STUDIES ON POLY(ADP-RIbose) SYNTHESIS IN LYMPHOCYTES OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS

THESIS

Presented to the Graduate Council of the University of North Texas in partial fulfillment of the requirements for the Degree of

MASTER OF SCIENCE

By

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A method for assaying poly(ADP-ribose) polymerase (PADPRP) activity in lymphocytes of systemic lupus erythematosus (SLE) patients has been developed. Using this method, PADPRP activity has been studied in lymphocytes from 15 patients and 13 controls. The mean activity in SLE lymphocytes was significantly lower than that in controls and 60% of the SLE patients demonstrated activities below the minimum of the control population.

Possible mechanisms for this altered metabolism were investigated. The $K_m$ app of PADPRP for NAD; size distribution, branch frequency, and rates of turnover of polymers; competition for substrate; and number of PADPRP molecules were studied. The data demonstrated that SLE lymphocytes have a decreased synthetic capacity rather than alterations in the substrate or in turnover of the product.
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TABLE OF CONTENTS

LIST OF TABLES .............................................. vi
LIST OF ILLUSTRATIONS ....................................... vii

Chapter
I. Introduction ....................................................... 1
   Poly(ADP-ribose) Metabolism
   Poly(ADP-ribosylation) in the Recovery of Mammalian Cells from DNA Damage
   Poly(ADP-ribose) Metabolism in Human Disease
   Abnormal Poly(ADP-ribose) Metabolism in SLE
   Research Prospectus

II. Materials ............................................................ 25

III. Methods ............................................................ 27
    Isolation of Peripheral Blood Lymphocytes
    Poly(ADP-ribose) Polymerase Assay in Lymphocytes
    Poly(ADP-ribose) Glycohydrolase Assay
    Characterization of Polymer Length and Degree of Branch Complexity in Poly(ADP-ribose)
    Synthesized by Lymphocytes In vitro
    Preparation of Samples for Quantification of Poly(ADP-ribose) Polymerase by Western Blot Analysis
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV. Results</td>
<td>33</td>
</tr>
<tr>
<td>Development of Poly(ADP-ribose) Polymerase Assay in Isolated Lymphocytes Analysis of an SLE Population for Poly(ADP-ribose) Accumulation in Isolated Lymphocytes Investigation of Possible Mechanisms of Decreased Accumulation of Poly(ADP-ribose) in SLE Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>V. Discussion</td>
<td>76</td>
</tr>
<tr>
<td>VI. Bibliography</td>
<td>93</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>I. Poly(ADP-ribose) Accumulation in SLE and Control lymphocytes</td>
<td>52</td>
</tr>
<tr>
<td>II. Stability of NAD in Poly(ADP-ribose) Polymerase Assay</td>
<td>62</td>
</tr>
<tr>
<td>III. Poly(ADP-ribose) Glycohydrolase Activity in Lymphocytes of Control and SLE Subjects</td>
<td>67</td>
</tr>
<tr>
<td>IV. Characterization of Poly(ADP-ribose) Polymerase in Lymphocytes from Control and SLE Subjects</td>
<td>71</td>
</tr>
<tr>
<td>V. Analysis of Activity and Amount of Poly(ADP-ribose) Polymerase in Lymphocytes</td>
<td>75</td>
</tr>
<tr>
<td>VI. Comparison of Assay Conditions for Poly(ADP-ribose) Polymerase</td>
<td>78</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Structure of poly(ADP-ribose)</td>
<td>4</td>
</tr>
<tr>
<td>2. Poly(ADP-ribose) metabolism cycle</td>
<td>6</td>
</tr>
<tr>
<td>3. Size distribution of freshly isolated lymphocytes</td>
<td>35</td>
</tr>
<tr>
<td>4. Size distribution of lymphocytes after 40 minutes of hypotonic shock</td>
<td>37</td>
</tr>
<tr>
<td>5. Effect of time in hypotonic shock buffer on size of lymphocytes</td>
<td>40</td>
</tr>
<tr>
<td>6. Effect of NAD concentration on accumulation of poly(ADP-ribose) in SLE and control lymphocytes</td>
<td>43</td>
</tr>
<tr>
<td>7. DNAse stimulated poly(ADP-ribose) accumulation in lymphocytes from a control subject</td>
<td>45</td>
</tr>
<tr>
<td>8. Poly(ADP-ribose) accumulation in the presence or absence of DNase</td>
<td>48</td>
</tr>
<tr>
<td>9. Time course of poly(ADP-ribose) accumulation in permeable lymphocytes from control subjects</td>
<td>50</td>
</tr>
<tr>
<td>10. Distribution of poly(ADP-ribose) accumulation in lymphocytes from an SLE and a control population</td>
<td>54</td>
</tr>
<tr>
<td>11. Distribution of poly(ADP-ribose) accumulation in lymphocytes from SLE patients with or without renal disease</td>
<td>57</td>
</tr>
</tbody>
</table>
12. HPLC analyses of radiolabeled nucleotides in the poly(ADP-ribose) polymerase assay................. 61
13. Analysis of the turnover of ADP-ribose polymers..... 65
14. Lineweaver-Burke analyses of poly(ADP-ribose) polymerase activity.............................. 70
15. The distribution of linear polymer chain lengths and of branched polymers in poly(ADP-ribose).... 73
CHAPTER I

INTRODUCTION

In recent years, several lines of evidence have suggested that patients with systemic lupus erythematosus (SLE) have altered poly(ADP-ribose) metabolism. The study presented here addresses the mechanism of altered poly(ADP-ribose) metabolism in SLE patients. Therefore this chapter reviews poly(ADP-ribose) metabolism and its relationship to SLE.

Poly(ADP-ribose) Metabolism

Poly(ADP-ribose) is a homopolymer synthesized from NAD$^+$ by the enzyme poly(ADP-ribose) polymerase (1). This enzyme transfers the ADP-ribose moiety from NAD to a protein acceptor or an existing (ADP-ribose) chain. ADP-ribose polymer was first described by Mandel's lab in 1963 (2). They found the incorporation of radiolabel from the adenine moiety of ATP into an acid-insoluble product when hen liver nuclei were incubated with nicotinamide mononucleotide. When this product was digested with snake venom phosphodiesterase (SVPD), three products were obtained: 1) 5'-AMP, 2) ribose 5'-phosphate bound to a protein acceptor, and 3) a nucleotide
Figure 1. Structure of poly(ADP-ribose).
and two molecules of phosphate (2). Shortly after, the structure of the homopolymer, termed poly(ADP-ribose) was elucidated by the same group (3). In contrast to DNA or RNA, this polymer has a ribose (1" - 2') ribose-phosphate-phosphate backbone as shown in Figure 1. The anomeric carbon of one ADP-ribose molecule is bound to the adenosine moiety of the next one via a 1"- 2' glycosidic linkage (4). A branching structure is formed via a ribose(1''' - 2") ribose(1"- 2')ribose linkage (5). The ADP-ribose polymer found in eukaryotic cells exhibits a wide range of sizes, from a few to over 250 residues (6,7). Polymers generated in vivo include both small polymers with low branching and long multibranched polymers (7).

Poly(ADP-ribose) polymerase is found in most eukaryotes. Exceptions of this are mammalian erythrocytes, mature granulocytes in peripheral blood and human myelocytes and thrombocytes (8). There are also reports of poly(ADP-ribose) polymerase activity in nuclei of plants and lower eukaryotes (9). No activity has been found so far in prokaryotic organisms.

An overview of poly(ADP-ribose) metabolism is shown in Figure 2. Poly(ADP-ribose) synthesis proceeds in three steps (10), namely initiation, elongation and branching of the polymer. The initiation reaction transfers an ADP-ribosyl moiety from NAD⁺ to the carboxylate group of glutamate or aspartate in an acceptor protein. The elongation reaction
Figure 2. Poly(ADP-ribose) metabolism cycle.
involves the sequential addition of ADP-ribose residues to the 2' hydroxyl group of the adenine-proximal ribose of the first residue. The branching reaction introduces an ADP-ribose residue which branches off from a linear portion of the polymer. It is generally accepted that all these reactions are catalyzed by poly(ADP-ribose) polymerase.

Poly(ADP-ribose) polymerase has also been called poly(ADP-ribose) synthetase or poly(ADP-ribose) transferase. It has been purified from various tissues (9). The size of the isolated enzyme from calf thymus is 120,000 dal and consists of three functionally different domains: the Mr 46,000 fragment for binding of DNA, the Mr 22,000 fragment for accepting poly(ADP-ribose) and the Mr 54,000 fragment for binding of the substrate (11,12). By energy dispersive X-ray fluorescence, calf thymus poly(ADP-ribose) polymerase has been found to bind two zinc ions per enzyme molecule (13). The zinc binding sites are localized in the DNA binding domain (13). It was estimated that the zinc fingers are involved in the recognition of DNA strand breaks and therefore in enzyme activation (13). This DNA binding domain also contains a nuclear location signal which targets proteins to the nucleus (14). Poly(ADP-ribose) can serve as an acceptor of ADP-ribose polymers (15) and this automodification can affect the kinetic properties of the enzyme, such as an increase in the Km for NAD and a lowering of Vmax has been observed (16). The DNA sequences of
poly(ADP-ribose) polymerase from bovine thymus, mouse, chicken and human have been cloned (17-21). Additionally, chromosomal mapping has localized a poly(ADP-ribose) polymerase hybridizing sequence on human chromosome 1 (the active gene) and on chromosomes 13 and 14 (processed pseudogenes) (18).

The activity of poly(ADP-ribose) polymerase is dependent on DNA and is stimulated by Mg$^{2+}$ and polycations, such as histones or polyamines (1). Berger and Petzold (22) showed that the enzyme can be activated with short, double-stranded DNA fragments such as the octamer d(GGAATTCC), which stimulated the enzyme even more than highly polymeric calf thymus DNA. This octamer has been used for poly(ADP-ribose) polymerase activity assays in vitro (23).

Inhibitors of poly(ADP-ribose) polymerase have become important tools in the study of the biological function of ADP-ribosylation reactions in intact cells. Purnell and Whish showed that substituted benzamides are potent inhibitors with Ki values of less than 2 μM (24). The studies of Sims et al. with benzamides and other inhibitors demonstrated that the aromatic nitrogen is not essential to effectively inhibit, but emphasized the importance of a carboxamide group for potent inhibition (25). The inhibitory constants for the most widely used classes of inhibitors range from 1 to 30 μM (1). Shall's group recently reported several new competitive inhibitors of poly(ADP-ribose)
polymerase including both 3-substituted benzamides and phthalazinone derivatives with $K_i$ values 7 to 24 times lower than 3-aminobenzamide (26).

The metabolism of ADP-ribose polymerase occurs in the nucleus and involves at least two other chromatin associated enzymes in addition to poly(ADP-ribose) polymerase (Figure 3). The hydrolysis of all polymer residues except the protein proximal residue is catalyzed by poly(ADP-ribose) glycohydrolase (10). The action of a protein-ADP-ribosyl lyase removes the protein proximal residue (10). Thus, ADP-ribose polymer metabolism constitutes a system for the reversible modification of chromatin proteins.

Poly(ADP-ribose) glycohydrolase was discovered by Miwa and Sugimura (27). This enzyme hydrolyzes the $(1'\rightarrow 2')$ alpha anomeric glycosidic bond of polymers but not the ribose-ribose bonds of phosphoribosyl AMP and diphospho-diribosyl AMP (28,29). In studying Hela cells, Tanuma et al. found two variants of poly(ADP-ribose) glycohydrolase which differed in molecular weight, pH optimum, $K_m$ and salt requirements for maximal activity. One of them was a tightly bound nuclear glycohydrolase and another was a soluble cytosolic activity (30). An action model of this enzyme has been reported by Hatakeyama et al. (31). Degradation of poly(ADP-ribose) seemed to occur in a biphasic manner. The large poly(ADP-ribose) bound to protein was first degraded rapidly and processed to small polymers. The small polymers and other protein-bound small polymers were degraded slowly. Recently,
Poirier et al. reconstituted a poly(ADP-ribose) turnover system *in vitro*. It consists of highly purified poly(ADP-ribose) polymerase and glycohydrolase in order to stimulate an *in vivo* situation. By developing a mathematical model to describe the kinetics of poly(ADP-ribose), nicotinamide and ADP-ribose accumulation in such a system, they concluded that poly(ADP-ribose) turnover can be considered as a coupled enzyme system in which the poly(ADP-ribose) polymerase serves as a substrate for the glycohydrolase (32).

**Poly(ADP-ribosylation in the Recovery of Mammalian Cells from DNA Damage**

Poly(ADP-ribose) has been proposed to participate in a number of biological functions. The important properties of poly(ADP-ribose) metabolism such as the large size and negative charge of the polymer, the nuclear location of the polymerase, the requirement of DNA strand breaks for the activity of this enzyme, and the association of the enzyme with nucleosomal chromatin structure have suggested that poly(ADP-ribosylation) may be involved in events which require modulation of chromatin structure including gene expression (33), DNA synthesis (34), and DNA repair (10).

The role of poly(ADP-ribose) synthesis in DNA repair is one of the most wildly studied topics in the field of poly(ADP-ribose) metabolism. Numerous experimental
approaches have demonstrated that poly(ADP-ribose) functions in DNA repair. The causal relationship between DNA damage, NAD\(^+\) depletion, and poly(ADP-ribose) biosynthesis was first shown in 1975. Two groups showed that NAD\(^+\) depletion caused by treatment of cells with DNA damaging agents was due to an enhanced utilization of NAD\(^+\) as a substrate for poly(ADP-ribose) biosynthesis (35, 36). Poly(ADP-ribose) polymerase activity depends on the number and type of DNA strand breaks (37). The double stranded restriction fragments with flush ends were more effective on poly(ADP-ribose) polymerase than overlapping ends (37). Xeroderma pigmentosum (XP) cells were found to be defective in the incision step in UV damage repair but not in alkylating agent damage repair (38). When these cells were exposed to UV, neither NAD\(^+\) depletion nor stimulation of poly(ADP-ribose) polymerase was observed (39). However, the alkylating agent N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) did stimulate the conversion of NAD\(^+\) into poly(ADP-ribose) in XP cells (40). These results confirmed that the production of DNA strand breaks is required for stimulation of poly(ADP-ribose) biosynthesis. In 1980 Shall and coworkers used 3-aminobenzamide subsequent to infliction of DNA damage by the alkylating agent dimethyl sulfate, concluded that poly(ADP-ribose) participates in DNA-excision repair by activating DNA ligase II (41). However other reports from several investigators did not support this conclusion. They pointed out that the elevation of DNA strand
break frequency in cells subjected to alkylating agents does not reflect blocked ligation activity but can result from an increased rate of incision (42). Studies of the clonal survival of cells at various stages of recovery from DNA damage showed that cells which were exposed to poly(ADP-ribose) synthesis inhibitors exhibited reduced survival capacity (43). Thus, it is important to consider the effects of these inhibitors on cell cycling which can affect cell survival independent of their actions on repair reactions. Boorstein and Fardee showed that the presence of 3-amino-benzamide following treatment with an alkylating agent caused an arrest in the G2 phase of the cell cycle with a delay in mitosis thereby preventing them from forming colonies (44). Similarly, Jacobson et al. showed that inhibitors such as nicotinamide analogs blocked the recovery of the cell division potential in C3H10T1/2 cells treated with MNNG (45). These experiments showed that following DNA damage poly(ADP-ribosyl)ation is required for normal cell cycle progression.

The role of poly(ADP-ribosyl)ation in strand breakage and ligation required for sister chromatid exchanges (SCE) has also been implicated. A positive correlation between the SCE inducing potential and inhibiting effects of inhibitors of poly(ADP-ribose) polymerase have been found by Oikawa et al. (46). Poly(ADP-ribose) synthesis inhibitors were also synergistic with various monofunctional alkylating agents in increasing SCE frequency (47). A correlation between the ability of 3-aminobenzamide to induce SCE and to delay cell
Cycle progression has been noted (48). Schwartz (49) demonstrated that the potentiation of alkylation-induced SCE frequency by 3-aminobenazamide was mediated at least in part by intracellular loss of NAD, which occurs due to transient membrane damage inflicted by some alkylating agents. However, much needs to be learned about the mechanism of SCE induction and how this relates to the biological role of poly(ADP-ribose).

There is no doubt that poly(ADP-ribose) metabolism is involved in DNA repair, but the precise mechanism is still unclear. One of the major hypotheses suggested by Shall and associates pointed out that poly(ADP-ribosyl)ation is a regulatory mechanism of ligation activity in the repair of alkylating damage in DNA (41). However, subsequent reports did not confirm such a specific role of poly(ADP-ribose) in DNA excision repair (42). Chromatin fractionation studies have shown that a major portion of endogenous polymers fractionate with the nuclear matrix (50). This raises the possibility that ADP-ribose polymer metabolism mediates the association of a damaged region of DNA with nuclear matrix proteins during the course of repair of DNA damage in chromatin. Recently a model involving a poly(ADP-ribose)-protein shuttle in chromatin has been accepted. This model was first suggested by Ebisuzaki and co-workers (51). Their study provided the basis for a shuttle mechanism in which poly(ADP-ribose) polymerase can move on and off DNA by the
action of poly(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase. Althaus summarized this working model as follows (52): "Following incision, poly(ADP-ribose) polymerase translocates from its "resting" position in the nucleosomal core to an adjacent high affinity binding site and becomes activated. At this stage, repair patches are formed and subsequently patch-containing DNA domains become preferentially unfolded by a mechanism involving poly(ADP-ribosyl)ation. Polymerase itself is the critical acceptor. The presence of the automodified polymerase may determine the sites of unfolding in damaged chromatin and coordinate unfolding with the incision reaction. Excision of adducts apparently requires the unfolded configuration. Following adduct removal and ligation, repaired DNA domains refold. The velocity of this unfolding may be regulated at the level of polymer turnover, which has been shown in vivo to depend on the amount of strand break induced polymers. Thus rapid turnover of polymers on poly(ADP-ribose) polymerase under high DNA damage conditions can rapidly restore the DNA binding properties as well as the activity of poly(ADP-ribose) polymerase which then becomes involved in a new cycle of unfolding/refolding."

**Poly(ADP-ribose) Metabolism in Human Diseases**

The study of molecular defects in human disease may provide very valuable insights into the physiological function of macromolecules. Unfortunately, no disease model
is known so far, which would clearly point to a vital role of poly(ADP-ribose) biosynthesis in the normal function and survival of mammals. Nevertheless, abnormalities of poly(ADP-ribose) metabolism have been noted in some human diseases.

Xeroderma pigmentosum (XP) has been a useful disease model to study the mechanism of DNA repair and poly(ADP-ribose) metabolism. Patients with XP are hypersensitive to UV light and epidermal carcinomas frequently develop at young age in individuals with this disease. Approximately 80% of these patients show a defect in the excision repair of pyrimidine dimers in DNA (incision step) (53). In 1980, Berger's group demonstrated the lack of responsiveness of poly(ADP-ribose) polymerase to UV irradiation in lymphocytes from XP patients (38). A normal UV response of the polymerase was obtained following supplementation of permeabilized cells with Micrococcus luteus UV endonuclease (39), suggesting the effect of DNA strand breaks in the activation of poly(ADP-ribose) polymerase in vivo. McCurry and Jacobson observed that fibroblasts from obligate heterozygote relatives of patients with XP show an intermediate type of NAD utilization for poly(ADP-ribose) biosynthesis following UV irradiation (40).

In 1985, Williams and collaborators described an abnormal catabolism of poly(ADP-ribose) in a case with a lysosomal storage disease. They purified a small molecular weight
compound from brain and kidney and elucidated its chemical structure as glutamyl ribose 5-phosphate, one of the known linkage groups of ADP-ribosé with acceptor proteins. Williams proposed that the molecular defect in this patient was a functional defect of ADP-ribosé protein lyase (54, 55). Later the characterization of the enzymology of poly(ADP-ribose) metabolism in this disease was reported by Jacobson et al. They found that cultured cells from this patient exhibited a greatly reduced accumulation of poly(ADP-ribose) in response to N-methyl-N'-nitro-N-nitrosoguanide treatment (56).

Another clue to better understanding of the physiological role of poly(ADP-ribose) may be derived from the demonstration of naturally occurring antibodies against poly(ADP-ribose) in the serum of patients with SLE. More details about this disease will be discussed in the following section.

Abnormal poly(ADP-ribose) metabolism in SLE

Clinical features of SLE. SLE (lupus) is a chronic immunological disorder and predominately a disease of young women, its peak onset being between ages 15 and 40 (57). An individual's potential to develop SLE is dependent on both inherited and environmental factors. Histopathological findings include fibrinoid necrosis and hematoxylin-positive bodies, which may be found in any organ.
The common symptoms of SLE are fatigue and general malaise, a low-grade fever, weight loss, joint pain, skin rashes and lesions, especially the "butterfly" rash across the bridge of the nose and on the checks. Forty percent of SLE patients exhibit photosensitivity (58). The organ involvement of this disease includes central nervous system (CNS) and renal disease. CNS damage in SLE patients can vary from insignificant to disabling (58). Renal disease is seen in half of all SLE cases. There is great variation in the characteristics of the renal histological lesions, in their clinical expression, in the clinical course of the kidney disease and in the pathogenetic mechanism that results in renal damage (59).

For diagnosis of SLE, no one clinical symptom or set of clinical expressions is specific (58). In 1982, American Rheumatism Association revised the criteria for classification of SLE: "a person is said to have SLE if any 4 or more of the 11 criteria are present, serially or simultaneously during any interval of observation" (60). For the treatment of mild cases adequate rest and salicylates, such as aspirin may be the only treatment necessary. For those with major organ system involvement, corticosterone and immuno-suppressive drugs are often implemented (58).

Multiple autoantibodies in the sera of SLE. SLE has been considered a prototypic autoimmune disease characterized by multiple autoantibodies, primarily to cellular constituents. In addition, primary B-cell defects, T-cell defects, and
impaired macrophage - T cell interactions have been demonstrated in SLE patients (61). Antibodies to a variety of nuclear antigens (ANA) including double stranded (ds) DNA, single-stranded (ss) DNA, histones, histone-DNA complexes, polyribonucleotides, both synthetic and naturally occurring, have been reported in SLE (62). As many as 95% of active untreated SLE patients are found to have positive ANA tests by indirect immunofluorescence (59). Levels of anti-DNA antibodies can serve as a guide to disease activity (57). In recent years, anticoagulant, antiphospholipid and anticardiolipid antibodies have become research fronts in lupus. These antibodies occur in a subset of patients often in the absence of any other demonstrable autoantibodies (57). In addition, anti-lymphocyte antibodies were also found in the great majority of SLE sera (63,64). Anti-lymphocyte antibodies have the capacity to alter immunological responses of normal cells in vitro in ways closely analogous to the functional abnormalities characteristic of lymphocytes from patients with SLE (65).

The mechanisms to generate these antibodies in SLE are still unclear. As one handbook (66) notes "at least 3 candidate models were suggested: 1) Autoantibody production is triggered by 'liberated' DNA which by itself is not highly immunogenetic. 2) Autoantibody production is the consequence of idioptypic cross-reactivity for anti-autoantibody production directed against environmental antigens with
autoantigens via use of particular immunoglobin variable regions present in susceptible individuals. 3) Autoantibodies represent the side effects of a network of interactions between isotypes, anti-idiotypes, and normal cellular components in the context of environmental insults."

**Anti-poly(ADP-ribose) antibodies.** In 1975, Kanai and collaborators first demonstrated that the sera of patients with SLE bound poly(ADP-ribose) (67). Later, they proved that the binding of sera of SLE patients with poly(ADP-ribose) was due to immunoglobin G (IgG) and that the binding was specific for poly(ADP-ribose) and for SLE (68). The specificity of antibody was studied in five cases of SLE showing high binding with poly(ADP-ribose) by inhibition experiments. In all the SLE sera, binding was inhibited by an excess amount of unlabeled poly(ADP-ribose) (69).

In 1979, Okolie and Shall confirmed this finding (70). They treated sera from SLE patients with snake venom phosphodiesterase and found a release of poly(ADP-ribose)-binding capacity. Their finding suggested the natural occurrence of poly(ADP-ribose), anti-poly(ADP-ribose) immune complex in SLE patients. In addition, antibodies to poly(ADP-ribose) were found only in SLE and three SLE-like rheumatic diseases. Anti-DNA antibodies, on the other hand, were found not only in the SLE and SLE-like diseases, but also in rheumatoid arthritis and chronic active hepatitis. Therefore, they emphasised the diagnostic value of poly(ADP-
ribose) antibodies, which they found more specifically related to SLE than anti-ss-DNA antibodies.

Morrow et al. examined 41 patients with SLE, 87 control with various diseases and 3 normal subjects. Elevated levels of poly(ADP-ribose) binding were found in 73% of the SLE patients compared with 58% who had elevated ds DNA binding (71). The correlation of an increased level of antibody to poly(ADP-ribose) with disease activity was made in 27 patients. Levels of anti-poly(ADP-ribose) antibodies were shown to correlate with clinical activity better than either anti-ds- or anti-ss-DNA antibodies (71). Clayton et al. reported a similar result (72). Sera from 61 SLE patients and 20 controls were examined. Fifty four percent of sera from patients showed positive in the poly(ADP-ribose) test (72). A population of SLE families was investigated by Shall's group. Thirty percent of the family members of SLE were found to have elevated anti-poly(ADP-ribose) levels (73). Antibodies to poly(ADP-ribose) were also found in drug induced lupus at a 75% positive rate (74).

There are several studies reported that detected and characterized anti-poly(ADP-ribose) antibody in animal models of lupus. In 1974, Kanai et al. first obtained precipitating antibodies to poly(ADP-ribose) in rabbits by injecting poly(ADP-ribose) mixed with methylated bovine serum albumin (MBSA). The antibody induced by the complexes consisted mainly of IgG and did not cross-react with related compounds such as DNA, RNA, and synthetic nucleic acid homopolymers.
The specificity of the antibody, confirmed by inhibition experiments using related compounds, showed that the antibody recognized the specific structure of poly(ADP-ribose) including its tertiary structure. This antibody did not react with ADP-ribose monomer and preferred the longer chain polymers of poly(ADP-ribose) for the antigen-antibody reaction (75). Kanai compared the antibodies to poly(ADP-ribose) in rabbits and patients with SLE. Poly(ADP-ribose) reactive antibodies were produced in rabbits by oligo(ADP-ribosyl)ated histone with two ADP-ribosyl units and by a complex of poly(ADP-ribose) with MBSA (76). Antibody against poly(ADP-ribose) found in 3 patients with SLE showed a similar inhibitory pattern to that of antibody produced in rabbits (76). Kanai and Fujiwara (77) also demonstrated the natural occurrence of antibodies to poly(ADP-ribose) in an animal model of SLE in MRL/MP-Ipr/Ilr mice. These antibodies also bound to ssDNA, suggesting that two antigens showed similar determinants (78). Even monoclonal antibodies produced by repeated cloning of hybridomas for their binding to poly(ADP-ribose) reacted strongly with ssDNA and Z-DNA, but not B-DNA, ssRNA or dsRNA (79). Silbley et al. reported similar findings in another animal model of SLE in NZB/W mice. Interestingly, they were able to raise antibodies of similar DNA cross-reactivity when they prepared monoclonal antibodies from C57bl/6 mice which had been immunized with poly(ADP-ribose). Comparison of specificities of the monoclonal
antibodies from the two groups of mice showed some striking similarities. In particular, three out of 11 antibodies from C57bl/6 mice preferred poly(dT) as judged by a solid phase radioimmunoassay. Similarly, 10 out of 17 antibodies from the NZB/W group showed the same type of specificity pattern. These results demonstrated that anti-DNA antibodies can be induced by poly(ADP-ribose) and that some of the autoimmune DNA-binding antibodies found in SLE may result from exposure to poly(ADP-ribose) (80). Based on these results, Sibley et al. speculated that a primary defect in poly(ADP-ribose) metabolism may cause the formation of anti-DNA antibodies in SLE. Therefore, they investigated the synthesis and degradation of poly(ADP-ribose) in isolated liver nuclei from autoimmune NZB/W mice and four strains of normal mice, compared to normal mice the maximum levels of incorporation of [3H]-NAD into poly(ADP-ribose) were increased about 2 fold in the autoimmune mice. The kinetics of incorporation suggested that this change was due to an increase in the activity of polymerase rather than a decrease in the level of degradative enzyme (81). However, when the same group examined the poly(ADP-ribose) synthesis in lymphocytes from SLE patients, a contrasting result was found. SLE patients showed a 70% decreased in poly(ADP-ribose) accumulation compared to normal controls (82). So far, the relationship between the elevated anti-poly(ADP-ribose) level and altered metabolism of poly(ADP-ribose) in SLE patients is still unclear.
Other abnormal metabolism in SLE. Apart from these abnormal antibodies, abnormal UV-repair response of SLE peripheral lymphocytes to inhibitors of poly(ADP-ribose) polymerase has been noted (83). In contrast to lymphocytes from healthy controls, enhancement of unscheduled DNA synthesis by nicotinamide or 3-aminobenzamide following UV irradiation was not detectable in SLE lymphocytes (83). It was also found that the ability of cultured human lymphocytes to divide in response to Con A after damage by MNU was significantly more impaired in patients with SLE then in healthy donors. The removal rate of O6-methyl guanine from the DNA was much lower than controls (84). Also it was reported that lymphocytes from SLE patients showed elevated frequencies of sister chromatid exchanges (85). The elevated sister chromatid exchange frequency is related to persistence of unrepaired DNA damage (86). It is not clear at this moment whether this observation is related to altered poly(ADP-ribose) metabolism in SLE cells.

Research Prospectus

The evidence described above implicated altered poly(ADP-ribose) metabolism in SLE. The study of Sibley et al. reporting decreased poly(ADP-ribose) synthesis in SLE lymphocytes (82) provided a clue for investigation of the altered metabolism of poly(ADP-ribose) in SLE patients. However, the poly(ADP-ribose) polymerase assay used in that
study (82) employed a substrate concentration 3000 to 5000 fold below the $K_m$. The low substrate concentration limited the assay sensitivity and may also have formed a product not representative of products formed at $K_m$ concentration (87). The aim of this study is to confirm the observation of Sibley et al. with an improved assay and to characterize the mechanism of this defect. An improved assay with increased sensitivity for analysis of poly(ADP-ribose) metabolism in lymphocytes from SLE and normal donors was developed. This was critical for characterizing this metabolism in the limiting volume of blood available from SLE patients. Poly(ADP-ribose) synthesis in peripheral lymphocytes was measured in a SLE population and in age and sex matched controls. In order to characterize the mechanism(s) of defective synthesis of poly(ADP-ribose) in SLE lymphocytes, several factors such as substrate stability, polymer turnover, glycohydrolase activity, $K_m$ app of poly(ADP-ribose) polymerase for NAD and size distribution of polymers were investigated.
CHAPTER II

MATERIALS

Subjects

Fifteen patients with systemic lupus erythematosus (SLE) were randomly selected from among available patients for study in the Texas College of Osteopathic Medicine, Internal Medicine Outpatient Clinic. All patients fulfilled 4 or more of the American Rheumatology Association (1982) revised criteria for SLE. Patients were excluded if they had been treated with a cytotoxic agent within one month. A sex and age matched control group was obtained from healthy volunteers.

Chemicals

The following chemicals were obtained from Sigma Chemical Co.: nicotinamide adenine dinucleotide (NAD), ADP-ribose, benzamide, deoxyribonuclease I (DNase I), albumin serum bovine (BSA), dextran, Ficoll, Tris-(hydroxymethyl)-aminomethane (Tris-base), Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), NP-40, sodium dodecyl sulfate (SDS), Triton X-100, and 2-mercaptoethanol. The following chemicals were purchased from Fisher Scientific: sucrose, disodium ethylenediamine
tetraacetate (EDTA), magnesium chloride, potassium hydroxide, perchloric acid (70%), phosphoric acid (85%) and trichloroacetic acid (TCA). Formic acid (96%), acetic acid and lithium chloride were from Mallinckrodt. Hypaque solution (50%) was purchased from Winthrop Laboratories. Acetone was purchased from Aldrich Chemical Co. Sodium fluoride and guanidine hydrochloride (GuHCl) were purchased from EM Sciences.

Radioisotopes

$^{32}$P-nicotinamide adenine dinucleotide ($^{32}$P-NAD) (specific activity = 30 Ci/mmol) was obtained from NEN Research Products. $^{32}$P-Poly(ADP-ribose) (specific activity = 38 $\mu$Ci/mm mol) was synthesized using $^{32}$P-NAD according to the procedure described by Menard and Poirier (88).

Other Supplies

Glass beads (4 mm diameter) were purchased from Fisher Scientific. Thin-layer plates (0.1 mm PEI cellulose F) were obtained from EM Science. A microbondapak C18 reversed-phase column was obtained from Waters Co. and a Progel$^{TM}$-TSK DEAE-NPR column was obtained from Supelco Inc. Dihydroxyboxyboronyl Bio-Rex 70 (DHB-B) was synthesized by a modification of the procedure described by Wielckens, et al. (89). Scintillation cocktail was purchased from ICN Radiochemicals.
CHAPTER III

METHODS

Isolation of Peripheral Blood Lymphocytes

Blood was collected in vacutainer tubes without anti-coagulant. Six ml of 6% dextran in saline (0.9% NaCl) and an equal volume of blood were swirled over 5 glass beads in a 50 ml Erlenmeyer flask to defibrinate the blood. Eight ml of defibrinated blood solution was placed on top of 5 ml of 6% Ficoll/10% Hypaque solution and centrifuged at 1200 x g for 30 min. The "buffy coat" containing lymphocytes was removed from the interface of the density gradient and diluted with 10 ml of phosphate buffered saline (PBS) (0.15 M NaCl, 0.01 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.2). A 0.1 ml aliquot was taken for cell counting (Coulter Counter model ZBI) and cell size analysis (Coulter Channelizer C1000). The remainder of the cell suspension was centrifuged at 1000 x g for 10 min. The supernatant was aspirated and the lymphocyte pellet was kept on ice for further assay.

Poly(ADP-ribose) Polymerase Assay in Lymphocytes

Lymphocytes were made permeable to NAD by a modification of the procedure of Berger et al. (90). A hypotonic shock
was employed by suspending the lymphocytes in 10 mM Tris-HCl, 1 mM EDTA, 4 mM MgCl$_2$ and 30 mM 2-mercaptoethanol at 2.5 x 10$^6$ cells/ml. The cells were held on ice for 40 min. The poly(ADP-ribose) accumulation assay was initiated by mixing 200 µl of cell suspension containing 0.5 x 10$^6$ cells with 50 µl of reaction mixture (750 µM NAD, 0.25% Triton X-100, 30 mM Tris-HCl, pH 7.8, 36 mM MgCl$_2$, 150 µg DNase and 1 µCi 32P-NAD). The reaction was carried out at 28°C for 10 min and terminated by the addition of 500 µl of 40% TCA (w/v). To facilitate recovery of the TCA pellets, 500 µg of bovine serum albumin was added to each tube. The suspension was chilled on ice for 15 min and centrifuged at 16,000 x g for 10 min. The pellet was dissolved in 97% formic acid and reprecipitated twice. The second precipitation decreased the background count from 2013 ± 1012 cpm to 501 ± 5 cpm. The final TCA pellets were dissolved in 100 µl of 97% formic acid and transferred to 4 ml of scintillation cocktail and counted. All reactions were carried out in duplicate. The data were calculated as specific activity where 1 unit represented pmol of ADP-ribose per million cells per 10 min incubation.

To measure ADP-ribose polymer turnover during the poly(ADP-ribose) polymerase assay, benzamide was added to the reaction to a final concentration of 500 µM after ten min of incubation to inhibit poly(ADP-ribose) polymerase. Net polymer synthesis in the presence of benzamide was measured
for up to 20 min.

For experiments to characterize the $K_m$ app of poly(ADP-ribose) polymerase for NAD, the velocities of poly(ADP-ribose) synthesis were measured at different concentrations of NAD as described above. $K_m$ app was estimated using the reciprocal plot and the Lineweaver-Burk equation below:

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right) \cdot \left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

**Poly(ADP-ribose) Glycohydrolase Assay**

Poly(ADP-ribose) glycohydrolase activity in lymphocytes was assayed by a modification of the procedure described by Jonsson et al. (91) and Menard et al. (88). Lymphocytes prepared as described above were suspended in 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10 mM NaF, 300 mM sucrose, 1 mM 2-mercaptoethanol and 0.1 % NP-40 at 12.5 x 10^6 cells/ml. The cell suspension was sonicated to obtain nuclei. Ten µl of reaction buffer (300 mM potassium phosphate buffer, 60 mM NaF and 60 mM 2-mercaptoethanol) and 10 µl of 32P-labeled poly(ADP-ribose) (60 µM) were prewarmed to 37°C, and 40 µl of cell suspension containing 5 x 10^5 cells was added. The mixture was then incubated for 10 min at 37°C and the reaction was terminated by adding 2 µl of 3% SDS. Three µl aliquots were applied to a thin-layer plate (0.1 mm PEI cellulose) together with unlabeled carrier ADP-ribose. The plates were developed, first in 100% methanol and then in 0.9% acetic acid, 0.3 M LiCl. After drying, the spots at the
origin, which represented the unconverted poly(ADP-ribose), and ADP-ribose spots were localized under UV light, cut, placed in 10 ml of scintillation cocktail, and counted. A unit of activity of poly(ADP-ribose) glycohydrolase was calculated as pmol of $^{32}$P-labeled poly(ADP-ribose) converted to ADP-ribose/10$^6$ cells/10 min.

**Measurement of NAD$^+$ by HPLC**

Reversed-phase HPLC was used to measure the NAD$^+$ in the acid soluble extract from the poly(ADP-ribose) polymerase assay as a function of time. The poly(ADP-ribose) polymerase assay was performed as described above and terminated by the addition of HClO$_4$ at a final concentration of 0.5 M at 0, 5 and 10 min intervals. The acid soluble fraction of HClO$_4$ extraction was neutralized to pH 7.0 with 1 M KOH, 0.33 M K$_2$HPO$_4$. For HPLC analysis, 1 ml of sample was filtered and injected to a Microbondapak C18 reversed-phase column. The nucleotides were separated with a running buffer of 100 mM potassium phosphate, pH 6.0, 0.3% methanol. The fractions were collected and the percentage of counts in NAD was determined by scintillation counting.

**Characterization of Polymer Length and Degree of Branch Complexity in Poly(ADP-ribose) Synthesized by Lymphocytes**

*In vitro*

ADP-ribose polymers were synthesized and precipitated using permeabilized lymphocytes as described above. Both
cell number and $^{32}$P-NAD specific activity were increased ten fold in the assay in order to obtain sufficient material for analysis of size distribution. Trichloroacetic acid (TCA) pellets were dissolved in 1 ml of 0.5 M KOH, 0.05 M EDTA and incubated for 1 hr at 37°C. ADP-ribose polymers were purified by DHB-B chromatography (92). Specifically, the sample was diluted to 12 ml with AAGE-9 buffer (1 M Guanidine-HCl, 250 mM NH$_4$OAc, 0.01 M EDTA, pH 9.0). The pH was adjusted to 9.0 ± 0.2, and the sample was applied to a 1 ml column of DHB-B that had been equilibrated with AAGE-9 buffer. The column was washed with 10 ml of AAGE-9 buffer followed by 10 ml of 1 M NH$_4$HCO$_3$, 0.1 M EDTA, pH 9.0. ADP-ribose polymers were eluted with 5.0 ml of H$_2$O. The purified ADP-ribose polymers were separated using a Progel™-TSK DEAE NPR column (93). A two ml aliquot of the purified polymers was injected and eluted using a nonlinear gradient of 0 to 0.6 M NaCl in a 25 mM Tris-Cl, pH 9.0 buffer. The fractions were collected and counted by scintillation spectroscopy. The percentage of total cpm in each fraction was calculated.

**Preparation of Samples for Quantification of Poly(ADP-ribose) Polymerase by Western Blot Analysis**

Poly(ADP-ribose) polymerase molecules in SLE and control lymphocytes were quantitated by Western blot analysis. A suspension of 2 x $10^6$ lymphocytes were precipitated with 0.5
M HClO₄ and kept on ice for 15 min. The pellet was collected by centrifugation at 14,000 rpm for 10 min and washed twice with 0.5 ml of acetone. The acid precipitable material was dissolved in 200 µl Laemmli buffer (62.5 mM Tris-HCl, pH 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.00125% (Bromophenol blue) and 6 M urea. Samples were frozen and shipped on dry ice for Western blot analysis.

**Statistical Analysis**

Statistical comparisons included the student's t-test and a one-way analysis of variance using Statview software.
CHAPTER IV

RESULTS

Development of Poly(ADP-ribose) Polymerase Assay in Isolated Lymphocytes

Permeabilization of lymphocytes. The method introduced by Berger et al. (99) to generate nucleotide permeable lymphocytes has been widely used in analysis of poly(ADP-ribose) synthesis in vitro. However, this method requires a centrifugation step to recover the nuclei after hypotonic shock. We found it was difficult to obtain a good recovery from centrifugation after the cells were permeabilized. It was also difficult to distribute the nuclei equally to different assay tubes, since the nuclei clumped after centrifugation. We studied the possibility of conducting the hypotonic shock at a cell concentration that did not require centrifugation to concentrate the cells prior to distribution into poly(ADP-ribose) polymerase assay tubes. A coulter counter and channelizer were used to follow the course of the hypotonic shock. A comparison of the size distribution of lymphocytes before and after hypotonic shock treatment is shown in Figures 3 and 4, respectively. Ninety six percent of the particles were observed in the size range of intact
Figure 3. Size distribution of freshly isolated lymphocytes. The peak at channel numbers 40 - 100 represents intact lymphocytes. The peak at Channel numbers 0 - 6 represents the platelets. The peak at channel numbers 10 - 20 represents red blood cells. Ninety six percent of particles were intact lymphocytes.
Figure 4. Size distribution of lymphocytes after 40 minutes treatment of hypotonic shock. Peak at channel number 0 - 20 represents platelets and lysed cells, 99.9% of particles were in this peak. The particles at channel numbers 40 - 100 represents intact lymphocytes. Zero point one percent of particles were in this size range.
lymphocytes at channel numbers 40-100 before hypotonic shock treatment. After 40 min of hypotonic shock, only 1% intact lymphocytes were observed. Experiments were conducted to determine the minimum dilution and time of incubation required to permeabilize the lymphocytes. The results of those studies are shown in Figure 5. The percent of cells appearing as intact lymphocytes decreased as a function of time. At a cell concentration of $2.5 \times 10^6$ cells/ml, 88% of lymphocytes were lysed after 30 min and 99% of cells were lysed after 40 min. When cells were suspended at a concentration of $5 \times 10^6$ cells/ml, cells lysed at a slower rate, 79% of cells at 30 min and 87% of cells at 40 min were lysed. Therefore all lymphocytes for poly(ADP-ribose) polymerase activity assays in this study were permeabilized by incubating with hypotonic buffer for 40 min on ice at a cell concentration of $2.5 \times 10^6$ cells/ml.

**Development of optimal reaction conditions for poly(ADP-ribose) polymerase assay.** Freshly isolated lymphocytes usually have low poly(ADP-ribose) polymerase activity (90) in the absence of DNA damage. Furthermore, the volume of blood available from SLE patients is usually limited and the yield of lymphocytes is often lower than in control subjects. Thus optimizing assay conditions in order to increase the sensitivity was the first objective of this study. The first variable studied was NAD concentration. Experiments were
Figure 5. Effect of time in hypotonic shock buffer on size of lymphocytes. Isolated lymphocytes were incubated in hypotonic buffer on ice. The size distribution of cells at different times were analysed by a Coulter counter and channelizer. Open circles, $2.5 \times 10^6$ cells/ml; closed circles, $5.0 \times 10^6$ cells/ml.
conducted to determine the NAD concentration which resulted in maximal accumulation of poly(ADP-ribose). The effect of varying NAD concentration ranging from 10-300 μM on net formation of ADP-ribose polymers in lymphocytes from two controls and two SLE subjects is shown in Figure 6. Polymer accumulation increased up to a maximum at 150 μM NAD. Poly(ADP-ribose) accumulation was increased 6 fold in controls and 5 fold in SLE subjects when the NAD concentration increased from 10 μM to 150 μM. Therefore, 150 μM NAD was chosen as a standard assay condition.

The second variable studied was DNase concentration. DNase was used to induce a direct and maximal stimulation of poly(ADP-ribose) polymerase. Thus the amount of DNase required to saturate this reaction was determined. Figure 7 shows that the accumulation of poly(ADP-ribose) increased with DNase concentration. The maximal stimulation of poly(ADP-ribose) polymerase activity occurred at 20 μg/ml DNase, which increased poly(ADP-ribose) polymerase activity 10 fold compared to the basal level of activity without DNase. We then assayed the basal levels of poly(ADP-ribose) accumulation in lymphocytes from 3 controls and 3 SLE subjects. It was found that there were very consistently low basal levels of poly(ADP-ribose) accumulation in both groups (25 ± 8 pmol/10 min/million cells in control and 18 ± 9 pmol/10 min/million cells in SLE). We also compared the
Figure 6. The effect of NAD concentration on accumulation of poly(ADP-ribose) in SLE and control lymphocytes. Permeabilized lymphocytes were incubated with different concentrations of NAD in the poly(ADP-ribose) polymerase assay containing 30 µg/ml DNase. Triangles, two different controls; Circles, two different SLE subjects.
Figure 7. DNAse stimulated poly(ADP-ribose) accumulation in lymphocytes from a control subject. Reactions were carried out using the poly(ADP-ribose) polymerase assay containing 150 µM NAD for 10 min.
poly(ADP-ribose) accumulation in control lymphocytes in the presence and absence of DNase. These data are shown in Figure 8. Even though the NAD concentration used was increased to 400 µM, the basal level of poly(ADP-ribose) polymerase activity was unchanged from that obtained using 150 µM NAD. Since the basal level of poly(ADP-ribose) accumulation was consistently low, subsequent assays measured only the DNase stimulated accumulation of poly(ADP-ribose). Thirty µg/ml DNase was used in all subsequent assays.

Using the optimal conditions described above for obtaining permeable cells and the standard NAD and DNase concentrations, we measured the time course of poly(ADP-ribose) accumulation in control lymphocytes. The data are shown in Figure 9. Under these conditions, the net product formation was nearly linear for at least 15 min. Thus, the standard poly(ADP-ribose) polymerase assay in this study was carried out for 10 min with 150 µM NAD and 30 µg/ml DNase in the buffer of Berger et al. (90). These assay conditions yielded maximal activity and thus provided increased sensitivity important for characterizing this metabolism in the limiting number of lymphocytes available from SLE patients.
Figure 8. Poly(ADP-ribose) accumulation in the presence or absence of DNase. Lymphocytes from control subjects were incubated in the poly(ADP-ribose) polymerase assay containing 400 μM NAD. Open circles, plus DNase 30 μg/ml; closed circles, minus DNase.
Figure 9. Time course of poly(ADP-ribose) accumulation in permeable lymphocytes from control subjects. Standard conditions for the poly(ADP-ribose) polymerase reaction were employed; NAD = 150 µM, DNase = 30 µg/ml.
Analysis of an SLE Population for Poly(ADP-ribose)

Accumulation in Isolated Lymphocytes

A population of 15 SLE subjects and 13 age and sex-matched controls were studied using the assay described above. The individual values obtained from poly(ADP-ribose) polymerase assays for this population are shown in Table I. The mean specific activity in the control group was $393 \pm 128$ units (pmol/10 min/million cells). The mean specific activity for the SLE group was $222 \pm 137$ units. A one way analysis of variance (ANOVA) ($0.0001 < p < 0.005$) and a student's unpaired t test ($0.0005 < p < 0.005$) showed the two groups to be significantly different. Some of the SLE and control subjects from each group were assayed on 2 to 3 different occasions. Multiple measurements for a given individual did not vary more than 21% of the mean in the control group. In the SLE group, the subjects with normal range activity (above 280 units) showed percent standard deviations between 2.1% and 13.5%. The subjects with abnormally low activity had higher standard deviations (24% - 126%). One subject's activity measured on two different occasions varied from 61 to 581 units.

Figure 10 shows the distribution of average poly(ADP-ribose) polymerase activity for each subject in the population studied. All controls tested, with one exception, showed a minimum activity of 280 units. However, the
Table I

Poly(ADP-ribose) Accumulation in SLE and Control Lymphocytes

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\(^1\) pmol of ADP-ribose polymerized/10^6 cells/10 min
Figure 10. Distribution of poly(ADP-ribose) accumulation in lymphocytes from an SLE and a control population. All reactions were carried out using standard conditions for the poly(ADP-ribose) polymerase assay. N = 15 for SLE and 13 for controls.
activities in the SLE population were very heterogeneous, ranging from 20 to 440 units. But interestingly, sixty percent of the SLE group demonstrated activities below the minimum value observed in the control group.

A clinical correlation for the decreased ADP-ribose accumulation in SLE patients was sought. Renal disorder is the major organ involvement in SLE patients (58). In this study, a possible correlation of decreased ADP-ribose accumulation and renal dysfunction in SLE patients was analysed. The SLE patients which met one of following criteria was defined as having renal disease, base on Criteria #7 of the American Rheumatology Association (ARA) (1982) revised criteria for SLE: 1) persistent proteinuria which had greater than 0.5 grams per day or greater than 3+ if quantitation not performed; 2) cellular casts, may be red cell hemoglobin granular, tubular or mixed. We defined patients without renal disease as those whose serum creatinine was < 1 mg/dl, and whose urinalysis demonstrated < 1+ protein and no casts on at least 2 occasions, or whose 24 hr urine protein concentration was < 500 mg. Figure 11 shows a comparison of poly(ADP-ribose) polymerase activities from 4 patients with renal disease and 7 patients without renal dysfunction as defined above. The mean for SLE with renal disease was significantly less than the mean for those without renal disease (149 ± 109 versus 298 ± 101, 0.01 < p < 0.025).
Figure 11. Distribution of poly(ADP-ribose) accumulation in lymphocytes from SLE patients with or without renal disease. n = 4 for SLE with renal disease and 7 for SLE without renal dysfunction.
Investigation of Possible Mechanisms of Decreased Accumulation of Poly(ADP-ribose) in SLE Lymphocytes

Because of the complexity of poly(ADP-ribose) metabolism, several possible mechanisms could account for the diminished accumulation of poly(ADP-ribose) observed in lymphocytes of SLE subjects. According to known requirements in poly(ADP-ribose) metabolism shown in Figure 2, the following possibilities were proposed: 1) decreased available substrate, NAD, due to increased competition by other enzymes in the assay; 2) increased polymer turnover due to elevated poly(ADP-ribose) glycohydrolase activity in the assay; 3) decreased synthesis due to reduced poly(ADP-ribose) glycohydrolase activity in situ; 4) decreased poly(ADP-ribose) polymerase activity.

The first mechanism investigated was the possibility of decreased accumulation of poly(ADP-ribose) in SLE samples due to increased competition by other enzymes for the radio-labelled substrate, $^{32}$P-NAD. NADases are known to be activated by disruption of plasma membranes and can rapidly deplete NAD in cell extracts (10). Furthermore, we observed that platelet contamination was significantly greater in SLE lymphocyte preparations than in control samples. The average purity of lymphocytes from the "buffy coat" in the SLE group was 41% ± 23, range was 9% to 81%. Compared with SLE patients, control subjects had an average purity of 61% ± 23,
range was 33% to 83%. The major contaminates were platelets and red blood cells. Membranes from these contaminating cells could contribute to NADase activity in the assay. In order to test this hypothesis, an SLE sample which was low in both poly(ADP-ribose) polymerase accumulation (80 units) and purity of lymphocytes (12.5%) was chosen for analysis of NAD concentration during the assay. The percentages of radioactivity in the NAD peak at 0, 5 and 10 min for control and SLE samples determined by HPLC analyses are shown in Figure 12 and Table II. These data showed that the concentration of $^{32}$P-NAD was not significantly consumed during the assay in either the control or SLE samples. Thus the observed decreases in poly(ADP-ribose) accumulation were not due to increased completion for NAD by other NAD consuming enzymes.

Poly(ADP-ribose) metabolism is a dynamic system in which the synthesis of ADP-ribose polymers catalyzed by poly(ADP-ribose) polymerase is in concert with the degradation of these polymers catalyzed by poly(ADP-ribose) glycohydrolase (10). The accumulation of poly(ADP-ribose) as measured in this study determined the net synthesis of poly(ADP-ribose). Therefore the second possible mechanism which could explain the observed decrease in polymer accumulation in SLE could be an increased turnover of the polymers due to elevated poly-(ADP-ribose) glycohydrolase activity in the assay. This study has examined this possibility by measuring the half
Figure 12. HPLC analyses of radiolabeled nucleotides in the poly(ADP-ribose) polymerase assay. Lymphocytes from control (A) and SLE (B) subjects were assayed. Open circles, 0 min; closed circles, 5 min; triangles, 10 min.
Table II

Stability of NAD in Poly(ADP-ribose) Polymerase Assay

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life of the ADP-ribose polymers in a control and a SLE sample. This was accomplished by adding benzamide to the assay at 10 min of incubation. The final concentration was 500 μM. This compound has been shown to inhibit 99% of poly(ADP-ribose) polymerase activity at this concentration (95). At the time of addition of benzamide to the incubation, 439 pmol of polymers in the control and 124 pmol in the SLE sample had formed. Ten min later, 99% of the polymers in control and 92% in SLE remained. These data are shown in Figure 13. The same analysis was done in 3 control and 3 SLE subjects. It was found that 98% ± 1 of polymers formed in controls and 91% ± 1 in SLE were stable under the conditions described above. These data show that poly(ADP-ribose) glycohydrolase was not active in this assay. Therefore, the differences observed in SLE samples could not be explained by increased poly(ADP-ribose) glycohydrolase activity.

The next possibility examined was that the chromatin of SLE lymphocytes may be more resistant to further modification by ADP-ribose polymers during the in vitro assay than controls. This could result from a decreased turnover of ADP-ribose polymers in situ if poly(ADP-ribose) glycohydrolase activity was lower in SLE lymphocytes than in controls. To test this hypothesis, the poly(ADP-ribose) glycohydrolase activities in extracts of lymphocytes from four control and five SLE subjects were measured. Three SLE
Figure 13. Analysis of the turnover of ADP-ribose polymers. At 10 min of incubation in the poly(ADP-ribose) polymerase assay, benzamide (final concentration = 500 μM) (open circles and triangles) or an equal volume of water (closed circles and triangles) was added to the incubation. The amount of ADP-ribose polymers remaining at various times was determined based on radioactivity. Circles, control; triangles, SLE.
subjects with abnormally low ADP-ribose polymer accumulation and two subjects with normal range values were studied. These data are shown in Table III. The mean poly(ADP-ribose) accumulation in this subset of SLE subjects was 234 ± 180, which was significantly lower than that in controls (414 ± 118 pmol). However, the poly(ADP-ribose) glycohydrolase activity was demonstrated to be the same in control (121 ± 6 units) and in SLE (120 ± 2 units). Thus no significant difference was observed between the control and SLE groups nor was there a significant difference between SLE samples with normal range versus diminished poly(ADP-ribose) accumulation (Table III).

The above studies established that the decreased accumulation of ADP-ribose polymers in SLE was not due to alterations in turnover or availability of substrate, but was the result of decreased ADP-ribose polymer synthesis. Several possible alterations in poly(ADP-ribose) polymerase could account for the decreased poly(ADP-ribose) synthesis. In this study we examined the possibility of 1) an altered affinity of the enzyme for the substrate, NAD; 2) an altered mechanism of elongation and branching of ADP-ribose polymers and 3) a decreased expression of poly(ADP-ribose) polymerase. These studies are described below.

The $K_m$ app for NAD for poly(ADP-ribose) polymerase was analysed in lymphocytes from two SLE patients and two
### Table III

**Poly(ADP-ribose) Glycohydrolase Activity in Lymphocytes of Control and SLE Subjects**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Glycohydrolase Activity(^1)</th>
<th>Poly(ADP-ribose) Accumulation(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>118</td>
<td>426</td>
</tr>
<tr>
<td>2</td>
<td>130</td>
<td>534</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>116</td>
<td>444</td>
</tr>
<tr>
<td>Mean</td>
<td>121 (\pm) 6</td>
<td>414 (\pm) 118</td>
</tr>
<tr>
<td>SLE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>152</td>
<td>440</td>
</tr>
<tr>
<td>2</td>
<td>128</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>106</td>
<td>108</td>
</tr>
<tr>
<td>4</td>
<td>115</td>
<td>420</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>110</td>
</tr>
<tr>
<td>Mean</td>
<td>120 (\pm) 2</td>
<td>234 (\pm) 180</td>
</tr>
</tbody>
</table>

\(^1\) pmol of ADP-ribose/10\(^6\) cells/10 min

\(^2\) pmol of ADP-ribose polymerized/10\(^6\)cells/10 min
controls. Figure 14 shows the plot of reciprocal velocity versus reciprocal substrate concentrations. The $V_{\text{max}}$ values were 825 and 977 pmol for controls and 159 and 78 pmol for SLE subjects and the $K_m$ values were 61 and 63 µM for controls and 56 and 83 µM for SLE subjects. These data showed that the $V_{\text{max}}$ values in the controls were 7.6 fold higher than in SLE but the $K_m$ values were very similar. Table IV summarizes these values.

The product of the poly(ADP-ribose) synthesis was characterized in order to understand the mechanism for decreased synthesis of the polymers. The size and degree of branch complexity of the polymers formed in SLE as well as controls lymphocytes were compared to polymers synthesized in vitro with purified poly(ADP-ribose) polymerase made by Kiehlbauch et al. (93). These data are shown in Figure 15. Using high resolution preparative anion exchange HPLC, poly (ADP-ribose) made in vitro with purified poly(ADP-ribose) polymerase were isolated as individual species and the multibranched polymers were isolated according to size and branching frequency (93). Interestingly, the polymers formed in lymphocytes lacked the most highly branched class of polymers compared to that synthesized by purified polymerase. However, both SLE and control lymphocytes synthesized other sizes of poly(ADP-ribose) in a similar ratio. These experiments showed that the products of the poly(ADP-ribose)
Figure 14. Lineweaver-Burke analyses of poly(ADP-ribose) synthesis. The poly(ADP-ribose) polymerase assays were conducted at 10, 37.5, 75, 150 and 300 µM NAD using standard assay conditions. Triangles, two different controls; circles, two different SLE subjects.
Table IX

Characterization of Poly(ADP-ribose) Polymerase in Lymphocytes from Control and SLE Subjects

<table>
<thead>
<tr>
<th></th>
<th>Specific Activity[^1]</th>
<th>V\text{max}</th>
<th>K\text{m app} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>318</td>
<td>825</td>
<td>61</td>
</tr>
<tr>
<td>Control 2</td>
<td>394</td>
<td>977</td>
<td>63</td>
</tr>
<tr>
<td>SLE 1</td>
<td>60</td>
<td>159</td>
<td>56</td>
</tr>
<tr>
<td>SLE 2</td>
<td>26</td>
<td>78</td>
<td>83</td>
</tr>
</tbody>
</table>

[^1]: pmol of ADP-ribose polymerized/10^6 cells/10 min
Figure 15. The distribution of linear polymer chain lengths and of branched polymers in poly(ADP-ribose) synthesized by purified poly(ADP-ribose) polymerase (A, data from Kiehlbauch et al., 1991) and in lymphocytes (B). Broken line, control; Solid line, SLE. The peaks under the area designed "branched polymers" represent fractions of polymers with increasing frequencies of branching from left to right.
polymerase reaction in SLE, even though it was only 5% of the amount in controls, distributed over similar polymer sizes and contained a similar pattern of branched polymers as controls, suggesting that the mechanism of the enzyme molecules in SLE is not altered, but that fewer active enzyme molecules are present.

Another possibility to explain the decreased poly(ADP-ribose) synthesis in SLE lymphocytes is decreased expression of poly(ADP-ribose) polymerase or increased turnover of the enzyme protein. The quantity of poly(ADP-ribose) polymerase in lymphocytes was assessed in nine SLE and four control subjects by Western blot analyses. A single experiment is shown in Table V. No significant difference between the SLE and control groups in the quantity of enzyme was observed. The average amount of enzyme protein was 18.6 ± 4.6 ng/10⁶ lymphocytes in the control group and 17.0 ± 8.4 ng/10⁶ lymphocytes in SLE. An unpaired t-test and a one way analysis of variance showed that p values are greater than 0.25. However the activity per nanogram of enzyme in SLE patients was significantly lower than that in control (9.8 pmols/ng ± 6.5 compared to 19.5 pmols/ng ± 8.4, 0.01 < p < 0.025). These data suggest a decreased poly(ADP-ribose) polymerase activity in SLE rather than a decreased number of polymerase molecules.
Table X

Analysis of Activity and Amount of Poly(ADP-ribose) Polymerase in Lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SLE</th>
<th>Unpaired t-test</th>
<th>Analysis of Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(ADP-ribose) polymerase</td>
<td>18.6</td>
<td>17.0</td>
<td>0.1 &lt; p &lt; 0.375</td>
<td>p &gt; 0.25</td>
</tr>
<tr>
<td>(ng/106 lymphocytes)</td>
<td>± 4.6</td>
<td>± 8.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity per ng enzyme</td>
<td>19.5</td>
<td>9.8</td>
<td>0.01 &lt; p &lt; 0.025</td>
<td>0.025 &lt; p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>± 8.4</td>
<td>± 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n =</td>
<td>4</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER V

DISCUSSION

The study presented here demonstrated that there were decreased accumulations of poly(ADP-ribose) in lymphocytes of SLE patients. Sixty percent of SLE subjects showed polymer accumulations below the range of control values. The mechanism of diminished ADP-ribose polymer accumulation in SLE was due to a decreased synthetic capacity for ADP-ribose polymers rather than alterations in the substrate or in the turnover of ADP-ribose polymers. Characterization of poly(ADP-ribose) polymerase in SLE lymphocytes showed that the $K_m$ for NAD was unaltered and that the size distribution and branch frequency of polymers were normal. The quantity of poly(ADP-ribose) polymerase in SLE and control lymphocytes suggested that the decreased polymer synthesis in SLE was due to decreased activity of poly(ADP-ribose) polymerase rather than a reduced number of polymerase molecules.

We have developed an assay for assessing poly(ADP-ribose) synthesis in isolated lymphocytes of SLE subjects. Poly(ADP-ribose) polymerase activity in freshly isolated lymphocytes is difficult to detect because of the low activity (90). In addition, because of the limited volume of blood and low
yield of lymphocytes from SLE patients, the number of cells available for each assay is also limited. Therefore, establishing a sensitive assay for measuring poly(ADP-ribose) polymerase activity in SLE lymphocytes was the first priority in this study. A technique to render cells permeable to nucleotides was developed in 1976 by Berger and associates (94) and was used to analyse the poly(ADP-ribose) polymerase activity in mouse L cells (96,97) and human lymphocytes (90). We modified Berger's assay in order to increase the sensitivity. Table VI compares the assay conditions of Berger with our assay. We used a similar buffer, but we optimized the NAD and DNase concentrations (which will be discussed later in this chapter). Several other modifications were made. The first modification concerned the method of hypotonic shock. In Berger's experiments, the length of hypotonic shock was 15 min (97), which they reported made all cells permeable as determined by uptake of trypan blue. Light microscopic examination showed that most of the cells were morphologically intact. In our assay, the time of hypotonic shock was increased from 15 min to 40 min since this was the time require to allow 99.9% of the cells to lyse as determined by size analysis. This modification assured access of the radiolabeled substrate, NAD, to the nuclear enzyme in the entire population. Secondly, since cells were not washed free of hypotonic buffer in our assay
Table VI

Comparison of Assay Conditions for Poly(ADP-ribose) Polymerase Activity

<table>
<thead>
<tr>
<th>Assay Condition</th>
<th>Berger Assay</th>
<th>This assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonic shock</td>
<td>15 min</td>
<td>40 min</td>
</tr>
<tr>
<td>Cell concentration</td>
<td>$1.7 \times 10^7$/ml (5 x 10^6/assay)</td>
<td>$2 \times 10^6$/ml (5 x 10^5/assay)</td>
</tr>
<tr>
<td>NAD, specific activity</td>
<td>$^3$H-NAD (2 x 10^4 dpm/nmol)</td>
<td>$^32$P-NAD (6 x 10^4 dpm/nmol)</td>
</tr>
<tr>
<td>NAD concentration</td>
<td>330 $\mu$M</td>
<td>150 $\mu$M</td>
</tr>
<tr>
<td>DNase</td>
<td>333 $\mu$g/ml</td>
<td>30 $\mu$g/ml</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.05%</td>
<td>0.05%</td>
</tr>
<tr>
<td>Tris-Cl, pH 7.8</td>
<td>33 mM</td>
<td>32 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>40 mM</td>
<td>32 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>0</td>
<td>0.8 mM</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>0</td>
<td>24 mM</td>
</tr>
</tbody>
</table>
as was the case for the Berger assay, the reaction contained 0.8 mM EDTA and 24 mM 2-mercaptoethanol. The presence of reducing agent has been shown to maintain the poly(ADP-ribose) polymerase in a more active state (98). Thirdly, in order to analyse the poly(ADP-ribose) polymerase activity in a small number of lymphocytes from SLE patients, the number of cells used in each reaction in our assay was ten times less than that in Berger's assay. Fourth, DNase concentration used in the assay were decreased from 330 μg/ml to 30 μg/ml, since it was reported that commercial preparations of DNase were contaminated with proteinase activities that degrade poly(ADP-ribose) polymerase in a dose-dependent manner (99). Compared to Berger's assay, our assay yielded 30 fold higher poly(ADP-ribose) polymerase activity in normal control lymphocytes. In Berger's assay, the maximal DNase (330 μg/ml) responsive poly(ADP-ribose) synthesis in freshly isolated normal lymphocytes was 17 pmol per 10^6 cells per 10 min. But in our assay, 30 μg/ml of DNase stimulated a maximal synthesis in control lymphocytes of 500 pmol poly(ADP-ribose) per 10^6 cells per 10 min. Therefore, the high dose of DNase used in Berger's assay may degrade poly(ADP-ribose) polymerase and decrease the enzyme activity in the assay in vitro.

The study of Sibley et al. (82) used an assay for poly(ADP-ribose) polymerase activity with a substrate
concentration of 0.03 μM NAD, which was 3000 to 5000 fold below the reported $K_m$ (98). The low substrate concentration allowed the use of NAD at a much higher specific activity but did not assay maximal velocity of the reaction and may also have formed a product not representative of products formed at $K_m$ concentrations with regard to size of polymers. It was reported (87) that purified poly(ADP-ribose) polymerase from calf thymus and rat liver synthesized unstable mono-ADP-ribose protein adducts ("initiator intermediates") when incubated with 100 nM or lower NAD concentrations. But at 100 μM NAD, a normal kinetics of polymer formation took place. Thus we re-examined poly(ADP-ribose) synthesis in lymphocytes of two SLE and two control subjects using improved assay conditions. We found the sensitivity of the assay for poly(ADP-ribose) polymerase activity was increased approximately 6 fold in both control and SLE samples by increasing NAD concentration from 10 μM to 150 μM. By measuring the poly(ADP-ribose) accumulation as a function of NAD concentration, we showed that the maximal reaction rate was achieved with 150 μM NAD. We chose this concentration to use in our subsequent assay because it allowed a higher specific radioactivity than that of Berger. Further it represents the approximate $K_m$ concentration of poly(ADP-ribose) polymerase purified from lymphoma (98).

Stimulation of poly(ADP-ribose) synthesis by DNase was first reported by Miller in Hela cell nuclei (39) and later
by Berger in L cells (96) and human lymphocytes (97). Berger et al. demonstrated that addition of 0.05% Triton X-100 to permeable cells allowed proteins such as DNase rapid and direct access to the DNA in the cell nuclei, and resulted in prompt degradation of cell DNA (90). The increase in poly(ADP-ribose) synthesis by adding DNase was due to the production of DNA strand breaks (10). It provided a useful technique to measure the stimulation of poly(ADP-ribose) synthesis. It is believed that the assay for DNase responsive poly(ADP-ribose) polymerase activity measures the total available enzyme in the cell; whereas the assay of the basal activity measures the physiologic activity of the enzyme at the time that the cells were permeabilized (96). In our assay, the addition of 30 µg/ml DNase in the assay increased by 10 fold the poly(ADP-ribose) accumulation in normal lymphocytes. This was similar to an other report which showed a 13 fold increase in the activity upon DNase stimulation (96). We also demonstrated that the basal level of poly(ADP-ribose) polymerase activity in isolated lymphocytes was very low and consistent in both control and SLE subjects (25 ± 8 pmol in control and 18 ± 9 in SLE). These findings were similar to the observations of Berger (90). Sibley's experiments (82) did not demonstrate a stimulatory effect of DNase on poly(ADP-ribose) polymerase. We believe it was because the sonication of the cells prior to addition of DNase resulted in a maximal number of DNA
strand breaks.

The study of 15 SLE and 13 controls using the improved assay for poly(ADP-ribose) accumulation described above demonstrated that the diminished accumulation of poly(ADP-ribose) in lymphocytes occurred in 60% of SLE patients (Table I). This result was consistent with the observation of Sibley et al. that the mean activity of an SLE population was decreased compared to controls (82). Sibley's study measured 18 patients with SLE. While the data was not shown for each individual in the study, the average activity of the population was lower compared to that reported here. The poly(ADP-ribose) accumulation in SLE was 30% of that in controls, and the percent standard deviation was less than 20. In the population studied here, a more heterogeneous population response was observed. Even though the mean accumulation in SLE lymphocytes was significantly lower than that of controls (56% of control), forty percent of the patients showed values which overlapped the normal range of poly (ADP-ribose) accumulation. The percent standard deviation from the mean was 62. One of the explanations for this difference between Sibley's study and this one could be in the different assay conditions employed. As discussed earlier, the low NAD concentration used in Sibley's assay may have resulted in ADP-ribose polymers which were abnormal in size. Another possible difference between the observation of Sibley's and ours may be due to the way in which the data
were presented. The activity of poly(ADP-ribose) polymerase was expressed as "a percentage of the maximum level of incorporation by the control samples". It is not clear why this method was chosen. In our assay, pmol of poly(ADP-ribose) formed in each sample was calculated, which subtracted the background and expressed the actual accumulation of poly(ADP-ribose). It is also possible that there was a difference in the SLE populations studied, since these studies took place in two very different geographical areas and climates.

Several possible mechanisms could account for the diminished accumulation of poly(ADP-ribose) observed in SLE lymphocytes. Sibley's study (82) addressed two potential explanations. First, the possibility that an inhibitor of poly(ADP-ribose) activity could be present in SLE lymphocytes was investigated. Control and SLE lymphocytes were mixed in varying ratios. The polymerase activity increased only in proportion to the number of normal lymphocytes added. These data suggested that the presence of an inhibitor in SLE lymphocytes is unlikely. Secondly, the possibility of decreased initiation sites for new poly(ADP-ribose) synthesis was examined. The addition of exogenous histone to the poly(ADP-ribose) assay did not increase synthesis. These data suggest that it is not potential sites of synthesis that are limiting.

In this study, we investigated further the possible
mechanisms of altered poly(ADP-ribose) metabolism. We proposed the following possible mechanisms for the diminished poly(ADP-ribose) accumulation in SLE lymphocytes:

1. Increased competition for NAD by other enzymes;
2. Decreased synthesis due to reduced poly(ADP-ribose) glycohydrolase activity in situ;
3. Increased turnover due to elevated poly(ADP-ribose) glycohydrolase activity in the assay;
4. Altered $K_m$ app of poly(ADP-ribose) polymerase for NAD;
5. Altered elongation or branching of ADP-ribose polymers;
6. Decreased number of poly(ADP-ribose) polymerase molecules.

We have conducted a series of experiments to investigate these possible mechanisms. One possible explanation for the difference in poly(ADP-ribose) accumulation in SLE and control lymphocytes could be due to different intracellular NAD concentrations. However, the NAD concentration in normal lymphocytes was reported to be 100 pmol/10$^6$ lymphocytes (100). Since the poly(ADP-ribose) synthesis reaction contained 5 x 10$^5$ cells, the lymphocytes could have contributed approx. 50 pmol of NAD to the incubation mixture. The final concentration of exogenous NAD in the reaction mixture was 150 μM, a total of 37.5 nmol in 250 μl. Thus the 50 pmol of NAD in 5 x 10$^5$ cells contributed less than 0.13% of the 37.5 nmol of NAD in the reaction mixture. This would not be expected to have any significant effect on the
incorporation of the exogenously supplied $^{32}$P-NAD. Therefore the diminished synthesis of poly(ADP-ribose) can not be explained by decreased intracellular NAD in SLE lymphocytes. However, degradation of exogenous radiolabeled NAD could have decreased the available substrate for poly(ADP-ribose) synthesis and might account for the diminished accumulation of poly(ADP-ribose) (possible mechanism #1). This possibility was excluded by our results which showed that the concentration of NAD during the poly(ADP-ribose) accumulation assay did not change and that the distribution of radioactivity in SLE and control samples was the same (Table II and Figure 12). Berger et al. also demonstrated that 93% of the remaining substrate was still in the form of $^3$H-NAD in a 30 minute incubation of the poly(ADP-ribose) polymerase assay (96). Our results indicated that there was no significant contaminating NADase activity in the poly(ADP-ribose) synthesis assay in either SLE or control samples.

Eukaryotic cells contain a poly(ADP-ribose) glycohydrolase capable of degrading ADP-ribose polymers (27). Poly(ADP-ribose) polymerase and glycohydrolase are a coupled enzyme system (32). The poly(ADP-ribose) glycohydrolase activity is necessary for the normal function of poly(ADP-ribose) polymerase (32). A decreased glycohydrolase activity in vivo may reduce the number of available sites on nuclear proteins for modification by poly(ADP-ribose) (possible mechanism #2). To test this hypothesis, the activity of
poly(ADP-ribose) glycohydrolase was examined in control and SLE lymphocytes. Our data (Table III) demonstrated that the poly(ADP-ribose) glycohydrolase activity was unaltered in SLE lymphocytes relative to controls.

Poly(ADP-ribose) accumulation in this study was a measure of the net synthesis and degradation of these polymers. Either a decrease in activity of poly(ADP-ribose) polymerase or an increase in activity of poly(ADP-ribose) glycohydrolase could produce a diminished accumulation of poly(ADP-ribose) in SLE lymphocytes (possible mechanism #3). In this study, we examined the turnover of poly(ADP-ribose) during the assay. When 500 μM benzamide was added to the reaction mixture, no new polymer synthesis occurred in either SLE or control lymphocytes, nor was the existing polymer degraded (Figure 13). These data showed that our assay for poly(ADP-ribose) accumulation did not possess a functional polymer turnover system. A similar situation was also reported by Benjamin (101) in the SV40 transformed Balb/c3T3 fibroblast cells and by Berger (96) in mouse L cell. Berger's study proposed an explanation for the inactive glycohydrolase in the poly(ADP-ribose) polymerase assay. In their experiments, the cells were allowed to synthesize poly(ADP-ribose) for 30 min at 30°C. When a portion of cells were washed free of the poly(ADP-ribose) synthesis mix and resuspended in buffer, the poly(ADP-ribose) was rapidly degraded. However, when another portion of cells were resuspended in buffer containing 330 μM
non-radioactive NAD, there was essentially no degradation of the poly(ADP-ribose). This experiment argued that NAD at this concentration completely inhibited the poly(ADP-ribose) glycohydrolase activity. Considering the fact that intracellular NAD concentration was reported to be about 300 µM (102), the NAD concentration in Berger's assay was approximately the same as the intracellular concentration. It is not clear why poly(ADP-ribose) glycohydrolase would be inhibited by 330 µM NAD in the permeable cell assay, but be active under similar NAD concentrations in situ.

The above studies established that the differences in the accumulation of poly(ADP-ribose) between the control and SLE lymphocytes were due to differences in poly(ADP-ribose) polymerase activity and not due to alterations in turnover or availability of substrate. Considering the three functional domains of poly(ADP-ribose) polymerase: NAD binding domain, DNA binding domain and automodified domain (9), the abnormal activity of poly(ADP-ribose) polymerase could be caused by an alteration in any of these functional domains. One domain dysfunction can interfere with another domain's function (9). It was found that altered binding of poly(ADP-ribose) polymerase to DNA can effect the enzyme Kₘ for NAD (9). Therefore the mechanism of decreased poly(ADP-ribose) polymerase activity is complicated. In this study, we examined the Kₘ app for poly(ADP-ribose) polymerase for NAD in
SLE and control lymphocytes (possible mechanism #4). Lineweaver-Burke analyses showed that the \( V_{\text{max}} \) of the enzyme in two SLE lymphocyte populations were only 9% and 18% of controls, but the \( K_m \) app values were demonstrated to be similar (66 \( \mu \text{M} \pm 12 \) vs 62 \( \mu \text{M} \pm 1 \)) (Table IV). The \( K_m \) of poly(ADP-ribose) polymerase for NAD was reported to be approximately 100 \( \mu \text{M} \) in SV40 Balb/c 3T3 fibroblasts (102) and 180 \( \mu \text{M} \) for an enzyme purified from human lymphoma (98). Most purified poly(ADP-ribose) polymerases exhibited a \( K_m \) for NAD around 50 ± 30 \( \mu \text{M} \) and differed depending on the cell source, the kind and amount of DNA and histone in the reaction (9).

The initiation, elongation and branching of ADP-ribose polymers catalyzed by poly(ADP-ribose) polymerase are important components of the enzyme activity. In this study, we characterized the product of the reaction by analysis of the length and branch complexity of ADP-ribose polymers formed in SLE and control lymphocytes (possible mechanism #5). Even though the SLE sample had only 5% of the amount of poly(ADP-ribose), the size distribution of linear and branched polymers was the same as in controls (Figure 15). This experiment suggested that the diminished synthesis of poly(ADP-ribose) was not due to a specific defect in elongation or branching of polymers.

Another possible explanation for fewer active poly(ADP-ribose) polymerase molecules in SLE is a decreased expression of the enzyme or an increased rate of turnover of the protein
which could lead to a decreased number of enzyme molecules (possible mechanism #6). Proteolytic degradation of poly(ADP-ribose) polymerase in permeabilized lymphocytes was noted by Berger and associates (103). In the process of poly(ADP-ribosylation) of the polymerase itself, a number of polypeptides with relative molecular masses of 96,000, 79,000, and 62,000 were found. The proteolysis was activated by exogenously added diadenosine 5',5'''-P1,P4- tetraphosphate (Ap4A) and other nucleotides containing pyrophosphate bonds (103). Any factor which increased proteolytic digestion of poly(ADP-ribose) polymerase would decrease the number of the active enzyme molecules. The preliminary data obtained in this study did not support this hypothesis, because the quantity of poly(ADP-ribose) polymerase in SLE lymphocytes migrating at the appropriate molecular mass on gels used for Western blot analysis was not significantly lower than that in controls. It is also possible that the SLE lymphocytes contained a normal number of enzyme molecules, but altered posttranslational modification. It was demonstrated that the poly(ADP-ribosylation) on poly(ADP-ribose) polymerase itself can lower the enzyme activity (16). If the extent of automodification of poly(ADP-ribose) polymerase in SLE lymphocytes were higher than that in controls, it could lead to lower activity. But since normal glycohydrolase activity in SLE lymphocytes was demonstrated in this study, this possibility seems unlikely.
Other modifications like mono-ADP-ribosylation on the arginine-specific sites of poly(ADP-ribose) polymerase (104) have been suggested as a modulator of enzyme activity.

In recent years, the effects of oxidative stress on DNA repair, carcinogenesis and poly(ADP-ribose) polymerase activity have been widely studied. Pero et al. found that oxidative stress markedly inhibited the poly(ADP-ribose) polymerase activity in human peripheral mononuclear leukocytes (105). This finding provided an interesting possible explanation for the decreased synthesis of poly(ADP-ribose) in SLE lymphocytes reported in this study. Considering a defective DNA repair has been reported in patients with SLE (83, 84) and SLE patients have a high incidence of cancer (98), it is possible that oxidative stress in SLE lymphocytes may inactivate poly(ADP-ribose) polymerase. The specific effects of oxidative stress that inactivate poly(ADP-ribose) polymerase are unknown. Berger et al. have demonstrated that the essential sulfhydryl groups on the enzyme are critical for the activity of the purified human lymphoid poly(ADP-ribose) polymerase (98). Considering the zinc finger structure of poly(ADP-ribose) polymerase, cysteine is the major residue involved in forming the zinc metal coordination complex important in DNA binding (13). The sulfhydryl group of cysteine is highly reactive. Oxidative stress may inactivate the poly(ADP-ribose) polymerase by oxidizing the thiol residues and damaging the zinc finger structure. This
hypothesis requires further investigation.

Besides the poly(ADP-ribose) polymerase itself, the characteristics of lymphocytes in SLE patients should be included in consideration of the mechanism of diminished poly(ADP-ribose) synthesis in SLE lymphocytes. SLE patients usually have a hyperactive B-cell system and an imbalance of T helper cells and T suppressor cells (65). On the other hand, it was reported that the expression of the poly(ADP-ribose) polymerase gene in B lymphocytes was lower than that in T lymphocytes (106). Thus it is possible that the diminished synthesis of poly(ADP-ribose) synthesis in SLE lymphocytes is due to an imbalance distribution of B and T lymphocytes in SLE patients. Experiments testing this hypothesis are in progress in our laboratory. Furthermore, it was demonstrated that both the mRNA level of poly(ADP-ribose) polymerase and polymerase protein were increased 3- to 10- fold over the 24 to 72 h period after phytohemagglutinin (PHA) stimulation (107). Therefore the difference in proliferation of lymphocytes between SLE and control lymphocytes, if any, could explain the different poly(ADP-ribose) polymerase synthesis in this two groups. This hypothesis is also under investigation by our group.

The relationship of decreased poly(ADP-ribose) polymerase activity to the clinical manifestations of SLE are unclear. In fact, the clinical symptoms of SLE are extremely heterogeneous. It is therefore not surprising that the pattern of
decreased poly(ADP-ribose) synthesis was also found to be heterogeneous in our SLE population. In Sibley's study (82) no correlation between disease activity and poly(ADP-ribose) polymerase activity could be identified. But in our preliminary attempt to identify a clinical correlation to our finding, we observed that four patients with evidence of renal disease demonstrated significantly lower levels of activity than patients without renal disease (Figure 11). Since the number of patients examined in this study was small, the relationship of altered ADP-ribose metabolism to this manifestation is not yet established. But this link warrants further investigation. Decreased poly(ADP-ribose) polymerase activity, like anti-poly(ADP-ribose) antibodies and other anti-nuclear antibodies may one day serve as a marker for major organ system involvement or as an indicator of other disease activity in SLE.


64. Winfield, J. B., Winchester, R. T., and Wernet, P. (1975) *Arthritis Rheum.* 18, 1


