN LIPOCORTIN-1

Recombinant human lipocortin-1 was produced in *Escherichia coli*¹⁰ and purified ($>99\%$) by methods described previously.¹⁶ The purified protein was supplied in 25 mM TRIS-HCl buffer pH 7.7 with

0.1–5 mM EDTA and 0.1 mg/ml human serum albumin (essentially globulin free). Preparations contained less than 50 pg/ml endotoxin. *E coli* of the same strain, but lacking the lipocortin-1-containing plasmid, were processed in parallel using a method identical with that used for recombinant lipocortin-1. This was used in the ELISA as a 'sham' control coating material.

ELISA FOR ANTILIPOCORTIN-1 ANTIBODIES

Plastic microwell plates (Immulon I, Nunc, Denmark) were coated with 100 μ l of 0.05 M carbonate-bicarbonate buffer pH 9.6 containing 1 μ g of recombinant human lipocortin-1 (Biogen Research Corp, Cambridge, Mass, USA) per well at 4°C for 18 hours. After a wash phase with carbonate-bicarbonate buffer each well was blocked with 100 μ l phosphate buffered saline (PBS) pH 7.2 containing 1% fetal calf serum (Gibco, Paisley, Scotland) for one hour at 37°C. An optimum range of dilutions of patient (1:20–1:1000) and control (1:20) sera in PBS containing 1% fetal calf serum were then incubated on the plate as were doubling dilutions of a positive serum standard and negative control blank wells. Serum incubation for one hour at 37°C was followed by a wash of PBS containing 0.05% v/v Tween 20 (BDH, Poole, Dorset, England). Each well was subsequently reacted at 37°C with 1:1000 dilution of rabbit antihuman IgG or antihuman IgM polyclonal antibody conjugated to alkaline phosphate (Sigma Chemical Co, Poole, Dorset, England). After a further PBS-Tween 20 washing step colour was developed with 5 mg/ml disodium-nitrophenyl phosphate (Sigma Chemical Co, Poole, Dorset, England) for one hour at 20°C. Absorbance values at 405 nm (A_{405}) were obtained with a Dynatech MR580 automated ELISA plate reader, and results were expressed in ELISA units. An ELISA unit was defined by the following equation:

$$\text{Elisa unit} = \frac{A_{405}(\text{sample}) \times \text{sample dilution}}{\text{reaction vol (ml)}}$$

A correction was made for day to day variability by assaying positive reference sera with defined activity on each plate.

ELISA VALIDATION

The specificity of the assay system for detecting antilipocortin-1 antibodies was assessed by the ability of soluble or phospholipid bound lipocortin-1 to compete with plate bound lipocortin-1 for antibody binding. *E coli*, AB1157 K-12 strain, grown under standard culture conditions and auto-

claved for 15 minutes at 121°C were washed in 10 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) buffer and resuspended at 1×10^{10} /ml in the same buffer containing sodium azide 1 µg/ml before being coated with 100 µg/ml lipocortin-1 for 30 minutes at 37°C. In brief, 50 µl of optimum dilutions of serum samples containing high levels of IgG or IgM antilipocortin-1 binding were preincubated for 30 minutes at 37°C with an equal volume of RPMI 1640 medium+10% fetal calf serum containing either 5 µg soluble lipocortin-1 or 5×10^8 lipocortin-1 coated bacteria. Control absorptions were performed with uncoated cells to determine any non-specific binding of antibodies to *E. coli*. Serum samples alone, together with those containing soluble lipocortin-1 or supernatants resulting from either one or three absorptions with *E. coli* were then assayed by the ELISA according to the previously described procedure. Table 3 shows the relatively poor competition of soluble lipocortin-1 in fivefold excess for antibody binding to lipocortin-1 absorbed onto plastic. When bound to *E. coli* membranes, however, lipocortin-1 successfully competed for between 46% and 89% of antibody binding after

three absorptions. Similar results for competition of IgG antilipocortin-1 binding have been obtained by absorbing positive sera with phospholipid micelles coated with lipocortin-1 (data not shown). This suggests that the antibody binding epitope on lipocortin-1 is most readily expressed when the molecule is bound to membranes or other solid phase matrices.

STATISTICAL METHODS

Clinical and ELISA data were non-parametric and therefore the Mann-Whitney U test was used for intergroup comparisons and the Wilcoxon signed rank test for intragroup significance testing. Correlation between variables was assessed by the Spearman rank correlation method.

Results

Control sera from blood donors (median age 39 years) and elderly healthy subjects (median age 70 years) were not significantly different in the antilipocortin-1 binding detected: IgM 20 units/ml (11–30), IgG 13 units/ml (9–28) for healthy controls

Table 3 Antilipocortin antibody competition

Patient No	Antibody class	Antilipocortin-1 antibody levels (ELISA units/ml)*	Soluble lipocortin-1 (5 µg/well) (%)†	E. coli + lipocortin-1 (%)†	
				Absorption 1	Absorption 3
1	IgG	329	2	4	46
2	IgG	346	0	27	53
3	IgM	465	27	16	51
4	IgM	822	23	19	89

*Normal range 0–64 ELISA units/ml serum.

†Percentage fall in absorbance values (405 nm) related to control wells without lipocortin.

Table 4 Corticosteroid treatment and antilipocortin antibodies. Values are expressed as median (interquartile range)

Group†	n	Total duration of disease (months)	Duration of treatment with prednisone >7.5 mg/day (months)	Antilipocortin antibodies (ELISA units/ml)	
				IgM	IgG
Healthy controls	67	0	0	20 (11–30)	13 (9–28)
PMR	26	36 (12–72)	4 (2–8)	25 (13–41)	16 (9–25)
COAD	13	60 (30–96)	30 (12–60)	13 (7–24)	26 (9–52)
RA-NIL	19	0	0	27 (17–41)	13 (6–33)
RA-LOW	28	68 (20–180)	3 (0–6)	31 (20–57)*	24 (15–38)*
RA-HIGH	16	150 (75–228)	15 (3–40)	136 (42–312)***	37 (15–50)*
SLE-NIL	11	0	0	61 (20–118)**	38 (24–62)*
SLE-LOW	11	23 (4–119)	9 (3–17)	31 (13–67)	16 (11–31)
SLE-HIGH	14	42 (24–127)	24 (12–30)	95 (30–156)***	73 (36–93)***

*Higher than healthy controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Mann-Whitney U test).

†PMR=polymyalgia rheumatica; COAD=chronic airways disease; RA=rheumatoid arthritis; SLE=systemic lupus erythematosus; NIL=LOW, HIGH—see footnotes to Tables 1 and 2.

and IgM 17 (11–23), IgG 21 (16–25) for elderly controls respectively, expressed as median (interquartile range).

Patient groups with RA (Table 1) were matched for age, sex distribution, active joint inflammation, and the presence of erosive disease, extra-articular manifestations, and rheumatoid factor. Similarly the patient groups with SLE (Table 2) were matched for age, sex distribution and number of American Rheumatism Association criteria. The SLE-NIL and the SLE-HIGH groups had more active disease, however, than the SLE-LOW group. The median age of the group with chronic airways disease (10 men, three women) was 65 years, and the median duration of disease was 24 years (interquartile range 11–35 years). None had an autoimmune disease. The group with polymyalgia rheumatica (five men, 21 women) were older (median age 70 years) and had shorter duration of disease (median three years, interquartile range 2–6 years).

Despite prolonged corticosteroid treatment in the groups with chronic airways disease and polymyalgia rheumatica (Table 4) and high corticosteroid requirements, particularly in the group with chronic airways disease (median prednisolone dose 15 mg/day), levels of antilipocortin-1 antibodies did not differ from control values (Figs 1, 2). Antilipocortin-1 binding levels in RA-NIL patients were also similar to those in healthy controls. Patients with RA taking corticosteroids, however, had significantly increased antilipocortin-1 levels, particularly IgM, notably in the RA-HIGH group. Levels of IgM antilipocortin-1 binding were associated with daily prednisolone dose and with duration of treatment with prednisolone >7.5 mg/day

($p<0.05$). Total duration of corticosteroid use was longer in the RA-HIGH group than for the RA-LOW patients (Table 4), but this difference was not statistically significant. There were no correlations between antilipocortin-1 IgM or IgG levels and age, rheumatoid disease activity assessed clinically or biochemically (plasma viscosity, C reactive protein), or other autoantibodies, including serum rheumatoid factor, antinuclear antibodies, and anti-dsDNA binding. In addition, the presence of IgM rheumatoid factor in combination with raised levels of IgG antilipocortin-1 was not associated with the presence of high IgM antilipocortin-1 activity. Thus measurement of the latter was not susceptible to interference by rheumatoid factor binding to IgG antilipocortin-1 in the ELISA assay.

In SLE significantly increased binding of both IgM and IgG antilipocortin-1 antibodies was present not only in the SLE-HIGH group but also in the patients with SLE who had never received corticosteroids (Table 4; Figs 1, 2). Furthermore, raised levels of IgM antilipocortin-1 were significantly associated with inflammatory disease activity ($p<0.01$) in the overall SLE population. Eighteen patients with SLE with inactive disease had a median IgM antilipocortin-1 level of 27 (interquartile range 16–59) ELISA units/ml, whereas 18 patients with active SLE had a median level of 97 (61–158) ($p<0.01$, Mann-Whitney U test). No correlations were detected between antilipocortin-1 antibodies and corticosteroid dosage, disease duration, or serological parameters (antinuclear antibodies, anti-dsDNA, anticardiolipin levels, or immune precipitins to extractable nuclear antigens). Concurrent immunosuppressive or second line agents, including

Fig. 1 Serum IgM antilipocortin antibody levels (vertical axis) in patient and control groups (see text). For statistical analysis, see Table 4. Ab=antibodies; COAD=chronic airways disease; PMR=polymyalgia rheumatica; RA=rheumatoid arthritis; SLE=systemic lupus erythematosus.

Fig. 2 Serum IgG antilipocortin antibody levels (vertical axis) in patient and control groups (see text). For statistical analysis, see Table 4. Ab=antibodies; COAD=chronic airways disease; PMR=polymyalgia rheumatica; RA=rheumatoid arthritis; SLE=systemic lupus erythematosus.

azathioprine, D-penicillamine, or gold, did not affect antilipocortin-1 antibody levels in patients with either RA or SLE.

Discussion

In patients with RA a striking association was found between raised IgM (and less dramatically IgG) antilipocortin-1 autoantibody levels and prolonged, high dose corticosteroid treatment. This association may be due to two related phenomena. Firstly, subsets of patients with RA have been shown to have enhanced phospholipase A₂ activity in peripheral blood mononuclear cells,¹⁷ increased prostanoïd release from monocytes,¹⁸ greater circulating numbers of activated HLA-DR⁺ monocytes,¹⁹ and enhanced spontaneous immunoglobulin production.^{20, 21} These features seem to be independent of drug treatment and indicate a 'primed' immune system in some patients with RA. This may result in presentation of increased amounts of self antigens, such as steroid induced lipocortin-1, with stimulation of autoantibody formation. Secondly, the type of corticosteroid presentation is also probably important as short duration corticosteroid treatment may reduce serum immunoglobulin concentrations, whereas chronic oral prednisolone may enhance spontaneous immunoglobulin synthesis.^{22, 23} Thus repetitive antigen challenge may lead to the generation of high levels of autoantibodies directed against lipocortin-1. We considered that active RA alone was insufficient to account for raised antilipocortin-1 levels as the occurrence of high antilipocortin-1 antibody levels in steroid treated patients with RA

was independent of age, active synovitis, and extra articular disease manifestations.

In contrast with RA, raised IgM antilipocortin-1 levels in SLE were associated with active inflammatory disease and were independent of corticosteroid treatment. When inflammatory features in SLE were controlled by corticosteroids lower binding levels similar to background control values resulted. The different pattern of autoantibody responses between RA and SLE patient groups may reflect important differences in disease expression with more widespread fundamental alterations of immune regulation occurring in active SLE.^{24, 25} Although our present findings confirm those of Hirata *et al*,⁹ there are several important differences. Firstly, the strong association between antilipocortin-1 antibodies and corticosteroid treatment was not found by Hirata *et al* in six rheumatoid patients who had such antibodies detected by bioassay methods. This discrepancy may result from the small number of serum samples analysed by these authors. Secondly, the present ELISA method allows quantitative assessment of antibody levels whereas the bioassays used previously may have been less sensitive; the ELISA method may allow better discrimination between individual patients with RA receiving corticosteroid treatment.

The class of antilipocortin-1 antibody binding was predominantly IgM in both RA and SLE. There was no correlation, however, between antilipocortin-1 antibody levels and anti-dsDNA, antinuclear antibodies, anticardiolipin antibody levels, or immune precipitins to extractable nuclear antigens in SLE, nor was there any correlation with serum

rheumatoid factor in RA. This confirms earlier work⁹ and suggests that IgM and IgG antilipocortin-1 antibodies are distinct autoantibodies. The reasons for the predominant IgM response are unclear but may be related to repetitive antigen challenge, resulting in repeated synthesis of primary IgM class autoantibodies. Alternatively chronic prednisolone treatment itself may be relevant to this phenomenon as it has been shown to potentiate the primary immune response to keyhole limpet haemocyanin immunisation.²⁶

Despite prolonged, high dose maintenance corticosteroid treatment in chronic airways disease (and in polymyalgia rheumatica) these patients did not have raised levels of antilipocortin-1 antibodies (Table 4). This suggests that other mechanisms are responsible for corticosteroid resistant asthma.¹⁴ Recent evidence suggests that defective monocyte responsiveness to corticosteroids may be important in reducing their therapeutic effect in these asthmatic patients.^{27, 28} These observations, together with normal antilipocortin-1 autoantibody levels in steroid treated patients with polymyalgia rheumatica, indicate that the nature of the underlying autoimmune disorder is important in the generation of such autoantibodies.

We suggest that antilipocortin-1 antibodies are clinically significant in patients with RA by impairing the lipocortin-1 mediated inhibition of phospholipase A₂ activity. This would result in a functional corticosteroid insufficiency with an increased corticosteroid requirement and thus relative 'steroid resistance'. Evidence supporting a functional role for these antibodies includes work by Hirata *et al.*,⁹ who showed that antilipocortin-1 antibodies potentiated phospholipase A₂ activity and enhanced the stimulatory activity of bradykinin on the release of arachidonic acid in vitro, whereas addition of purified lipocortin-1 overcame these effects. This work, together with observations of enhanced phospholipase A₂ activity¹⁷ and increased prostanoid release in RA peripheral blood monocytes,¹⁸ suggests that antilipocortin-1 autoantibodies may have important functions in RA inflammatory processes and immune phenomena. Longitudinal studies are in progress to examine autoantibody induction at the start of corticosteroid treatment in patients with RA, as well as the relation between IgM antilipocortin-1 antibody levels and disease activity in SLE. This information may lead to better use of corticosteroids in the treatment of these diseases.

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