

HIDDEN AUTOANTIBODIES AGAINST COMMON SERUM
PROTEINS IN MURINE SYSTEMIC LUPUS
ERYTHEMATOSUS

Detection by In Vitro Plaque-forming Cell Assay

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The serum of humans and of mice with systemic lupus erythematosus (SLE) contains autoantibodies of many specificities, nearly all cell associated (1). The preferential detection of autoantibodies to cellular antigens may not be due solely to a special predilection to produce such antibodies in SLE, but might be also a consequence of in vivo absorption of other autoantibodies by self-antigens present in serum in high molar concentrations. Cell-associated autoantigens, in contrast, would be either relatively inaccessible to circulating autoantibodies (if intracellular) or present in sufficiently low serum concentrations to escape in vivo absorption.

To detect autoantibody specificities that would otherwise be obscured in serum, modified Jerne plaque assays were developed to measure cellular secretion of antibodies against common serum proteins in a serum-free in vitro system. The results indicate that the spleens of autoimmune mice of several strains contain large numbers of cells secreting autoantibody to mouse albumin and transferrin, while these antibodies are present only in small amounts of serum.

Materials and Methods

Mice. MRL/Mp-*lpr/lpr*, MRL/Mp-*+/+*, and BXSB mice were from a colony maintained at the University of North Carolina. CBA, B10-congenic, and A-congenic mice were obtained from Dr. Geoffrey Houghton, University of North Carolina. NZB mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. Mouse, human, and bovine albumins and human transferrin were purchased from Sigma Chemical Co., St. Louis, MO. Mouse, rabbit, hamster, and dog transferrins were purchased from Cappel Laboratories, Cochranville, PA. Bovine transferrin was obtained from U.S. Biochemical Corp., Cleveland, OH. Ovalbumin was purchased from Mann Laboratories, New York.

Assay for Antibody-forming Cells. Cellular secretion of antibody to autologous proteins was quantitated using a microtiter Jerne plaque method (2). Purified mouse proteins were attached to sheep erythrocytes (ASA Biologicals, Winston-Salem, NC) using a chromic chloride technique (3, 4). One part of washed, packed erythrocytes was added to one part

This work was supported by grants R01-AM26574 and P60-AM30701 from the U. S. Public Health Service, and by a grant from the North Carolina United Way. P. Cohen and R. Eisenberg are Senior Investigators of the Arthritis Foundation.

of protein at a concentration of 1 mg/ml in normal saline. Chromic chloride (Mallinckrodt, Inc., St. Louis, MO) was then added, generally at 1 mg/ml in normal saline, although optimal concentrations were determined empirically for each protein. After 15 min at room temperature, the erythrocytes were washed three times before use. The presence of protein on the conjugated erythrocytes was determined for each batch by passive hemagglutination using appropriate goat or rabbit reference antisera against mouse serum, mouse transferrin (U.S. Biochemical Corp.), or human, bovine, dog, or hamster serum (Cappel Laboratories). Spleen cells to be assayed for autoantibody plaque-forming cells (PFC) were suspended to 1×10^6 /ml in Hanks' balanced salt solution (University of North Carolina Cancer Center, Chapel Hill, NC). 100 μ l of cell suspension were mixed with 10 μ l of 10% erythrocyte suspension in the wells of flat-bottom microtiter plates (Costar, Data Packaging, Cambridge, MA) and the plates spun for 10 min at 200 g and then for 5 min at 400 g. The resulting suspensions of lymphocytes and erythrocytes were allowed to incubate for 1 h, at which point was added 10 μ l of a 1:10 dilution of the IgG fraction of a rabbit anti-mouse IgG antiserum, together with sufficient guinea pig complement to achieve a final 1:13 concentration. After 1 h incubation at 37°C, hemolytic plaques were scored with a stereo microscope. Controls, i.e., uncoupled sheep erythrocyte suspensions and wells without added lymphocytes, were performed for each experiment to assure that plaques were not artifactual.

Results

Quantitation of PFC Against Autologous Proteins. The spleens of MRL/Mp-*lpr/lpr*, BXSB, and NZB mice were found to contain substantial numbers of PFC against purified mouse albumin and mouse transferrin (Table I). All antitransferrin and antialbumin PFC required rabbit anti-mouse IgG for visualization and thus represented IgG PFC. In contrast, much smaller numbers were found in spleens of mice that did not suffer from autoimmune disease. A requirement for protein synthesis was indicated by the finding that 4-h pretreatment of spleen cells with cycloheximide (100 μ g/ml) decreased PFC ~85%.

Table II shows the numbers of anti-mouse transferrin plaques observed in MRL/Mp-*lpr/lpr* mice of varying ages. There was a steady increase in antitransferrin plaques with age, coincident with the development of autoimmunity in

TABLE I
Strain Survey for PFC Against Mouse Serum Proteins

Strain	Age	PFC per 10^6 spleen cells	
		Albumin	Transferrin
	<i>mo</i>		
MRL/Mp- <i>lpr/lpr</i>	6	495	2,351
BXSB	5	293	2,188
NZB	8	71	1,675
MRL/Mp-+/+	9	23	210
B10.D2	18	8	22
CBA	3	ND	17
A.BY	3	ND	106
A.CA	3	ND	58
A.SW	3	ND	79
B10.A	3	ND	31

PFC directed against mouse albumin- or mouse transferrin-coated sheep erythrocytes were enumerated in a hemolytic plaque assay (see Materials and Methods). The small number of PFC directed against sheep erythrocytes was determined in parallel assays, and has been subtracted from the data shown. Data represent a single experiment in which all plaque assays were done simultaneously. The figures for mouse albumin and transferrin PFC are representative of experiments involving 26 MRL/Mp-*lpr/lpr* mice and 9 BXSB mice. 10 additional experiments using normal mice yielded similar low numbers of PFC.

TABLE II
Age Dependence of Anti-Mouse Transferrin PFC in
MRL/Mp-*lpr/lpr* Mice

Age <i>d</i>	PFC per 10 ⁶ spleen cells
13	213
56	1,131
91	2,500
120	>5,000

PFC were simultaneously determined in spleen cell suspensions derived from the MRL/Mp-*lpr/lpr* mice of the indicated ages. Data are from a single experiment in which assays were performed simultaneously. Two similar experiments with *lpr* mice of various ages gave the same results.

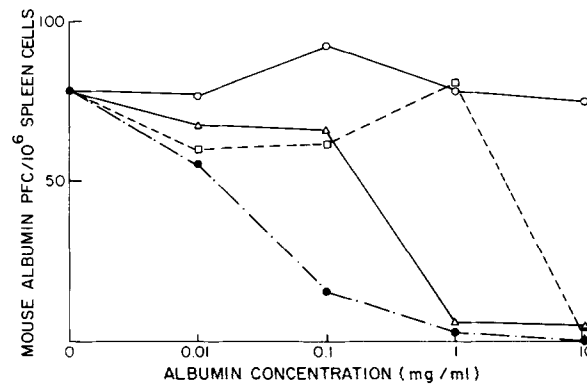


FIGURE 1. Data represent the effect of adding graded amounts of albumin from various sources on the number of PFC directed against mouse albumin-coated sheep erythrocytes. Simultaneous determination was made of PFC directed against uncoated sheep erythrocytes and these (small) numbers have been subtracted from the data. (●) Mouse albumin; (Δ) cow albumin; (□) human albumin; (○) egg albumin.

these mice. Similar data were obtained with anti-albumin PFC in both MRL/Mp-*lpr/lpr* and BXSb mice.

Specificity of PFC Against Serum Proteins. To determine whether the anti-mouse protein PFC were primarily directed against murine autoantigens, we performed inhibition studies. The specificity of anti-mouse albumin PFC in MRL/Mp-*lpr/lpr* mice is shown in Fig. 1. In this experiment, graded amounts of albumin from various species were added to the assay system in an effort to inhibit plaques with soluble antigen. Mouse albumin was the most inhibitory preparation, followed by cow and human; no inhibition of plaques was seen with ovalbumin. Thus, antibody secreted was relatively specific for mouse albumin and therefore truly autoreactive. Similar experiments (Fig. 2) were performed in the mouse transferrin system. It was noted that, while human transferrin did not inhibit mouse transferrin PFC even at concentrations >1 mg/ml, bovine transferrin was approximately equivalent to mouse transferrin in inhibiting mouse transferrin PFC, indicating crossreactivity between the anti-mouse transferrin autoantibody and bovine transferrin. Similar results were obtained when bovine transferrin was used on the indicator erythrocytes (not shown). Therefore, bovine transferrin appeared to react extensively with mouse autoantibody to transferrin, while human transferrin was not crossreactive.

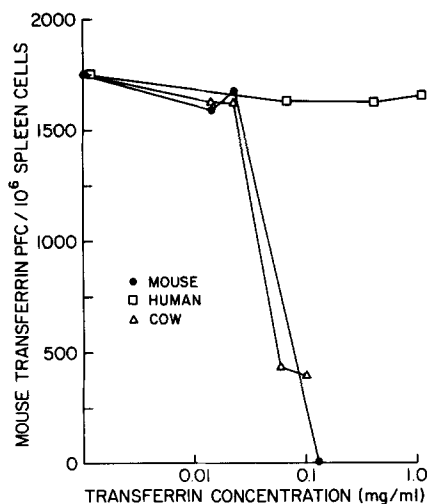


FIGURE 2. Data represent the effect of adding various concentrations of transferrin derived from different species on the number of anti-mouse transferrin PFC. This is representative of two similar experiments. Background PFC against sheep erythrocytes have been subtracted as in Fig. 1.

Comparison of PFC Against Foreign Serum Proteins With Autologous PFC. Polyclonal activation of B cells has been shown to result in formation of autoantibodies (5). If this mechanism contributed to the formation of autoantibodies to transferrin, independent of autoantigen, that it would be anticipated that autoantibodies to transferrins of other species would also be produced as a consequence of the generalized activation of B cells. Alternatively, if a process other than global activation of B cells (i.e., an antigen-driven immune response) were responsible for the production of anti-mouse transferrin in autoimmune mice, then relatively few PFC directed against heterologous noncrossreactive transferrin would be seen. In Fig. 3, it is seen that, while roughly equivalent numbers of PFC were present for the crossreactive mouse and bovine transferrins, there were few PFC seen against human transferrin. Rabbit, hamster, and dog transferrins, which are partially crossreactive with the mouse autoantibody (based on their capacity to inhibit mouse transferrin PFC), when coupled to sheep red blood cells, were able to detect varying numbers of PFC. The results provide evidence against polyclonal B cell activation as the mechanism of autoantibody formation, since PFC were seen mainly against those transferrins known to crossreact with the true autoantibody.

Detection of Serum Antialbumin and Antitransferrin by ELISA Assay. Assays were developed to detect circulating antibody against mouse albumin and transferrin, using sensitive enzyme-linked immunosorbent assay (ELISA) technology. Sera from *lpr* mice reproducibly gave more binding to mouse albumin- or transferrin-coated plates than did normal mouse serum, yet normal samples always contained some transferrin-binding immunoglobulin. Extensive efforts were made to inhibit the binding both of normal and *lpr* sera with free transferrin, at concentrations as high as 1 mg/ml. In no case could the binding be inhibited, indicating that the circulating antibody probably was of low avidity.

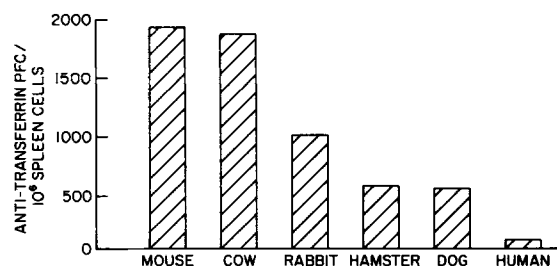


FIGURE 3. PFC were enumerated against erythrocytes coated with the indicated transferrins. PFC against uncoated erythrocytes, which were negligible, have been subtracted.

Discussion

The present data indicate that there is substantial spontaneous production of autoantibody to serum proteins in spleens of autoimmune mice. Although small amounts of such antibodies are detectable in the serum, the inability of free antigen to inhibit them indicates that the free antibody is of low avidity.

The observation that the autoimmune mice produce antibodies to serum proteins as well as to cell-bound antigen suggests that autoantibody production in murine lupus is a more generalized phenomenon than previously believed and that the spectrum of SLE autoantibodies should be widened to include not only those directed against constituents of cells, but also those directed against serum proteins. The data contrast with recent views (6) that the autoantibodies observed in SLE may represent a much more limited repertoire of molecules with interesting polyspecificities.

The present study also shows that the autoantibodies detected here at the cellular level are relatively specific for autologous proteins. The hypothesis that autoantibody production in those strains is merely a consequence of diffuse polyclonal B cell activation cannot explain these findings, since one would then anticipate equivalent production of antibodies to transferrins of different species. Furthermore, while it is true that the production of polyclonal antibodies to many exogenous antigens is characteristic of murine lupus, such antibodies are generally elevated only two- or threefold (7). For both quantitative and qualitative reasons, such a global increase in antibody production would not explain the production of autoantibodies to serum proteins that are specifically directed against autologous serum proteins. The mechanism responsible must be able to cause general production of autoantibodies that are specific for the species. These data are best explained by assuming that autoantigens themselves drive the autoantibody response and that, in the presence of a regulatory abnormality, the production of each individual autoantibody is due to antigen-specific events rather than diffuse activation of B cells by polyclonal B cell activators of T or non-T cell origin.

Since the autoantibodies against serum proteins described herein are probably incorporated into immune complexes shortly after antibody secretion because of the availability of antigen, such complexes may be involved in the genesis of glomerular injury and other immune complex effects. In addition, it is possible that some species of transferrin autoantibodies interfere with iron binding, or with the cell growth-regulating properties of transferrin, and thus possibly contribute to anemia or disordered cell replication in SLE.

Summary

The autoantibodies found in human and murine systemic lupus erythematosus (SLE) are generally directed against cells or components of cells such as nuclear antigens. This predilection may be due to the unusual immunogenicity of certain autoantigens, or to unusual patterns of antibody crossreactivity. Alternatively, the observed spectrum of reactivities may reflect the *in vivo* absorption of those autoantibodies directed against soluble antigens. To test whether hitherto undetected autoantibodies against serum proteins might exist in murine SLE, we developed assays that were independent of the possibility of absorption of autoantibodies by serum autoantigens; large numbers of plaque-forming cells (PFC) directed against mouse albumin and mouse transferrin were easily detected in the spleens of MRL/Mp-*lpr/lpr*, BXSB, and NZB mice. The secreted antibodies were relatively specific for the mouse proteins, since only limited cross-reactivity was seen with albumin and transferrins of other species in inhibition experiments. The production of these hidden antibodies could not be the result of diffuse polyclonal B cell activation, since the PFC to mouse transferrins and albumin were not always accompanied by comparable numbers of PFC against related albumins and transferrins. The results indicate that autoantibody production in murine lupus is a generalized phenomenon, not limited to the production of autoantibodies to nuclear or other cell-bound antibodies. However, the relative specificity of the autoantibodies for self-antigens indicates that diffuse polyclonal B cell activation cannot be the mechanism responsible, and argues that a selective mechanism, probably driven by antigen, accounts for production of autoantibodies in SLE.

Received for publication 3 December 1984 and in revised form 8 March 1985.

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