accumulate progressively in selected neurons during aging of the human brain, particularly in senile dementia of the Alzheimer type, are highly insoluble complexes whose bonds are not broken by SDS-BME, 9.5*M* urea, 2 percent Triton X-100, 1 percent NP40, 6*M* guanidine hydrochloride, and 0.2*N* HCl or NaOH. This unexpected finding may explain our earlier report of no differences in the amount of neurofilament proteins or other neuronal cytoskeletal proteins between electrophoretograms of AD cortical fractions rich in neurofibrillary tangles or PHF and electrophoretograms of control fractions (*8*). It suggests that the established gel electrophoretic techniques used in previous attempts to characterize PHF composition did not allow entry into the gels of the principal polypeptides making up the PHF. This observation provides a caveat regarding the use of gel electrophoresis in the search for protein abnormalities in diseased or aged brain tissue.

The finding of the insolubility of PHF after heating in detergents and reducing agents strongly suggests that covalent bonds other than disulfide (*12*) cross-link specific amino acids of the individual filaments into a rigid intracellular polymer. Precedent for this type of cross-linking of structural proteins exists in descriptions of unusual, covalently bound insoluble polymers in human erythrocytes, skin keratinocytes, and senile cataracts (*12, 13*). Our data provide evidence that such high molecular weight, covalently cross-linked proteins can occur in nerve cells. Different protein polymers in senile cataracts, terminally differentiated epidermal cells, and red blood cells are covalently cross-linked by γ-glutamyl-ε-lysine side-chain bridges (*13, 14*). In each case, the presence of ε-(γ-glutamyl)lysine cross-links was postulated on the basis of insolubility in SDS and reducing agents and was subsequently confirmed. These cross-links are believed to be assembled under the catalytic influence of a Ca^{2+}-dependent transglutaminase (*12–14*). Activation of transglutaminase by an increase in cytoplasmic availability of Ca^{2+} and subsequent protein cross-linking may play a general role in some remodeling reactions during cell aging (*13*). We recently demonstrated that transglutaminase is present in the human brain and can cross-link normal human neurofilament proteins into an insoluble high molecular weight filamentous polymer (*15*). On the basis of these precedents and our findings, we propose a testable model for age-related fibrillary degeneration of human neurons that involves cross-linking of neuronal intermediate filaments [perhaps by ε-(γ-glutamyl)lysine bonds formed by transglutaminase] and consequent accumulation of permanent, highly rigid fibrous polymers that may irreversibly alter the dynamics of the neuronal cytoskeleton and the maintenance of axons and dendrites.

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16. We thank C. Abraham for expert technical assistance, W. Hamilton for preparation of sections for electron microscopy, W. Busse for photographic preparation, and K. Kosik and A. Pope for helpful comments. Supported by NIH grant AG02126 and by a teacher-investigator award (D.J.S.) from the National Institute of Neurological and Communicative Disorders and Stroke.

27 October 1981

Autoantibodies to Nucleosomal Proteins: Antibodies to HMG-17 in Autoimmune Diseases

Abstract. *The relative amounts of autoantibodies against defined nucleosomal proteins present in serums from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD) have been examined by an enzyme-linked immunoassay. Autoantibodies to nucleosomal proteins were detected in 45 percent of the patients with SLE, 18 percent of the MCTD patients, and none of the RA patients. The results suggest that, in SLE, antibodies are formed against a subset of nucleosomes which contain protein HMG-17.*

Serums from patients with the clinically distinct systemic rheumatic diseases—systemic lupus erythematosus (SLE), progressive systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis (RA), and mixed connective tissue disease (MCTD)—contain autoantibodies to a variety of nuclear macromolecules. Nuclear molecules recognized as antigens in these autoimmune diseases include single- and double-stranded DNA, RNA, ribonucleoproteins, deoxyribonucleoproteins, nucleosomes, histones, and nonhistone proteins (*1*). One of the central questions in studies of antibodies to nuclear antigens is whether the serological specificities can aid in the clinical diagnoses of systemic rheumatic diseases. Definition of the specificities of the serum antibodies in these patients by accurate, sensitive, and convenient immunoassays could aid in answering the above question.

We have used an enzyme-linked immunoassay (ELISA) (*2*) technique to quantify the relative amounts of antibodies against the four nucleosomal histones, and against nucleosome associated nonhistone chromosomal proteins HMG-1, HMG-2, and HMG-17 in serums from patients with SLE, RA, and MCTD. We find that (i) antibodies to some of these proteins are present in both SLE and MCTD but not in RA. (ii) Antibodies to protein HMG-17 are significantly more prevalent than antibodies to

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HMG-1 and HMG-2. (iii) Among the positive SLE serums there is a definite pattern in the specificity toward the nucleosomal histones. Most of the positive serums displayed strong reactivities toward histones H2A and H2B, whereas only a few displayed much weaker activity against H3 and H4.

All patients studied conformed to diagnostic criteria or description of the diseases (*3*). Antibodies to nuclear material were detected by immunofluorescence of mouse liver and found in all the patients with SLE and MCTD and in 50 percent of the RA patients. Precipitating antibodies to double-stranded DNA were found exclusively in the SLE patients whereas the MCTD patients had high titers of antibodies to a ribonuclease-sensitive, extractable, nuclear antigen. Antibodies to γ-globulins (rheumatoid factors) as detected by the bentonite flocculation test were found in high titers in 11 of the RA patients. Nonhistone chromosomal proteins HMG-1, -2, and -17 and the four nucleosomal histones H2A, H2B, H3, and H4 were purified from calf thymus (*4*). Because chromosomal proteins are conserved during evolution (*5*), it was expected that the calf proteins would be recognized by the human antibodies. The seven chromosomal antigens tested were indeed free of detectable contaminants (Fig. 1, right). The presence of antibodies to the various chromosomal proteins in the serums of the 54 patients tested was detected by ELISA with alkaline phosphatase–labeled goat immunoglobulin G (IgG) to human IgG. The applicability of the ELISA to the study of histones and HMG proteins has been tested with antibody elicited in rabbits (*5*) (Fig 1). The amount of substrate hydrolyzed (detected as absorbance at 405 nm) is dependent on the concentration of both the antigen and the antiserums and was free of nonspecific effects. The assay, which is performed in microtiter plates, is useful at antigen concentrations ranging from 5 μg to 1×10^{-5} μg per well at serum dilutions between 1×10^{-2} to 1×10^{-6}. All human antiserums were diluted 1:400 and tested with 1.0 μg antigen per well. The human IgG bound to the plates was detected by the addition of alkaline phosphatase–labeled goat antiserum to human IgG (diluted 1:500), and the substrate paranitrophenol (1.0 μg/ml). The color (absorbance, 405 nm) which developed after 1 hour at 23°C (Table 1) was measured with a spectrophotometer. Under these conditions the assay is very sensitive with minimal background. Control normal serums or those obtained from nonaffected patients (those without antibodies to the antigens tested) developed an absorbance of 405 nm of 0.01 or less. Antibodies to at least one of the chromosomal proteins tested were present in 15 of the 54 serums examined. The relative amounts of the various antibodies present in the positive serums can be deduced from the absorbancies (Table 1). The strongest (absorbance at 405 > 0.15) and the most prevalent reaction

Table 1. Relative content of autoantibodies to chromosomal proteins in the 15 positive human serums. Microtiter plates were coated with 1.0 μg of antigen in 100 μl of phosphate buffer. The plates were incubated with 1:400 dilutions of the human serums. Alkaline phosphatase–labeled goat antiserum to human IgG (Miles), diluted 1:500, and the substrate *p*-nitrophenol (1.0 mg/ml) were used to detect the human IgG bound.

Patient	Absorbancy at 405 nm when antigen is						
	HMG-17	HMG-1	HMG-2	H2A	H2B	H3	H4
	Systemic lupus erythematosus						
1	0.13	0	0	0.07	0.15	0	0
8	0.06	0.36	0	1.10	0.66	0	0
10	0.11	0	0	0.11	1.01	0	0
16	0.29	0	0	0	0.52	0.03	0.03
18	1.32	0	0	0.10	0.39	0.10	0.46
20	2.5	0	0	2.5	0.75	0.07	0.05
26	0.22	0	0.11	0.42	0.15	0	0
34	0.08	0	0	0.19	0	0	0
42	0	0	0	0.23	0	0	0
44	0.85	0	0	1.41	1.47	0.17	0
46	0	0.11	0.48	0.24	0.05	0	0
52	0.14	0.39	0	0	0.36	0	0.02
53	0	0	0	0	0.13	0	0
	Mixed connective tissue disease						
13	1.62	0	0	0	0.05	0	0
49	0	0	0	0	0.40	0.09	0

Table 2. Correlation between clinical diagnosis and the presence of autoantibodies to nucleosomal proteins.

Clinical diagnosis	Tested (No.)	Positive (No.)	Number of patients with antibodies to						
			HMG-17	HMG-1	HMG-2	H2A	H2B	H3	H4
SLE	29	13	10	3	2	10	11	4	4
MCTD	11	2	1				2	1	0
RA	14	0							

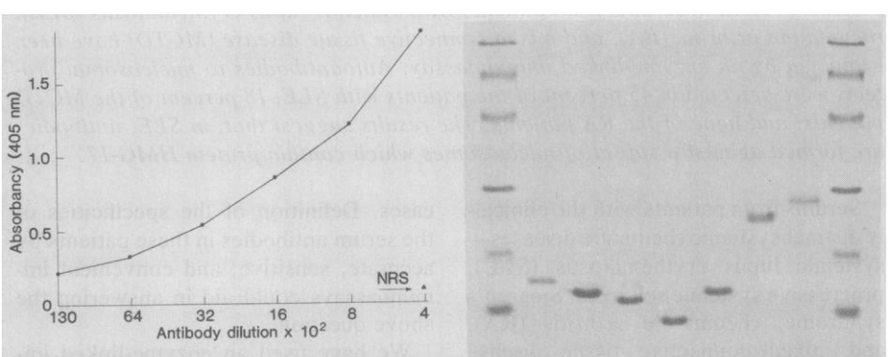

Fig. 1. (Left) Enzyme-linked immunoassay for chromosomal proteins. An example with 50×10^{-8} g per well of chromosomal protein HMG-1 antigen and the indicated serum dilutions. The antigen is added to microtiter plates in 0.01*M* sodium phosphate buffer (*p*H 6.8) and 0.15*M* NaCl. The remaining reactive sites on each well of the microtiter plate are blocked with 1 percent horse serum. Alkaline phosphatase–labeled goat antiserum to rabbit IgG (Miles) diluted 1:600 and *p*-nitrophenol (Sigma) (1.0 mg/ml) are used to determine the amount of rabbit IgG bound. The absorbancy at 405 nm developed at 23°C about 1 hour after substrate addition was measured directly in the microtiter plates with a Titertek Multiscan (Flow); *NRS*, normal rabbit serum. (Right) The purity of the nucleosomal proteins used as antigens is demonstrated by electrophoresis in 18 percent polyacrylamide gels containing 0.1 percent sodium dodecyl sulfate. The columns indicate: *m*, molecular weight markers from top to bottom as follows: phosphorylase, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,000; α-lactalbumin, 14,400. *a*, histone H3; *b*, H2A; *c*, H2B; *d*, H4; *e*, HMG-17; *f*, HMG-2; and *g*, HMG-1.

was observed when the antigen was histone H2B. Only three patients had detectable antibodies to both histones H3 and H4. With one exception (antibody for H4 in patient No. 18) the values were extremely low. Similarly, the response to HMG-1 and HMG-2 was also very infrequent. In contrast, 11 of the 15 patients had antibodies to chromosomal proteins HMG-17.

Grouping of the serums tested according to the clinical diagnosis (Table 2) reveals a correlation between detectable amounts of antibodies to chromosomal proteins and the diagnosis of SLE. None of the serums from the 14 patients with RA contained detectable amounts of antibodies against the seven nuclear proteins screened. Only two of the MCTD patients displayed antibody activity, and of these two, one (No. 13) had a strong response to HMG-17 and the other (No. 49) a moderate response to H2B and a weak response to H3. In contrast, 13 of the 29 SLE patients (45 percent) had antibodies against at least one of the chromosomal proteins tested, and 11 of these 13 displayed multiple specificities. Previous studies revealed that, in chromatin and nucleosomes, antigenic sites of histone H2B are more exposed to antibody binding than antigenic sites of histones H3 and H4 (*6*). Rekvig and Hannestad detected human autoantibodies that react with core mononucleosomes and provided evidence that the antigenic determinant resides in the trypsin-sensitive regions of histones H2B and H2A of the native histone octamer (*7*). Thus one of the immunogens in SLE may be the intact nucleosome. The weak response to HMG-1 and HMG-2, which presumably reside in the linker region between adjacent nucleosomes, further supports this possibility. A significant number of patients showed antibodies to HMG-17, which is found in chromatin in significantly lower amounts than HMG-1 and HMG-2 (*8*). It has been suggested that HMG-17 is associated with nucleosomes in the transcribable regions of the genome (*9*). Since HMG-17 is found only on a subset of the nucleosomes, one of the immunogens in SLE may be a subset of those nucleosomes that contain specialized DNA sequences.

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10. We thank Dr. K. Kraemer for helpful discussions. Request reprints from M.B.

2 September 1981; revised 5 November 1981

DNA Strand Breakage in Human Leukocytes Exposed to a Tumor Promoter, Phorbol Myristate Acetate

Abstract. *The phorbol myristate acetate–stimulated respiratory burst in human peripheral blood leukocytes is associated with extensive DNA strand breakage. Damage to DNA occurs before gross cellular damage is evident and may be related to the action of phorbol myristate acetate as a skin tumor promoter in animals.*

Phagocytic cells constitute a key element in the body's defense against infection (*1, 2*). In addition to the capacity for engulfing particles (phagocytosis), these cells have the capacity to produce potentially toxic active oxygen species (such as O_2^- and H_2O_2) in response to appropriate stimuli, a process termed the respiratory burst (*2, 3*). Active oxygen species kill microbes, but are also potential-

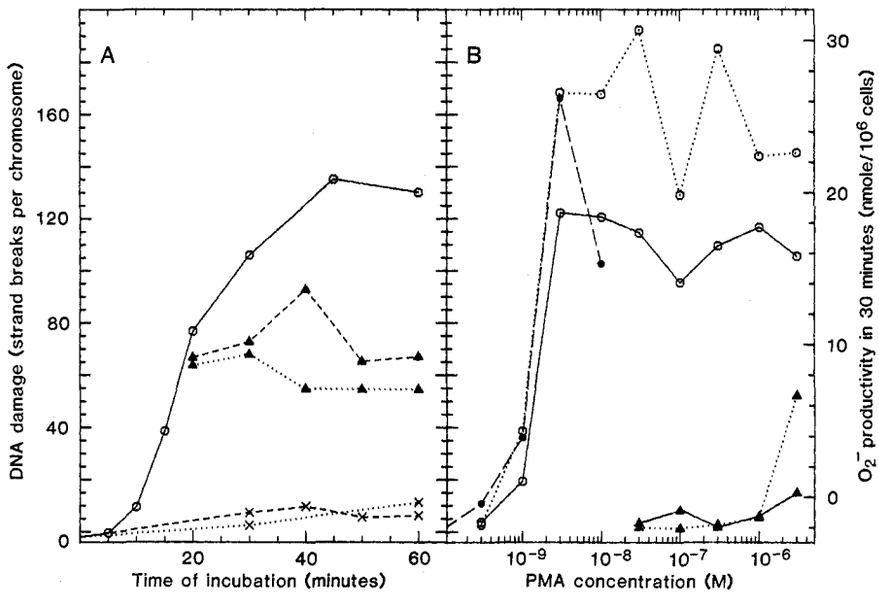

Fig. 1. (A) Time course of DNA damage after addition of PMA and effect of 2-deoxyglucose. Ten milliliters of a suspension of human leukocytes (2×10^6 per milliliter, rich in polymorphonuclear leukocytes) was incubated at 37°C with PMA (10^{-8} *M*). At the indicated times, samples were removed, chilled to 0°C, and analyzed for DNA strand break damage (○). Other samples were treated in a similar fashion, except that 2-deoxyglucose (1 m*M*) was added at 0 time (x) or at 20 minutes (▲), and fructose was substituted for glucose in the incubation medium. DNA strand break damage was determined as described in (*10*). The number of induced strand breaks was calculated by reference to the effect on DNA unwinding rate of ^{60}Co gamma rays and the assumption that 38 mGy causes 46 single-strand DNA breaks per cell or an average of one break per chromosome. (B) DNA damage with (○) PMA and (▲) PMA-ME, and (●) O_2^- production in human leukocytes exposed to PMA. Cells were incubated at 37°C with the indicated concentrations of PMA or PMA-ME for 40 minutes to assess DNA damage as in (A), and for 30 minutes to monitor O_2^- as the superoxide dismutase–inhibitable reduction of cytochrome C. The solid and broken lines indicate results with blood from two different donors; O_2^- production was measured in a third donor.

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