Isoaspartyl Post-translational Modification Triggers Autoimmune Responses to Self-proteins*

Mark J. Mamula‡§, Renelle J. Gee‡, James I. Elliott§, Alessandro Sette§, Scott Southwood¶, Paul-James Jones**, and Peter R. Blier**

From the ‡Section of Rheumatology and §Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520, ¶Epimmune, Inc., San Diego, California 92121, and the **Department of Analytical Sciences and Immunological Diseases, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut 06877

The normal functioning immune system is programmed to attack foreign pathogens and other foreign proteins while maintaining tolerance to self-proteins. The mechanisms by which tolerance is broken in the initiation of autoimmunity are not completely understood. In the present study, mice immunized with the murine cytochrome c peptide 90–104 showed no response by the B or T cell compartments. However, immunization with the isoaspartyl form of this peptide, where the linkage of Asp93 to Leu94 occurs through the β-carboxyl group, resulted in strong B and T cell autoimmune responses. Antibodies elicited by immunization with the isoaspartyl form of self-peptide were cross-reactive in binding to both isoforms of cytochrome c peptide and to native cytochrome c self-protein. In a similar manner, immunization of mice with the isoaspartyl form of a peptide autoantigen of human systemic lupus erythematosus (SLE) resulted in strong B and T cell responses while mice maintained tolerance to the normal aspartyl form of self-antigen. Isoaspartyl linkages within proteins are enhanced in aging and stressed cells and arise under physiological conditions. These posttranslationally modified peptides may serve as an early immunologic stimulus in autoimmune disease.

The immune system has evolved to be tolerant of self-proteins by the deletion of autoreactive cells in the thymus or bone marrow and by the establishment of B and T lymphocyte energy in the peripheral circulation (1–6). These mechanisms are based on the presentation of a vast array of self-peptides to the immune system. Despite the efforts to instruct the immune system to ignore self-tissues, the appearance of various autoimmune diseases demonstrates that tolerance to self-antigens is not perfect. Flaws in the development of immune tolerance can be revealed by the immunization of animal models with a variety of self-peptides leading to B and T cell autoimmunity as well as autoimmune-mediated pathology (7–12).

How tolerance is broken in the initiation of autoimmunity is not completely understood. The immunization of mice with a single self-peptide, the amino-terminal 11 amino acids of myelin basic protein (MBP)† in complete Freund’s adjuvant can elicit pathology resembling that of human multiple sclerosis (7). The induction of disease requires a post-translationally acetylated form of MBP peptide 1–11. While this disease can be elicited with a single self-peptide or even with T cells of a single specificity, the autoimmune response diversifies to many sites on the MBP over the course of the disease. T cell responses originate with the dominant single self-peptide but rapidly evolve to include other cryptic peptide epitopes within MBP. Similar observations of determinant spreading have been made in murine models of diabetes and systemic lupus erythematosus (SLE), two diseases arising spontaneously in susceptible strains of mice (8, 11, 13).

Antinuclear autoantibodies specific for double-stranded DNA and the U1/Sm ribonucleoprotein particle (snRNP) are diagnostic markers of SLE. The snRNP particle is an RNA-protein complex essential for the splicing of pre-mRNA (14). Proteins designated B, B’, and D comprise the target proteins of anti-Sm autoantibodies in SLE patients. It is not known how high affinity autoantibodies and autoreactive T cells arise to these intracellular proteins. The mature phenotype of autoantibodies found in diseases such as SLE indicates that autoimmunity is driven by helper T lymphocytes and a source of antigen (15–20).

While it is clear that autoimmunity can spread to several sites on an autoantigen over the course of experimentally induced disease models, the initiating antigenic peptide in naturally arising disease is unknown. A hypothesis of molecular mimicry implies that foreign pathogens that share amino acid sequences with self-peptides can break immunologic tolerance in the induction of autoimmunity. However, no pathogen has been unambiguously linked with the induction of any human autoimmune syndrome. Alternatively, we have initiated studies to consider forms of self-antigens that can be viewed as “foreign” by the immune system. As would be expected, we have demonstrated that the immune system does not respond to immunization with selected peptides from self-proteins. However, when the same self-peptides are converted to the isoaspartyl isiform, vigorous autoimmune responses develop upon immunization. After initiation by the isoaspartyl peptide isoforms, autoimmunity is amplified to other peptides on the autoantigen.

Isoaspartyl peptides arise spontaneously under physiologic conditions and are particularly elevated in cells undergoing stress and in aging cells (21–25). The presence of isoaspartyl

* This work was supported by the Arthritis Foundation, the Ethyl F. Donaghue Foundation, and National Institutes of Health Grant AI36529 (to M. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Yale University School of Medicine, 333 Cedar St., LCI 610, P.O. Box 208031, New Haven, CT 06520-8031. Tel.: 203-737-2840; Fax: 203-785-7053; E-mail: mark.mamula@yale.edu.

† The abbreviations used are: MBP, myelin basic protein; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein particle; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
peptides have been observed as a major component of the amyloid-containing brain plaques of patients with Alzheimer’s disease (24). With relevance to immune responses, it is possible that tolerance to these forms of self-proteins fails to occur early in lymphocyte development. Based on the enhanced immunity to some isoaspartylated self-peptides, it is possible that an accumulation of these aberrant peptides may be an early stimulus for an autoimmune immune response.

**EXPERIMENTAL PROCEDURES**

**Antigens**

Aspartyl and isoaspartyl forms of murine snRNP D peptide 65–79 ([IRYFILPDSLPLD]), murine cytochrome c 90–104 (ERADILIAYLKATNE), and murine cytochrome c 81–104 ([PIAGIKKGERADILAYLKATNE]) were synthesized by Fenoc (N-9-fluorenylmethoxycarbonyl) biochemistry in the Yale University/W.M. Keck Biotechnology Resource Laboratory. Isoaspartyl residues of aspartic acid were introduced at the residues designated in bold (as indicated above). Peptides were purified by reverse phase high performance liquid chromatography to single peaks and were analyzed by mass spectroscopy, amino acid analysis, amino acid sequencing, and nuclear magnetic resonance (NMR, as described below).

Native murine U1/Sn snRNPs were purified as described previously (26). In brief, agarose bound mouse monoclonal anti-trimethylguanosine (Oncogene Sciences, Manhasset, New York) was used for affinity purification of snRNP particles. Nuclear extracts were added to the anti-trimethylguanosine column at a rate of 2 ml/min and bound snRNPs competitively eluted under nondenaturing conditions using 20 mM 7-methylguanosine. Eluted snRNPs were dialyzed against a buffer containing 20 mM Hepes, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol, concentrated, and stored at −70 °C for future use. The efficiency of binding to and elution from the anti-trimethylguanosine column was assessed using both RNA and protein gel electrophoresis. The 70,000, A, B, C, and D proteins of the U1 snRNP particle were approximately equal in these preparations. Native murine and pigeon cytochrome c was purchased from Sigma and repurified by ion exchange chromatography.

**Animals**

B10.A, B10.BR, and MRL lpr/lpr mice were purchased from the Jackson Laboratory, Bar Harbor, ME.

**NMR Analysis**

Samples of either peptide were dissolved in H2O/D2O (90:10) to a final concentration of 9 mM (for aspartyl 90–104) or 1.6 mM (for isoaspartyl 90–104). No pH adjustments were made to neutralize trifluoroacetic acid in the preparation. Two-dimensional phase-sensitive 1H–1H ROESY spectra were acquired on a Bruker AM-500 spectrometer at ambient temperature. Both spectra consisted of a 4096 × 256 point 2D phase-shifted sine-bell function, and then Fourier transformed. Prior to Fourier transformation in F2, the data were zero filled to 4048 points in F2, and then apodized with a 2048 point 1/2 phase-shifted sine-bell function. Spectra were referenced in F1 and F2 by setting the water resonance to 4.7 ppm.

**Quantitative Detection of Isoaspartyl-modified Residues**

Isoaspartyl residues were detected by the enzyme, protein isoaspartyl methyltransferase, according to the manufacturer’s protocol (ISO-QUANT™). Protein Demidification Detection Kit, Promega Corp., Madison, WI. In brief, samples and control peptides are incubated 30 min at 30 °C in a reaction mixture containing protein isoaspartyl methyltransferase, [1H5-adenosyl]-t-methionine, and cold S-adenosyl-t-methionine. The reaction is stopped at pH 10 on ice and volatile [1H]methanol is condensed in the reaction vessel. Fifty μl of sample is adsorbed to a sponge attached to the cap of a scintillation vial. The sample is incubated 60 min at 40 °C to volatile [1H]methanol into the scintillation mixture and counted for counts/min. Positive controls include those provided in the ISOQUANT™ kit as well as synthetic peptides described above containing 1 isoaspartyl residue/peptide (1 pmol of [1H]methanol/μmol of protein).

In some experiments, isoaspartyl levels were examined in mitogen-activated B and T lymphocyte populations. In brief, freshly isolated splenic or lymph node B and T cells (5 × 10^6/ml) were cultured with either lipopolysaccharide (10 μg/ml) for 48 h or with concanavalin A (10 μg/ml) for 24 h at 37 °C. Cell pellets (10^6 cells) were collected from each culture, lysed by sonication in 100 μl of PBS/Tween, and assayed for isoaspartyl content as described above. Control cultures were incubated under identical conditions in the absence of lipopolysaccharide or concanavalin A.

**T Cell Proliferation Assays**

B10.A mice were immunized subcutaneously with 100 μg of either isoform of the murine snRNP D or cytochrome c peptide in PBS emulsified in complete Freund’s adjuvant (Difco). After 10 days, the draining lymph nodes (popliteal, inguinal, and periaortic) were excised and single cell suspensions were prepared. Cells were cultured in triplicates (5 × 10^5 cells/well) in 200 μl of Clics medium (Irvine Scientific) supplemented with 5% fetal bovine serum, 1-galactamine, 5 × 10^-5 M 2-mercaptoethanol, and antibiotics. Antigen stimulation was provided by adding the aspartyl or isoaspartyl isoforms of the snRNP D or cytochrome c peptides, whole mouse cytochrome c (Sigma), or purified native mouse snRNPs, as indicated. After 3 days, cultures were pulsed with 1 μCi of [3H]thymidine, harvested 16 h later onto glass fiber filters, and counted in a BetaPlate liquid scintillation counter (LKB Wallac). Bar graphs represent cultures in which the deviation was less than 10% of the mean counts/min of triplicate cultures. Individual experiments utilized two to three mice immunized with each peptide. The data are representative of at least six individual proliferation experiments.

**Autoantibody Analysis**

Groups of four to six B10.A mice were immunized at day 0 with 50 μg of the indicated peptide emulsified in complete Freund’s adjuvant and boosted with the same peptide in incomplete Freund’s adjuvant at day 21. Mice were bled at day 28 and at weekly intervals thereafter.

Antibody binding to individual peptides was measured by ELISA and reported as optical density (405 nm). Polystyrene plates were pre-treated with 0.2% glutaraldehyde in 100 mM phosphate buffer at pH 5.0 for 20 min at room temperature. After washing with PBS, peptides were added at a concentration of 5 μg/ml PBS (pH 8.0) for 2 h at room temperature. Plates were blocked with 1% bovine serum albumin in PBS before use. All subsequent wash steps used PBS with 0.05% Tween 20. Serum was diluted 1/200 in PBS/Tween with 0.1% bovine serum albumin and incubated in wells for 4 h at room temperature. In some experiments, serum dilutions were preincubated for 4 h at room temperature with selected peptides or proteins (as indicated in figures) in order to examine specific solution phase inhibition of antibody responses. After a first incubation of plates with primary antibody, plates were washed three times with PBS/Tween. Bound antibody was quantitated by sequential incubations with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) and 5-nitrophenyl phosphate in diethanolamine buffer as chromogenic substrate. NMS indicates the use of preimmune mouse serum. Anti-double-stranded DNA binding was similarly examined by commercially available ELISA (Arlington Scientific Inc., Arlington, TX) according to the manufacturer’s instructions. All data points and percent inhibitions were calculated from the mean of triplicate wells in which individual standard deviation was less than 15% of the mean O.D. (405 nm) signal.

**Class II Peptide Binding Assays**

Celle.—The B cell lymphoma CH-27 was used as a source I-A^b and I-E^k MHC class II molecules. The cell line was maintained in vitro by culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml 2-mercaptoethanol, 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA), and 100 units/ml penicillin (Life Technologies, Inc., Grand Island, NY). Large quantities of recombinant mouse I-E^k MHC class II were eluted with 0.05 M diethylamine in 0.15 M NaCl at 50 °C and then passed over the appropriate antibody columns, which were developed under identical conditions in the absence of lipopolysaccharide or concanavalin A.

**Affinity Purification of Class II Molecules**—Mouse class II molecules were purified as described previously (42, 43) using the 10.3.6 monoclonal antibody I-A^k-specific (BDH) coupled to Sepharose 4B beads. Lysates were filtered through 0.8- and 0.4-μm filters and then passed over the appropriate antibody columns, which were then washed with 15 column volumes of 0.5% Nonidet P-40, 0.1% SDS, and 2 column volumes of PBS containing 0.4% n-octylglucoside. MHC class II was eluted with 0.05 M diethylamine in 0.15 M NaCl containing 0.4% n-octylglucoside (pH 11.5). A 1/20 volume of 1.0 M Tris, 0.02 M sodium EDTA, 0.1 M sodium chloride, and 1 M glycine, pH 12.5. At 20°C, 0.2 ml of B10.A cell lysate (grown in spinner cultures. Cells were lysed at a concentration of 10^8 cells/ml in PBS containing 1% Nonidet P-40, 1 m M phenylmethylsulfonyl fluoride, 5 mM sodium orthovanadate, and 25 mM iodoacetamide. The lysates were cleared of debris and nuclei by centrifugation at 10,000 × g for 20 min.

**Indirect Binding Assay**—Cells were grown in spinner cultures. Cells were lysed at a concentration of 10^8 cells/ml in PBS containing 1% Nonidet P-40, 1 m M phenylmethylsulfonyl fluoride, 5 mM sodium orthovanadate, and 25 mM iodoacetamide. The lysates were cleared of debris and nuclei by centrifugation at 10,000 × g for 20 min.
1.5 mM NaCl (pH 6.8) was added to the eluate to reduce the pH to ~7.5, and then concentrated by centrifugation in Centriprep 30 concentrators (Amicon, Beverly, MA).

**Class II Peptide-binding Assays**—Purified mouse class II molecules (5–500 nM) were incubated with 1–10 nM radiolabeled peptides for 48 h in PBS containing 5% dimethyl sulfoxide in the presence of a protease inhibitor mixture. Purified peptides were iodinated using the chloramine-T method (44). Radiolabeled probes used were HEL p46–61 for I-A^a^ and l-repressor 12–26 for I-E^a^ and I-E^f^ and I-E^f^ assays were performed at pH 5.0 (45).

Peptide inhibitors were typically tested at concentrations ranging from 120 μg/ml to 1.2 ng/ml. The data were then plotted and the dose yielding 50% inhibition (IC_{50}) was measured. In appropriate stoichiometric conditions, the IC_{50} of an unlabeled test peptide to the purified MHC is a reasonable approximation of the affinity of interaction (K_d). Peptides were tested in two to four completely independent experiments.

Class II peptide complexes were separated from free peptide by gel filtration on TSK2000 columns (TosoHaas, Montgomeryville, PA), and the fraction of bound peptide calculated as described previously (43). In preliminary experiments, each of the I-A and I-E preparations were titered in the presence of fixed amounts of radiolabeled peptides to determine the concentration of class II molecules necessary to bind 10–20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed using these class II concentrations.

**RESULTS**

**Isoaspartyl Forms of Self-peptides Elicit Autoimmunity**—In previous studies using cytochrome c as a model autoantigen, immunization with a cryptic self-peptide from the carboxyl terminus of mouse cytochrome c (residues 81–104) can elicit strong autoimmune responses (10). Shorter cytochrome c peptides within p81–104 were then synthesized to more accurately identify the range of T cell immune tolerance to self-cytochrome c peptides. In the course of these studies, two separate preparations of murine cytochrome c 90–104 were synthesized approximately 4 months apart. B10.A mice were immunized individually with either preparation of self-peptide emulsified in complete Freund’s adjuvant. We discovered that one preparation of the cytochrome c peptide produced strong T cell responses in a proliferative assay, whereas a second preparation, synthesized 4 months later, elicited no B or T cell responses in a manner identical to Fig. 1A, left (isoaspartyl) and right (aspartyl) sets of columns.

We next sought to determine the biological properties that conferred immunity to one peptide preparation while a second preparation of the same self-peptide elicited no responses. Both cytochrome c peptide preparations had identical compositions by amino acid analysis and mass spectrometry but had different retention times by reverse-phase high performance liquid chromatography (data not shown). Amino acid sequencing revealed that the non-immunogenic peptide contained the predicted sequence of mouse cytochrome c while the immunogenic peptide sequence as predicted to the site of an aspartic acid at residue 4 from the amino terminus (data not shown). Thereafter, the sequencing reaction was blocked.

**Formation of Isoaspartyl Peptides**—Both peptide preparations were analyzed by two-dimensional NMR to determine the structural basis for this difference in immunogenicity (Fig. 2). The analysis demonstrated that in the immunogenic cytochrome c peptide, Asp^{iso} was joined to Leu^{iso} through an amide bond involving the carboxyl group of the side chain (27). We did not detect any other physical or biochemical differences between the two peptides. As described earlier, isoaspartyl post-translational modifications occur through a cyclic imide intermediate formed by the attack of peptide bond nitrogen on the side chain carboxyl (27). Under cellular physiologic conditions, hydrolysis of the cyclic intermediate yields either the isoaspartyl peptide or conversion back to the normal aspartyl form.

T lymphocyte responses after immunization with isoaspartyl and aspartyl forms of cytochrome c 90–104 are illustrated in Fig. 1A. As demonstrated, T cell responses arise only after immunization with the isoaspartyl cytochrome c peptide while tolerance (or unresponsiveness) is maintained to the aspartyl form. T cell responses elicited by the isoaspartyl cytochrome c are not cross-reactive and do not respond to stimulation with the aspartyl peptide or with native cytochrome c protein. Immunogenicity was not related to MHC binding properties since it was determined that the aspartyl and isoaspartyl isoform peptides had virtually identical affinity for the restricting I-E^k^ class II molecule (Table I).

**Lupus Autoimmunity Initiated by Isoaspartyl Self-peptides**—Since the isoaspartyl form of self-peptides can arise physiologically in cells, we wanted to determine if specific T cells and/or autoantibodies of autoimmune disease could be initiated by post-translationally modified peptides. We examined the immunogenicity of an aspartyl and an isoaspartyl peptide of the D protein component of the U1/Sm snRNP, an autoantigenic target of human SLE and in murine models of this disease. Normal, non-autoimmune prone strains of mice were immunized with either form of the peptide emulsified with complete Freund’s adjuvant. In a manner similar to immunization with isoforms of self-cytochrome c, T cells from mice immunized with the isoaspartyl form of the snRNP D peptide responded to the
Isoaspartyl Self-peptides Elicit Autoimmunity

Fig. 2. NMR analysis demonstrates that the immunogenic self-peptide contains an isoaspartyl residue. A portion of the two-dimensional ROESY spectra of aspartyl (panel A) and isoaspartyl (panel B) isoforms of cytochrome c peptide 90–104, demonstrating the presence of an Asp93 Hb1, Hb2-Leu94 HN cross-peak for β-90–104 (boxed, panel B) and its absence in the α-isofom (panel A).

Table I

| Peptide                  | Kd (µM) |
|-------------------------|---------|
| Cytochrome c 81–95      | 239     |
| Aspartyl cytochrome c 90–104 | 2.2     |
| Isoaspartyl cytochrome c 90–104 | 2.0     |
| λ Repressor 12–26       | 0.29    |

Isoaspartyl peptide (Fig. 1B) but not to the aspartyl (normal) form of the same peptide or to the native snRNP particle. In contrast, no T cell response was observed when the aspartyl form of the snRNP D peptide was used as the immunogen.

We next investigated the humoral immune responses in mice immunized with either the normal or isoaspartyl forms of cytochrome c peptides or the snRNP D peptides (Fig. 3). Antibodies were elicited by immunization with the isoaspartyl form of cytochrome c with specific binding to the isoaspartyl immunogen as well as to the aspartyl form of peptide from either the p81–104, p90–104, and also to the native cytochrome c protein. In contrast to T cell immune responses, antibodies elicited by the isoaspartyl peptide are cross-reactive in their ability to bind either isoform of self-peptide. As a control, antibodies did not bind to an irrelevant cytochrome c peptide 52–67. Immunization with the aspartyl peptide did not elicit detectable antibodies at any time point even when followed to more than 6 months post-immunizations.

Solution-phase inhibition studies were performed to better elucidate the fine specificity and/or degree of epitope spreading of antibody responses to the isoaspartyl cytochrome c isoform peptide (Table II). Anti-isoaspartyl cytochrome c 90–104 antibodies were preincubated with specific peptides in solution prior to their binding by ELISA to the longer isoforms of cytochrome c peptide 81–104. As a control, the unrelated cytochrome c peptide, 52–67 failed to inhibit binding to either cytochrome c isoform of 81–104. In contrast, isoaspartyl p91–104 significantly inhibited binding to either cytochrome c 81–104 isoform. The ability of native cytochrome c or the aspartyl forms of p81–104 and p90–104 to primarily inhibit binding to the aspartyl isoform indicates that subsets of autoantibodies exist that selectively bind either the isoaspartyl or aspartyl structure individually. The lack of complete inhibition in these specific studies may be due to antibodies elicited to amino acid sequences flanking the aspartyl site.

Immunization with the isoaspartyl snRNP peptide elicited anti-nuclear antibodies by indirect immunofluorescence typical of those diagnostic of human SLE while the corresponding normal isoform of the peptide elicited no detectable antibody responses (data not shown). Similar to responses observed to the cytochrome c model peptides, serum antibodies were detected only after immunization with the isoaspartyl form of the snRNP D peptide (Fig. 3B). Anti-snRNP autoantibodies were cross-reactive in their ability to bind either isoforms of the snRNP peptide or in binding to the native snRNP particle. In addition, we observed diversification of the immune response to include double-stranded DNA-binding antibodies. The latter response may be attributed to cross-reactive binding of some populations of anti-snRNP autoantibodies as described previously (19, 28). Moreover, sera from MRL lpr/lpr mice, the spontaneous murine model of human SLE, possess autoantibodies binding the isoaspartylated snRNP peptide (data not shown).

Isoaspartyl Levels Are Elevated in Activated Lymphocytes—A
Isoaspartyl self-peptides elicit autoimmunity

number of autoimmune syndromes, such as multiple sclerosis and SLE, are mediated by activated B and T lymphocytes in peripheral lymphoid compartments. Since the exact source of self-antigen in autoimmunity is not known, we examined cellular isoaspartyl levels in resting and activated B and T lymphocytes from MRL lpr mice, the murine model of human SLE, and from non-autoimmune prone (B10.A) mice (Table III). Although the source of cells did not influence isoaspartyl levels, activated B lymphocytes possess approximately 3-fold increased isoaspartyl levels as compared with resting cells. Sim-

FIG. 3. Serum antibodies from mice immunized with the isoaspartyl form of cytochrome c or snRNP D peptides elicit autoantibodies. B10.A mice were immunized and boosted with isoaspartyl or aspartyl isoforms of cytochrome c p91–104 (panel A) or snRNP D p65–79 (panel B) linked to ovalbumin. Serum was collected 28 days post-immunization and examined by ELISA for binding to individual peptides, as indicated. Standard deviation of each data set was less than 15% of the mean OD405 nm of triplicate wells (see “Experimental Procedures”).

TABLE II

Competitive inhibition of antibodies raised to isoaspartyl cytochrome c 90–104

Serum was collected from mice immunized with the isoaspartyl cytochrome c 90–104 peptide (see “Experimental Procedures”). Serum dilutions were incubated overnight at 4 °C with 50 µg/ml inhibitor peptides (as indicated) prior to ELISA analysis on plates coated with aspartyl or isoaspartyl forms of cytochrome c 81–104. % Inhibition was calculated as: [1 – (inhibited OD/uninhibited OD)] × 100.

| Inhibitor          | % Inhibition of binding to |
|--------------------|----------------------------|
|                    | Aspartyl 81–104 | Isoaspartyl 81–104 |
| Aspartyl 90–104    | 74             | 40             |
| Isoaspartyl 90–104 | 80             | 84             |
| Aspartyl 81–104    | 70             | 36             |
| Isoaspartyl 81–104 | 74             | 91             |
| Native cytochrome c| 77             | 11             |
| Cytochrome c 52–67 | 9              | 5              |

isoaspartyl levels as compared with resting cells. Numbers in bold represent significant changes as compared to resting lymphocyte samples (p < 0.05).

TABLE III

Isoaspartyl levels in resting and mitogen-activated lymphocytes

Splenic B or T lymphocytes were purified from autoimmune MRL lpr/lpr or non-autoimmune prone B10.A mice. Cell lysates (106 cells) were assayed for total isoaspartyl content (see “Experimental Procedures”). Numbers in bold represent significant changes as compared to resting lymphocyte samples (p < 0.05).

| Cell source      | Cell type | Activation state | cpm   | Isoaspartyl |
|------------------|-----------|------------------|-------|-------------|
| MRL lpr          | B         | Resting          | 1,330 | 7.1         |
| MRL lpr          | B         | LPS activated    | 4,309 | 23.0        |
| MRL lpr          | T         | Resting          | 656   | 3.5         |
| MRL lpr          | T         | ConA activated   | 2,409 | 14.8        |
| B10.A            | B         | Resting          | 952   | 5.0         |
| B10.A            | B         | LPS activated    | 3,273 | 17.4        |
| B10.A            | T         | Resting          | 2,000 | 10.6        |
| B10.A            | T         | ConA activated   | 3,817 | 20.3        |
| Negative control |           |                  | 586   | 3.1         |
| Positive control |           |                  | 10,793| 57.5        |

Isoaspartyl peptides in the native snRNP autoantigen—We next examined native snRNP complexes and native mouse cytochrome c for the presence of isoaspartylated forms of protein (Table IV). We observed that native murine cytochrome c protein did not possess isoaspartyl residues while the intact snRNP particle, purified from cell lysates, possessed isoaspartyl residues above those signals found in negative control peptides (Table IV; p < 0.01). These studies, however, do not locate the exact site of isoaspartyl modification but instead represent post-translational modifications present in any of the snRNP proteins. The inability to sequence through isoaspartyl modifications makes it a difficult task to precisely determine the location of these residues in native proteins.

DISCUSSION

Our results demonstrate that the isoaspartyl form of a self-peptide can be immunogenic under conditions where T and B cells are unresponsive to the corresponding normal aspartyl form of the peptide. In this system, no cross-reactivity between the isoforms was observed in the T cell response. T cells elicited with the isoaspartyl form of self-peptide fail to respond to the normal peptide form. However, isoaspartyl-specific T cells can drive autoantibodies that are promiscuous in their ability to bind either the aspartyl or isoaspartyl form of self-antigen (Fig. 3). This finding implies that isoaspartyl forms of autoantigens occurring in vivo could induce the formation of autoantibodies capable of recognizing the normal isoforms of these proteins. In this way, autoimmunity initiated by a single self-determinant could expand and potentially diversify to other sites on autoantigens, an observation known to occur in autoimmune states (7–12). Do these observations have relevance to the in vivo development of autoimmunity? Isoaspartyl forms of self-proteins are not uncommon inhabitants of cells. At physiological pH, Asp and Asn residues can nonenzymatically cyclize with the adjacent (C-proximal) peptide bond to form an aspartimide; however, they then either fall under or are an aspartyl or isoaspartyl residue (29–31) (Fig. 2). Aspartimide formation is associated with the aging of cellular proteins and has been described in erythrocytes and many other cell types. Events that create cell stress, such as heat shock, increases the measured number of isoaspartyl residues leading to the inactivation of cellular proteins (27, 32, 33). We have also found that mitogen-stimulated B and T lymphocytes have from 2 to 5 times the endogenous levels of

Isoaspartyl Peptides in the Native snRNP Autoantigen—We next examined native snRNP complexes and native mouse cytochrome c for the presence of isoaspartyllated forms of protein (Table IV). We observed that native murine cytochrome c protein did not possess isoaspartyl residues while the intact snRNP particle, purified from cell lysates, possessed isoaspartyl residues above those signals found in negative control peptides (Table IV; p < 0.01). These studies, however, do not locate the exact site of isoaspartyl modification but instead represent post-translational modifications present in any of the snRNP proteins. The inability to sequence through isoaspartyl modifications makes it a difficult task to precisely determine the location of these residues in native proteins.

DISCUSSION

Our results demonstrate that the isoaspartyl form of a self-peptide can be immunogenic under conditions where T and B cells are unresponsive to the corresponding normal aspartyl form of the peptide. In this system, no cross-reactivity between the isoforms was observed in the T cell response. T cells elicited with the isoaspartyl form of self-peptide fail to respond to the normal peptide form. However, isoaspartyl-specific T cells can drive autoantibodies that are promiscuous in their ability to bind either the aspartyl or isoaspartyl form of self-antigen (Fig. 3). This finding implies that isoaspartyl forms of autoantigens occurring in vivo could induce the formation of autoantibodies capable of recognizing the normal isoforms of these proteins. In this way, autoimmunity initiated by a single self-determinant could expand and potentially diversify to other sites on autoantigens, an observation known to occur in autoimmune states (7–12).

Do these observations have relevance to the in vivo development of autoimmunity? Isoaspartyl forms of self-proteins are not uncommon inhabitants of cells. At physiological pH, Asp and Asn residues can nonenzymatically cyclize with the adjacent (C-proximal) peptide bond to form an aspartimide; however, they then either fall under or are an aspartyl or isoaspartyl residue (29–31) (Fig. 2). Aspartimide formation is associated with the aging of cellular proteins and has been described in erythrocytes and many other cell types. Events that create cell stress, such as heat shock, increases the measured number of isoaspartyl residues leading to the inactivation of cellular proteins (27, 32, 33). We have also found that mitogen-stimulated B and T lymphocytes have from 2 to 5 times the endogenous levels of

number of autoimmune syndromes, such as multiple sclerosis and SLE, are mediated by activated B and T lymphocytes in peripheral lymphoid compartments. Since the exact source of self-antigen in autoimmunity is not known, we examined cellular isoaspartyl levels in resting and activated B and T lymphocytes from MRL lpr mice, the murine model of human SLE, and from non-autoimmune prone (B10.A) mice (Table III). Although the source of cells did not influence isoaspartyl levels, activated B lymphocytes possess approximately 3-fold increased isoaspartyl levels as compared with resting cells. Sim-
Isoaspartyl Self-peptides Elicit Autoimmunity

The antibody cross-reactivity observed in the present study suggests that isoaspartyl epitopes could stimulate the B-cell-mediated diversification of autoimmunity observed by us in our previous work with cytochrome c and lupus autoantigens (snRNPs) and by others in work with myelin basic protein (38-41). Even in the absence of epitope spreading, our results support the hypothesis that naturally arising autoantibodies could develop from exposure of the immune system to isoaspartyl forms of self-peptides.

Acknowledgments — We thank Drs. Charlie Janeway and Mark Shlomchik for their careful review of this work. We also thank Promega Corp. for contributions of the ISOQUANT™ Protein Deamidation Detection Kit.

REFERENCES

1. Billingham, R. E., Brent, L., and Medawar, P. B. (1956) Nature 172, 603–606
2. Kappler, J. W., Roehm, N., and Marrack, P. (1987) Cell 49, 273–280
3. Bretscher, P., and Cohen, M. (1970) Science 169, 1042–1049
4. Jenkins, M. K., and Schwartz, R. H. (1987) J. Exp. Med. 165, 302–319
5. Mueller, D. L., Jenkins, M. K., and Schwartz, R. H. (1989) Annu. Rev. Immunol. 7, 445–480
6. Schiéd, H., Hotschke, O., Kalscher, H., and Rammensee, H. G. (1990) Science 247, 587–1589
7. Lehmann, P. V., Forsthuber, T., Miller, A., and Sercarz, E. E. (1992) Nature 358, 155–157
8. Kaufman, D. L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G. S. P., Robinson, P., Atkinson, M. A., Sercarz, E. E., Tobin, A. J., and Lehmann, P. V. (1993) Nature 360, 67–73
9. Lou, Y., and Tung, K. S. (1993) J. Immunol. 151, 5790–5799
10. Mamula, M. J. (1993) J. Exp. Med. 177, 567–571
11. Bockenstedt, L. K., Lee, G., and Mamula, M. J. (1995) J. Immunol. 154, 3536–3542
12. Garza, K. M., Griggs, N. D., and Tung, K. S. K. (1997) Immunity 6, 89–96
13. Fatenejad, S., Brooks, W., Schwartz, A., and Craft, J. (1994) J. Immunol. 152, 5523–5531
14. Wassarman, D. A., and Steitz, J. A. (1992) Science 257, 1918–1925
15. Steinberg, A. D., Roths, J. B., Murphy, E. D., Steinberg, R. T., and Rapeche, E. S. (1980) J. Immunol. 125, 871–873
16. Ducrot, J. R., Portunova, J. P., and Routin B. L. (1988) J. Exp. Med. 167, 713–721
17. Jevnikar, A. M., Grusby M. J., and Glimcher L. H. (1994) J. Exp. Med. 179, 1137–1143
18. Smito, T. J., Portunova, J. P., and Routin B. L. (1988) J. Exp. Med. 167, 713–721
19. Shlomchik, M. J., Marshak-Rothstein, A., Wolfszcz, C. B., Rothstein, T. L., and Weigert, M. G. (1987) Nature 329, 805–811
20. Bloom, D. D., Davignon, J., Cohen, P., Eisenberg, R. A., and Clarke, S. H. (1993) J. Immunol. 150, 1579–1590
21. Tan, E. M. (1989) Adv. Immunol. 44, 93–151
22. Aswad, D. W. (1996) In Deamidation and Isoaspartylation Formation of Peptides and Proteins (Aswad, D. W., ed.) pp. 31–46, CRC Press, Boca Raton, FL
23. Galletti, P., Ingrosso, D., Mannina, C., Clemente, G., and Zappia, V. (1995) Biochem. J. 306, 313–325
24. Najbauer, J., Orpiszewski, J., and Aswad, D. W. (1996) Biochemistry 35, 5183–5190
25. Roher, A. E., Loewenson, D. J., Clarke, S., Wolwoc, G., Wang, R., Cotter, R. J., and Reardon, I. M., Zurcher-Neely, H. A., Heinricsson, R. L., Ball, M. J., and Greenberg, B. D. (1995) J. Biol. Chem. 270, 3072–3083
26. Tsai, W., and Clarke, S. (1994) Biochem. Biophys. Res. Commun. 203, 491–497
27. Bringmann, P., Rwe, J., Appel, B., Reuter, R., and Luhrmann, R. (1988) EMBO J. 2, 1129–1135
28. Chazin, W., Kordel, J., Thulin, E., Hofmann, T., Drakenberg, T., and Forsen, E. S. (1980) J. Immunol. 125, 6132–6137
29. Lu, Y., and Tung, K. S. (1993) J. Exp. Med. 177, 263, 2924–2931
30. Mamula, M. J., and Janeway, C. A. (1993) Immunol. Today 14, 1137–1143
31. Voorter, C. E. M., de Haard-Hoekman, W. A., van den Oetelaar, P. J. M., van den Dijcke, E. P., and van den Oetelaar, P. J. M. (1995) J. Immunol. 154, 4857–4864
32. Mamula, M. J. (1993) Annu. Rev. Biochem. 54, 479–506
33. Luria, R., and Schirch, B. (1988) Biochemistry 27, 7671–7677
34. Voorter, C. E. M., de Haard-Hoekman, W. A., van den Oetelaar, P. J. M., van den Dijcke, E. P., and van den Oetelaar, P. J. M. (1995) J. Immunol. 154, 4857–4864
35. Mamula, M. J. (1993) Annu. Rev. Biochem. 54, 479–506
36. Mamula, M. J. (1995) Immunol. Rev. 144, 301–314
37. Roth, R., and Mamula, M. J. (1996) J. Immunol. 157, 2924–2931
38. Mamula, M. J. (1997) J. Biol. Chem. 272, 16087–16094
39. Mamula, M. J., Fatenejad, S., and Craft, J. (1994) J. Immunol. 152, 35–40
40. Gerg, J. C., Herje, V., Johnson, D. R., Raghupathy, R., and Streminger, J. L. (1987) J. Biol. Chem. 262, 16087–16094
41. Sette, A., Buus, S., Colon, S., Miles, C., and High, G. M. (1989) J. Immunol. 142, 35–40

### TABLE IV

| Protein          | Picomoles of CH₃/pmol total protein |
|------------------|------------------------------------|
| No protein       |                                    |
| Tp1 (pos control)| 0.04                               |
| Tp1 (pos control)| 0.08                               |
| Mouse cytochrome | 0.18 (NS)*                          |
| Pigeon cytochrome| 0.12 (NS)*                          |
| Isoaspartyl cytochrome c (p81)| 0.91                               |
| Aspartyl cytochrome c (p81)| 0.09                               |
| Isoaspartyl snRNP-D| 0.81                               |
| Aspartyl snRNP-D | 0.11                               |
| Native snRNPs    | 0.38 (p < 0.01)*                    |

* Student t test, NS, not significant (p > .15).
Isoaspartyl Self-peptides Elicit Autoimmunity

44. Buus, S., Sette, A., Colon, S. M., Miles, C., and Grey H. M. (1987) Science 235, 1353–1358
45. Sette, A., Southwood, S., O’Sullivan, D., Gaeta, F. C., Sidney, J., and Grey, H. M. (1992) J. Immunol. 148, 844–851
46. Molberg, O., McAdam, S. N., Korner, R., Quarsten, H., Kristiansen, C., Madson, L., Fugger, L., Scott, H., Noren, O., Roeperstorf, P., Lundin, K. E. A., Sjoestrom, H., and Solid, L. M. (1998) Nature Med. 6, 713–717
47. Meadows, L., Wang, W., denHaan, J. M. M., Blokland, E., Reinhardus, C., Drijfhout, J. W., Shabanowitz, J., Pierce, R., Agulnik, A. I., Bishop, C. E., Hunt, D. F., Goulmy, E., and Engelhardt, V. H. (1997) Immunity 6, 273–281
48. Skipper, J. C. A., Hendrickson, R. C., Guldén, P. H., Brichard, V., Van Pel, A., Chen, Y., Shabanowitz, J., Walfel, T., Slingluff, C. L., Boon, T., Hunt, D. F., and Engelhardt, V. H. (1996) J. Exp. Med. 183, 527–534
49. Wood, P., and Elliott, T. (1998) J. Exp. Med. 188, 773–779