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Immunology

Molecular mimicry: Histone H3 and mycobacterial protein epitopes

(histone-like proteins/Freund's adjuvant/antiserum specificity/amyloid)

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ABSTRACT **A 15-kDa protein detected initially in amyloidotic ileum from a transgenic mouse and subsequently in control (nontransgenic) ileum by various polyclonal rabbit antiserums applied to electroblots of extracts derived from these tissues was identified by partial sequence analysis as histone H3. Antiserums were made against immunogens unrelated to the histone, but they recognized calf thymus histone H3 (14.7 kDa) on Western blots. The bacterial component of the Freund's medium used as an adjuvant for the immunogens was either *Mycobacterium butyricum* or *Mycobacterium smegmatis*. Absorption tests with histone H3 and sonicated *M. butyricum* substantiated the presence of anti-histone H3 activity in the antiserums. These findings indicate that the two mycobacterium species make a protein with epitopes perceived as nonself by recipient rabbits but sufficiently similar to epitopes of mammalian histone H3 that the rabbits produced antibodies cross-reactive with the histone.**

The concept of molecular mimicry has gradually become disseminated among workers in the field of molecular biology since the term was introduced in 1968 (1), but the potential for such mimicry may not yet be fully appreciated. Here we describe an instance of its unanticipated emergence in an experimental setting.

During investigations undertaken to identify the characteristic protein of an amyloid deposit in the ileum of a transgenic mouse, we found, in extracts derived from control as well as from the amyloidotic ileum, a 15-kDa constituent recognized by several polyclonal antiserums raised against different amyloid proteins or synthetic fragments thereof when the antiserums were applied to electroblots of the tissue extracts. The 15-kDa protein proved to be histone H3 and was found to be reactive with antiserums not expected to have antibodies capable of such recognition. The evidence indicating molecular mimicry is presented in the following sections. The amyloid aspect of the work will be described elsewhere.

MATERIALS AND METHODS

Tissue Extraction. Characterization of the 15-kDa protein was done on tissue fractions prepared according to methods that have been used to extract different amyloid proteins from a variety of tissues.

Amyloidotic ileal tissue was removed from transgenic mice, and normal (control) ileal tissue was removed from their nontransgenic sibs; the genotypes of the mice consisted of various admixtures of DBA/2 and C57BL/6 (2). Pieces of the tissue were pulverized in liquid nitrogen; the pulverized tissue was washed by successive suspension/centrifugation (≈100 mg of tissue per 5 ml) in phosphate-buffered saline (PBS) (three times), 15 mM phosphate buffer (pH 8.0), and 10 mM acetic acid (pH 3.1), and then suspended in 6 M urea/10 mM HCOONa/HCOOH, pH 2.9. After overnight stirring at 4°C the suspension was centrifuged for 15 min at 8500 × *g*. The supernate (acid urea extract) and pellet (suspended in a small volume of water) were dialyzed in 3.5-kDa cutoff tubing (Spectrapor no. 3; Spectrum Medical Industries) against 20 mM acetic acid and lyophilized. Part of the lyophilized pellet from the acid urea extraction was suspended (≈1.5 mg/ml) in 75% (wt/vol) HCOOH; the suspension was stirred overnight at 4°C, diluted severalfold with water, and centrifuged for 15 min at 600 × *g*. The supernate (formic acid extract) was lyophilized, and the residual pellet was discarded.

Alternatively, the pulverized tissue was washed four times by suspension (≈200 mg of tissue per 10 ml) and centrifugation in PBS and then extracted with distilled water according to the method of Pras (21), with minor modifications (3). Four sequential 70,000 × *g* water extracts of the PBS-washed tissue were made. The pellets produced in these extracts by centrifugation for 1 hr at 100,000 × *g* were suspended in small volumes of water and lyophilized; the second water extraction was the only one that yielded sufficient material (control and transgenic) to meet the needs of analysis.

Antiserum Production. Antibodies were raised in rabbits by one or more multiple-site injections of immunogen in aqueous medium emulsified with complete Freund's adjuvant (CFA); the experimental immunogens were mouse amyloid A (AA) protein (3), human serum amyloid A protein (4), and a synthetic peptide (Peptide Synthesis Service, Howard Hughes Medical Institute, University of Washington) corresponding to residues 651–676 of the 695 isoform (5) of the Alzheimer amyloid precursor protein (βPP; ref. 6). These injections were followed by or interspersed with administration of the immunogen emulsified with incomplete Freund's adjuvant. Serum samples were obtained 1–2 weeks before the first injection (preimmune serum) and 7–10 days after a series of injections was completed. The bacterial component (killed) of the CFA was either *Mycobacterium butyricum*, purchased as a dried preparation (Difco) and added (10 mg/ml) to incomplete Freund's adjuvant from the same vendor, or *Mycobacterium smegmatis*, present (0.5 mg/ml) in a commercially obtained CFA (GIBCO).

The antiserums chosen for study were drawn from our bank of specimens, which were stored at −20°C. They and the preimmune serums were used at a 200-fold dilution.

Depletion of Antibodies in Antiserum by Absorption with Antigen. (*i*) A 300-μg portion of lyophilized calf thymus histone H3 (Boehringer Mannheim) was dissolved in 30 μl of 25 mM acetic acid (pH 2.9). The solution was neutralized with 30 μl of 100 mM Na_2HPO_4 (pH 9.1) and then treated with 30 μl of rabbit antiserum to mouse AA protein. The preparation was stirred magnetically at 4°C; a control preparation, lacking only the histone H3, was stirred at the same time. During stirring the histone-containing preparation became cloudy.

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Abbreviations: AA, amyloid A protein; βPP, Alzheimer amyloid precursor protein; CFA, complete Freund's adjuvant.

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After 24 hr, each preparation was diluted with 30 μl of blocking buffer (see *Western Blotting*, below) and centrifuged for 1 min at 13,000 rpm in a microcentrifuge; the supernatant solutions were used for immunoblotting. (*ii*) A 6.0-mg portion of dried *M. butyricum* (see *Antiserum Production*, above), suspended in 120 μl of PBS, was disrupted (three times for 20 sec; full scale on the low-power setting) with the small probe of a Braun-Sonic 2000 sonicator (Braun Biotech, Allentown, PA). The suspension was then treated with 60 μl of rabbit antiserum to mouse AA protein and stirred at 4°C. A similarly diluted but otherwise untreated sample of the antiserum was maintained alongside the treated sample. After 24 hr, each preparation was diluted with 60 μl of blocking buffer and centrifuged as described in *i*.

Molecular-Sizing Electrophoresis. Samples were analyzed by urea/SDS/PAGE, essentially as described (7), in a vertical minigel apparatus (Mighty Small II; Hoefer); the total acrylamide concentration was 13.5% (17:1, acrylamide/*N*,*N*′-methylene-bisacrylamide), and the urea concentration was 6.4 M. Before application to the gel, samples were heated for 4 min at 100°C in sufficient sample buffer (7% 2-mercaptoethanol added) to provide a severalfold excess of SDS over protein. Molecular mass standards were, in addition to commercially available purified proteins, monkey amyloid A protein [8.6 kDa (8)] and duck amyloid A protein [11.6 kDa (9)].

Amino Acid Sequence Determination. Sequence analysis, performed in an Applied Biosystems model 470 instrument (10), was applied directly to protein contained in a band excised from a lightly stained (Coomassie blue R-250) poly(vinylidene difluoride) membrane (Immobilon-P, Millipore) used to capture the protein by electroblotting from a urea/SDS/acrylamide gel in 25 mM Tris/192 mM glycine/7% (vol/vol) methanol, pH 8.0. Sequence analysis was also applied to peptides generated by in-gel tryptic digestion (11) of protein resolved by urea/SDS/PAGE; subsequent separation of the peptides by reversed-phase HPLC in an acetonitrile gradient in 0.1% trifluoroacetic acid [2.1 × 100 mm Brownlee Aquapore RP-8 (C_8) column, Hewlett–Packard 1090 M chromatograph] provided a profile of peaks from which a selection for sequencing was made on the basis of a background profile provided by similar digestion of a blank area adjacent to the band of interest.

Western Blotting. Samples resolved by urea/SDS/PAGE were transferred (100 mA, 4 hr), in 25 mM Tris/192 mM glycine/7% methanol, pH 8.0, to nitrocellulose (0.2-μm pore size; Schleicher & Schuell). The resulting blot, after treatment with 1% bovine serum albumin/0.05% Tween 20/150 mM NaCl/50 mM Tris·HCl, pH 7.7 (blocking buffer), was exposed overnight at 4°C to primary antiserum (diluted in blocking buffer) and developed with peroxidase-conjugated goat anti-rabbit IgG followed by 4-chloro-1-naphthol/hydrogen peroxide (12).

RESULTS AND DISCUSSION

Western blot analysis (Fig. 1) of samples of acid urea extracts of washed (successively in saline, phosphate buffer at pH 8, and 10 mM acetic acid) homogenates of transgenic and control mouse ileums revealed an ≈15-kDa doublet, recognized by antiserum prepared against the synthetic peptide corresponding to residues 651–676 of the βPP 695 isoform (5) and administered with CFA. The trailing member of the doublet persisted on blots of the formic acid extracts of the residues remaining after acid urea extraction. The doublet, with the trailing member predominating, was a prominent constituent on blots of the 100,000 × *g* pellets derived from water extracts of multiply washed (saline) homogenates of the control and transgenic ileums. In addition to showing bands of different degrees of intensity at ≈15 kDa, some of the extracts of the ileal tissue showed immunoreactions, generally minor, corresponding to higher or lower molecular masses. Edman degradation of the small amount of the 15-kDa protein contained on a band excised from a poly(vinylidene difluoride) membrane used to capture the protein by electroblotting of a sample of formic acid extract of transgenic ileum yielded a partial amino-terminal sequence (XXTKQTAXXST, where X denotes an unidentified residue) in which the identified residues are common to mouse and bovine histone H3 (13). Sequence analyses of peptides separated by HPLC of an in-gel tryptic digest of the protein isolated by urea/SDS/PAGE of a sample of such a formic acid extract further identified the protein as histone H3. Peptide sequences found were EIAQDFK, STGGXAPR, STELLIR, and YRPGTVALR; two of the six chromatographic peaks analyzed yielded no sequence. Recognition, by the antiserum, of the principal constituent [14.7 kDa, calculated from sequence (14)] of a sample of calf thymus histone H3 included in the electroblot (Fig. 1) confirmed the presence of antibodies to histone H3 in the antiserum.

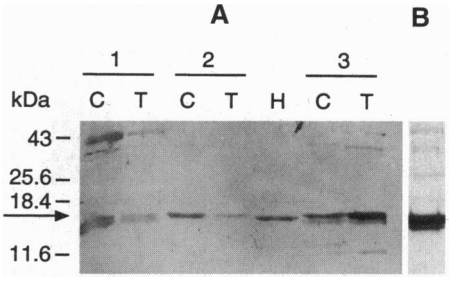

FIG. 1. Recognition of histone H3 in extracts of mouse ileum by antiserum from a rabbit administered *M. smegmatis* in CFA. (*A*) Western blots of calf thymus histone H3 (H), acid urea (lanes 1) and 75% HCOOH (lanes 2) extracts of control (C) and transgenic (T) mouse ileum, and lyophilized 100,000 × *g* pellet material (lanes 3) derived according to Pras (see text) from these tissues. Antiserum was obtained 8 weeks after initial injection of experimental immunogen (synthetic peptide 651–676 of βPP 695 isoform) and only injection of CFA. Initial sample loadings: lane H, ≈5 μg; lanes 1 and 2, ≈30 μg; lanes 3, ≈20 μg. (*B*) Sample (≈10 μg) of calf thymus histone H3 (14.7 kDa) on a Coomassie-stained gel. The arrow indicates the calf thymus H3 position.

The unexplained presence of antibodies to histone H3 in the antiserum and other antiserums that we later examined suggested to us that the source of the immunogen responsible for this immunoreactivity could be the bacterial component (*M. butyricum* or *M. smegmatis*) of the CFA used in the production of the antiserums. Weak to moderate reactivities shown by preimmune rabbit serums against electroblots of histone H3 were enhanced in serum specimens obtained after exposure of the rabbits to the CFA administered in conjunction with the experimental immunogens (Fig. 2). The degree of enhancement varied among the individual rabbits and, according to the limited data available, was greater in response to injection of *M. butyricum* than of *M. smegmatis*. A systematic comparison of the effects of the two species and of different immunization protocols was not made.

A strong immunoreaction shown by calf thymus histone H3 against antiserum to mouse AA protein was markedly diminished in intensity when a duplicate sample of the histone was tested after absorption of the antiserum with the identical histone; in the same test, a similar diminution of intensity was shown by the ≈15-kDa bands in samples of acid urea extracts of mouse ileum (Fig. 3*A*). Absorption did not diminish the intensity of the immunoreaction shown against the antiserum by a sample of mouse AA protein. Absorption of the same antiserum with dried, sonicated *M. butyricum* resulted in less marked diminutions of intensity in the immunoreactions of the ≈15-kDa bands in these samples; again, absorption did not diminish the intensity of the immunoreaction shown against

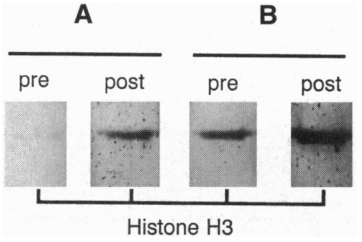

FIG. 2. Binding of antibodies in preimmune and postimmune rabbit serums. (*A*) Electroblots of calf thymus histone H3 tested with preimmune serum (pre) and postimmune serum (post) from the same rabbit after immunization against synthetic peptide 651–676 of βPP 695 isoform, administered with *M. smegmatis* in Freund's adjuvant three times over a period of 16 months. Initial histone loading, ≈3 μg per lane. (*B*) Electroblots as in *A*, tested with preimmune serum from another rabbit and postimmune serum from this rabbit after immunization against mouse AA protein, administered with *M. butyricum* in Freund's adjuvant three times over a period of 2 weeks.

the antiserum by a sample of mouse AA protein (Fig. 3*B*). These results further confirm the anti-histone generating capability of a mycobacterium; also, they show that any histone H3 epitopes that might have been present in the mouse AA protein preparation were below the limit of detection. Western blots (data not shown) of samples of dried *M. butyricum* and *M. smegmatis* tested with the anti-mouse AA before and after absorption with histone H3 revealed no evidence of band-intensity reduction as a consequence of the absorption, at least insofar as major or well-defined bands are concerned. A possible explanation for this result is that the epitopes responsible for generation of anti-histone H3 reside on proteins insufficiently concentrated for positive detection, too large to enter the acrylamide gel, or poorly transferred to the blotting membrane.

Evidence for the immunogenicity of *M. butyricum* and *M. smegmatis* in the generation of immunoreactivity other than that against histone H3 is provided in Fig. 4. Both generated antibodies that recognized a diffuse region of antigenicity, from ≈50 kDa to >100 kDa, in samples of the dried micro-

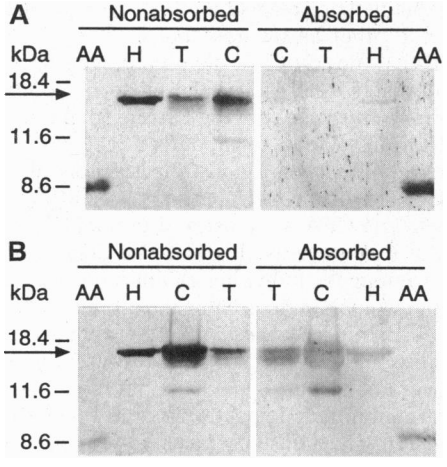

FIG. 3. Depletion of antibodies by absorption of antiserum with calf thymus histone H3 or *M. butyricum*. (*A*) Electroimmunoblots of histone H3 (H) and acid urea extracts of control (C) and transgenic (T) mouse ileum. The antiserum (nonabsorbed and absorbed with the histone) was from a rabbit immunized against mouse AA protein (see legend to Fig. 2*B*; different rabbit); a sample of the protein (AA) was included as a positive control. Initial sample loadings: lanes H, ≈5 μg; lanes C and T, ≈90 μg; lanes AA, ≈2 μg. (*B*) Same as in *A*, except that the antiserum was absorbed with *M. butyricum* and the initial AA loading was ≈1 μg. The arrow indicates the calf thymus histone H3 position.

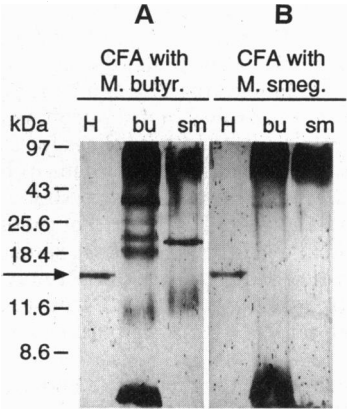

FIG. 4. Response of rabbits to administration of mycobacteria as constituents of CFA in the production of antiserums. (*A*) Western blots of calf thymus histone H3 (lane H), dried *M. butyricum* (lane bu), and dried *M. smegmatis* (lane sm). The antiserum was made against human serum amyloid A protein and administered with CFA containing *M. butyricum*; serum was obtained after six injections over a period of 32 days. Initial sample loadings: lane H, ≈3 μg; lanes bu and sm, ≈100 μg. (*B*) Samples were the same as in *A*; antiserum was made against the aforementioned βPP synthetic peptide, which was administered with CFA containing *M. smegmatis* (see legend to Fig. 2*A*). Arrow indicates calf thymus histone H3. Dried *M. butyricum* was purchased (see text); dried *M. smegmatis* was a gift from Cheng-Mei Shaw (University of Washington).

organisms. Both generated antibodies that recognized, in a *M. butyricum* sample only, antigens of ≈35 kDa (very faint vs. anti-*M. smegmatis*) and <8 kDa; in addition, *M. butyricum* generated antibodies that recognized, in a *M. smegmatis* sample only, antigens of ≈21 kDa (sharp) and ≈13 kDa (diffuse). Also, *M. butyricum* generated antibodies that recognized, in a *M. butyricum* sample only, antigens of ≈26 (faint), ≈22, ≈19, and ≈12 (diffuse) kDa.

A noteworthy feature of the ≈15-kDa ileal proteins and of calf thymus histone H3 was their poor electrophoretic transferability from an acrylamide gel to a blotting membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid/10% (vol/vol) methanol/NaOH, pH 10.5. Transfer was markedly improved, although not complete, when 25 mM Tris/192 mM glycine/7% methanol, pH 8, was used instead of the sulfonate buffer. Migration from the gel to the membrane was toward the anode. When a second membrane was used, on the cathodic side of the gel, little transfer was noted by immunodetection, no more than could be explained by diffusion alone. Histone H3, with its large excess of basic over acidic amino acid residues (14), should have a net positive charge at pH 8 and therefore should migrate toward the cathode; the anodic migration suggests retention of sufficient SDS from the SDS/PAGE buffer to provide a net negative charge.

That bacteria make histone-like proteins has been known for well over a decade (15). In view of the conserved nature of histones in general and the highly conserved nature of histone H3 (14), it is understandable that antiserums produced with the stimulation provided by mycobacteria in CFA may contain antibodies reactive against mammalian histones. The evidence accumulated in this study verifies the induction of such immunoreactivity. In addition to *M. butyricum* and *M. smegmatis*, other mycobacterium species, notably *Mycobacterium tuberculosis*, will probably be found capable of inducing antibodies to histones in recipient rabbits. The presence of anti-histone H3 activity in preimmune serums suggests natural exposure of the hosts to microorganisms having histone-like proteins in their composition.

Histone H3 in baker's yeast (*Saccharomyces cerevisiae*) contains a 6-aa sequence that occurs also in the uveitopatho-

genic peptide M of retinal S-antigen, which has been found to induce experimental autoimmune uveitis in Lewis rats (16, 17). This apparent involvement of histone H3 in the mechanism of induction of an autoimmune disease may be explained, as the investigators suggest, on the basis of molecular mimicry, and would therefore be representative of a "biologically meaningful mimicry" (18). The induction of antibodies to histone H3 in rabbits injected with CFA almost certainly rests on a sequence similarity between epitopes of histone H3 and a protein constituent of the bacterial component of the CFA; the similarity falls short of the degree that would allow immunological tolerance to intervene; whether the induction is accompanied by some manifestation of autoimmunity, we do not know.

Our aim in this communication is to draw attention to the possible presence of unsuspected immunoreactivities in antiserums whose production involves use of CFA and to the need of characterizing the resulting antibodies. Although this work focuses attention on histone H3, sequence similarities between mammalian and mycobacterial proteins other than histones and histone-like proteins have been reported, and instances of antigenic mimicry and autoimmune diseases in a growing variety of situations are being recognized (19, 20).

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