

Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases

(systemic lupus erythematosus/IgM/prostaglandins/arachidonic acid/monoclonal antibody)

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ABSTRACT The activity of phospholipase inhibitory protein, lipomodulin, partially purified from rabbit neutrophils, was markedly decreased after treatment with sera from patients with rheumatic diseases such as systemic lupus erythematosus, rheumatoid arthritis, and dermatomyositis. The decrease of the protein's inhibitory activity on phospholipase A₂ paralleled the amount of [³⁵S]methionine-labeled lipomodulin precipitated by the sera. Absorption of patients' sera with anti-human IgM (μ chain) or protein A-agarose, but not with anti-human IgG (γ chain), decreased their ability to decrease the activity of lipomodulin on phospholipase A₂ or to precipitate the radioactive lipomodulin. The IgM fraction of patients' sera could precipitate [³⁵S]methionine-labeled lipomodulin (40,000 daltons) which comigrated with highly purified lipomodulin on gel electrophoresis with sodium dodecyl sulfate. All of these observations suggest that the sera of many patients with rheumatic diseases contain autoantibody against lipomodulin. A monoclonal antibody against lipomodulin was also obtained. Stimulating human fibroblasts with bradykinin in the presence of monoclonal antilipomodulin antibody markedly enhanced arachidonic acid release due to the activation of phospholipase(s) in the intact cells, and this stimulatory effect was blocked by adding purified lipomodulin. These findings suggest that lipomodulin regulates the activity of phospholipase(s) on the cell surface and that autoantibodies against lipomodulin may play a role in certain symptoms of rheumatic diseases, especially by the formation of prostaglandins and other metabolites of arachidonic acid.

A phospholipase A₂ inhibitory protein, which we propose to name "lipomodulin," was recently isolated from neutrophils treated with glucocorticoids (1). This protein was found to inhibit phospholipase A₂ (phosphatide 2-acylhydrolase, EC 3.1.1.4), an enzyme that cleaves the ester bond in the β-position of phosphatidylcholine, to liberate unsaturated fatty acids (mainly arachidonic acid) and lysophosphatidylcholine. Lipomodulin was shown to be induced by glucocorticoids in a receptor-mediated fashion (1). The inhibitory action of glucocorticoids on prostaglandin formation has been assumed to be due to induction of this inhibitory protein, which decreases the availability of the precursor of prostaglandins, arachidonic acid, by inhibiting phospholipase A₂ (1, 2).

Recently, autoantibodies for cell surface molecules have been detected in patients with myasthenia gravis, Graves disease, insulin-resistant diabetes, or asthma (3-6). These antibodies are assumed to be closely related to the pathogenesis of the symptoms that these diseases produce. Because prostaglandins and

hydroxyl compounds of arachidonic acid are thought to play a role in the pathophysiology of rheumatic diseases (7), we initiated a search for autoantibodies against lipomodulin.

We report here the presence of autoantibodies against lipomodulin, a cell surface protein, in the sera of many patients with rheumatic diseases such as systemic lupus erythematosus and rheumatoid arthritis, chronic inflammatory diseases.

EXPERIMENTAL PROCEDURES

Purification and Assay of Lipomodulin. Lipomodulin was purified, by a procedure to be described elsewhere, from 400 ml of medium (RPMI-1640) in which rabbit neutrophils (10⁷ cells per ml) had been incubated for 16 hr with 1 μM flucinolone acetonide. Briefly, the concentrated medium was chromatographed sequentially on DEAE-cellulose and Sephadex G-75; active fractions were pooled and concentrated. One microgram of this preparation inhibited approximately 0.4 μg of porcine pancreatic phospholipase A₂. This preparation (12 μg) migrated as a single protein band on sodium dodecyl sulfate gel electrophoresis (staining with Coomassie blue). Radioactive lipomodulin was isolated from 100 ml of the medium in which rabbit neutrophils had been incubated with [³⁵S]methionine (20 μCi/ml; 1 Ci = 3.7 × 10¹⁰ becquerels) after pooling with medium from a preparation as described above.

Lipomodulin was assayed by its ability to inhibit pancreatic phospholipase A₂ (assay I) or by its ability to inhibit bradykinin-induced release of [³H]arachidonate from prelabeled fibroblasts (assay II). For assay I, phospholipase A₂ activity was measured *in vitro* with and without lipomodulin. The reaction mixture contained, in a total volume of 25 μl, 25 mM Tris/glycylglycine buffer (pH 8.0), 0.25 μg of porcine pancreatic phospholipase A₂ (Sigma), and 0.2 mM α-palmitoyl-β-[1-¹⁴C]arachidonylphosphatidylcholine (55 μCi/mol; Applied Science, State College, PA), as described (1). For assay II, human fibroblasts (≈10⁶ cells per well) were grown in 35-mm wells in 2 ml of Eagle's minimal essential medium supplemented with Earle's salts and 10% fetal calf serum. The medium was changed after 4 days, and the experiments were carried out 3 days later. The cells were labeled with 5 μCi of [5,6,8,9,11,12,14,15-³H(N)]arachidonic acid (100 Ci/mmol; New England Nuclear) in 1 ml of serum-free Dulbecco's minimal essential medium per well. After incubation for 1 hr at 37°C in an atmosphere of 95% O₂/5% CO₂, cells were

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washed twice with 1 ml of Dulbecco's medium, and incubated for 15 min at 37°C in 1 ml of the same medium with or without lipomodulin and other additions as indicated. Bradykinin was then added (final concentration, 0.5 $\mu\text{g}/\text{ml}$) and, after 30 min at 37°C, the incubation was stopped by adding 0.5 ml of 6 M formic acid. A 1-ml sample of medium was centrifuged at 27,000 $\times g$ for 25 min, and 0.5 ml of the supernatant was used to measure [^3H]arachidonate and its metabolites.

Lipomodulin Antibodies. A monoclonal antilipomodulin antibody was identified among antibodies raised against rat cerebral cortical synaptic plasma membranes (8). Splenic lymphocytes from immunized BALB/c mice were fused with mouse myeloma cells (P3X63Ag8) to yield hybridomas producing monoclonal antibodies. Among 44 monoclonal antibodies studied, one, designated 4-4C3, prevented lipomodulin's inhibition of phospholipase A_2 *in vitro*. The isolation and characterization of the antibodies will be described in detail elsewhere.

Clotted blood from healthy subjects or patients diagnosed as having rheumatic diseases (e.g., systemic lupus erythematosus) was centrifuged at 1600 $\times g$ for 10 min. Some sera were separated into IgM and IgG fractions by column chromatography on Bio-Gel A-200. Sera from MRL/1 and NZB (NZB \times NZW) F_1 mice were obtained as described (9).

Rabbit anti-mouse IgG (heavy and light chains) and anti-human IgG (heavy and light chains) were purchased from Miles. Rabbit anti-human IgM (μ chain) and anti-human IgG (γ chain) were from Boehringer Mannheim.

For the experiments shown in Figs. 2 and 3, samples of lipomodulin (2–3 μg) were preincubated at 37°C for 30 min with samples of serum from patients with rheumatic diseases in a total volume of 26 μl containing 0.1% Trasylol (a protease inhibitor) and 25 mM Tris-HCl buffer (pH 7.0). Anti-human IgG (light and heavy chains) (4 μl) was added, and the incubation was continued for another 30 min. The tubes were centrifuged in a Beckman Microfuge B for 2 min and an aliquot (10 μl) of the supernatant was used for the lipomodulin assay described above (assay I). When [^{35}S]methionine-labeled lipomodulin was used, the volume of the mixture was doubled and the precipitate was washed twice with 0.5 ml of 150 mM NaCl/10 mM sodium phosphate, pH 7.4. The immunoprecipitates were dissolved in 100 μl of 1% sodium dodecyl sulfate/10 mM sodium phosphate, pH 7.0/0.1% 2-mercaptoethanol. A sample (50 μl) was used for radioassay and the remaining 50 μl was subjected to gel electrophoresis as described (10). The molecular weights were de-

termined by using a calibration protein kit (Combithek; Boehringer Mannheim).

RESULTS

Specificity of Monoclonal Antibody for Lipomodulin. When purified lipomodulin was preincubated with the monoclonal antibody (4-4C3) followed by precipitation of the antigen-antibody complexes with anti-mouse IgG (heavy and light chains), phospholipase A_2 inhibitory activity in the supernatant was markedly decreased. The monoclonal antibody pretreated with anti-mouse IgG failed to decrease phospholipase A_2 inhibition by lipomodulin. Neither the monoclonal antibody nor anti-mouse IgG activated or inhibited phospholipase A_2 activity. The ability of the monoclonal antibody to decrease lipomodulin activity was a function of the amounts of either lipomodulin or antibody (Fig. 1). The activity of phospholipase A_2 was inhibited by lipomodulin in a dose-dependent manner. After pretreatment of lipomodulin with monoclonal antibody, the dose-response curve was shifted to the right. At a fixed concentration of lipomodulin, increasing amounts of monoclonal antibody resulted in a decrease in lipomodulin activity. All of these data suggest that this monoclonal antibody is specific for lipomodulin. It should be noted that, without the precipitation by the second antibody, the soluble monoclonal antibody-lipomodulin complex could inhibit phospholipase A_2 activity *in vitro*, suggesting that the monoclonal antibody and the phospholipase interact with different domains of lipomodulin.

Activation of Phospholipase(s) in Fibroblasts by Monoclonal Antibody. Fibroblasts release arachidonic acid and its metabolites in response to proteins and peptides such as bradykinin (11). When human fibroblasts were treated with the monoclonal antibody, the basal level of arachidonate release was slightly increased (Table 1). In the presence of bradykinin, the antibody increased arachidonate release 2- to 3-fold. Lipomodulin inhibited the bradykinin-induced arachidonate release from fibroblasts but had no effect on the basal release. These results suggest that lipomodulin is involved in the regulation of phospholipase(s) activity by bradykinin and that the monoclonal antibody enhances the stimulatory effect of bradykinin by interacting with lipomodulin on the surface of the cells. This was further supported by the observation that, when purified lipomodulin was added together with the antibody, the arachidonate release stimulated by bradykinin was almost completely

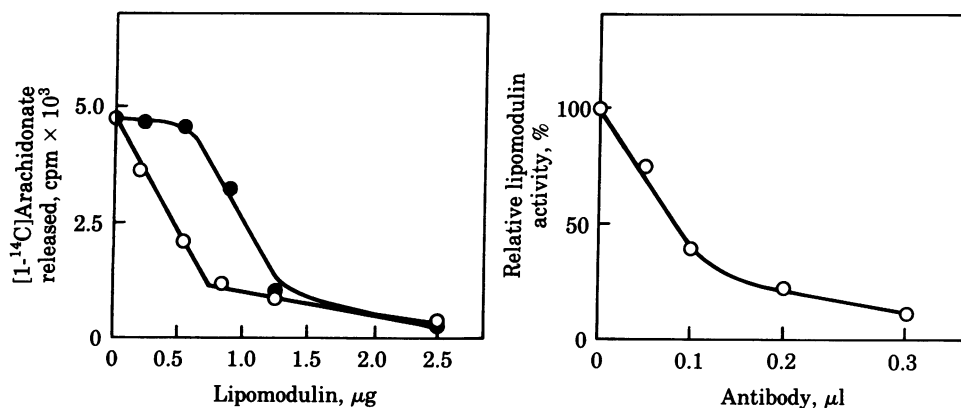


FIG. 1. Reduction of lipomodulin activity by monoclonal antibody. (Left) Phospholipase A_2 (0.25 μg) was assayed with the indicated concentrations of purified lipomodulin, either untreated (\circ) or previously incubated with monoclonal antibody (1:100 dilution) followed by precipitation of the antigen-antibody complex with anti-mouse IgG (heavy and light chains) (\bullet). (Right) Lipomodulin (1 $\mu\text{g}/4 \mu\text{l}$) was treated with 5 μl of monoclonal antibody, diluted as indicated, followed by addition of 1 μl of anti-mouse IgG (heavy and light chains) and centrifugation. Inhibition of phospholipase A_2 by samples of supernatant is expressed relative to that produced by an equivalent amount of untreated lipomodulin (100%).

Table 1. Bradykinin stimulation of arachidonate release from fibroblasts pretreated with monoclonal antibody against lipomodulin

Addition	³ H]Arachidonate released, cpm	
	Control	Bradykinin added
None	362 ± 58	1210 ± 120
Antibody	1070 ± 91	4160 ± 260
Antibody plus lipomodulin	1110 ± 101	1250 ± 102
Lipomodulin	462 ± 82	560 ± 62

After incubation with [³H]arachidonate, fibroblasts were incubated for 15 min with or without monoclonal antibody (diluted 1:1000) and lipomodulin (2 μg/ml), as indicated, and then for 30 min with or without bradykinin (0.5 μg/ml). The results are expressed as mean ± SEM (*n* = 3). The data were typical among three independent experiments.

suppressed. The antibody–lipomodulin complex may be able to inhibit phospholipase A₂ *in vitro* and also to inhibit arachidonate release stimulated by bradykinin in intact cells. The antibody–lipomodulin complex, however, did not change the arachidonate release in the absence of bradykinin. This might be due to the receptors for proteins which contain phospholipase as a part of their constituents and whose phospholipase A₂ is directly activated by binding as reported in B lymphocytes (12).

Presence of Lipomodulin Antibody in Sera from Patients with Rheumatic Diseases. Sera were screened for antibodies on the basis of their ability to counteract the inhibitory effect of lipomodulin on phospholipase A₂ (assay I) or on bradykinin-induced arachidonate release from fibroblasts (assay II). A number of patients with systemic lupus erythematosus, rheumatoid arthritis, or dermatomyositis had antilipomodulin antibodies in their sera (Table 2). No difference in incidence was detected between the patients with rheumatoid arthritis who were treated or not treated with glucocorticoids (3/6 and 3/6, respectively). Patients with active systemic lupus erythematosus had higher titers of antibody than did patients in an inactive phase of the disease. This also was the case with the same patients in the different phases. Sera from healthy persons did not give any positive results by either assay. Sera from patients with acute inflammatory diseases such as traumatic arthritis gave negative results. Like patients with systemic lupus erythematosus, MRL/l (*n* = 8) and (NZB × NZW)F₁ (*n* = 12) mice, which spontaneously develop an illness resembling human systemic

Table 2. Detection of antilipomodulin antibody in sera from patients with rheumatic diseases

Serum	No. positive sera/ no. tested	
	Assay I	Assay II
Normal	0/20	0/10
Systemic lupus erythematosus	16/26	*4/10
Rheumatoid arthritis	7/13	*3/6
Dermatomyositis	2/5	—
Periarteritis nodosum	2/5	—

The clinical diagnoses were made in clinics of the National Institute of Arthritis and Metabolic Diseases and of the Juntendo University Medical Hospital (Tokyo). When more than 25% of lipomodulin activity was inhibited by assay I and assay II under the standard assay conditions, we concluded that the serum contained antibody.

*These sera were also positive in assay I, and one sample from each diagnostic group that was negative in assay II was positive in assay I.

Table 3. Effect of various antisera on antilipomodulin activity in serum from a patient with lupus erythematosus

Treatment of serum	Lipomodulin activity*	
	cpm	%
No serum	5300	100
Untreated serum	2200	42
Serum treated with:		
Anti-human IgG (heavy and light chains)	5190	98
Anti-mouse IgG (heavy and light chains)	2560	48
Anti-human IgG (γ chain)	2070	39
Anti-human IgM (μ chain)	4820	91
Protein A-agarose	4960	94

Samples of lipomodulin (2 μg) were incubated in a total volume of 30 μl for 30 min with or without serum from a patient with lupus erythematosus that had been treated as indicated. After centrifugation, 10 μl of supernatant was assayed for lipomodulin activity with phospholipase A₂ (assay I). Serum (30 μl) was treated with 10 μl of the indicated antibody or agarose (20 mg/ml) for 30 min at 37°C. After centrifugation, 26 μl of the supernatant was incubated with lipomodulin as described above. In the absence of serum, arachidonate released by hydrolysis of radioactive phosphatidylcholine was 1920 cpm with untreated lipomodulin and 7220 cpm with no lipomodulin. The results shown are means of duplicates. Essentially similar results were obtained with seven different sera.

*Decrement, in cpm, of [³H]arachidonate released by lipomodulin treated as described. The column headed % shows activity relative to that of untreated lipomodulin (100%).

lupus erythematosus (9, 13), all had varying amounts of antilipomodulin antibodies in their sera. To differentiate antilipomodulin antibody from antibodies against IgG or DNA which are also increased in rheumatic diseases (13), positive sera were incubated with an excess of heat-denatured calf thymus DNA and heat-denatured human gamma globulins for 1 hr at 37°C and 48 hr at 4°C, respectively, after which the supernatant was examined for antilipomodulin activity. Although all anti-DNA and all anti-gamma globulin activities were removed, the antilipomodulin activity remained.

A positive serum from a patient with systemic lupus erythematosus was treated with various anti-human immunoglobulins and their effects on lipomodulin were measured. Anti-human IgM (μ chain) and protein A-agarose but not anti-human IgG (γ chain) bound and removed the antibody activity against lipomodulin (Table 3). Essentially similar results were obtained with seven patients. Neither the patient's serum nor rabbit anti-human IgG activated or inhibited the phospholipase A₂ activity in the assay system. The data suggested that patients with rheumatic diseases have IgM autoantibody against lipomodulin. Consistent with this interpretation was the observation that the IgM fraction, but not the IgG fraction, from a positive serum removed lipomodulin activity.

Identification of Lipomodulin Precipitated by Monoclonal Antibody and Serum from Rheumatic Patients. Incubation of [³⁵S]methionine-labeled lipomodulin with serum from a patient with systemic lupus erythematosus followed by the addition of anti-human IgG (heavy and light chains) led to precipitation of radioactivity in amounts dependent on the volume of the serum added (Fig. 2 *Left*). Serum from a normal subject did not precipitate any radioactive material. The radioactivity in the immunoprecipitates paralleled the decrease in lipomodulin activity in the supernatant (Fig. 2 *Right*). To identify the antigen, the immunoprecipitates from the crude preparation of [³⁵S]methionine-labeled lipomodulin with patient serum or monoclonal antibody plus anti-mouse IgG were subjected to gel

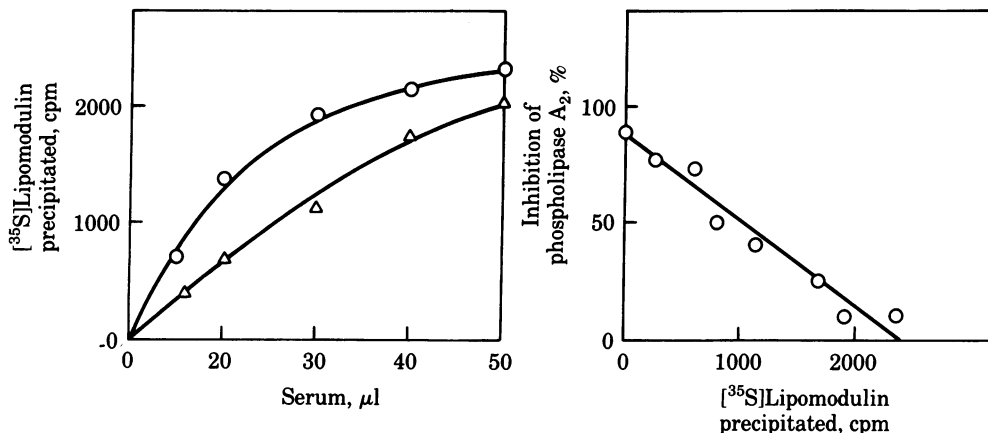


FIG. 2. Precipitation of ^{35}S methionine-labeled lipomodulin by serum from two patients with lupus erythematosus. ^{35}S Methionine-labeled lipomodulin ($2\ \mu\text{g}$, 2600 cpm) was incubated in a total volume of $100\ \mu\text{l}$ with the indicated volume of serum. For control, $150\ \text{mM NaCl}/10\ \text{mM}$ sodium phosphate, pH 7.4, was used to replace serum. The immunoprecipitate and supernatant were obtained as described in the text. (Left) Radioactivity in immunoprecipitates vs. μl of serum. \circ , Serum 1; Δ , serum 2. (Right) Inhibition of phospholipase A_2 by samples of supernatants after immunoprecipitation vs. cpm in precipitate.

electrophoresis with sodium dodecyl sulfate. The immunoprecipitates with either monoclonal antibody or positive serum from a patient showed a major radioactive peak, which, like a purified lipomodulin, migrated as a peptide of 40,000 daltons (Fig. 3). In addition, small amounts of radioactivity were detected in areas corresponding to peptides of 24,000 and 16,000 daltons. The peaks in these areas might be due to proteolysis of lipomodulin during the immunoprecipitation because the presence of soybean trypsin inhibitor (0.1%) in the reaction mixture prevented the appearance of radioactivity in these areas. These data demonstrate that the monoclonal antibody and patient sera can precipitate lipomodulin.

DISCUSSION

Lipomodulin is a protein that inhibits phospholipases such as phospholipase A_2 and whose synthesis is induced by glucocorticoids (1, 2). Phospholipase A_2 has been recognized as an important enzyme in the release of prostaglandin precursors, especially arachidonic acid, although recent work with platelets implicated a phosphatidylinositol-specific phospholipase C that yields diacylglycerol and, subsequently, arachidonic acid (7). The present report demonstrates that a substantial proportion of patients with one of four different rheumatic diseases spontaneously produce antibodies against lipomodulin. These antibodies interfere with lipomodulin's inhibition of arachidonic acid release. Thus, in such patients, the antilipomodulin antibody increases the formation of arachidonic acid and, subsequently, the formation of inflammatory prostaglandins. This concept is supported by the observation that inhibitors of prostaglandin synthesis such as chloroquines and glucocorticoids are effective therapeutically in such patients (13, 14). In addition, the present experiments showed that anti-lipomodulin antibody present in sera of patients with systemic lupus erythematosus potentiated the stimulatory activity of bradykinin on the release of arachidonic acid, whereas purified lipomodulin overcame this effect.

Rheumatic arthritis and systemic lupus erythematosus are diseases of chronic inflammation. Prostaglandins appear to play an important role in chronic inflammation in these diseases as well as altering lymphocyte function (13, 14). It therefore is not surprising that antibodies against lipomodulin were detected in patients with these so-called autoimmune diseases. The relationship between antibodies against lipomodulin and autoim-

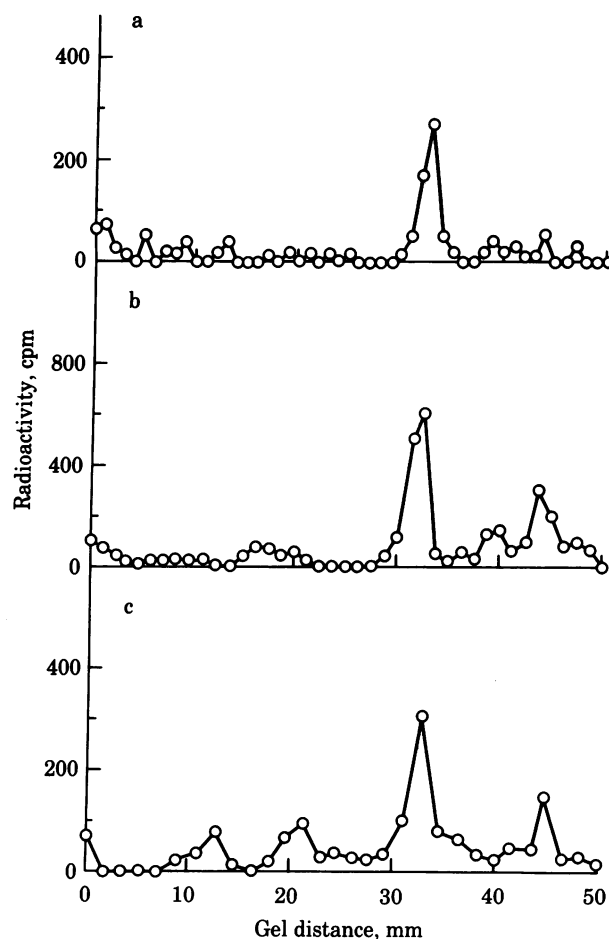


FIG. 3. Sodium dodecyl sulfate electrophoresis of purified and immunoprecipitated lipomodulin. ^{35}S Methionine-labeled lipomodulin was purified, and $1\ \mu\text{g}$ of it (2400 cpm) was subjected to electrophoresis (a). For the immunoprecipitation, the radioactive medium in which neutrophils were incubated were concentrated to 1/10th the original volume and used as crude lipomodulin. Crude lipomodulin ($30\ \mu\text{l}$) was incubated with $70\ \mu\text{l}$ of patient's serum (b) or with $70\ \mu\text{l}$ of diluted (1:20) monoclonal antibody (c) at 4°C for 16 hr. Anti-human IgG (light and heavy chains) or anti-mouse IgG (light and heavy chains) ($10\ \mu\text{l}$) was added to these reaction mixtures and incubation at 4°C was continued 24 hr. After centrifugation, the immunoprecipitates were washed and subjected to electrophoresis. The gels were sliced from the bottom to the front (marker dye, bromophenol blue).

mune diseases is further supported by the finding of such antibodies in the sera of NZB and MRL/l mice, models of systemic lupus erythematosus. The detection of antilipomodulin antibody in only half of the patients with rheumatic diseases might be due to lack of sensitivity of the assay we used. Alternatively, such antibodies may be important in some, but not all, patients. In those patients with antibody, however, the titer of antibody correlated with the severity of the disease. In addition, among patients studied only once, those in the active phase of disease had considerably higher titers than did inactive patients.

The antibody nature of the lipomodulin-binding proteins was demonstrated by specific interaction with antibodies against human immunoglobulins. The greater part of the antibody activity was found in the IgM fraction, and anti-IgM antibody specifically depleted the relevant antilipomodulin activity. These results are consistent with the large number of autoantibodies produced by patients with various autoimmune diseases. Moreover, adsorption experiments clearly demonstrated that the antilipomodulin antibodies were distinct from antibodies against DNA and human gamma globulins. Thus, the major autoantibodies produced by patients with systemic lupus erythematosus or rheumatoid arthritis could not be implicated by crossreactivity. It appears that the antibodies against lipomodulin may represent a new autoantibody type in these patients.

Preliminary experiments have suggested that the antilipomodulin antibody favors IgE-potentiating factor synthesis whereas lipomodulin favors IgE-suppressive factor production. IgE-potentiating factor(s) was also induced upon the incubation of rat lymphocytes with melittin, phospholipase A₂ activator, or lysophosphatidylcholine or its analogues (unpublished data). These results are consistent with the reciprocal effects of antilipomodulin antibody and glucocorticoids on phospholipase activation. Thus, in addition to its potential role in augmenting inflammation, antilipomodulin antibody might bring about an apparent deficiency in suppressor function (13, 14). Although

the antibodies against lipomodulin could play a role in both inflammatory and lymphocyte abnormalities in patients with rheumatic diseases, the discovery of these antibodies does not yet shed light upon the pathogenesis of these disorders. Nevertheless, understanding the mechanisms of induction of inflammation in these disorders should lead to better approaches to therapy.

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