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Monoclonal Antibodies to Human Protamines

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ABSTRACT

Nine monoclonal antibodies to human protamine, hup1a, 1b, 1c, 1d, 1e, 2a, 2b, A, and B, have been isolated and partially characterized. Enzyme-linked immunoabsorption assay analyses with HPLC-separated human protamine 1 and protamine 2+3 mixture identified five of these antibodies as specific for human protamine 1, two antibodies specific for protamine 2+3 mixture and two monoclonal antibodies reactive with all three human protamines. These findings were confirmed by immunoblotting. None of the antibodies reacted with poly-arginine or somatic histone proteins. Additional analyses with bull, boar, and ram protamines indicated that all of the monoclonal antibodies except hupA are specific for human protamine. HupA reacted with protamines from all of the species tested. These studies suggest that each of the antibodies recognizes one of at least four distinct epitopes on protamine.

INTRODUCTION

During spermatogenesis, a complex sequence of biochemical and morphological events transform the round-spermatid nuclei into highly condensed sperm heads that display species-specific shapes. Protamine, the major sperm nuclear protein of mammals and other animals, was first identified and characterized in fish by Miescher (1) and Kossel (2). Since then protamines have been found in most vertebrates, some invertebrates and even some plants (3,4). The complete amino acid sequences are available for 5 mammalian species: boar (5), bull (6,7), ram (8), mouse (from cDNA, 9), and man (10-13). In addition, partial amino acid sequences are available from rat (14) and stallion (15). Protamines are highly basic proteins containing, depending on the species, 44 to 52% arginine and 12 to 18% cysteine. All five mammalian protamine 1 proteins completely sequenced to date are 50 amino acids in length and are identical at 20 amino acid positions including a conserved N-terminal hexapeptide (ala-arg-tyr-arg-cys-cys) and an internal stretch of 5 arginines. Both mouse and man have more than one protamine type. In mouse, protamine 2 is 65 amino acids long, accounts for about 70% of the nucleoprotein mass (16), and shows very little homology with protamine 1 (17). Human sperm contain three protamines (12,18,19). Amino acid composition (19) and sequence data (10,12,13) suggest that human protamines 1 and 2 are different proteins.

Protamine is involved in the condensation and stabilization of sperm DNA. Several models have been proposed for the interaction between protamine and DNA in species containing only one protamine type (see review by Balhorn, [20]). Balhorn (20) presented a model for bull sperm chromatin in which the N-terminus of protamine is folded back over the rest of the molecule and the protamine molecules are aligned in tandem in the minor groove of DNA. In this organization arginine residues neutralize adjacent DNA charges and cysteines form both intra and intermolecular S-S bridges. However, we have essentially no understanding of nucleoprotein organization in species containing more than a single protamine type,

such as man. Immunological probes for specific protamines will provide a new approach that can be used to characterize the organization of human sperm chromatin.

Here are described nine monoclonal antibodies (MAbs) against human protamines. We demonstrate that human protamines are immunologically reactive, supporting earlier findings of autoantibodies to protamines in an infertile male (19,21), of immunological cross-reactivity among several mammalian protamines (22), and of the presence of anti-protamine antibodies in experimental animals and humans receiving protamine insulins (23,24). In addition, the antibodies described here are shown to discriminate among protamine types of man and of other mammals.

MATERIALS AND METHODS

Sperm Sources

Pooled samples of semen from bull (breed unspecified), from boar (Yorkshire breed) and from Corriedale rams, were purchased from American Breeders Service (DeForest, WI), Swine Genetics International (Cambridge, IO), and as a gift from Duane Garner (University of Nevada, Reno, NV) respectively. Sperm samples were washed three times in Tris-saline and used immediately or stored as frozen pellets at -20°C.

Preparation of Human Sperm Nuclei

Amembranous human sperm nuclei (hereafter referred to as sperm nuclei) were prepared from pooled ejaculates collected as part of a local program involving anonymous donors. Semen samples were allowed to liquefy at room temperature for 1 to 4 hrs then frozen at -4°C for storage up to 2 weeks. Groups of 5 to 12 samples were thawed, pooled, and centrifuged at 4000 x g for 5 min. The sperm pellet was resuspended in Tris-saline, ultrasonicated briefly with a Branson sonicator (Shelton, CT, setting 3 for 30 sec), and centrifuged at 4000 x g for 3 min. The pellet was resuspended in 20 ml of 1% cetyltrimethylammonium bromide (CTAB), 0.01M dithiothreitol (DTT), 0.01M Tris, pH 8.0, sonicated briefly, and allowed to sit at 4°C for 30 min to dissolve the tail, acrosome and membranes surrounding the chromatin. The sperm nuclei were rinsed twice in Tris-saline containing 0.1% CTAB and stored at 4°C. After storage, brief ultrasonication was used to resuspend the nuclei.

Protamine Extraction

Whole human protamine (i.e. the mixture of protamines 1, 2, and 3) was isolated from sperm nuclei using a modification of the procedure for the mouse (16) as described by Balhorn *et al.* (12). The protamines of bull, boar, and ram were prepared in similar fashion.

Protamine Separation

The human protamines were separated by high-performance liquid chromatography (HPLC) as described by Mazrimas *et al.* (25). Prior to chromatography, the protamine was reduced for 18 hr with a 50-fold excess of DTT in 6M GuCl, 0.002M ethylenediaminetetraacetic acid (EDTA) and dialyzed at least 1.5 hr against 200 volumes of 10 mM HCl, 1 mM DTT. The ion-pairing agent, trifluoroacetic acid (TFA), was added to a final concentration of 0.1% and the reduced protamine was chromatographed on a Nucleosil RP-C18 column (7.5 mm I.D. X 300 mm). Human protamine 1 was separated from protamines 2 and 3 using a linear, 12 to 24% acetonitrile gradient in 0.1% TFA, run at a rate of 2 ml/min for 50 min. The fractions containing the protamines were frozen and lyophilized. HPLC peaks were identified by electrophoresis of the proteins in acid-urea gels (16).

Immunizations

For cell fusion #1, six-month-old BALB/cBkl mice (Bantin and Kingman, Fremont, CA) and Biozzi high-responder strain mice (26) were immunized weekly for 3w by intraperitoneal (IP) injections of 50 μg of whole human protamine in complete Freund's adjuvent (1:1). Serum was obtained 14d after the last IP injection and tested for anti-protamine activity in an enzyme-linked immunosorption assay (ELISA), as described below, and the animal with the highest serum titer selected for cell fusion (Balb/c mouse #3, see Table 1). Seven days later, this animal was injected intravenously (IV) on 3 consecutive days with 50 μg of human protamine in sterile saline. The next day, the spleen was removed, minced, and placed in 25 mM Tris-HCl, 0.15M NH4Cl, pH 7.5 solution to lyse the red blood cells. This animal yielded MAbs hup1a, 1b, 1c, 1d, 1e, A, and B.

For fusion #2 a separate group of BALB/c mice were immunized weekly for 3w by IP injections of 50 μg of whole human protamine precipitated with calf thymus DNA (protamine/DNA) and mixed with complete Freund's adjuvent (1:1). The animals were then rested for 2 months and immunized once a month for the next two months with 50 μg of whole protamine/DNA in adjuvent. They were then rested one month and immunized monthly for 5 months by IP injections of 2 x 10^5 sperm nuclei. One month following the last IP

immunization, an animal was selected and given a final immunization of 50 μg of whole human protamine in sterile saline. Four days later its spleen was removed and fused as described below. This fusion yielded MAbs hup2a and hup2b.

TABLE 1
Serum Titers in Two Mouse Strains

		ANTIGENS	
Strain	**Mouse**	**Protamine**[1]	**Sperm Heads**[2]
Balb/c	1	128[3]	32
	2	128	64
	3	256	64
	4	32	64
	5	64	16
Biozzi	1	0	nd[4]
	2	0	nd
	3	0	nd
	4	0	nd
	5	0	nd

1. Whole human protamine
2. Amembranous sperm nuclei (see Methods).
3. Expressed as the reciprocal of the dilution at which twice background activity was observed (i.e., the endpoint titer). A zero indicates no activity at a serum dilution of 1:1, the highest concentration used.
4. Not done.

Cell Fusion and Screening

SP2/0 myeloma cells were grown in M3 medium (27) containing 2% fetal bovine serum. Splenocytes were fused with an equal number of SP2/0 myeloma cells according to Oi and Herzenberg (28), as modified by Bigbee *et al.* (29). Cells were plated without a feeder layer in equal parts of M3 and Hana HB101 serum-free media (Hana Biologicals, Berkeley, CA) containing 30 mM hypoxanthine, 40nM aminopterin, 30 μM thymidine, and 2% fetal bovine serum (27).

The ELISA described by Jensen *et al.* (30), with the following modifications, was employed to screen and characterize antibody-secreting clones. Microtiter plates were coated with 0.25 μg/well of antigen (whole human protamine). The blocking solution (3% ovalbumin in PBS) containing 10 μg/ml calf thymus DNA (Sigma, St Louis, MO). The presence of anti-protamine antibodies was detected using peroxidase-conjugated anti-mouse immunoglobulin anti-serum (1/500, U.S. Biochemicals, Cleveland, Ohio) and 2,2 azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) as substrate. Absorbence measurements of 405 nm were collected as a function of time, and the data were analyzed using the "Cyberdoma" ELISA software described by Slezak *et al.* (31).

The cells from ELISA-positive wells were expanded and subcloned at least twice by limiting dilution to insure monoclonal origin. MAbs were produced in ascites tumors generated by injection of 1 x 10^7 hybridoma cells into Balb/c mice which had received 500 rad gamma radiation the previous day (27). Isotype analysis was done by ELISA using immunoglobulin heavy-chain, and light-chain-specific anti-serum (Miles Laboratories, Elkhart, IN and Southern Biotechnology Assoc., Birmingham, AL). Crude ascites fluids were used unless otherwise indicated. In those cases antibodies were purified from ascites fluid by hydroxylapatite chromatography (32).

Electrophoresis and Immunoblotting

Protamines were separated on acid-urea gels (16). Slab gels were pre-electrophoresed overnight at 20 V (constant voltage), and the protamines separated (20 μg/lane) by electrophoresis at 20 mA constant current for 105 min using a Protean I electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) and transferred to nitrocellulose (NC) filters as follows. The gels were soaked in transfer buffer (0.7% acetic acid, 1M urea) for 0.5 h, placed against NC in a Bio-Rad Transblot unit (Bio-Rad Laboratories, Richmond, CA), and the proteins transferred at 100 Volts for 2 h at 4°C in transfer buffer (under these

conditions protamines migrate towards the cathode). After transfer the NC filters were dryed and stored at room temperature (RT). Active sites on the NC filters were blocked at RT by a 1h incubation in 3% ovalbumin in PBS (pH 9.0) followed by 0.5 h in a solution containing 10 µg/ml calf thymus DNA in PBS (pH 7.2). Blocked filters were incubated for 1 hr in primary antibody, washed with 0.01% tween-water, incubated at RT in a 1/500 dilution of peroxidase-conjugated anti-mouse immunoglobulin (United States Biochemicals, Cleveland, OH), washed again, and developed in HRP color-development reagent (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Differential Immunization Response of Mice

Both Biozzi high-responder and BALB/c mice were immunized with whole human protamine. Acid-urea gel electrophoretic analysis of this mixture (Figure 1, tract a) revealed protamines 1, 2, and 3 as the major basic proteins. The levels of serum antibodies reactive to whole human protamine and to sperm nuclei immobilized in ELISA wells are shown in Table 1. Serum titers against both human protamine and sperm nuclei were observed in all of the BALB/c mice (end-point titers ranged from 1:16 to 1:256). However, no detectable antibody titer was observed in any of the Biozzi high-responder mice. BALB/c mouse #3 (Table 1) had the greatest serum titer and its spleen cells were fused with SP2/0 myeloma cells for fusion #1.

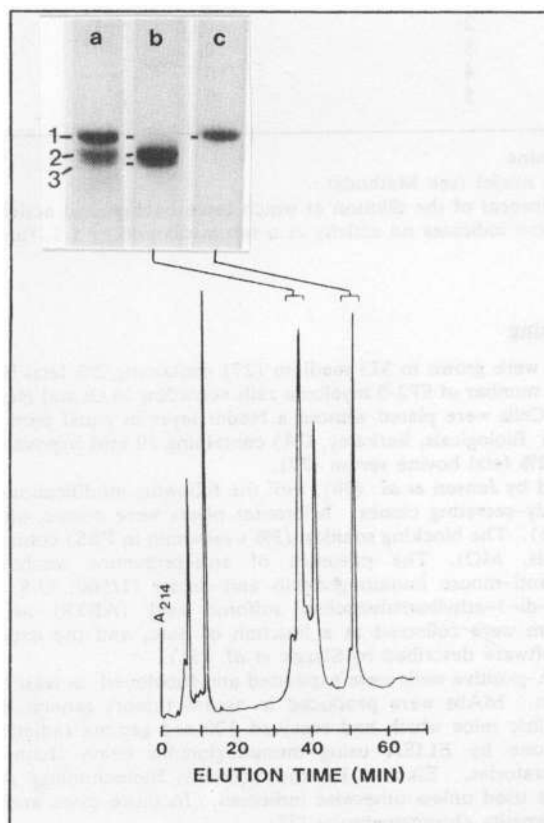

FIGURE 1: Separation of whole human protamine by HPLC and gel electrophoresis. Whole human protamine was fractionated on an HPLC column (see methods section). Samples from the designated peaks were analyzed by acid-urea gel-electrophoresis: Lane b, a mixture of protamine 2+3 obtained from the indicated chromatogram peak; lane c, protamine 1 from indicated peak. The gel pattern for whole human protamine is shown for reference (lane a). Protamine 1, 2, and 3 are indicated to the side of lane a. The gel lanes are representative of the purity of the samples used for immunizations and ELISA.

Hybridoma Isolation

Fusion #1: Growing hybridomas were observed in approx 90% of the wells 12d after cell fusion. Culture fluid from 59 of 1920 wells gave positive responses when screened against whole human protamine. Cells from 7 wells showing the greatest activity were cloned by limiting dilution and used to generate MAb-containing ascites fluid. These 7 MAbs were named hup1a, hup1b, hup1c, hup1d, hup1e, hupA, and hupB. Cells from the remaining 52 wells were expanded in 24-well plates and frozen.

Fusion #2: The product of the second fusion was derived from spleen cells of a BALB/c mouse using a different immunization protocol (see Methods Section). Culture supernatants from 2880 wells were screened, and 6 wells were observed to produce antibodies reactive with whole human protamine. Two stable MAbs were subcloned and named hup2a and hup2b.

Isotyping

Analysis using heavy-chain-specific antisera from two independent sources indicated that hup1a, 1b, 1c, 1d, 1e, A, B, and 2b are of IgG1 type while hup2a is of the IgG2b type. All had kappa light chains.

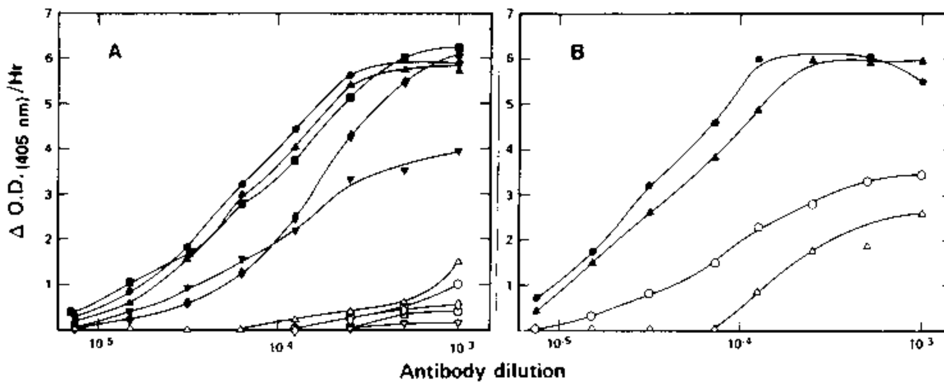

FIGURE 2: ELISA titration curves of the anti-protamine antibodies against human protamine 1 and protamine 2+3 mixture. Ascites fluid was diluted as indicated. Solid symbols represent protamine 1, open symbols represent protamine 2+3 mixture. Panel A, hup1a ○ ●; hup1b △ ▲; hup1c □ ■; hup1d ▽ ▼; and hup1e ◊ ♦. Panel B, hupA ○ ●; and hupB △ ▲. ELISA activity is expressed as the rate (change in optical density per hour) of substrate conversion.

Antibody Specificity

The ability of hup1a, 1b, 1c, 1d, 1e, A, and B to discriminate human protamine 1 from the other human protamines was tested by ELISA using HPLC-purified human protamine 1 and human protamine 2+3 mixture as the immobilized antigens (Figure 1).

Results of typical titration experiments are shown in Figures 2 and 3. MAbs hup1a, 1b, 1c, 1d, and 1e (Figure 2A) showed strong activity to human protamine 1, but little (e.g., hup1b) or no activity (e.g. hup1d) to the human protamine 2+3 mixture. In contrast, hupA and hupB (Figure 2B) recognized both human protamine 1 and the 2+3 mixture, although both MAbs appeared to prefer human protamine 1. Similar titrations of hup2a and hup2b (Figure 3) showed that they recognized the human protamine 2+3 mixture and not human protamine 1.

TABLE 2
ELISA Activity[1] Of Undiluted Culture Medium From Wells with Anti-Protamine Activity

Fusion No.	Well No.[3]	ANTIGENS[2] Protamine 1	ANTIGENS[2] Protamine 2+3 Mixture
1	52	8.6	6.9
	6A	5.6	4.8
	28	8.1	4.8
	44	6.2	4.7
	45	8.4	4.2
	51	7.6	3.4
	3A	10.3	3.1
	25	7.9	2.7
	42	6.5	2.4
	53	2.3	1.8
	7A	7.2	1.5
	47	5.9	1.2
	26	4.7	0.9
	9	4.1	0.0
	57	3.9	0.0
	56	2.0	0.0
	31	1.7	0.0
	17	6.2	0.0
	12	3.5	0.0
	25A	3.4	0.0
	44A	7.9	0.0
	48	3.3	0.0
	39	6.3	0.0
	29	6.3	0.0
	27	5.0	0.0
	22	3.9	0.0
2	5	0.6	7.2
	6	0.4	3.0
	27	0.0	5.5
	28	0.0	6.1
	32	0.0	4.2
	33	0.0	3.6

[1]. Change in optical density/hour (i.e., the slope) of ELISA.
[2]. HPLC-purified human protamine 1 or a mixture of human protamine 2 and 3 (see Figure 1).
[3]. Arbitrary number used to distinguish wells with a positive response in the initial screen. Cells in these wells were transferred to a 24-well plate, allowed to grow, and then frozen. Frozen samples were thawed and the cells grown in 24-well plates. The culture fluids from these wells were assayed individually.

To determine whether the specificity of hup1a, 1b, 1c, 1d, 1e, A, and B was representative of the hybridomas derived from fusion 1, the cells from 26 of the original 59 antibody-producing wells were thawed and the antibodies produced were examined for their ability to discriminate between the human protamines. The reactivities of these uncloned cultures against protamine 1 and the protamine 2+3 mixture are shown in Table 2. We found 13 cultures which recognized protamine 1 specifically and another 13 cultures that recognized both protamine 1 and the protamine 2+3 mixture. None of these cultures secreted antibodies specific for the protamine 2+3 mixture. Thus, hup1a, 1b, 1c, 1d, 1e, A, and B are a representative sampling of fusion 1. Likewise, the 6 protamine-antibody-secreting cultures of fusion 2 were assayed against protamine 1 and the protamine 2+3 mixture, and all reacted strongly to the protamine 2+3 mixture (Table 2).

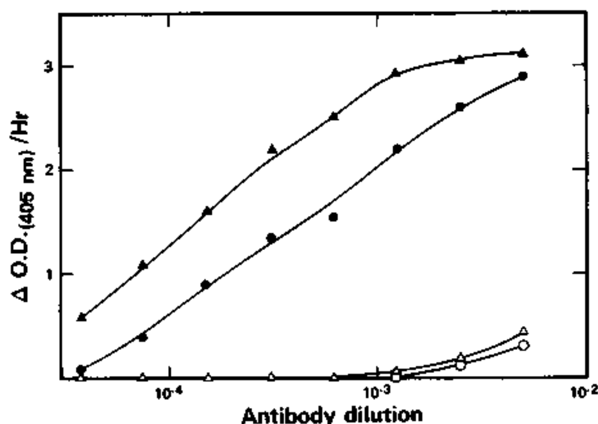

FIGURE 3: ELISA titration curves of hup2a and hup2b ascites fluids against human protamine 1 and protamine 2+3 mixture. The solid symbols represent protamine 2+3 mixture, open symbols represent protamine 1. Hup2a ○ ●; Hup2b △ ▲.

Electrophoresis and immunoblotting were used to confirm the antibody specificity indicated by ELISA and to determine whether hupA, B, 2a and 2b could distinguish between human protamines 2 and 3. Human protamines 1, 2, and 3 were separated by gel electrophoresis (Figure 4), transferred to NC filters, and replicate filters probed individually with each MAb. The results, shown in Figure 4, confirm the ELISA findings. Hup1a, 1b, 1c, 1d, and 1e labeled only protamine 1. HupA and hupB labeled both protamine 1 and protamine 2. In the blot shown, protamine 3 was labeled weakly by hupA and hupB, but in replicate blots protamine 3 was labeled more intensely. In a separate experiment, also shown in Figure 4, hup2a and hup2b strongly labeled protamine 2 and protamine 3 with little reactivity to protamine 1.

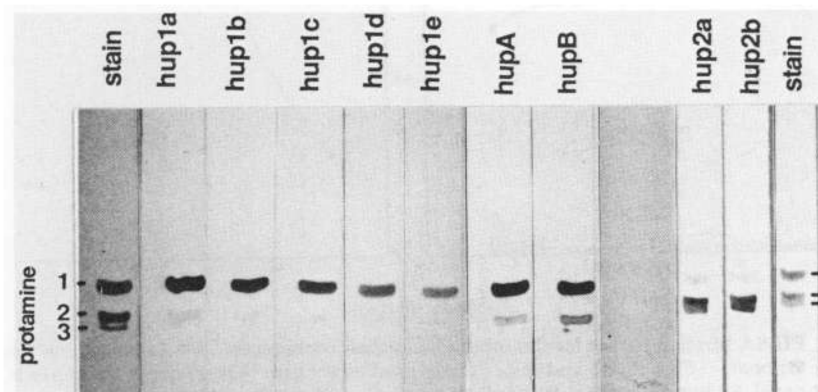

FIGURE 4: Immunoblot of human protamines separated by acid urea gel electrophoresis. Whole human protamine was separated on acid-urea gels and transferred to nitrocellulose filters. Individual lanes were probed with the antibodies indicated. Lanes designated 'stain' represent filters stained with amido schwartz. Protamines 1, 2, and 3 are indicated.

Species Specificity

The relative specificity of the antibodies against human, bull, boar, and ram protamines was determined in ELISA (Figure 5). Hup1e which had a strong preference for human protamine 1 (Figure 2A) did not recognize any of the nonhuman protamines (Figure 5A). Hup1a, 1b, 1c, and 1d each displayed this same differential species response, i.e., recognition of human protamine 1 only (data not shown). Hup2a and 2b which had a strong preference for human protamines 2+3 (Figure 3) also did not recognize any of the

nonhuman protamines (Figure 5D, data shown only for hup2a). However, hupA and B which were the two antibodies reactive against all three human protamines (Figure 2B) showed differing species specificities. HupA recognized human, bull, boar, and ram protamine (Figure 5B) but hupB appeared to be human specific (Figure 5C). The nine MAbs also were titrated in an ELISA using poly-arginine as immobilized antigen and none reacted (data not shown). Likewise the antibodies were analyzed for reactivity to calf thymus histone proteins and none was observed (data not shown).

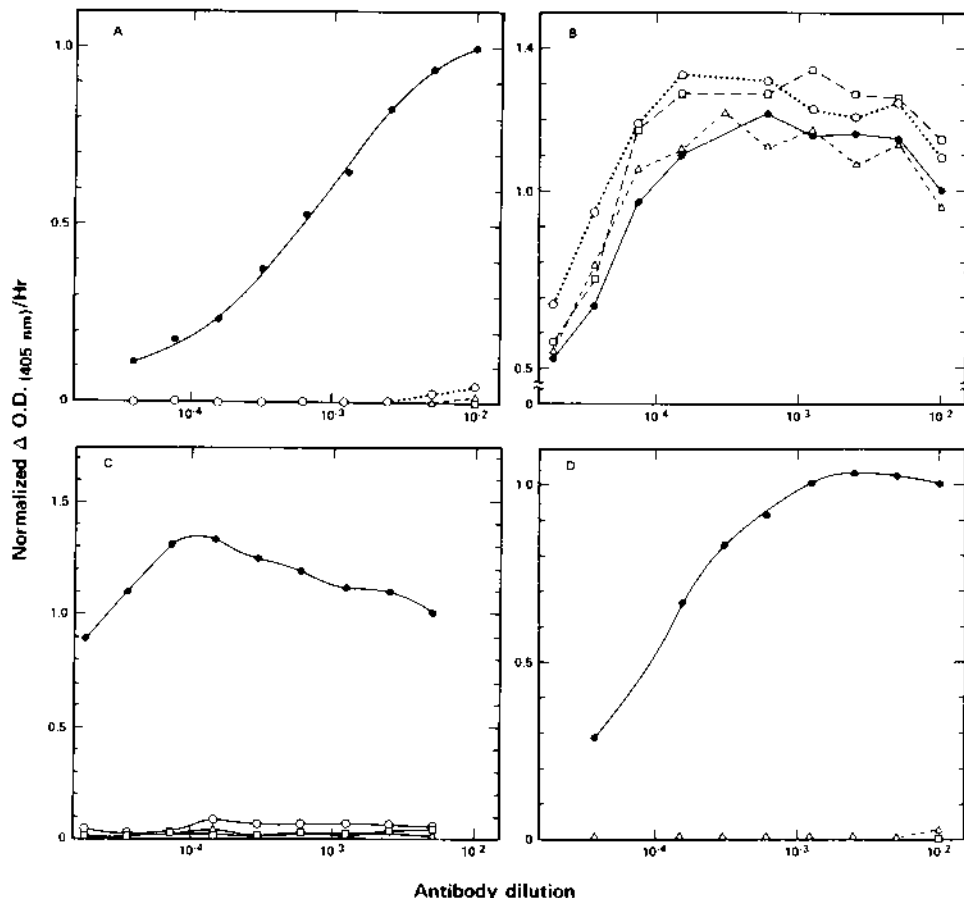

FIGURE 5: ELISA titration curves for the other mammalian protamines. Whole protamine from human ●; boar, ○; bull Δ; and ram □, was used as antigen. The response of hup1e is shown in panel A, hupA in panel B, hupB in panel C, and hup2a in panel D.

DISCUSSION

Nine anti-protamine MAbs were obtained from two independent fusions of mouse splenocytes and SP2/0 myeloma cells. With our immunization strategies human protamine was not highly immunogenic in Biozzi high-responder mice but elicited an immune response in BALB/c mice. Such differential immune responses are not surprising, since both intrinsic (e.g., accessibility and mobility) and extrinsic host factors (e.g., tolerance, immune response genes, idiotype networking, and structural gene repertoire) are known to affect the immune response (33, 34). The antibodies from fusion 1 either specifically recognize protamine 1 or cross reacted with all 3 human protamines. In contrast, the antibodies isolated from fusion 2 all appeared to be specific for human protamines 2 and 3. These differing responses may be the result of (a) the use of differing immunogens (i.e., whole protamine for fusion 1 versus a combination of sperm nuclei and precipitated whole protamine for fusion 2), (b) the difference in immunization schedules, or (c) individual animal differences in immune response. Our experiments were not designed to discriminate among these factors.

Antibody specificity was evaluated in ELISA using HPLC-purified human protamine 1 and protamine 2+3 mixture as the immobilized antigens. MAbs hup1a, 1b, 1c, 1b, and 1e recognize human protamine 1 but not human protamine 2 or 3. Hup2a and hup2b were specific for protamine 2 and/or protamine 3. To resolve the ambiguities introduced by using a protamine 2+3 mixture, protamines 1, 2, and 3 were separated electrophoretically, transferred to nitrocellulose filters and probed with the MAbs. HupA and hupB recognized all 3 human protamines (albeit protamine 3 only weakly), while hup2a and hup2b recognized both protamine 2 and 3. The weak reactivity of hup2a and hup2b to protamine 1 in the immunoblots was not observed in the ELISA. It is possible that cryptic sites on protamine 1 are available when the protein is denatured and transferred to filters. Mendelsohn *et al.* (35) observed similar inconsistencies between immunoblots and ELISA's for a group of anti-histone MAbs. An alternate explanation for this result may involve the nonspecific sticking of antibody to protamine. We observed significant nonspecific binding by irrelevant MAbs when filters were probed with peroxidase-conjugated anti-mouse antibody in the absence of the anti-protamine MAbs. This nonspecific binding was completely eliminated by blocking with a DNA solution prior to probing with the anti-protamine probes. These nonspecific reactions between protamine and antibody are probably related to the highly basic nature of protamines (i.e., isoelectric points near 12). At the pH commonly used for immunoassays (i.e. pH range 7 to 8), protamines have a strong positive charge, and nonspecific ionic interactions with other proteins may occur. Charged regions on protamine should be effectively neutralized by DNA, the substance normally bound by protamine. Thus, the weak reaction of hup2a and hup2b to human protamine 1 may be due to insufficient blocking of the filters or protein exchange after blocking. Pesce *et al.* (36) reported nonspecific antibody binding to a number of cationized proteins (proteins with high isoelectric points) and the elimination of this binding by treatment with polyanions. In ELISA, nonspecific binding was not as obvious if exogenous DNA was omitted from the blocking solution. The MAbs specifically recognized immobilized protamine as well as the protamine-DNA complex. The inability of hup2a and hup2b to distinguish between protamine 2 and 3 is not surprising since recent sequence studies (12, 13) indicate identical primary amino acid sequences for these two protamines except for the first three amino acids.

The results of our analyses of the ability of these MAbs to bind protamine from bull, boar, and ram indicate that the antibodies recognize at least 4 epitopes represented by the 4 panels in Figure 5. Hup1a, 1b, 1c, 1d, and 1e are specific for human protamine 1, with little or no reactivity to the other protamines (eg., hup1e in Figure 5A) and thus may all recognize the same epitope. In addition, the antibodies did not react to poly-arginine in ELISA suggesting that they do not recognize the poly-arginine-rich core region of protamine. HupA recognized all protamines tested (Figure 5B) but not poly-arginine or somatic histones. In contrast, hupB recognized human protamines 1, 2, and 3 but not those from the other species tested (Figure 5C). Thus, hupA and hupB probably recognized different epitopes. Finally, hup2a and hup2b appear to be specific for human protamine 2 and 3, showing no cross-reactivity against the other mammalian protamines tested (Figure 5D). Thus, these two MAbs recognize yet another epitope.

The nature of the epitopes recognized by these MAb's is not clearly resolved by the results reported here. Whether they recognize sequential or conformational epitopes has yet to be determined. Studies are underway to more clearly elucidate the epitopes (e.g., titrations against peptides derived from enzymatic digestion of protamine). A clear understanding of the epitope recognized by these antibodies is vital if they are to be utilized as reagents in studies of sperm chromatin structure.

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