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IDIOTYPIC ANALYSIS OF ANTIBODIES TO HEN EGG-WHITE LYSOZYME (HEL)

I. Occurrence of Species-Specific Cross-Reactive Idiotypes of Antibodies Directed to Distinct Regions of HEL in Guinea Pigs[1]

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The successful isolation of two distinct antibody populations with different specificity, one recognizing the loop region (peptide 8, P8) of hen egg-white lysozyme (HEL) and the other recognizing the N- and C-terminal region (peptide 17, P17) of HEL, from the antiserum of a single animal (No. 10) of strain 13 (ST13) guinea pig immunized against intact lysozyme molecule enabled us to test the occurrence of idiotypes of both antibodies in sera of several animal species immunized against HEL.

ST13 anti-P8 antibody (No. 10) and ST13 anti-P17 antibody (No. 10) were inoculated into rabbits to induce anti-idiotypic sera. The rabbit antisera were extensively absorbed with normal ST13 immunoglobulins to render them idiotype specific.

The reaction between ^{125}I-ST13 anti-P8 idiotype (No. 10) and its anti-idiotypic antiserum was completely inhibited by the cold ST13 anti-P8 idiotype (No. 10) and not by ST13 anti-P17 antibody (No. 10). Conversely, cold ST13 anti-P17 antibody (No. 10) caused a complete displacement of the ^{125}I-ST13 anti-P17 (No. 10)/anti-idiotype reaction, whereas cold ST13 anti-P8 antibody (No. 10) caused little or no inhibition.

The ST13 anti-P8 idiotype (No. 10) was found in the anti-HEL antibodies of all individuals of three strains of guinea pig tested. In contrast, the levels of the ST13 anti-P17 idiotype (No. 10) detected in the anti-HEL antibodies of guinea pigs were much less when compared with anti-P8 idiotype (No. 10). In addition, no significant levels of both ST13 anti-P8 idiotype (No. 10) and ST13 anti-P17 idiotype (No. 10) were detected in A/J mice, BALB/c mice, CD (SD) rats, goats, or sheep.

These results suggest that antibodies against different determinants carried by HEL molecule have different idiotypic specificities and that idiotypes of antibody directed to the loop region of HEL occur more frequently than that of antibody directed to the N-/C-terminal part of the same protein molecule, although both ST13 idiotypes of antibodies with different specificities are expressed only in guinea pigs.

The variable regions of immunoglobulin molecules carry unique antigenic determinants called idiotypes (1–4). Idiotypes have been recognized in several myeloma proteins and various conventional antibody populations in human and animals (1–6) as well as in the membrane receptors of T and B lymphocytes (7–13).

The term "idiotypic determinants" was originally proposed by Oudin to designate those antigenic determinants in a population of antibody molecules that are not found in other immunoglobulins of the donor animal or in antibody directed to the same antigen from other animals of that species (3, 14). There is much evidence, however, that cross-idiotypic specificity is observed between immunoglobulin molecules of different individuals of the same species (15–28). Some instances of interspecies cross-reactive idiotypes have also been reported (29–32). Moreover, idiotypic cross-reactivity between antibodies of different specificities has been demonstrated (33–36), although a number of studies have demonstrated that antibodies of different specificities do not share common idiotypes (1–4, 14).

In general, several haptens or carbohydrates share the characteristic of inducing an inherited idiotype in at least one mouse strain (for reviews see 3, 37). In contrast, investigations of inheritance of idiotypes of antibodies directed to natural protein antigens are limited, probably because proteins have complex structures and there is not enough information available on the antigenic determinants in proteins to understand their nature. However, it is noteworthy that the occurrence of both anti-KLH idiotype (27) and anti-nuclease idiotype (28) have been proven to be present in sera of mice having the same immunoglobulin allotype as A strain mice immunized against those antigens.

Our interest in investigating the idiotypic nature of antibodies specific to regions of hen egg-white lysozyme (HEL)[3] is based

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[3] Abbreviations used in this paper: ST13, strain 13 guinea pig; HEL, hen egg-white lysozyme; P8, HEL derivative peptide of sequence 57-107 bearing a split between 84 and 85; P17, HEL derivative peptide of sequence 1-27 and 123-129 linked by a single S-S bond; PloopI·II, same sequence as P8 but has no split along entire sequence; PIb, HEL derivative peptide of sequence 29-54 and 109-129 linked by a single S-S bond; PBS, 0.15 M NaCl-0.01 M potassium phosphate, pH 7.40; BBS, 0.15 M NaCl-0.1 M sodium borate, pH 8.20; BSA, bovine serum albumin; BBS-BSA, BBS containing 0.2% BSA; BBS-BSA-Az, BBS-BSA containing 0.04% sodium azide.

on the following: a) HEL is a naturally occurring protein antigen of vertebrates, the sequence and crystallographic structure of which have been well characterized (38, 39); b) antigenic determinants of HEL have been extensively characterized by us and other investigators (40–47); and c) antibodies to HEL-regions have been successfully isolated by affinity chromatography. These observations have allowed us to ask whether antibodies directed to distinct regions of a protein (HEL) molecule have similar or disimilar idiotypic specificities. In addition, we can ask whether idiotypes of antibodies directed to distinct regions of a protein antigen (HEL) can be expressed similarly in sera of animals of different genetic background in response to immunization with the protein antigen.

In the present study we compare the distribution of idiotypes of strain 13 guinea pig antibodies with different specificities, one specific to the loop region of HEL and the other specific to the N- and C-terminal region of HEL.

MATERIALS AND METHODS

Animals. Inbred strain 13 (ST13), closed colony of JY1 strain, and outbred strain Hartley guinea pigs of female weighing 300 to 400 g were used in these experiments. Outbred guinea pigs were purchased from Nippon Clea Inc., Osaka, Japan; breeding stock for ST13 and JY1 guinea pigs were obtained from National Institutes of Health, Tokyo, Japan and bred in our Institute. Male and female CRJ:CD (SD) rats weighing 200 to 220 g were purchased from Nippon Charles River Ltd., Kanagawa, Japan. Female A/J and BALB/c mice from Shizuoka Experimental Animals Ltd., Shizuoka, Japan, were used.

Purification of HEL. Six times recrystallized HEL was purchased from Seikagaku Fine Biochem. Co. Ltd., Japan. For preparing the derivative peptides, it was used without further purification. For preparation of immunizing antigen, crystalline HEL was further purified by passage through SE-Sephadex C-25 and then through QAE-Sephadex C-25 to remove possible contaminating acidic proteins of egg-white as described previously (42).

Preparation of peptide fragments of HEL. N- and C-terminal containing peptide 17 (P17: 1 through 27 and 123 through 129 peptides are linked by a single S-S bond) was purified from a pepsin digest of HEL as described (45). The catalytic site associated peptide Ib (PIb: 29 through 54 and 109 through 129, linked by a single S-S bond) was prepared by a limited digestion of HEL with thermolysin as described (44).

Two kinds of peptide, the peptide 8 (P8: sequence 57 through 107 bearing a split between 84 and 85) and the peptide loopI·II (PloopI·II: same sequence as P8 but without any split within the sequence), were prepared as follows.

Limited digestion of HEL by pepsin was performed as described previously (45). The pH of the hydrolysate was adjusted to 5.5 by 4 N NaOH and diluted 2-fold with 0.1 N acetic acid. The diluted hydrolysate (prepared from 10 g of HEL) was applied to a SE-Sephadex C-25 column (2 × 40 cm) at 25°C. The column was developed at 70 ml/hr with 3 liters of 0.05 M sodium acetate buffer, pH 4.5, in 8 M deionized urea. Fractions of 10 ml were collected. Two peak fractions (fraction 1: 140 through 188 and fraction 2: 200 through 258) were pooled separately (Fig. 1).

Each fraction was diluted 2-fold with 0.1 N acetic acid and applied to a separate SP-Sephadex C-25 column (2 × 40 cm) that was equilibrated with 1.0 M sodium acetate buffer, pH 3.5. Each column was developed with a linear gradient formed by using 2 liters each of 1.0 M sodium acetate buffer, pH 3.80, and 1.0 M sodium acetate buffer, pH 4.19. The peak fraction in each chromatography was collected.

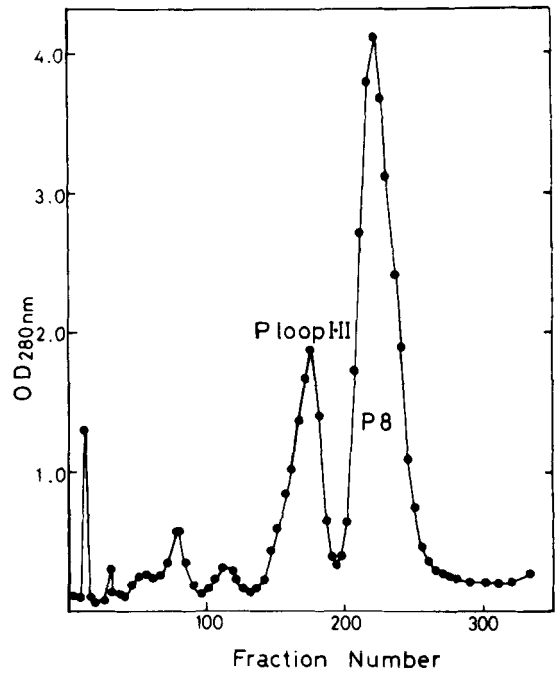

Figure 1. Separation of two kinds of loop peptide. Details are given in the text.

Amino acid analyses were performed according to the method of Simpson *et al.* (48). The amino acid composition of fraction 1 and fraction 2 were as follows. Fraction 1: Trp, 1.94 (2); Lys, 1.90 (2); Arg, 2.82 (3); S-sulfoCys, 3.86 (4); Asp, 9.83 (10); Thr, 1.82 (2); Ser, 6.02 (6); Glu, 1.07 (1.0); Pro, 2.19 (2); Gly, 4.00 (4); Ala, 3.81 (4); Val, 1.83 (2); Met, 0.84 (1); Ile, 3.76 (4); Leu, 2.86 (3). Fraction 2: Trp, 1.86 (2); Lys, 1.94 (2): Arg, 2.86 (3); S-sulfoCys, 3.92 (4); Asp, 9.88 (10); Thr, 1.93 (2); Ser, 6.02 (6); Glu, 1.05 (1); Pro, 2.09 (2); Gly, 4.00 (4); Ala, 3.92 (4); Val, 1.90 (2); Met, 0.86 (1); Ile, 3.82 (4); Leu, 2.98 (3). The N-terminal residue of each peptide was determined by the dansyl method (49). Dansyl-peptide was hydrolyzed at 105°C for 18 hr, and dansyl-amino acids were identified by two-dimensional chromatography on polyamide sheet (50). Only glutamine was found as N-terminal residue of fraction 1, whereas glutamine and serine were found as N-terminal residues of fraction 2. The results of amino acid analyses and N-terminal determination of peptides indicate that both peptides are derived from the sequence of 57 through 107, but fraction 1 (PloopI·II) has no split along entire sequence, whereas fraction 2 (P8) has a split between Leu (84)-Ser (85). These results were further confirmed by analyzing the reduced and alkylated products of both PloopI·II and P8 (Data are not shown).

All peptide preparations were passed through two serial columns (3 × 140 cm each) of Bio Gel P-10 in 10% (v/v) acetic acid to remove traces of intact lysozyme. Enzymic activity of each peptide at 5 mg/ml concentration was routinely measured using *Micrococcus lysodeikticus* as substrate (40). No peptide preparations used in this series of experiments had any enzymic activity and therefore were considered to contain less than 0.01% of intact HEL. Immunologic activities of P8 were reported previously by us (40–42).

Immunization. Guinea pigs were immunized i.p. weekly, each time with 0.5 mg of purified HEL adsorbed on Bentonite (1 mg/ml of 1% Bentonite in PBS[3]). In addition to the HEL-Bentonite injection, 0.5 ml of complete Freund's adjuvant (CFA) emulsified in PBS (1:1) were injected i.p. at first injection and once every 10 weeks thereafter. Blood was taken once a week starting 10 weeks after the first injection. Mice were immunized weekly

by i.p. inoculations of HEL (0.5 mg per injection, in 0.02 ml of PBS emulsified with 0.18 ml of CFA as described by Tung and Nisonoff (18). Goat and sheep were immunized as follows: the first injection was given subcutaneously with 10 mg of HEL in CFA and followed by injections of 10 mg of HEL in incomplete Freund's adjuvant once every 5 weeks.

Purification of antibodies directed to two distinct regions, P8 and P17, of HEL from a single ST13 guinea pig No. 10. Antibodies to P8 and P17 were isolated by immunoabsorbents as previously described (41, 45). Immunoabsorbents were prepared by the method of Axen *et al.* (51) by using Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and cyanogen bromide. The peptide fragments coupled to Sepharose were P8 and P17. Briefly, region-specific antibodies were prepared from 10 ml of a pooled antiserum bled between 17 and 22 weeks containing 170 mg of precipitable anti-HEL antibody from ST13 guinea pig No. 10. The antibody was absorbed on and eluted by HEL solution from P17 coupled to Sepharose 4B. The eluate was concentrated by lyophilization and applied to a Sephadex G-150 column equilibrated with 0.1 N acetic acid. The antibody fraction was dialyzed successively against 0.2 M acetate buffer, pH 4.3, and 0.01 M Tris-HCl buffer, pH 8.0. The antibody preparation was passed over a goat anti-HEL antibody coupled to Sepharose CL-4B to remove a small amount of contaminating HEL in the preparation. The unbound fraction from the P17-Sepharose immunoabsorbent was used for preparation of anti-P8 antibody from the same animal, ST13 guinea pig No. 10. The procedure was the same as above, except that P8-conjugated Sepharose was used as an immunoabsorbent.

Preparation of anti-idiotypic antibodies. Rabbits were immunized with five subcutaneous injections of specifically purified HEL-region-specific antibodies at 2-week intervals, each time with 500 μg of protein in CFA. Blood was taken 8 days after the last injection. The rabbit antibodies to normal ST13 guinea pig immunoglobulins were removed by absorption on Sepharose CL-4B column to which normal ST13 serum had been coupled. The absorption was carried out until the antisera no longer reacted with normal guinea pig immunoglobulins at 4°C.

Labeling of purified antibodies with ^{125}I. Purified ST13 antibodies were iodinated by a slight modification of the chloramine T method (52). Routinely, 25 μg of antibody were iodinated with 0.1 mCi of carrier-free Na[^{125}I] (New England Nuclear, Boston, Mass.) and chloramine T at a 25-fold molar excess over antibody. The reaction was terminated after 30 min at 0°C by addition of 25 μl of a 5 mM sodium bisulfite solution. After addition of 10 μl of 1 M KI solution, the reaction mixture was chromatographed on Sephadex G-25 column equilibrated with BBS (see Abbreviations) containing 2 mg/ml of BSA (BBS-BSA). Specific activity was usually 2×10^6 cpm/μg. Iodinated antibodies were stored at 4°C in BBS-BSA containing 0.04% of sodium azide (BBS-BSA-Az).

Radioimmunoassay for specificity of the purified antibodies to HEL regions. A solid phase radioimmunoassay was performed by using polyvinyl microtiter plates (model 220-24; Cooke Laboratory Products Div., Dynatech Lab. Inc., Alexandria, Va.) as previously described by Klinman *et al.* (53). Briefly, individual wells were filled with various kinds of HEL derivative peptides at a concentration of 0.4 mg/ml in BBS-Az, pH 8.2. After standing overnight at 20°C, the wells were thoroughly washed with BBS-BSA-Az, and then filled with a 1% solution of BSA in BBS-Az, pH 8.2. After 4 hr at 20°C, the wells were washed four times with BBS-BSA-Az, and 100 μl of specifically purified ^{125}I-labeled antibodies, 0.25 μg in each well, were added. After standing overnight at 20°C, the plates were thoroughly washed with BBS-BSA-Az, individual wells were cut out, and radioactivity was determined with a gamma scintillation counter.

Idiotype assay. A double antibody radioimmunoassay was performed (54). Typically, 20 μl of ^{125}I-labeled specifically purified ST13 antibody (0.05 μg), 100 μl BBS-BSA-Az, pH 8.2, 30 μl of diluted normal rabbit serum (corresponding to 3 μl serum), and 50 μl of diluted rabbit anti-idiotypic antiserum (corresponding to 1 μl antiserum and diluted by Sepharose absorption, see above) was incubated 30 min at room temperature, and this was followed by adding 150 μl of goat antiserum to rabbit immunoglobulin. After 50 min at 37°C and about 18 hr at 4°C, the immune precipitates were washed once in the cold with BBS-BSA-Az and counted in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The goat antiserum to rabbit immunoglobulin was routinely absorbed with normal ST13 serum (usually by passing 100 ml antiserum over 50 ml Sepharose CL-4B containing about 3 ml normal ST13 serum). For inhibition assays, various inhibitors (unlabeled purified HEL-region specific antibodies, or various anti-HEL antisera) were added to anti-idiotypic serum; then after 30 min the ^{125}I-labeled idiotype was added and the rest of the assay was as described above.

RESULTS

Specificity of antibody preparations directed against two distinct regions of HEL. As mentioned in *Materials and Methods*, ST13 guinea pig antibodies directed to P8 and to P17 regions have been fractionated from a single animal (ST13, No. 10) on immunoabsorbent columns bearing selected polypeptide fragments (P8 and P17) of HEL. The successful isolation of anti-P8 antibody and anti-P17 antibody from a single guinea pig, ST13 (No. 10), enabled us to examine the distribution of these idiotypes in various anti-HEL sera in the present paper. First, the specificity of these two antibody preparations was determined by a direct solid phase radioimmunoassay as described in *Materials and Methods*. The interactions of ^{125}I-anti-P8 (No. 10) and ^{125}I-anti-P17 (No. 10) with HEL and its derivative polypeptides are shown in Figure 2. Each antibody preparation is completely specific for the corresponding peptide fragment. Thus, ^{125}I-labeled anti-P8 antibody (No. 10) resulted in efficient binding to the wells coated with P8, but not to the wells coated with P17 or PIb that are unrelated peptides of HEL. As is shown in Figure 2, the anti-P17 antibody preparation was also specific. Another finding in these experiments was that both anti-P8 and anti-P17 antibodies interacted most efficiently with native HEL molecule compared with peptide fragments.

Specificity of anti-idiotypic antisera. Indirect radioimmunoassay was utilized to analyze two rabbit anti-idiotypic antisera used in the present experiment: one recognizing idiotypes of ST13 anti-P8 (No. 10) and the other recognizing idiotypes of ST13 anti-P17 (No. 10). As is shown in Table I, the binding of the isolated radiolabeled anti-P8 antibody by the rabbit anti-idiotypic antiserum is inhibited equally well—more than 90% inhibition with 1 μg of either cold anti-P8 antibody (No. 10) or 1 μl of donor (ST13, No. 10) anti-HEL serum. Moreover, absorbed rabbit antiserum to anti-P8 (No. 10) was clearly anti-idiotype. Thus, the reaction with ^{125}I-anti-P8 (No. 10) was not inhibited by the preimmune serum of the donor, normal sera from various animal species including other guinea pigs of the same strain, or anti-P17 antibody of ST13, No. 10 (Table I). Removal of anti-HEL antibody from the donor serum (No. 10) by HEL-coupled Sepharose CL-4B immunoabsorbent also

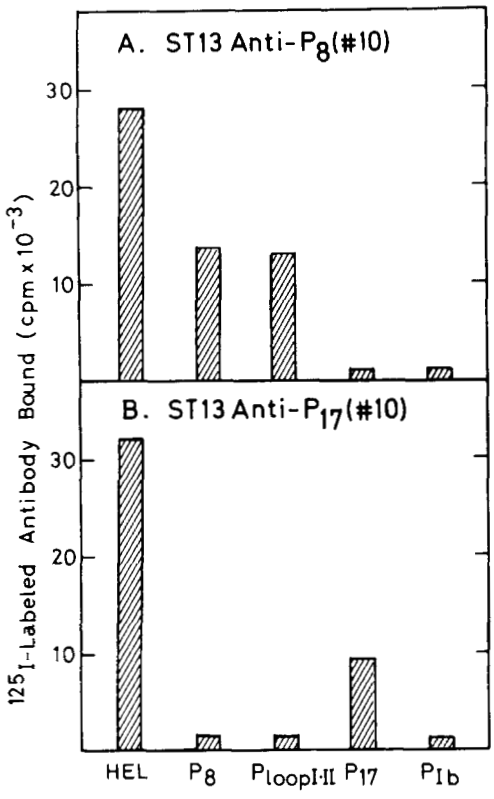

Figure 2. Specificity of anti-P8 antibody and anti-P17 antibody preparations purified from the serum of a single guinea pig, ST13 (No. 10), immunized against native hen egg-white lysozyme (HEL). Specificities were measured by a solid phase radioimmunoassay: ^{125}I-labeled anti-P8 antibody (No. 10) and ^{125}I-labeled anti-P17 antibody (No. 10) were reacted in the polyvinyl microliter plate wells coated with various distinct region polypeptide fragments isolated from HEL molecules digested with proteases.

failed to inhibit the anti-P8/anti-idiotype reaction.

Similar results by using an absorbed rabbit antiserum to ST13 anti-P17 (No. 10) in the indirect radioimmunoassay were obtained as shown in Table II. The specificity of the anti-idiotypic serum was demonstrated by the finding that ^{125}I-anti-P17/anti-idiotypic reaction was inhibited effectively by the antiserum of the donor as well as the cold anti-P17 idiotype but not by the preimmune serum of the donor, anti-P8 idiotype from ST13 (No. 10), or HEL-absorbed donor serum. These results indicated that the activity of both anti-idiotypic antisera was directed against the variable regions of two distinct polypeptides—the P8 and P17 binding immunoglobulins from a single animal.

Distribution of ST13 anti-P8 idiotype (No. 10) in several different anti-HEL antisera. Several different anti-HEL antisera were tested as inhibitors in the ST13 anti-P8 (No. 10)/anti-idiotype radioimmunoassay to examine the extent of the idiotype cross-reactivity among antibodies produced by individuals of various animal species. First, a qualitative screening was carried out by using 10 μl of various antisera containing 30 to 150 μg of antibody depending on the antiserum. The results in Table III indicated the degree of inhibition of ^{125}I-anti-P8 (No. 10) binding to its anti-idiotypic antibody. As expected, it was found that the hyperimmune donor serum (ST13, No. 10) was the most effective inhibitor: thus, the anti-idiotype reaction was inhibited almost completely by a small amount of the anti-HEL antiserum. In contrast, the individual anti-HEL sera from different ST13 guinea pigs were less effective. Similar results were

TABLE I

Specificity of an anti-idiotypic rabbit antiserum directed to anti-P8 antibody from a ST13 guinea pig No. 10

Inhibitor	Amount of Inhibitor	^{125}I-Anti-P8 (No. 10) Precipitated[a]
		% of control
Preimmune-ST13 serum (No. 10)	10 μl	98
Normal ST13 serum (pool)[b]	10 μl	94
ST13 7S immunoglobulin[c]	120 μg	98
Normal Hartley serum (pool)	10 μl	96
Normal JY1 serum (pool)	10 μl	96
JY1 7S immunoglobulin[c]	130 μg	96
Hyperimmune donor serum (No. 10)	1 μl	8
Purified anti-P8 antibody (No. 10)	1μg	9
Purified anti-P17 antibody (No. 10)	1 μg	95
Normal A/J serum (pool)	10 μl	98
Normal BALB/c serum (pool)	10 μl	95
Normal rat serum (pool)	10 μl	103
Preimmune-sheep serum (No. SL60)	10 μl	99
Goat anti-*E. coli* serum (pool)	10 μl	99
Hartley anti-RNase antibody	98 μg	92
Mouse (A/J) anti-RNase antiserum	10 μl	100

[a] In testing for inhibitory activity, a sensitive indirect radioimmunoassay was carried out with 0.05 μg of the labeled, specifically purified anti-P8 antibody, as described in the text. In the uninhibited control, 23% of the labeled antibody was precipitated.

[b] Pooled from the sera of several nonimmunized animals.

[c] Forty-five per cent saturated fraction of ammonium sulfate of unimmunized guinea pig serum was further purified on Sephadex G-200 column.

TABLE II

Specificity of an anti-idiotypic rabbit antiserum directed to anti-P17 antibody from a ST13 guinea pig No. 10

Inhibitor	Amount of Inhibitor	^{125}I-Anti-P17 (No. 10) Precipitated[a]
		% of control
Preimmune-ST13 serum (No. 10)	10 μl	92
Normal ST13 serum (pool)[b]	10 μl	89
ST13 7S immunoglobulin[c]	120 μg	92
Normal Hartley serum (pool)	10 μl	89
Normal JY1 serum (pool)	10 μl	92
JY1 7S immunoglobulin[c]	130 μg	90
Hyperimmune donor serum (No. 10)	1 μl	0
Purified anti-P17 antibody (No. 10)	1 μg	11
Purified anti-P8 antibody (No. 10)	1 μg	90
Normal A/J serum (pool)	10 μl	94
Normal BALB/c serum (pool)	10 μl	90
Normal rat serum (pool)	10 μl	102
Preimmune-sheep serum (pool)	10 μl	96
Goat anti-*E. coli* serum (pool)	10 μl	91
Hartley anti-RNase antibody	98 μg	88
A/J anti-RNase serum (pool)	10 μl	98

[a] A double antibody radioimmunoassay was used as in Table I with 0.05 μg of the labeled, specifically purified anti-P17 antibody (see details in the text). In the absence of inhibitor 18% of the labeled antibody was precipitated.

[b] Pooled from several unimmunized animals.

[c] Forty-five per cent ammonium sulfate cut of normal serum was passed over a Sephadex G-200 column.

obtained when antisera from two other strains of guinea pigs, JY1 and Hartley strain, were tested. The degree of the inhibition by different guinea pig antisera besides the autologous antiserum could not reach above 60% even if a large amount (50 μl) of the test serum was added to the system. The inhibitory activities of the guinea pig antiserum have been proven to be

TABLE III

Distribution of ST13 anti-P8 idiotype (No. 10) in various anti-HEL antisera

Unlabeled Inhibitor	No. of Antisera Tested	Amount of Inhibitor	% Inhibition of ^{125}I-Anti-P8 Binding[a]	
			Range	Mean
		μl		
A. Guinea pig				
Hyperimmune donor serum No. 10		1		89
		0.1		62
		0.01		6
ST13 anti-HEL antiserum	9	10	18–41	32
JY1 anti-HEL antiserum	5	10	35–43	38
Hartley anti-HEL antiserum	5	10	24–47	38
B. Mouse				
A/J anti-HEL antiserum	5	10	5–15	8
BALB/c anti-HEL antiserum	5	10	3–8	5
C. Rat				
SD anti-HEL antiserum	5	10	1–7	4
D. Goat				
Goat anti-HEL antiserum (pool)	2	10		5
E. Sheep				
Sheep anti-HEL antiserum	2	10	4, 5	4

[a] A radioimmunoassay with 0.05 μg of ^{125}I-anti-P8 was carried out. Twenty-three percent of the radioactive ligand was precipitated in the uninhibited control. Details are given in the text.

due to the anti-HEL antibody. The inhibitory activity was completely absorbed by HEL-Sepharose immunoabsorbent (data not shown). It was also shown in Table III that the ST13 anti-P8 idiotype (No. 10) was not detected in various anti-HEL antisera tested from mouse, rat, goat, and sheep. The above results suggested the occurrence of the ST13 anti-P8 idiotype (No. 10) cross-reactivity among antibody populations just in guinea pigs.

The following experiments were carried out by a quantitative assay, as shown in Figure 3, to establish the presence of the species-specific cross-reactive idiotypes in several anti-HEL antisera. As shown in Figure 3*A*, diluted antiserum containing a fixed amount (25 μg) of anti-HEL antibodies of various animal species was added to the ^{125}I-ST13 anti-P8 (No. 10)/anti-idiotype radioimmunoassay system to determine their effectiveness as inhibitors. Again, the hyperimmune antiserum of the autologous guinea pig completely displaced the ^{125}I-labeled ST13 anti-P8 antibody (No. 10) from its anti-idiotypic antibody. Diluted anti-HEL antisera containing an equal amount (25 μg) of antibody from different individuals of ST13, JY1, and Hartley strain of guinea pigs were effective inhibitors, whereas antibodies from other animal species immunized against HEL had little or no inhibitory effect. These results clearly indicated the presence of the species-specific, cross-reactive idiotype in guinea pigs.

Distribution of ST13 anti-P17 idiotype (No. 10) in several different anti-HEL antisera. It was of interest to examine the inheritance of the idiotypes of antibody populations with specificity for different antigenic determinants of a protein antigen produced by an animal immunized against the native protein molecule. The successful isolation of two distinct antibody

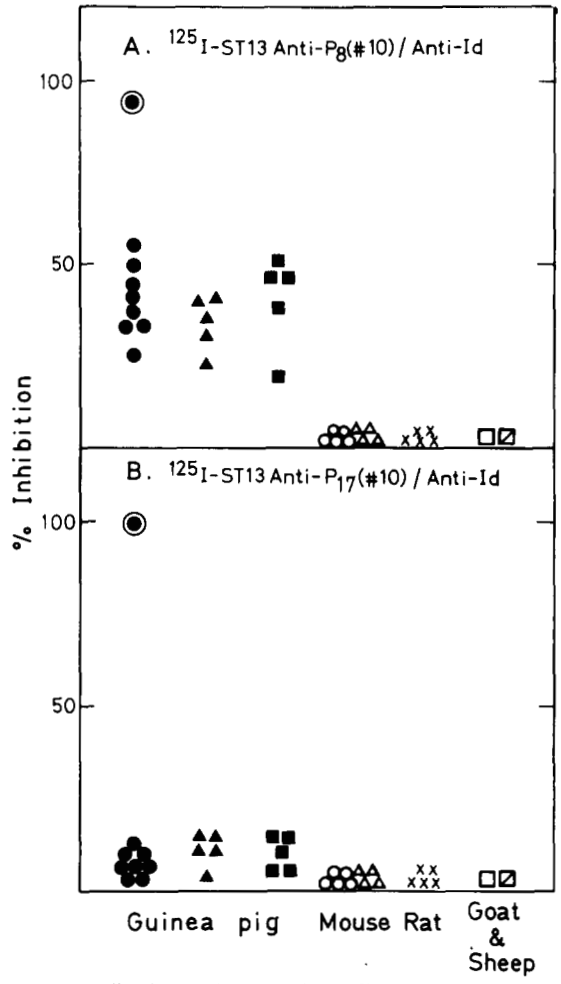

Figure 3. Distribution of ST13 anti-P8 idiotype (No. 10) and ST13 anti-P17 idiotype (No. 10) in several different anti-HEL antisera of various animal species. In the radioimmunoassay, ^{125}I-ST13 anti-P8 antibody (No. 10) (*A*), or ^{125}I-ST13 anti-P17 antibody (No. 10) (*B*), 0.05 μg each, and their corresponding rabbit anti-idiotypic antisera were utilized. The inhibitory capacities were measured by diluted anti-HEL antisera containing a fixed amount (25 μg) of antibody. Inhibitor antisera were: Autologous (ST13, No. 10) (⊙); ST13 (●); JY1 (▲); Hartley (■); A/J (○); BALB/c (△); rat (×); goat (□); sheep (▨). In the uninhibited controls, 23% of the labeled ligand in *A* and 20% of the labeled ligand in *B* were precipitated.

populations with different specificity, one recognizing the loop region (peptide 8, P8) of HEL and the other recognizing the N- and C-terminal part (peptide 17, P17) of HEL, from a single guinea pig (ST13, No. 10) immunized against intact lysozyme molecule enabled us to test the occurrence of both antibody idiotypes in sera of various animal species immunized against HEL.

As described above, careful measurement of ST13 anti-P8 idiotype (No. 10) in various anti-HEL antisera showed that the idiotype was present in every guinea pig antiserum tested but not in antisera of many other animal species tested. In contrast, the level of ST13 anti-P17 idiotype (No. 10) in guinea pigs was much lower than that of ST13 anti-P8 idiotype (No. 10).

Preliminary efforts to test the inhibitory capacities of antibodies raised in various animal species immunized against HEL on the reaction between ^{125}I-ST13 anti-P17 idiotype (No. 10) and its anti-idiotypic antiserum suggested that the occurrence of ST13 anti-P17 idiotype (No. 10) was rather restricted in the donor antiserum (Table IV). It should be noted that the amount

of antiserum added as inhibitors was the same as that for testing the distribution of anti-P8 idiotype (No. 10), as was shown in Table III. Table IV demonstrated that a small amount of the donor antiserum was able to inhibit completely the ^{125}I-labeled ST13 anti-P17 (No. 10) binding to its anti-idiotypic antibody. However, anti-HEL antisera other than the donor's did not show strong inhibition at high concentrations of inhibitors, ranging from 30 to 150 μg of antibody. Moreover, the finding that the inhibitory activity by guinea pig antisera (2 to 20%, Table IV) did not change when 5 times the volume of the antisera were added to the radioimmunoassay system indicated maximal inhibition had been achieved.

More quantitative measurements of the distribution of ST13 anti-P17 idiotype (No. 10) were carried out by utilizing a diluted antiserum containing a fixed amount (25 μg) of each anti-HEL antibody tested as an inhibitor (Figure 3*B*). The amount of the inhibitor antibody corresponding to 500 times the radioactive ligand antibody was the same as in the case of ST13 anti-P8 idiotype (No. 10) assay (Fig. 3*A*). Again, the unique nature of the ST13 anti-P17 idiotype (No. 10) was suggested. Autologous antiserum (ST13, No. 10) could inhibit completely the idiotype binding to its anti-idiotypic antibody, whereas antisera of other guinea pigs tested had much less inhibitory activity and antisera of mouse, rat, goat, or sheep had little or no effect on the anti-idiotypic reaction.

Inhibitory ability of purified HEL-region specific antibody populations on the anti-idiotypic reactions. The effect of specifically purified antibody preparations directed toward two distinct regions of HEL molecule on the reaction between idiotypes and anti-idiotypes was determined (Fig. 4). Autologous donor antibodies caused powerful inhibition with a small amount of the cold proteins in both systems: ^{125}I-ST13 anti-P8 (No. 10)/anti-idiotype and ^{125}I-ST13 anti-P17 (No. 10) anti-idiotype reactions. Anti-P8 antibody preparations isolated from other individuals (ST13, No. 9 and No. 15) of the same strain as the donor and from a pooled serum of Hartley strain also caused inhibition (18 to 20%) at a level (5 μg) corresponding to 100 times the ^{125}I-ST13 anti-P8 antibody (No. 10), whereas anti-P17 antibodies had no effect on the anti-idiotypic reaction (Fig. 4*A*). In contrast, the anti-P17 antibodies isolated from sera of other guinea pigs did not inhibit the ^{125}I-ST13 anti-P17 (No. 10)/anti-idiotype reaction at a 5 μg level (Fig. 4*B*). It should be pointed out that the sensitivity of the ^{125}I-ST13 anti-P17 idiotype (No. 10)/anti-idiotype system capable of detecting the idiotype is even higher than that of ST13 anti-P8 idiotype (No. 10). Thus, 0.1 μg of cold ST13 anti-P17 antibody (No. 10) caused 45% inhibition of the anti-P17 anti-idiotype reaction whereas the same amount of cold ST13 anti-P8 antibody (No. 10) caused 25% inhibition of the anti-P8 anti-idiotype reaction. These findings utilizing purified antibody populations confirmed the sensitivity of the anti-P17 reaction and suggested that the ST13 anti-P8 idiotype (No. 10) is present in the guinea pig species much more frequently than ST13 anti-P17 idiotype (No. 10).

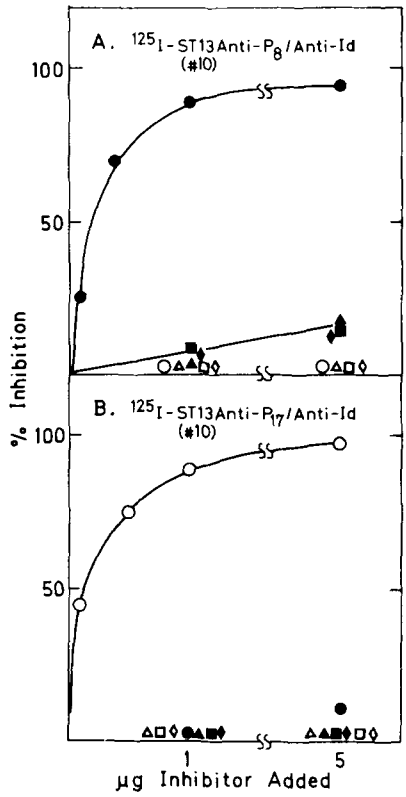

Figure 4. Effect of specifically purified HEL region recognizing antibody preparations on the reactions between idiotypes and anti-idiotypic antibodies. Competitive inhibition on the ^{125}I-ST13 anti-P8 idiotype (No. 10)/anti-idiotype reaction (*A*) and on the ^{125}I-anti-P17 idiotype (No. 10)/anti-idiotype reaction (*B*) were measured by the radioimmunoassay using 0.05 μg of each radiolabeled ligand. Inhibitor antibodies were: ST13 anti-P8 (No. 10) (●); ST13 anti-P8 (No. 9) (▲); ST13 anti-P8 (No. 15) (■); Hartley anti-P8 (pool) (◆); ST13 anti-P17 (No. 10) (○); ST13 anti-P17 (No. 9) (△); ST13 anti-P17 (No. 15) (□); Hartley anti-P17 (pool) (◇). Percent of ^{125}I-labeled ligand precipitated in the uninhibited controls were: 22% in *A* and 19% in *B*.

TABLE IV

Distribution of ST13 anti-P17 idiotype (No. 10) in various anti-HEL antisera

Unlabeled Inhibitor	No. of Antisera Tested	Amount of Inhibitor	% Inhibition of ^{125}I-Anti-P17 Binding	
			Range	Mean
		μl		
A. Guinea pig				
Hyperimmune donor serum No. 10		1		100
		0.1		77
		0.01		23
ST13 anti-HEL antiserum	9	10	2–13	7
JY1 anti-HEL antiserum	5	10	4–19	11
Hartley anti-HEL antiserum	5	10	7–20	13
B. Mouse				
A/J anti-HEL antiserum	5	10	0–2	1
C. Rat				
SD anti-HEL antiserum	5	10	0	0
D. Goat				
Goat anti-HEL antiserum (pool)	2	10		0
E. Sheep				
Sheep anti-HEL antiserum	2	10	0, 2	1

[a] A radioimmunoassay with 0.05 μg of ^{125}I-anti-P17 (No. 10) was carried out as in Table II. In the uninhibited control, 18% of the labeled antibody was precipitated. Details are given in the text.

DISCUSSION

Our experiments were designed to answer the following questions: a) Would antibodies to distinct antigenic determinants carried by the same protein molecule have the same or similar

idiotypic specificities suggested by other investigators (34, 35)? b) What is the frequency of occurrence in other individuals of the same species and other species of common idiotypes of antibodies directed to different antigenic determinants of a protein molecule that arise on immunization with the protein?

To answer those questions, first, it is desirable to choose a suitable antigenic material that is well characterized, homogeneous, and that is a molecule able to be fractionated into fragments bearing different antigenic determinants. Second, it is desirable to be able to isolate a considerable amount of antibody directed to distinct regions of a protein molecule in high purity from a single animal of inbred strain. HEL is such a protein, and guinea pigs make significant amounts of antibody.

The data presented in this paper demonstrate that two distinct populations of antibodies could be isolated from a single ST13 guinea pig. One population is directed to the loop region (P8) of HEL and the other to the N-/C-terminal region (P17) of HEL. These populations have different idiotypic specificities, and both idiotypes are present in sera of all individuals of three strains of guinea pigs immunized with HEL. The experiments also indicate that neither ST13 anti-P8 (No. 10) nor ST13 anti-P17 idiotype (No. 10) were detectable in sera of A/J mice, BALB/c mice, CD (SD) rats, goats, or sheep. Thus, the cross-reactive idiotype with those antibodies are likely to be classified as species-specific idiotypes. Moreover, the frequency of occurrence of ST13 anti-P8 idiotype (No. 10) appears to be higher than that of ST13 anti-P17 idiotype (No. 10). Our results document the first report, suggesting that antibodies against distinct antigenic determinants carried by the same protein molecule have different idiotypic specificities and that the frequency of appearance of cross-reactive idiotypes of antibodies directed against different antigenic determinants of a protein is quite different.

The results presented in this paper establish the anti-idiotypic activity of two antisera prepared in rabbits immunized against purified anti-P8 and anti-P17 antibodies from a single guinea pig (ST13, No. 10). The specificity of the purified anti-P8 and anti-P17 antibodies used for the experiments was determined by radioimmunoassay. These HEL-region specific antibodies reacted only with corresponding polypeptides. The specificity of the idiotypic system defined was established by using radioimmunoassay. The absorbed antiserum was shown to bind only purified ^{125}I-labeled ST13 anti-P8 antibody (No. 10) and not nonspecific immunoglobulins. In addition, anti-HEL serum (ST13, No. 10) completely inhibited the idiotype/anti-idiotype binding. In contrast, a serum directed to an unrelated antigen, bovine pancreatic ribonuclease, did not show significant inhibitory capacity. More imporant, ST13 anti-P17 antibody (No. 10) at 20 times the amount over ^{125}I-labeled ST13 anti-P8 antibody (No. 10) did not significantly inhibit the anti-P8 idiotype reaction. Similar results by using an absorbed rabbit antiserum to ST13 anti-P17 idiotype (No. 10) were obtained.

The results that have been presented clearly establish the occurrence of intraspecies idiotypic cross-reactivity between antibody populations of identical specificity, anti-P8, from guinea pigs. Quantitative studies of this cross-reactivity, assayed by radioimmunoassay, demonstrate that this sharing is only apparent. Thus, the inhibition of the binding of radiolabeled autologous idiotype required much less cold idiotype than homologous anti-HEL antibodies (Fig. 3*A*). Since the inhibition by 0.25 μg of the cold autologous idiotype gave approximately the same level of inhibition of the idiotype binding by 25 μg of guinea pig anti-HEL antibodies, the difference of inhibitory capacity is approximately 100-fold. In contrast, the concentration of ST13 anti-P17 idiotype (No. 10) in sera of other guinea pigs immunized against HEL seemed much less. In support of this suggestion is the finding that even 500 times the amount (25 μg) of anti-HEL antibodies over ^{125}I-ST13 anti-P17 idiotype (No. 10) showed only 10 to 20% inhibition of the idiotype binding (Fig. 3*B*), whereas 0.1 μg of autologous cold idiotype gave 45% inhibition. Judging from the fact that 25 ng of cold ST13 anti-P17 (No. 10) gave 15% inhibition on the idiotype binding, the frequency of occurrence of the cross-reactive idiotype present in other guinea pig anti-HEL antibodies could be less than one out of a thousand molecules.

The striking difference in occurrence of common idiotypes of antibodies directed against P8 and P17 seems not to be a special case. We have isolated three additional antibody populations directed to P8 and P17, two antibodies specific to P8 and one antibody specific to P17, from an individual serum of a ST13 guinea pig immunized against HEL. By using the three anti-idiotypic antisera as probes, we have found results similar to those presented in this paper (Sakato, N. *et al.*, manuscript in preparation).

It should be noted that the striking difference in occurrence of common idiotypes of antibodies directed against distinct regions of HEL molecule is not due to the different concentrations of each antibody present in the test sera, since nearly the same amount of both antibody populations could be isolated from any guinea pig anti-HEL serum tested.

Oudin and Cazenave (34, 35) have presented evidence that idiotypes of rabbit antibodies directed to distinct regions of other protein antigens, such as human serum albumin and human fibrinogen, may have the same or very similar idiotypic specificities. In their experiments, homologous (rabbit anti-rabbit) anti-idiotypic sera were used. In contrast, heterologous (rabbit anti-guinea pig) anti-idiotypic sera were utilized in the present experiments. Therefore, it is possible that the mode of recognition of their homologous anti-idiotypic sera could be quite different from that of our heterologous anti-idiotypic sera, although further work will be necessary to clarify this point. Studies are planned to produce homologous anti-idiotypic sera against HEL-region specific antibodies. This will aid in understanding the reason for differences in conclusions between their results and ours.

In addition, we are now investigating the idiotypes of murine antibodies directed against HEL-regions to elucidate the nature and role of idiotypes in immune system in response to HEL.

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Note Added in Proof. Harvey *et al.* (55) have recently reported the evidence that idiotypes of murine antibodies directed against N-/C-terminal did not share by the idiotypes of antibodies directed to the rest region of HEL molecule.

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