PROTAMINE-REACTIVE NATURAL IgM ANTIBODIES IN HUMAN SERA

Characterization of the Epitope Demonstrates Specificity of Antigenic Recognition; Occurrence Indicates Obscurity of Origin and Function

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Antibodies detected, in broad occurrence, in normal human sera are classified as natural antibodies (1, 2). As the technology has progressed, an ever-increasing number of such antibodies have been detected (3–14). In many instances, the antigenic sites recognized by those antibodies have been identified as components of autogenous molecules and, thus, the designation "autoantibodies" has been applied and various immunoregulatory or housekeeping functions have been assigned. A long-held hypothesis has recently been restated and supporting evidence presented that the molecules with which certain natural antibodies are antigenically reactive may provide the immunogenic stimulus for their induction or expansion (6).

We report here the identification of a specific set of IgM antibodies that are present in all sera from a representative population of normal individuals, thus fulfilling the criterion of natural antibodies. However, since those antibodies are present in sera of individuals whose stage of development assures that they have not been exposed to the self-proteins with which the antibodies are antigenically reactive, that hypothesis of origin may not apply and the designation "autoantibodies" may not be appropriate.

We have detected antibodies of the IgG class reactive with purified protamines of human sperm in all sera of 140 normal subjects including neonatal, pediatric, and adult males and females. Similarly reactive IgM antibodies were detected in all except the neonatal sera (Table I).

Protamines are small highly basic nucleoproteins that occur in no cells other than spermatids and spermatozoa. Those proteins are synthesized *de novo* and incorporated into the nuclei of spermatogenic cells during spermiogenesis, a progression that takes place in the immunologically sequestered lumena of the seminiferous tubules of the postpuberal testis (15). Although that structural immunologic barrier does not continue into the excurrent ducts of the male tract, there is evidence that immunosuppressive substances are introduced as the semen is formed, and under normal conditions, immunity to sperm-unique

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components may be inhibited (16). Similarly, it is logical to accept the premise that immunosuppressive factors may be present in the female genital tract to protect against the potential immunogenicity of components of the ejaculate (17).

In the context of the foregoing, therefore, it seems unlikely that the protamine-reactive antibodies in all of the normal sera examined are attributable to induction by protamines. Moreover, even if it is possible that protamines, released from degenerating sperm, may escape surveillance and suppression and, therefore, protamine-induced antibodies could be present in the sera of postpuberal males and sexually experienced females while the protamine-reactive IgG in the early pediatric sera could be attributed to placentally transferred maternal IgG, it is clear that the protamine-reactive IgM antibodies in pediatric sera may not, in any way, be attributable to induction by protamines. Commonality between the protamine-reactive IgM antibodies of adult and pediatric sera is established in this report. It appears, therefore, that protamines are not the immunogenic stimulant for those antibodies. The questions then arise as to whether those antibodies are induced by other autogenous proteins that are homologous with protamines or arise by some not yet recognized immunologic mechanism and whether the antigenic targets of that set of natural antibodies are protamines or other molecules.

As the first address to those questions, with the aid of a mouse monoclonal antibody (mAb) raised to human sperm protamines, we have characterized the antigenic site recognized by the protamine-reactive IgM antibodies of human sera.

Materials and Methods

ELISA. The assay method by which the data of Table I were assembled and which was used throughout this study has been described (18, 19). Briefly, 50 μl of antigen (specified concentration) was placed in each well of a 96-well flat-bottomed microtiter plate, covered, and held at room temperature for 4 h. The wells were washed (20×) with 0.01% Tween-20/PBS, then filled to the rim with 5% BSA, covered and held at 5°C overnight. The plates were brought to room temperature and washed 20× and 50 μl of serum or mAb (diluted with 1% BSA) was added to each well. After 2 h at room temperature, the wells were washed 20× and 50 μl of peroxidase-conjugated second antibody [goat $F(ab')_2$ anti-human IgM μ chain specific, or goat $F(ab')_2$ anti-mouse IgA, IgG, and IgM] was added. The plates were covered, held at room temperature for 1.5 h, and washed 20×. 50 μl of substrate (ortho-phenylene diamine) was added to each well and, after 30 min, the reaction was stopped by the addition of 50 μl of H_2SO_4 (2.5 N). The optical density of the colored solution in each well was read at 490 nm in an automated plate reader. That protocol was developed to maximize sensitivity and to minimize ambiguity arising from serum background and plate-to-plate differences in binding efficiency (19). The total IgM and IgG titers of each serum were derived in reference to a single standard serum for which the absolute weights of IgM and IgG were determined.

Antigens. Purified protamine 1 (P1)[1] and protamine 2 (P2) were prepared as described (20) with modifications. Briefly, spermatozoa were isolated from pooled ejaculates and sonicated to separate heads from tails. The heads were collected and, by treatment with Triton-X, were separated into a membrane protein fraction and a DNA-nucleoprotein

[1] *Abbreviations used in this paper:* HPmAb, mAb to human sperm protamines; P1 and P2, purified protamine 1 and 2.

fraction. The latter was solubilized in 4 M guanidinium chloride (GuCl), 1% 2-ME and treated with ethylene-imine to aminoethylate the cysteinyl residues of the protamines, thereby preventing formation of S-S bonds, then dialyzed against 50 mM HCl and chromatographed on a BioRex 70 column with stepwise elution by ascending concentrations of GuCl. Pure P1 appears in the 23% eluate, Pure P2 (a and b) appears in the 27% eluate, while a series of phosphorylated variants of P2 together with a portion of the P1 appear in an intermediate pool (25% eluate). The P1 and P2 (23 and 27% eluates) were each dialyzed overnight against 50 mM HCl, followed by 2–3 h dialysis against PBS, pH 7.2, and the concentration of each was measured by a dye-binding assay (21). The synthetic peptides were prepared by the Protein Sequencing Laboratory at The Rockefeller University by the method of Merrifield (22) and purified by reverse-phase HPLC.

Polyarginine (10,000–20,000 mol wt) was obtained from Chemical Dynamics Corp. (Plainfield, NJ) and polylysine (15,000–30,000 mol wt) was from Sigma Chemical Co. (St. Louis, MO).

Purified calf thymus histones and pure histone H_3 were a gift from Dr. Richard Sterner (The Rockeller University, New York, NY).

Monoclonal Antibodies. The mAb to human sperm protamines (HPmAb) was obtained by immunization of a BALB/c mouse with the nuclear protein fraction of human sperm and fusion of the spleen cells with a nonsecreting mouse myeloma. In preparation for this study the hybridoma culture was recloned by limiting dilution to assure monoclonality. The HPmAb was isolated from ascites fluid by ammonium sulfate precipitation and purified by dialysis against PBS followed by DEAE column chromatography.

The mAb against chick myosin, used as control, was a gift from Dr. Anuradha Saad (Cornell University Medical College, New York, NY) and was similarly prepared from ascites fluid.

Human Sera. The sera were either donated by the authors and other laboratory personnel or obtained as clinical specimens identified only with regard to date of birth, sex, and "no findings."

Immunoabsorptions. For each of the immunoabsorption procedures the weight of absorbing protein stated in the figure legend was coupled to 0.5 gm cyanogen bromide–activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) in an 0.7 × 10 cm column. 40–50 μl serum, diluted 1:50 with 1% BSA/sodium borate buffer, pH 7.5, was layered on the column, which was then mixed end over end, overnight at 4°C. The absorbed serum was drained out of the column and tested by ELISA at the noted dilutions for reactivity with the designated antigens.

Homology Search. A search for proteins bearing a six residue segment designated as one with a minimum of four arginyl residues including a cluster of three was carried out (9/11/87) by the Protein Identification Source of the National Biomedical Research Foundation, and augmented by our own survey of the current literature.

Results

Occurrence of Protamine-reactive Antibodies in Human Sera. The data of Table I show that protamine-reactive antibodies are present in all sera of a cohort of normal subjects aged 0–60 yr. Protamine-reactive IgG antibodies are present in all sera and IgM antibodies are present in those whose immune systems have matured sufficiently to produce a significant titer of IgM (23). A proportionality of the total IgM represented by the protamine-reactive IgM was computed for each serum of a group from each subject category (Fig. 1). We have not been able, thus far, to isolate the protamine-reactive IgM antibodies from human sera, with activity intact. The computation, therefore, does not designate the actual weight of protamine-reactive IgM but does define the titer of protamine reactive antibody, compared with the titer of total IgM, within a similar assay protocol. The values derived for that proportionality, i.e., ratio of protamine-reactive IgM

TABLE 1
Occurrence of Human Serum Antibodies Reactive with Human Sperm Protamines

Age	Number tested	IgM	IgG
0 (cord blood)	1	0	1
1 d	2	0	2
2 d	2	0	2
4 d	3	0	3
5 d	2	0	2
7 d	3	2	3
10 d	3	2	3
12–28 d	11	11	11
1–12 mo	9	9	9
1–2 yr	4	4	4
adult ♂	41	41	41
adult ♀	59	59	59

All sera were assayed at 1:100, 1:500, 1:1,000 with pooled human protamines as antigen. All sera designated as positive displayed reactivity at 1:500 dilution. Titers of IgM and IgG reactivity with protamines were computed relative to that of a reference serum (18, 19). Negative IgM values were <1% of reference serum; positive IgM values for pediatric sera ranged from 15% (7-d neonate) to 65% of reference serum; IgG values were 40–90% of reference serum. For adult sera, the range of IgM reactivity with protamine was 54–180% of reference serum and that of IgG reactivity was 50–200%. Total IgG and IgM titers for all sera were within normal range for the respective age groups (23).

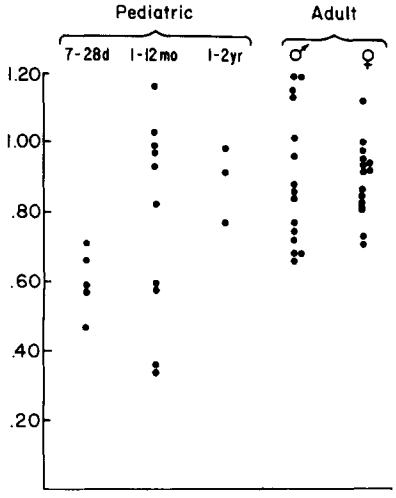

FIGURE 1. Proportionality of protamine-reactive IgM to total IgM in human sera. A value (x) for total IgM for each serum was derived as percentage of a reference serum (see Materials and Methods) and a value (y) for protamine-reactive IgM was derived as percentage of the same reference serum. The plotted values are $\frac{x}{y}$ for each serum.

to total IgM, for all population subgroups falls within a narrow range (Fig. 1), suggesting that the protamine-reactive antibodies represent a specific, and perhaps obligatory, component of the natural antibody repertoire of normal human serum.

Identification of Human Sperm Protamines. The nuclear proteins of human sperm include two protamines, designated P1 and P2. Determination of the

amino acid sequences has shown that P2 exists as two variants differing only in that P2a has three additional residues at its NH_2 terminus (Table II*A*) (24, 25). We have recently demonstrated that P1 and P2a,b are obtainable, in purified form and in high yield, separately from a group of phosphorylated variants of P2 (26). Those preparations of P1 and P2 are in "ideal state" for use as antigen in solid-phase assay since each is a small molecule with high positive charge favoring linear orientation on the polystyrene surface and there is little possibility of intramolecular crosslinkage since, in the preparative process, the cysteinyl residues are amino-ethylated (see Materials and Methods), thus blocking formation of disulfide bonds.

Specificity of HPmAb for Protamines: Definition of the Epitope. We have prepared an mAb (HPmAb), using the nucleoprotein fraction of human sperm as the immunogen, that is reactive with P1 and P2 and the phosphorylated variants of P2 and with no other component of human sperm (Fig. 2). Repeated assays, using different batches of purified P1 and P2 have confirmed that, although the reactivity with each is of the same order of magnitude, HPmAb displays greater reactivity with P1 when assayed against molar equivalents of P1 and P2. Inspection of the primary structure of those molecules (Table II*A*) reveals that there is not extensive sequence homology between the two; positional homology at four residues within a six residue piece is displayed in only three segments: 7–12 of P1 with 20–25 of P2b, 29–34 of P1 with 34–39 of P2b, and 45–50 of P1 with 49–54 of P2b. However, for each, P1 and P2b, arginine comprises 48% of the amino acid composition, and, in each, a number of the arginyl residues are clustered. Although a greater proportion of the arginyl residues of P2 is grouped in clusters of two or more residues, P1 has a single cluster of six arginyl residues. That inspection of the amino acid sequences suggested that recognition of the protamines by HPmAb might be dependent upon arginine density. The HPmAb, therefore, was assayed against three synthetic decapeptides (Table II*B*) representing segments of P2 selected for differential density of arginine and for variety of other amino acids, and against polyarginine, representing maximum arginine density. The high reactivity of HPmAb with polyarginine (Table III *A*) supports the proposition that the recognition is dependent upon clustered arginyl residues and suggests that other amino acids are not essential for recognition of the epitope. Peptide 1 shows no reactivity with HPmAb, suggesting that a cluster of two arginyl residues is inadequate for recognition while the modest, but real, reactivity of peptide 2 suggests that a density of four arginyl residues, including a cluster of three, within a six residue piece is recognized (Table III*A*). The apparent inconsistency of the display of reactivity by peptide 3 (Table III*A*), in which the four arginyl residues are single, was readily resolved. When the synthetic peptides were used as synthesized, i.e., not treated to block the cysteine–cysteine crosslinkage, peptide 3 showed reactivity with HPmAb. However, when the three peptides were treated, as in the preparation of the protamines (see Materials and Methods), to block the formation of S-S bonds between the cysteinyl residues, the reactivity of peptide 3 with HPmAb was eliminated (Table III*B*). We interpret that observation to indicate that, in the untreated state, the two cysteinyl residues in peptide 3 are crosslinked, resulting in a conformation in which the four single arginyl residues are brought into juxtaposition so that

TABLE II

Amino Acid Sequences

	A Human sperm protamines
P1	A R Y R C C R S Q S R S R Y Y R Q R Q R S R R R R R S C Q T R R R A M R C C R P R Y R P R C R R H
P2a	R T H G Q S H Y R R R H C S R R R L H R I H R R Q H R S C R R R K R R S C R H R R R H R R G C R T R K R T C R R H
P2b	G Q S H Y R R R H C S R R R L H R I H R R Q H R S C R R R K R R S C R H R R R H R R R G C R T R K R T C R R H

	B Synthetic peptides (P2b fragments)
peptide 1 (P2b 16–25)	H R I H R R Q H R S
peptide 2 (P2b 26–35)	C R R R K R R S C R
peptide 3 (P2b 42–51)	R G C R T R K R T C

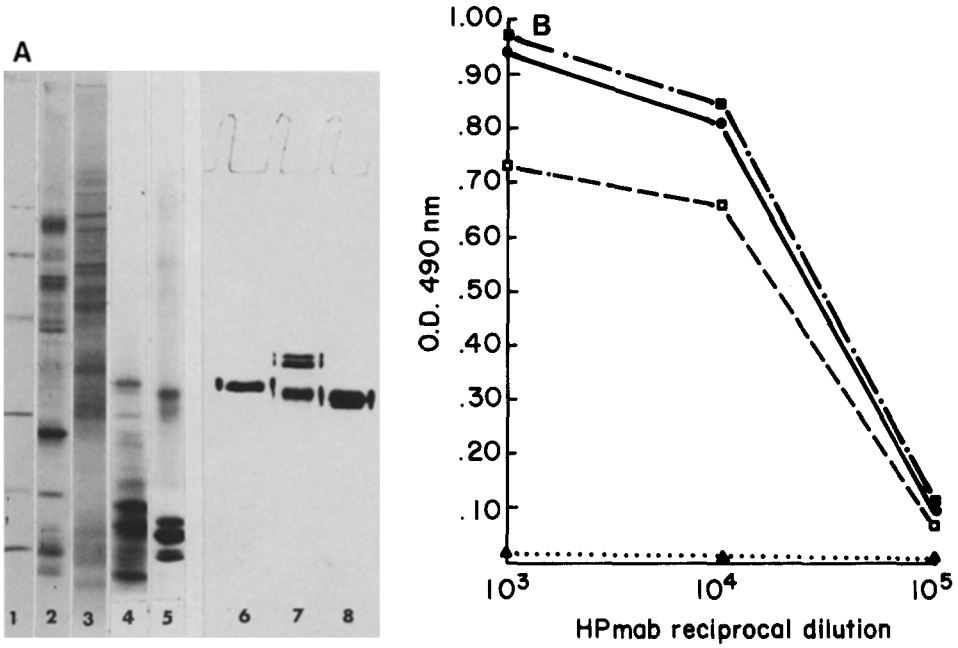

FIGURE 2. (*A*) Fractions of human sperm proteins. (Lanes *1–5*) SDS PAGE; (lanes *6–8*) acetic acid–urea PAGE. (Lane *1*) Mol wt markers, 14.4, 20, 30, 43, 67, 94 × 10^3; (lane *2*) tail proteins; (lane *3*) Triton-X soluble proteins of head membranes; (lane *4*) 8% eluate of BioRex-70 chromatogram of nuclear proteins; (lane *5*) 16% eluate; (lane *6*) 23% eluate (P1); (lane *7*) 25% eluate (P1 and phosphorylated P2); (lane *8*) 27% eluate (P2). (*B*) ELISA values of HPmAb reactivity with each sperm protein fraction. (Lanes *2–5*) ····; (lane *6*) —·—·—; (lane *7*) ——; (lane *8*) ---. HPmAb is reactive only with the three protamine fractions (lanes *6*, *7*, and *8*).

those residues are in adequate proximity or orientation for antibody recognition to take place. With the cysteinyl residues blocked, that conformation was not induced and peptide 3 was not reactive with HPmAb (Table III*B*). Consonant with that observation, the reactivity of peptide 2, putatively dependent upon a density of four arginyl residues including a triplet, and the absence of reactivity of peptide 1 were not altered by treatment to block cysteine–cysteine crosslinkage (Table III*B*).

The specific requirement of the HPmAb for an epitope including an arginine triplet was further documented when the HPmAb displayed no reactivity (data not shown) against the more prevalent set of basic nuclear proteins, the histones and, specifically, against the arginine-rich histone H3, in which there are two arginine doublets, residues 52, 53 and 128, 129, but no arginine triplet (27).

The possibility that recognition by HPmAb of a cluster of three or more arginyl residues is due solely or mainly to a concentrated high positive charge was probed further by comparing the reactivity of HPmAb with polyarginine, representing maximal net positive charge as well as maximal arginine density, with that of polylysine, representing very nearly as high net positive charge (Fig. 3). The assay data of Fig. 3 indicate that the specificity of the HPmAb is not that of recognition of an epitope with high positive charge but is related to unique

TABLE III
Reactivity (OD_{490}) of HPmab with Synthetic Peptides (Table II) and Polyarginine

	Antigen (μg/ml)	HP mab 1:1,000	Myosin mab 1:1,000	Nonimmunized mouse serum 1:100
A	Peptide 1 (50)	0.06	0.06	
	Peptide 1 (10)	0.00	0.01	
	Peptide 2 (50)	0.33	0.05	
	Peptide 2 (10)	0.27	0.01	↓
	Peptide 3 (50)	0.25	0.05	<0.04
	Peptide 3 (10)	0.11	0.01	↑
	Polyarginine (20)	0.82	0.10	
	Polyarginine (10)	0.80	0.08	
B	Peptide 1 (50)	0.06	0.04	
	Peptide 2 (50)	0.32	0.03	
	Peptide 3 (50)	0.05	0.04	

(A) Peptides untreated. (B) Peptides amino-ethylated to block formation of S-S bonds between cysteinyl residues. (A) HPmab is reactive with peptides 2,3 (untreated) and with polyarginine. (B) Amino-ethylation of peptides results in elimination of reactivity of HPmab with peptide 3 and no change in reactivity of peptide 1 or 2. Control mab (anti-myosin) and non-immunized mouse serum are not reactive with the peptides or polyarginine.

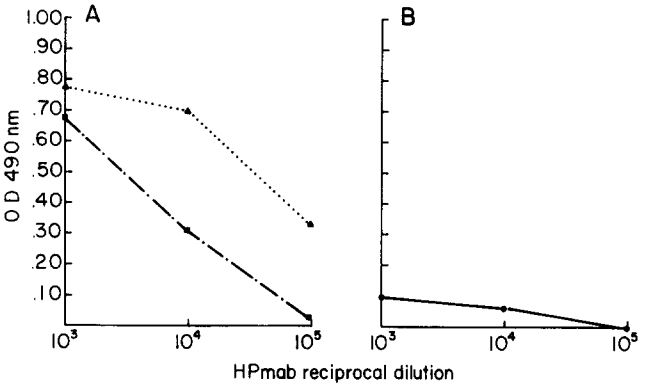

FIGURE 3. Reactivity of HPmAb with (*A*) polyarginine and (*B*) polylysine at ascending antigen concentrations. 2 μg/ml polyarginine (—·—·—·); 5, 10, or 20 μg/ml polyarginine (····); 2, 5, 10 or 20 μg/ml polylysine (——). Each plotted value represents the mean of four assays, carried out in duplicate.

properties inherent in a cluster of arginyl residues. The nature of those properties has not yet been determined.

Comparison of the reactivity curves (Figs. 3 and 4) indicates greater similarity in the binding kinetics of HPmAb with P1 and polyarginine than with P1 and P2. For both P1 and polyarginine, HPmAb appears to be saturated at low antigen concentration. For polyarginine that might be postulated to be due to a higher order of reiteration of the epitope, but in both P1 and P2 the putative minimum epitope of six amino acids (28), in this instance a six amino acid piece with four arginyl residues including a cluster of three, appears twice. The greater reactivity of HPmAb with P1 (Fig. 4), therefore, may be due to the cluster of six arginyl residues, a density of arginine not present in P2 (Table II*A*).

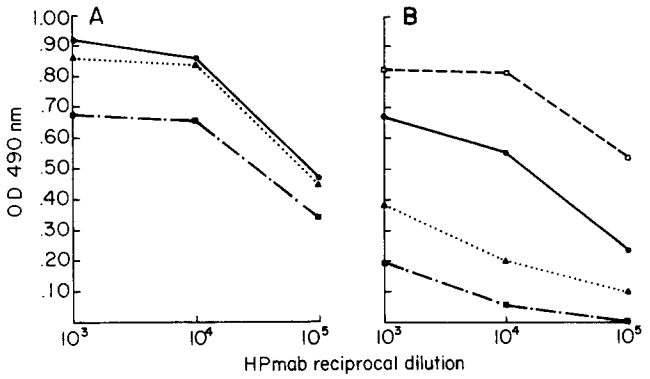

FIGURE 4. Reactivity of HPmAb with (*A*) P1 at 2 μg/ml (—·—·—·); 5 μg/ml (····); 10 or 20 μg/ml (——); (*B*) P2 at 2 μg/ml (—·—·—·); 5 μg/ml (····); 10 μg/ml (——); 20 μ/ml (——). See text for discussion. Each plotted value represents the mean of five assays, each carried out in duplicate. With molar equivalents (equivalent weights) of P1 and P2, reactivity of HPmAb is greater with and saturated at lower antigen concentrations of P1.

An overview of the foregoing data leads to the proposition that the HPmAb reacts with clustered arginyl residues, with activity strength related to arginine density, with a minimum requirement of four within a six residue epitope with three of the arginyl residues in series or in juxtaposition to provide a specific configuration of three arginyl residues; the precise geometry of that configuration has not been defined.

Protamine-reactive Antibodies in Human Serum. The properties of the human serum protamine-reactive IgM antibodies (Table I) were investigated by comparison with those established for HPmAb and by determining whether the reactivity resides in a specific subset of human IgM antibodies.

The relative reactivities of a representative group of human sera (adult male, adult female, and pediatric) with P1 and P2 and polyarginine were assayed (Fig. 5). Despite the expected differences in absolute titer, the similarity among the sera in rank order of activity with the three antigens, in the slopes of serum dilution versus reactivity curves and in the response to increasing antigen concentration suggests that the serum reactivity is that of a discrete set of IgM antibodies present in all normal sera. The data of Fig. 5 also confirm that the protamine-reactive IgM antibody set of pediatric sera has the same properties as that of adult sera.

Concordance between the immuno-recognition properties of the human IgM antibodies and those of the HPmAb was demonstrated by competition ELISA (Fig. 6). The inhibition by HPmAb of the serum antibody reactivity with both P1 and P2 was proportionate to the concentration of HPmAb. Nearly 50% inhibition of the serum reactivity with P1 was achieved by HPmAb dilution of 1:1,000. Consonant with the observation that the HPmAb displays greater reactivity with P1 than with P2 (Figs. 2*B* and 4), the data of Fig. 6 also indicate that the HPmAb displays greater competition with the P1 reactivity of the serum antibodies than with the P2 reactivity.

Assurance that the blocking of the serum IgM reactivity with P1 and P2 was due to specific epitope recognition by the HPmAb was provided by parallel assays carried out with a mouse mAb to chick myosin and with normal (nonimmunized) mouse serum, with no inhibition of human serum IgM reactivity with P1 and P2 exhibited by either (data not shown).

The converse competition assay, i.e., blocking of the HPmAb with human

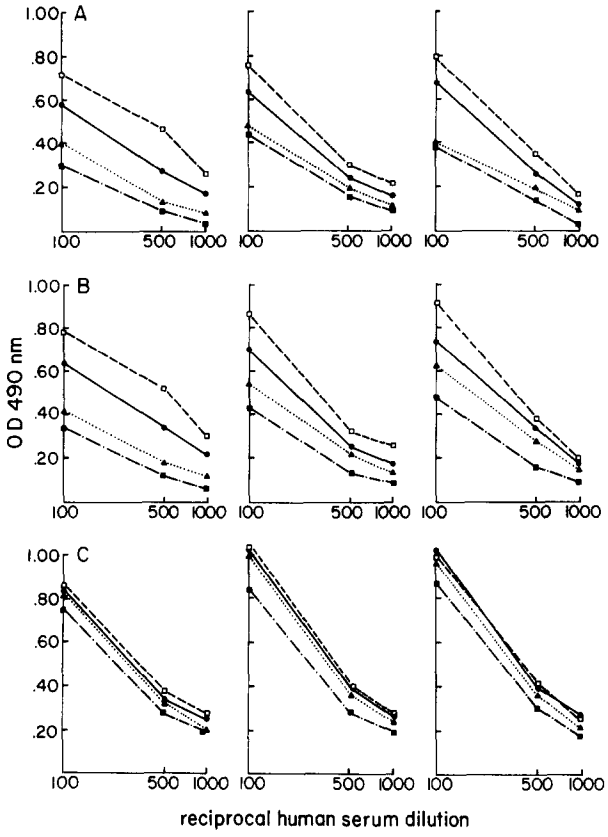

FIGURE 5. Reactivity of human serum IgM with (*A*) P1, (*B*) P2, (*C*) polyarginine at 2 μ/ml (·–·–·); 5 μg/ml (·····); 10 μg/ml (——); 20 μg/ml (– – –) of each antigen. (*Left panels*) pediatric (6 mo) serum; (*middle panels*) adult male; (*right panels*) adult female.

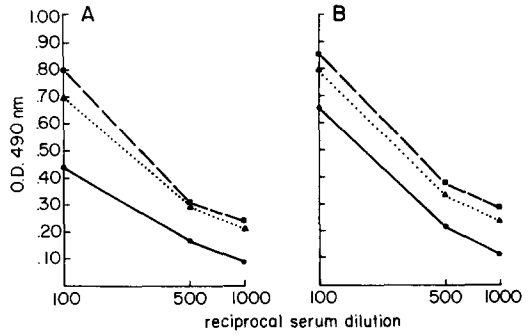

FIGURE 6. Competition by HPmAb with human serum IgM reactivity against (*A*) P1 and (*B*) P2. The standard ELISA protocol (see Materials and Methods) was carried out with the following modification: Before the addition of human serum to the antigen-bound wells, 50 μl HPmAb at dilution 1:1,000 (——), 1:10,000 (· · · ·), no HPmAb (– – –) was placed in each well for 1 h, then washed out and followed by human serum in the noted dilutions; reactivity was detected by use of peroxidase-conjugated anti-human IgM. (*A*) HPmAb 1:1,000 blocked ~45% of serum reactivity, at 1:100 and 1:500 of serum, with P1. (*B*) HPmAb 1:1,000 blocked ~25% of serum reactivity with P2 when the serum was 1:100 and ~45% of reactivity when the serum was 1:500.

serum, also indicated that the epitope recognized by the HPmAb displays homology with that recognized by the human protamine-reactive IgM antibodies (Table IV). Although the maximum inhibition by human serum that was displayed in the solid-phase blocking assay used in this study was only ~15% (Table IV) similar blocking activity was effected by a representative serum from each of

TABLE IV

Competition by Human Sera (Adult ♂, Adult ♀, Pediatric) with Reactivity of HPmab against (A) P1 (10 μg/ml) and (B) P2 (10 μg/ml)

	Serum (1:100)	HPmab 1:1,000		HPmab 1:10,000	
		OD	Decrease	OD	Decrease
			%		%
A	0	0.79	—	0.74	—
	Adult ♂	0.71	10	0.65	12
	Adult ♀	0.70	12	0.65	12
	Pediatric	0.68	14	0.63	15
B	0	0.64	—	0.57	—
	Adult ♂	0.61	—	0.53	—
	Adult ♀	0.62	—	0.51	11
	Pediatric	0.58	10	0.51	11

The ELISA protocol (see Materials and Methods) was carried out with the insertion of a single step: before addition of HPmab, 50 μl of human serum was added to each well, and after 1 h (room temperature) was removed and the wells were washed. The reactivity was detected by peroxidase-conjugated anti–mouse IgA, IgG, IgM.

TABLE V

Reactivity (OD_{490}) of Human Sera IgM with Synthetic Peptides (Table II) (A) Peptides Untreated (B) Peptides Amino Ethylated

		Serum					
		Adult ♂		Adult ♀		Pediatric	
		1:100	1:500	1:100	1:500	1:100	1:500
A	Peptide 1	0.12	0.09	0.02	0	0.07	0.02
	Peptide 2	0.72	0.24	0.70	0.29	0.63	0.19
	Peptide 3	0.82	0.24	0.47	0.18	0.56	0.20
B	Peptide 1	0.06	0.02	ND	ND	ND	ND
	Peptide 2	0.78	0.24	ND	ND	ND	ND
	Peptide 3	0.05	0.01	ND	ND	ND	ND

Human serum IgM antibodies are not significantly reactive with peptide 1 untreated or treated, are reactive with peptide 2 when both untreated and treated, and are reactive with peptide 3 untreated, but not when the peptide is treated to prevent cysteine–cysteine crosslinkage (see Table III).

the human population classes, adult male, adult female, and pediatric. That observation is in accord with the display of similar discrimination and activity of adult and pediatric sera with the synthetic peptides used to define the epitope for HPmAb (compare Tables III and V). The data of Table V, showing that the reactivity of the serum antibodies with the synthetic peptides parallels that of the HPmAb, also provide further support for the consideration that the epitopes for the two categories of antibodies are similar. The IgM antibodies of all three sera recognized, as did the HPmAb (Table III), peptide 2 in either ethylated or nonethylated state (cysteine-cysteine crosslinkage blocked or unblocked), did not

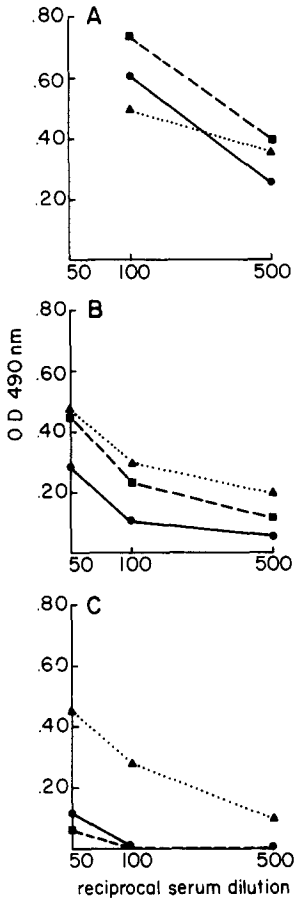

FIGURE 7. Reactivity of human serum IgM with P1 (——); P2 (—), and sperm head membrane proteins (· · · ·) after absorption of serum on pooled protamines. (*A*) Nonabsorbed serum; (*B*) serum absorbed on 100 μg of protamines; (*C*) serum absorbed on 200 μg of protamines.

recognize peptide 1 in either state, and recognized peptide 3 when nonethylated, but not when ethylated (Table V).

To assure that the serum reactivity with protamines is attributable to a circumscribed set of IgM antibodies, a series of affinity absorption procedures was carried out. First, it was demonstrated that those antibodies distinguish between a fraction of pooled protamines and a fraction of sperm membrane proteins which, as shown previously (29), includes eight or nine proteins that are reactive with naturally occurring human serum antibodies. After absorption on an affinity column to which ascending amounts of pooled protamines were bound, the serum reactivity with protamines was progressively reduced to zero while only minor reduction of serum reactivity with the membrane protein fraction was observed (Fig. 7).

As in the analysis of the epitope for the HPmAb, the possibility was considered that the reactivity of the serum antibodies was primarily that of a charge-dependent reaction. Also, since autoantibodies to histones have been identified in both normal and pathologic human sera (30) an assessment was made of the ability of human serum IgM antibodies to discriminate between protamines and histones. In affirmation of the high sensitivity of the ELISA method used in this

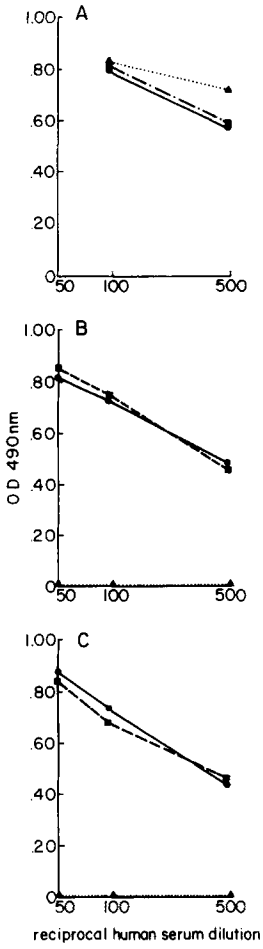

FIGURE 8. Reactivity of human serum IgM with P1 (—·—·—·—·), P2 (——), purified calf thymus histones (including equimolar weights of H1, H2a, H2b, H3, and H4) (· · · ·), after absorption on histones. (*A*) Nonabsorbed serum, (*B*) absorbed on 200 μg of histones, (*C*) absorbed on 500 μg of histones.

study (18, 19) a high titer of IgM antibodies reactive with purified calf thymus histones was detected in the test serum (Fig. 8*A*). After absorption of an aliquot of the serum on a histone bound affinity column, however, that reactivity was eliminated while reactivity of the histone-absorbed serum against protamines was virtually unchanged (Fig. 8, *B* and *C*).

Further assurance that the protamine reactivity of a subset of IgM antibodies in human serum is not that of charge attraction was obtained by the data of Fig. 9. When tested with polylysine, IgM antibodies of human serum did bind to that antigen. However, when the serum was absorbed on a polylysine-bound affinity column, reactivity with polylysine was deleted while reactivity with protamines and with polyarginine was retained (Fig. 9*B*). Conversely, when the serum was absorbed on polyarginine, reactivity with polylysine was retained while reactivity with protamine 1, protamine 2 and polyarginine was deleted (Fig. 9*C*).

Antigenic Sites Recognized by HPmAb and Human Serum Ig Antibodies are Similar (Perhaps Identical). Table VI is a summary comparison of the reactivity of HPmAb, a mouse IgG secreted by a hybridoma of reasonably assured monoclonality (see Materials and Methods) and the reactivity of a specific subset of

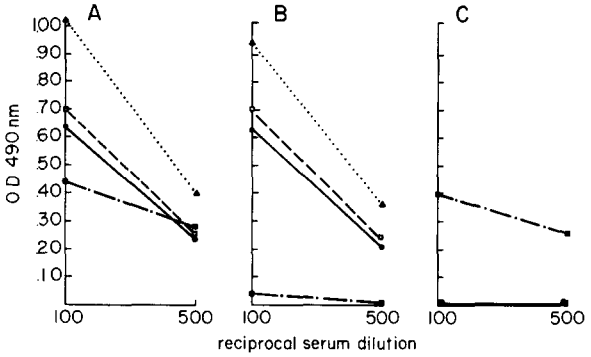

FIGURE 9. Reactivity of human serum IgM with P1 (——), P2 (---), polyarginine (····), and polylysine (—·—·—·—·). (*A*) Non-absorbed serum; (*B*) serum absorbed on 200 μg polylysine; (*C*) serum absorbed on 200 μg polyarginine.

TABLE VI

Summary of Concordance of Antigen Recognition by HPmab and Protamine Reactive Serum IgM Antibodies

Antigen	ELISA		Protamine-reactive serum IgM
	HPmab	Total serum IgM	
P1	++	+	
P2	+	++	
Polyarginine	++	++	
Polylysine	−	+	—*
Peptide 1	−	−	
Peptide 1, ethylated	−	−	
Peptide 2	+	+	
Peptide 2, ethylated	+	+	
Peptide 3	+	+	
Peptide 3, ethylated	−	−	
Pooled CTH	−	+	—‡
Histone H3	−	+	—§
Sperm membrane proteins	−	+	—‖

* Serum absorbed on polylysine: polylysine-reactive antibodies deleted, protamine-reactive antibodies retained.

‡ Serum absorbed on CTH: histone-reactive antibodies deleted, protamine-reactive antibodies retained.

§ By inference from ‡.

‖ Serum absorbed on pooled protamine: protamine-reactive antibodies deleted, sperm membrane protein-reactive antibodies retained.

natural IgM antibodies in human sera. That comparison indicates that the epitopes for the two antibody sets show a high degree of homology. The principle difference detected by the experimental protocols of this study is that of apparent rank order of reactivity with the three antigens, human sperm protamine 1, human sperm protamine 2, and polyarginine (compare Figs. 3 and 4 with Fig. 5). One possible explanation for that difference would be that the protamine-reactive human IgM antibodies include several subsets with modestly different preferences for intra-epitope arrangement of arginyl residues, or for extra-epitope factors that influence binding patterns (28, 31). It is apparent, however,

that the dominant and common characteristic of the epitope recognized by both HPmAb and serum IgM antibodies is that of clustered arginyl residues.

Since the protamine-reactive IgM antibodies are present in sera of individuals who have had no exposure to sperm or protamines, the possibility was considered that other proteins autogenous to humans with segments homologous to that putative epitope might be related either to origin (immunogenic stimulation) or to role (antigenic recognition) of that set of natural IgM antibodies. A search was made of protein sequence data bases for segments of six amino acids with four arginyl residues, including a cluster of three. That sequence was identified in all of the vertebrate and invertebrate protamines or sperm-specific histones that have been sequenced, in a few bacterial proteins mainly of *E. coli*, and in a number of viral proteins (Table VII). The only human autogenous proteins displaying that sequence, other than protamines, are two complement precursors, two T cell glycoproteins and one (inferred) variant of the γ chain of class II histocompatibility antigens (Table VII).

Discussion

We have detected a set of natural IgM antibodies in sera of normal human males and females of all age groups, that are reactive with human protamines, the basic nuclear proteins of sperm, and we have identified the minimum antigenic site of six residues as one with four arginyl residues including a cluster of three. Since those antibodies are present in sera of pediatrics, who have had no exposure to protamines, immunogenic stimulation by protamines as the origin of those antibodies is argued against. Within the context of currently recognized immunologic mechanisms, the origin of those antibodies, therefore, may be attributed to other autogenous molecules with a sequence homologous with the protamine antigenic site or to idiotopes on other Igs bearing the image of the protamine antigenic site. Studies directed to the latter possibility are in preliminary status, hampered by the general difficulty of isolating specific subsets of IgM antibodies from human sera (32) and the particular difficulty in isolating the protamine-reactive antibodies with activity retained; those antibodies appear to show very high binding strength to the conventional types of binding matrices (our unpublished observations).

The possibility that those antibodies are induced by any of the segments of the human proteins listed in Table VII is intriguing since all are believed to be involved in immunoregulatory mechanisms and all may be present in the nascent immune system. On the other hand, since the neonatal gastro-intestinal tract is populated with *E. coli*, the possibility that the *E. coli* proteins (Table VII) provide the initial stimulus for induction of the protamine-reactive antibodies also may be considered and, in fact, a hypothesis of long-standing states that natural antibodies may arise from stimulation by intestinal tract flora (2).

Perhaps even more provocative for consideration is the possible role of the protamine-reactive antibodies, particularly in view of the limited spectrum of the proteins listed in Table VII, many of which are of viruses with human host specificity. The possibility that viral proteins may be the antigenic target of those antibodies is reminiscent of an early hypothesis that one role of natural antibodies may be that of defense against potential infectious agents (1).

TABLE VII

Proteins, Other than Protamines and Sperm-specific Histones, with a Segment of Six Amino Acids Including Four Arginyl Residues, Three of which Are Arranged in a Cluster

	Protein	Residues
Human	Complement C3 precursor	668–673
	Complement C4A	1,426–1,431
	T cell surface glycoprotein CD8 precursor	208–213
	T cell surface glycoprotein CD4 precursor	422–427
	Inferred precursor of γ chain of histocompatibility antigen	
Bacteria		
Escherichia coli	Replication initiation protein	57–62
		65–70
	Transposase	139–144
	nus B protein	3–8
	30 S ribosomal protein	2–7
	Alanyl-tRNA synthetase	430–435
	Diaminopimelate decarboxylase	403–408
	DNA-directed RNA polymerase	197–202
		1,368–1,373
Serratia marcescens	Anthranilate synthetase (component II)	157–163
Viruses		
Herpes simplex (type 1)	Kinase-related transforming protein	141–146
Herpes simplex (type 2)	Glycoprotein D	364–369
Papillomavirus (type 6b)	Probable L2 protein	4–9
Papillomavirus (type II)	Probable L2 protein	3–8
		438–443
Papillomavirus (type 33)	Probable L2 protein	450–455
	Probable E6 protein	140–145
Papillomavirus (type 8)	Probable L2 protein	318–323
		327–332
Hepatitis B virus (subtype AYW and 6 subtypes)	Core antigen	150–155
		155–160
		164–169
		171–176
Epstein-Barr virus (strain B95-8)	Hypothetical BHLF 1 protein	157–162
		282–287
		407–412
		532–537
		2,085–2,090
	Hypothetical BORF 1 protein	9–14
	Probable glycoprotein	528–533
		837–842
	Hypothetical BSLF 1 protein	859–864
	Hypothetical BLLF 2 protein	53–58
	Hypothetical BERF 3 protein	90–95
		362–367
	Hypothetical BRLF 1 protein	86–91
Adenovirus 2	Minor core protein	315–320
		331–336
	Probable early E4 34K protein	265–270
	Probable early E4 13K protein	70–75
	Probable early E1B 21K protein	157–162

TABLE VII—*Continued*

	Protein	Residues
	Late L1 52K protein	53–58
	Early E2A DNA-binding protein	46–51
	Major core protein precursor	100–105
		152–157
	Terminal protein	349–354
		366–371
Adenovirus 5	Probable early E1B 21K protein	157–162
	Late L1 52K protein	54–59
	Early E2A DNA-binding protein	46–51
Adenovirus 7	Terminal protein	338–343
		355–360
Rhinovirus 2	Genome polyprotein	1,396–1,401
Yellow fever virus (strain 17D)	Genome polyprotein	1,702–1,707
Sindbis virus (two strains)	Structural polyprotein	22–27
	Nonstructural polyprotein	1,886–1,891
T cell leukemia virus (HTLV II)	env polyprotein	305–310
HIV (HTLV III)	Trans-activating transcriptional regulatory protein: version 1	61–66
	version 2	39–44

These studies have provided a definition of the antigenic site with which a set of natural IgM antibodies of human sera are reactive. Thus far, however, neither the origin nor the role of that set of antibodies is clear. The prevalence of those antibodies in normal sera and the relatively high and constant proportion of the total circulating IgM in which they are present suggest that such clarification would be a significant contribution to the understanding of innate, in contrast to induced, immunologic defense systems.

Summary

We have identified a set of natural IgM antibodies in human serum that are reactive with protamines, a class of low molecular weight basic nucleoproteins that are synthesized *de novo* in the postpuberal testis and are unique to sperm. Those antibodies were detected by ELISA in significant titer in all of 100 sera of normal adult males and females and in 26 of 28 sera of normal pediatrics aged 7 d to 2 yr. Commonality between the protamine-reactive IgM antibodies of pediatric and adult sera was established by the demonstration of similarity in antigen recognition and reaction kinetics. Therefore, the role of protamines as either immunogenic stimulus or antigenic target of that set of natural antibodies is not likely.

The antigenic site recognized by the protein-reactive serum IgM antibodies was characterized by comparison with the pattern of antigen recognition by a monoclonal antibody to human sperm protamines (HPmAb). By the use of synthetic peptides simulating the amino acid sequences of various segments of human protamine 2 and of polyarginine, polylysine, and histones as test antigens, the principle characteristic of the antigenic site recognized by both HPmAb and the serum IgM antibodies was inferred to be that of clustered arginyl residues with an apparent minimum requirement of four arginyl residues, including a

triplet, within a six residue piece; for both, the reaction was shown to be not dependent upon charge attraction. A series of immunoabsorption procedures indicated that the protamine-reactive serum IgM antibodies are a discrete set with a high order of specificity.

A search of protein data bases revealed that the putative minimum epitope is present in four or five human autogenous proteins, all moieties of the immune system, and in a number of viral proteins. The possible implications of those findings are discussed in the light of early hypotheses concerning the origin and function of natural antibodies and the many recent reports of identification of natural antibodies in normal human sera. The set of natural antibodies identified in this study may be unique or may represent a class of antibodies present in the repertoire that, by virtue of the obscurity of their origin or function, have not been previously or extensively recognized.

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