

## SIX BALB/c IgA MYELOMA PROTEINS THAT BIND $\beta$ -(1 $\rightarrow$ 6)-D-GALACTAN

### PARTIAL AMINO ACID SEQUENCES AND IDIOTYPES

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Plasmacytomas are readily induced in the inbred BALB/c strain of mice by the intraperitoneal injection of mineral oils or branched chain alkanes such as 2, 6, 10, 14 tetramethylpentadecane (pristane). These tumors provide a source for a large number of different homogeneous immunoglobulins which are the products of a nonpolymorphic mammalian genome. Myeloma proteins in our laboratory and in the laboratory of Dr. Melvin Cohn of the Salk Institute have been screened for binding activity with a variety of antigens. From these studies have emerged groups of proteins that bind the same antigens. Further immunochemical analysis of the binding interactions has revealed that an antigen bound by several proteins is usually bound via the same haptenic group. Three major groups of antigen-binding myeloma proteins, defined by their haptening activity are, the  $\alpha$ (1  $\rightarrow$  3) dextran group (1, 2), the phosphorylcholine group (3, 4, 5), and the  $\beta$ -(1  $\rightarrow$  6)-D-galactan group (6, 7). With the  $\alpha$ (1 $\rightarrow$ 3) dextran group all of the proteins have been shown to contain lambda type light chains of identical amino acid sequence (2, 8). The proteins, however, differ from each other by possessing individual antigenic (idiotypic) specificities and different V<sub>H</sub> polypeptide chains (2, 8). In the phosphorylcholine group at least three distinct subgroups of light chains can be found based on widely different NH<sub>2</sub>-terminal sequences (9); one of these subgroups contains eight very closely related proteins that also share the same individual antigenic specificity suggesting structural identity (4, 5). In the present study we describe structural and antigenic studies on six IgA myeloma proteins of BALB/c origin with anti- $\beta$ -(1  $\rightarrow$  6)-D-galactan activity.

#### *Materials and Methods*

*Origin of Tumors.*—The plasmacytomas JPC1, SAPC 10, XRPC 24, XRPC 44, TEPC 191, and J539 which produced the corresponding myeloma proteins (J1, S10, X24, X44, T191, and J539) used in this study were induced in strain BALB/c mice by three bimonthly intraperitoneal injections of 0.5 ml mineral oil or pristane (Aldrich Chem. Co., Inc., Milwaukee, Wis.) (10, 11). The J539 plasmacytoma was induced at the Salk Institute and was kindly given to us

for this study by Dr. Melvin Cohn. The other five tumors were induced at the National Institutes of Health.

*Detection of Galactan-Binding Activity.*—The specificity of the J539 myeloma protein was discovered by screening against protein antigens to which *p*-azophenyl- $\beta$ -D-galactoside had been covalently bonded (12). Subsequently, we found that the J539 protein also precipitated with larchwood arabinogalactan and gum ghatti, both polysaccharides having multiple  $\beta$ -(1  $\rightarrow$  6)-D-galactopyranosyl linkages (6). The myeloma proteins in this laboratory were identified in screening studies using larchwood arabinogalactan (Calbiochem, San Diego, Calif.) and gum ghatti solutions (Fisher Scientific Co., Pittsburgh, Pa.).

Ascites or serum from mice with primary plasmacytomas was reacted by the micro-Ouchterlony method with arabinogalactan (2 mg/ml) or gum ghatti (1 mg/ml) and found to give strong lines of precipitation. Other antigens which are precipitated by these proteins have been isolated from natural products in the laboratory and include an extract of the hardwood bedding used in the mouse cages and an extract of milling wheat (6) (Fig. 1).

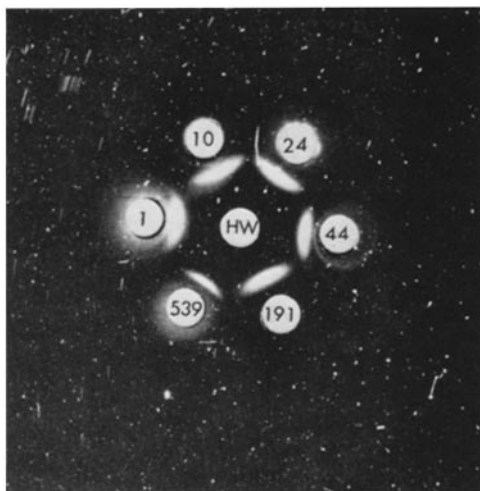


FIG. 1. Precipitation of six antigenalactan IgA myeloma proteins with an extract of hardwood bedding.

*Source of Myeloma Proteins.*—To produce large quantities of myeloma proteins  $10^5$  to  $10^6$  plasmacytoma cells dissociated from a subcutaneous tumor were transplanted intraperitoneally into mice which had received 1 or 2 intraperitoneal injections of 0.5 ml pristane 7–60 days before transplantation (13). The tumor lines themselves were maintained by serial subcutaneous passage using the trochar method (14) in BALB/c mice.

For production of large amounts of ascites the tumors were grown in (BALB/c  $\times$  DBA/2) F<sub>1</sub> hybrids. These mice, when primed with pristane, produced voluminous quantities of ascites. The ascites was removed by paracentesis with a 16 gauge needle. Several collections were made from each mouse. An average mouse produced 20–30 ml of ascites.

The myeloma proteins used in the hemagglutination and amino acid sequence studies were isolated from whole ascites by affinity chromatography using Sepharose-bovine serum albumin (BSA)-*p*-azo phenyl  $\beta$ -D-thiogalactopyranoside columns according to the methods previously described (7, 15). The proteins isolated by this method were shown to be pure by immunoelectrophoresis using rabbit antimouse serum.

*Idiotypic Antisera.*—Idiotypic antisera were prepared to each of the six proteins by im-

munization of strain AL/N or A/J mice with myeloma protein which had been partially purified by ammonium sulfate precipitation from serum or by washed immune precipitates made by precipitating the myeloma protein from ascites with a solution of arabinogalactan (2 mg/ml). Preparations of myeloma proteins were mixed with complete Freund's adjuvant (CFA)<sup>1</sup> and were injected into each mouse at six different sites. For the initial injection each mouse received approximately 400  $\mu$ g. After three injections with CFA the rest of the injections were given with incomplete Freund's adjuvant.

Serum was harvested by orbital puncture after day 30 and was shown to precipitate with the immunizing protein. For each protein the sera from five or more mice were individually collected. Three different sera were evaluated for each protein in the inhibition of hemagglutination test.

*Heavy Chain Class of Myeloma Proteins.*—All six of the galactan-binding myeloma proteins in this study were IgA class proteins as determined by a specific rabbit antimouse IgA serum.

*Inhibition of Hemmagglutination (HI).*—Sheep red blood cells (SRBC) were coupled with immunoadsorbant-purified myeloma protein by the CrCl<sub>3</sub> method (16). 0.1 ml of a 10  $\mu$ g/ml solution of protein in 0.85% NaCl was added to 0.1 ml of 0.05% solution of CrCl<sub>3</sub> and to this mixture 0.1 ml of a 50% suspension of washed SRBC was quickly added and allowed to react for 2–4 min at room temperature.

Idiotypic antisera for each protein were tested for hemagglutinating (HA) activity in the microtiter system and the limiting dilution was determined. A concentration of serum four times the end point in the HA test was used in the HI studies. In the HI test purified myeloma proteins were serially diluted and to each dilution a constant amount of antiserum was added followed by the addition of coupled SRBC. The end point reported is the reciprocal of the highest log<sub>2</sub> dilution of myeloma protein that gave complete inhibition.

*Chain Separation.*—Heavy and light chains were separated by partial sulfitolysis<sup>2</sup> followed by chromatography on Sephadex G-100 in 6 M urea-1 M acetic acid. Chains were used for sequence studies either as the sulfitolyzed product or were fully reduced with 0.01 M dithiothreitol in 5 M guanidine-0.05 M Tris-HCl pH 8.2 (2 h, 37°C) and alkylated with 0.02 M <sup>14</sup>C iodoacetamide (30 min, 37°C).

*NH<sub>2</sub> Terminal Sequence Determination.*—Sequence analyses were performed on a Beckman Model 890C Protein Sequencer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using the standard Beckman DMAA peptide program. 10–15 mg of polypeptide chain were dissolved in 0.5 ml acetic acid for application to the sequencer. The thiazolinone derivative obtained after each degradation cycle was converted to the phenylthiohydantoin (PTH) by incubation in 1 N HCl at 80°C for 10 min. The PTH's were extracted twice with ethyl acetate and dried under a stream of N<sub>2</sub>.

PTH-amino acids were identified by gas chromatography on SP400 columns (17) before and after silylation and/or thin-layer chromatography (18), and/or amino acid analysis. PTH derivatives were hydrolyzed to their respective amino acids under vacuum in 6 N HCl containing 0.05% thiodiglycol for ~18 h at 140°C. Hydriodic acid hydrolysis was performed according to the method of Smithies et al. (19). Cysteine was identified by scintillation counting of the <sup>14</sup>C-labeled derivative.

## RESULTS

*Partial Aminoterminal Sequences.*—The NH<sub>2</sub>-terminal sequences of the light chains from the six galactan-binding proteins are presented in Table I. As may

<sup>1</sup> Abbreviations used in this paper: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; HA, hemagglutinating activity; HI, inhibition of hemmagglutination; PTH, phenylthiohydantoin; SRBC, sheep red blood cells.

<sup>2</sup> Appella, E. Personal communication.

TABLE I

*NH<sub>2</sub>-Terminal Sequences of Mouse Anti- $\beta$ →1.6 Galactan Proteins*

		V <sub>L</sub>																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
	Glu	Ileu	Val	Leu	Thr	Gln	Ser	Pro	Ala	Ileu	Thr	Ala	Ala	Ser	Leu	Gly	Gln	Lys	Val	Thr	Ileu	Thr	Cys									
S10																																
X24																																
X44																																
T191																																
J539																																
J1																																

		V <sub>H</sub>																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
	Glu	Val	Lys	Leu	Leu	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly	Ser	Leu	Lys	Leu	Ser	Cys	Ala	Ala (Ser)	Gly	Phe	Asp	Phe (Ser)			
S10																															
X24																															
X44																															
T191																															
J539																															
J1																															

be seen no differences were found in the first 23 residues of any of the light chains. The NH<sub>2</sub>-terminal sequences of the heavy chains from the six proteins were determined to residue 30 (Table I). Four of the chains (S10, T191, J539, and X44) had an identical sequence. The J1 heavy chain in contrast to the other five proteins had isoleucine instead of leucine at position five. Ileu was identified both by gas chromatography and amino acid analysis. Position 19 in the X24 H chain could not be identified. As Lys was identified at this position in the five other chains and since Lys is readily identified by amino acid analysis this position in X24 is occupied by an amino acid which we have not been able (for reasons not yet understood) to identify.

*Specificity of Antisera.*—The antisera to each myeloma protein were tested in the micro-Ouchterlony system for their ability to precipitate with ascites from each of the galactan-binding proteins (Fig. 2). AL or A/J mice strains used in preparing the antisera can form both idiotypic and anti-IgA allotypic antibodies to these proteins. Sera selected for the study precipitated strongly with the immunizing protein and weakly, if at all, with the other proteins. Only one antiserum (anti-J1) was purely idiotypic by the precipitin tests (Fig. 2). The anti-S10, T191, and anti-J539 antisera showed restricted activity and precipitated strongly with immunizing antigen and one other of the proteins. Several anti-S10 antisera precipitated T191 and S10 suggesting by this method shared idiotypic specificities. Anti-T191 occasionally showed weak precipitating activity with S10. The anti-J539 cross-reacted with X44. Antisera to X24 and X44 reacted with all of the proteins (Fig. 2). The precipitin reactions, however, utilize large amounts of antibody and do not reflect the specificity of diluted antiserum, which is revealed in the direct HA tests.

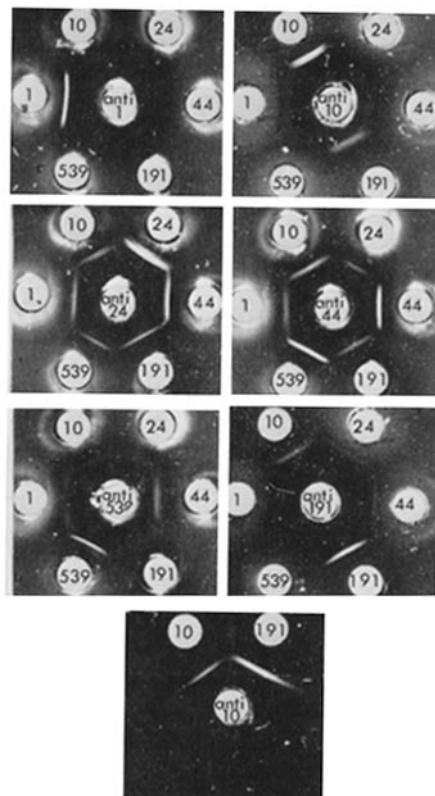


FIG. 2. Seven ouchterlony patterns showing precipitation of myeloma proteins with various antisera. In each of the patterns the unabsorbed antiserum was placed in the center well and serum or ascites from tumor-bearing mice placed in the outer well. Two anti-S10 antisera are illustrated, both of which show cross-reactions with T191. (For further description see text.)

Hemmagglutination titers were determined for 15 of the antisera in the study for five types of coupled cells (Table II). As may be seen, the highest titers were always to the immunizing protein indicating the preponderant antibody in any given serum was directed to idiotypic determinants. Some of the antisera were essentially specific for the immunizing protein while others with high titers for the immunizing protein cross-reacted at low titer with several but not all of the other proteins. Antisera prepared to the SAPC10 protein cross-reacted with TEPC191 as suggested by the precipitin studies.

*Inhibition of Hemagglutination by Antigalactan Myeloma Proteins.*—Using dilutions of the antisera ranging from  $1/800$  to  $1/50,000$  the antisera were found to contain only idiotypic specificities. First, at these dilutions none of the antisera were inhibitable by normal BALB/c serum. Second, other IgA myeloma proteins of BALB/c origin which did not have anti- $\beta$ -(1  $\rightarrow$  6)-D-galactan-binding

TABLE II  
*Direct Hemagglutination of SRBC Coupled with Myeloma Proteins*

Antiserum	No.	Dilution	Log <sub>2</sub> HA titer to cells coupled with myeloma proteins				
			J1	S10	X24	X44	T191
Anti-J1	1	1:10	12	5	0	0	0
	2	1:10	6	0	0	0	0
	3	1:10	15	5	3	0	0
Anti-S10	1	1:50	0	12	0	0	4
	2	1:10	0	14	0	0	3
	3	1:10	0	16	0	0	1
Anti-X24	1	1:50	0	0	18	0	0
	2	1:10	0	0	10	0	0
	3	1:10	0	5	10	11	0
Anti-X44	1	1:10	0	0	0	24	0
	2	1:10	0	0	0	20	0
	3	1:10	0	3	0	24	0
Anti-T191	1	1:10	0	6	0	0	15
	2	1:10	0	9	0	4	14
	3	1:50	0	8	0	3	10

activity were unable to inhibit the specific reactions. Finally, using concentrations of myeloma protein for inhibition ranging from 125 to 500  $\mu\text{g}/\text{ml}$ , all of the antisera were found to be individually specific (Table III). As may be seen in Table III most antisera at the concentrations tested were completely inhibited by dilutions that contained as little as  $10^{-1}$  to  $10^{-2}$   $\mu\text{g}$  of the specific myeloma protein. 1 thousand to 10 thousand-fold excesses of the other myeloma proteins in the antigalactan group failed to inhibit. The results clearly indicate that each of the antisera when used at limiting dilution identifies on its homologous immunizing myeloma protein an antigenic determinant not shared by any of the other proteins. One significant cross specificity was observed in one of the antisera prepared to S10 (Table III) that cross-reacted with T191.

#### DISCUSSION

The basic observations made in this study of six BALB/c IgA myeloma proteins with  $\beta$ -(1  $\rightarrow$  6)-D-galactan-binding activity were (a) all of the light chains had the same aminoterminal sequence to residue Cys23; (b) four of the proteins had the same V<sub>H</sub> aminoterminal sequence to residue 30, and the two other proteins differed from this sequence at only one position. The JPC1 protein has isoleucine at position five instead of leucine which is found in all of the others and the X24 protein has an unidentified residue at position 19; (c) each of the

TABLE III  
*Inhibition of Hemagglutination of Idiotypic Antisera with Coupled SRBC by Myeloma Proteins that Bind  $\beta 1 \rightarrow 6$  D-Galactan*

Idiotypic antiserum* to:	No. antisera	Max. conc. inhibitor	Log <sub>2</sub> titer of inhibitor giving complete inhibition					
			JPC-1	SAPC10	XRPC24	XRPC44	TEPC191	J539
		$\mu\text{g/ml}$						
JPC-1	1	125	8	—	—	—	—	—
	2	125	12	1	—	—	—	—
SAPC-10	1	500	—	14	—	—	8	—
	2	125	—	12	—	—	—	—
XRPC24	2	125	1	2	11	—	—	—
	1	125	—	3	12	—	—	—
XRPC44	1	125	—	2	—	12	—	—
	1	125	—	1	—	11	—	—
TEPC191	4	125	—	—	—	—	12-14	—
	1	500	—	3	—	—	11	—
J539	1	125	—	2	—	—	—	12
	2	125	—	2	1	—	—	10

— equals no inhibition observed.

\* Prepared in A/H mice.

proteins possessed a unique individual antigenic determinant which was demonstrable by an inhibition of hemagglutination method.

The observation that these six proteins are idiotypically different implies that they all have different primary structures. The similarity of the V kappa sequence up to the Cys23 residue suggests the light chains in these proteins could be the same or nearly so. Kappa chains with the same aminoterminal 23 residues have been shown to have 3-17 differences throughout the remainder of the chains (20, 21). These sequences, however, were done on light chains from myeloma proteins with no known antigen-binding activity. Proteins that all bind the same hapten may be much more similar to each other in primary structure due to the selective pressure to maintain the same antigen-binding specificity. Therefore, the fact that all of the antigalactan proteins have the same aminoterminal Cys23 sequence suggests that this particular V kappa polypeptide sequence is an essential component of anti- $\beta$ -(1  $\rightarrow$  6)-D-galactan-binding proteins in the BALB/c mouse. This is analogous to findings with the mouse lambda chains from proteins with  $\alpha$ (1  $\rightarrow$  3) dextran-binding activity described by Weigert et al. (2, 8). In the antidextran system all of the proteins thus far studied have identical lambda chains.

In contrast, light chains from phosphorylcholine-binding proteins are represented by at least three distinct germ line genes (9). We are currently undertaking a complete sequence analysis of the V regions of the light chains of the galactan-binding proteins to ascertain the types of variation (if any) which can be found among a group of apparently very similar proteins.

While less is known about heavy chain diversity in the mouse, partial amino acid sequences to residue 30 have indicated several subgroups, but the diversity in the amino terminal sequence appears to be far less than in the kappa chains (14, 20, 21). While the six  $V_H$  sequences were not identical they did appear to be closely related. In fact only two differences were noted among the six chains. A similar observation has been made for the heavy chains of phosphorylcholine-binding proteins. Of five chains studied four are identical through the first hypervariable region and the fifth has a single substitution at position 4.<sup>3</sup>

While these partial structural studies indicate that the proteins have extensive similarities it was demonstrated with the very sensitive inhibition of hemagglutination method that each protein possessed an individual antigenic specificity. This therefore requires that the proteins have other primary structural differences at some point beyond the aminoterminal sequence reported here. Thus, the antigalactan proteins do not resemble the group of BALB/c phosphorylcholine-binding myeloma proteins (the S63-TEPC15 group) which have the same individual antigenic specificities by both the precipitin (4) and the inhibition of hemagglutination techniques and which thus far have no demonstrable primary structural differences. It is possible that the S63-TEPC15 phosphorylcholine-binding proteins may in fact be composed of chains of identical primary structure (9).<sup>3</sup> It should be noted at this point that in the phosphorylcholine system several other protein types e.g. MOPC 167, McPC 603, and MOPC 511 have been identified which have light chains from completely different subgroups than the S63-T15 group. Clearly the antigalactan proteins differ. The reason for this variation is not clear. Some speculations can be offered as explanations. A basic assumption in the following remarks is that the plasmacytomas are related to normal natural antibody-producing cells. Indirect evidence supporting this notion has previously been described (22, 23). If so, then a specific antigen-binding myeloma population indirectly reflects an antigen selected population of cells.

It may be argued that the variations we have observed originated by a mechanism different from activation of unique pairs of germ line  $V_L$  and  $V_H$  genes. These changes may have resulted from some form of somatic mutational mechanism that is imposed on germ line genes. The sequence variations which we have found in the  $V_H$  chains in fact suggest this possibility since they can be interpreted as resulting from single point mutations in the existing genes. It is then conceivable that random mutations in the genes could affect the antigenicity, but not the antigen-binding specificity of a given protein. Variations of this type could also affect binding but not to the degree that the protein could no longer be selected for by specific antigen. In this connection it is interesting to note that in a previous study we have found differences in the fluorescence of

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<sup>3</sup> Barstad, P., S. Rudikoff, L. Hood, M. Potter, W. Konigsberg, and M. Cohn. Manuscript in preparation.



three of the six proteins reported here on binding with  $\beta$ -(1  $\rightarrow$  6)-D-galactotriose and galactotetraose (7).

From the differences observed in the fluorescence properties on binding of hapten (7) it seems safe to assume that some of the variation among these proteins will be found in residues that are either part of the binding site or which contribute indirectly to the structure of the combining site. In addition, other differences may be found in amino acids which do not contribute to the structure of the combining site. The two interchanges described in the heavy chain aminoterminal sequences might be examples of substitutions which are not concerned with the structure of the site. Most studies to date indicate that site-related amino acids, i.e., amino acids in the hypervariable regions and amino acids which have been affinity site labeled (24–28) are not located in the aminoterminal portion of either the light or heavy chain. Substitutions in the aminoterminal sequences of the heavy chain might, however, contribute to the individual antigenic specificity of these proteins.

It is interesting to compare the results obtained from the mouse phosphorylcholine and galactan systems with those of the human monoclonal cold agglutinin and antigamma globulin systems. Human cold agglutinins have been found to fall into three major groups sharing idiotypic specificities (29). Capra et al. (30) determined the aminoterminal sequences of 10 kappa chains from IgM cold agglutinins and found 7 to fall in the VK<sub>III</sub> subgroup. Of these three had identical sequences and three others differed by single substitutions.

Human proteins with antigamma globulin activity can also be divided into groups demonstrating shared idiotypic specificity. Although each protein has been shown to possess individual antigenic specificities, two groups have emerged with shared specificities (31). Two of the proteins found in the same idiotypic specificity group, Lay and Pom, with the exception of an unusual Ala NH<sub>2</sub> terminal residue in the Lay protein, have only a single difference in the first 40 residues of their heavy chains (which includes the first hypervariable region) although the light chains are from entirely different subgroups. Two other VK<sub>I</sub> light chains from antigamma globulin proteins, Dav and Fin, have identical sequences through the first hypervariable region (32). Thus, many similarities can be found in both the mouse and human systems. Detailed studies of these systems should provide further insight into structure-function relationships and the variations in antibody specificity.

#### SUMMARY

Six IgA myeloma proteins of BALB/c origin which bind antigens containing  $\beta$ -(1  $\rightarrow$  6)-D-galactan side chains have been isolated by affinity chromatography on galactoside-BSA-Sepharose columns. Partial amino acid sequences of the light chains to residue Cys23 and the heavy chains to residue 30 were determined on the automated sequencer. No differences were found among the six V<sub>K</sub> sequences. Among some 50 partial V<sub>K</sub> sequences that have thus far been de-

terminated these six chains are the only ones thus far identified in this subgroup; at least 25  $V_K$  subgroups in the mouse have been identified so far. The heavy chain partial sequences were also very closely related but two differences were found. One protein differed from the other five by having isoleucine instead of leucine at position 5, a second protein differed from the others by having an unidentified amino acid at position 19. Using the highly sensitive inhibition of hemagglutination method it was found that each of the proteins possessed a unique individual antigenic determinant.

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