ANTIHAPTEN ANTIBODY SPECIFICITY AND L CHAIN TYPE*

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(Received for publication 31 May 1967)

Two distinct types of L polypeptide chains (named κ and λ) have been identified in both guinea pig γ_1- and γ_2-immunoglobulins (1, 2). These two types of L chains have distinct antigenit determinants, slight differences in electrophoretic mobility, and display quite distinct peptide maps.[1] In normal guinea pig serum, approximately one-third of γ_2-globulin molecules bear the λ-type of L chains (L molecules) and all or most of the others bear κ-chains (K molecules). In specifically purified guinea pig antihapten antibodies, however, the proportion of K/L molecules may diverge markedly from that found in normal γ_2-globulins. Sometimes, as in purified anti-dinitrophenyl (DNP) antibodies obtained from animals immunized with DNP-protein conjugates, K molecules constitute 90–100% of the total antibody, while in purified anti-*p*-iodobenzenesulfonyl (pipsyl) antibodies there is an obvious increase in the amounts of L molecules which may, in some animals, reach as high as 80%. Observations of the same nature have been reported previously in studies of purified antibodies isolated from human sera (3–7).

These findings indicate a relationship between the immunological specificity of the antibodies and the type of L chain which they bear, and suggest that guinea pig anti-DNP antibody molecules bearing κ-chains might display a greater affinity for the DNP determinant than anti-DNP antibodies bearing λ chains. The decrease in L molecules in purified anti-DNP antibodies observed in the course of immunization with DNP-protein conjugates at the time when their binding affinities for the haptens are increasing favors this interpretation (2). In this paper, this hypothesis is verified by a study of the ratio of K and L molecules in specifically isolated anti-DNP antibodies with varying binding affinities for the hapten. The results obtained show that there is indeed a correlation between the type of L chain present in the immunoglobulins synthesized and their average binding affinity for the hapten.

Materials and Methods

Proteins.—Bovine gamma globulin (BGG) and bovine serum albumin (BSA) were obtained from Armour Pharmaceutical Co., Kankakee, Illinois. Human fibrinogen (HF) was purchased from Mann Research Laboratories, New York, N.Y.; and guinea pig γ_2-globulin

* Supported by United States Public Health Service Grant AI-04983 and the Health Research Council of the City of New York under Contract No. I-138.

[1] Lamm, M. E., B. Lisowska-Bernstein, and V. Nussenzweig. Comparison of guinea pig γ_1- and γ_2-globulins by peptide mapping. To be published.

from Pentex, Inc., Kankakee, Illinois. Guinea pig serum albumin (GPA) was prepared by starch block electrophoresis. Guinea pig γ_2-globulin was further purified by filtration through DEAE-cellulose as described in reference 8. Guinea pig γ_1-globulin was obtained from specifically purified guinea pig antihapten antibodies (anti-dinitrophenyl) by the methods described in reference 9. The $F(ab)'_2$ fragments of guinea pig γ_2-globulins were obtained by pepsin digestion as described in reference 10. Guinea pig purified L polypeptide chains were prepared as in reference 11.

Other Reagents.—2,4-Dinitrophenol (DNP-OH) was purchased from Mann Research Laboratories, New York, N. Y. and *N*,ε-2,4-dinitrophenyl-L-lysine (ε-DNP-L-lysine) was bought from Cyclo Chemical Co., Los Angeles. *p*-Iodobenzenesulfonyl (pipsyl) chloride and 1-fluoro-2,4-dinitrobenzene (DNFB) were obtained from K & K Laboratories, Plainview, New York. ε-Aminocaproic acid (EACA) was obtained from Mann Research Laboratories. Pipsyl-ε-aminocaproic acid (pipsyl EACA) was synthesized by reacting pipsyl chloride with a 10-fold molar excess of EACA at *p*H 10 (carbonate buffer). The compound was crystallized by acidification of the reaction mixture. The crystallization was repeated three times.

Preparation of Conjugates.—DNP and pipsyl protein conjugates were prepared as described in reference 12, by reaction of DNFB or pipsyl chloride with the proteins under alkaline conditions, followed by extensive dialysis. Protein concentrations were determined by Kjeldahl analysis and in the case of DNP conjugates the degree of substitution was calculated from the absorbancy at 360 mμ (ϵ_1 cm, at 360 mμ was 17,400 for free ε-DNP-L-lysine).

The degree of substitution of the pipsyl conjugates was not determined but was assumed to be high considering the relationship between the weight of pipsyl chloride and the proteins used. The following DNP conjugates were used: DNP_{44}-BSA, DNP_{59}-BGG, DNP_{150}-HF. The subscripts refer to the appropriate average number of haptens per molecules of the carrier.

Immunization of Guinea Pigs.—Hartley-strain guinea pigs were injected with doses of protein conjugates varying from 0.050 to 1 mg. The antigen solutions were emulsified with equal volumes of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit) and injected into the four footpads (total volume 0.4–0.6 ml). In a few experiments, which will be indicated in the text, the animals were twice boosted intradermally at four sites in the skin with a total of 0.2–0.4 mg of antigen, during the 2nd and 3rd wk after immunization. Blood was collected at different intervals and the serum kept frozen without preservatives.

Precipitation Reactions in Liquid Medium (13).—The reactions were carried out in a total volume of 0.5 ml. Increasing amounts of antigen were added to a constant volume of antiserum. Reaction mixtures were incubated at 37°C for ½ hr and for 1 to 2 days at 4°C. Washed specific precipitates were dissolved at 0.02 M lauryl sulfate and absorbancies read at 280 mμ. When DNP antigens were used, the dissolved precipitates were also read at 360 mμ. In this case the amount of antibody precipitated could be calculated from the absorbancies of the total dissolved precipitate at 280 mμ and the absorbancies of the DNP antigens at 360 mμ.

In the case of pipsyl antigens, the supernatants of the precipitation reactions were tested for excess of antigen. The amounts of antibody precipitated were then calculated by subtracting the amount of antigen added from the total protein precipitated in the tubes where no excess antigen could be found in the supernatant.

Purification of Antibody.—Before starting the purification procedure, an amount of Na_2-ethylenediaminetetracetate required to obtain a final concentration of 0.01 M was added to the immune serum. Anti-DNP antibodies were isolated by precipitation with DNP-HF followed by elution of the washed precipitate with 0.1 M, DNP-OH in the presence of streptomycin sulfate (35 mg/ml) (12). Anti-pipsyl antibodies were precipitated with pipsyl-HF and eluted with pipsyl-EACA (2×10^{-3} M) in the presence of streptomycin.

Labeling with ^{131}I.—Labeling of proteins with ^{131}I was carried out as described in reference

14. To 1 mg of protein sample in a carbonate buffer pH 10, was added 0.1 ml of a solution containing 2.5 μc of $Na^{131}I$ in a mixture containing 0.025 ml of a solution of I_2 in KI (I_2, 1.23, and KI, 1.57 mg/ml). The specific activity of the protein was approximately $2\text{–}3\times10^5$ cpm/mg.

Measurement of Antibody Binding Affinity (K_0) by Fluorescence Quenching.—The technique of titration and methods of calculation used are described in detail by Eisen and Siskind (15). In the present work, titrations were performed under the conditions described in reference 16, and K_0 calculated assuming a Q_{max} of 100%, which was shown in the case of anti-DNP guinea pig antibodies to be the value which agrees best with equilibrium dialysis measurements.

Rabbit Antisera.—Antisera containing high levels of antibodies against L chains were obtained by immunizing rabbits with guinea pig L chain as described in reference 1. These, antisera were made specific against λ-chain-bearing molecules (L molecules) by specific absorption with purified anti-DNP guinea pig antibodies isolated from animals hyperimmunized with DNP_{59}-BGG, which contain only K molecules. Other antisera specifically directed against guinea pig L molecules could also be obtained by similar absorption of sera from rabbits immunized with whole γ_2-globulins or their pepsin digested fragment $F(ab)'_2$ (8). These antisera, however, contained lower concentrations of anti-L molecules, and for this reason were very seldom used.

Rabbit antisera specifically directed against Fc fragments of guinea pig γ_1- and γ_2-globulins were prepared as follows:

(*a*) *Anti-Fc γ_1*: Rabbits were injected with the purified γ_1-globulin or with crystals obtained after papain digestion of γ_1-globulin (17). 1-2 mg of proteins incorporated in complete Freund's adjuvant were injected into the footpads (total volume 1.6 ml). Several bleedings were taken after 6 wk and the antisera were then absorbed with purified γ_2-globulin.

(*b*) *Anti-Fc γ_2*: These antisera were obtained by immunizing rabbits with DEAE purified normal γ_2-globulin. The animals were injected in the footpads with 1 mg of protein in complete Freund's adjuvant (total volume 1.6 ml) and bled 6 wk later. Their sera were then absorbed with $F(ab)'_2$ fragments.

The rabbit antisera against Fc γ_1 and Fc γ_2 were tested for specificity by precipitation reactions with purified γ_1- and γ_2-globulin labeled with ^{131}I.

The anti-Fc γ_1-antiserum precipitated approximately 85% of the purified γ_1-globulin preparation and 0 to 1% of the γ_2-preparation. The anti-Fc γ_2-antiserum precipitated approximately 95% of the γ_2-globulin and 10% of the purified γ_1-globulin preparation (this 10% precipitation is due to contamination with γ_2-globulins present to some degree in all our preparations of γ_1-globulin).

A rabbit antiserum directed against guinea pig γ_M-globulin was a gift of Dr. Ruben Binaghi, College de France, Paris.

Measurement of the Percentage of L Molecules in the Purified Immunoglobulin Preparations.—This measurement was made by specific precipitation of these ^{131}I-labeled proteins by antisera directed against L molecules. The reaction was carried out in the antibody excess zone. An aliquot of the labeled protein was brought to 0.2 ml with saline and usually 0.3 ml of the antiserum was added. After an incubation of 30 min at 37°C and 1 day at 4°C, the precipitates were washed three times with cold saline and then dissolved and brought to the original volume with 0.1 N KOH. The percentage of labeled protein precipitated was calculated by comparing the total number of counts in the tubes before the washings of the precipitate, with the total number of counts in the washed precipitate after it had been dissolved in KOH. All measurements were made in a well-type gamma counter.

Two types of controls were included for each measurement. They consisted of mixtures of the labeled protein with normal rabbit serum and with a rabbit antiserum against $F(ab)'_2$ fragments of guinea pig γ_2-globulin in the antibody excess zone. The percentage of globulin

precipitated by the normal rabbit serum (0–3%) was subtracted from the percentage values obtained with the specific antisera. The anti-F(ab)$'_2$ antiserum precipitates both γ_1- and γ_2-globulins and was employed to evaluate the total amount of these two immunoglobulins present in each preparation. The anti-F(ab)$'_2$ antiserum precipitated approximately 95% of every preparation used.

No purified antibody preparations which were studied contained γ_M-globulin in appreciable amounts. This was verified in two independent ways. The technique of double diffusion in agar gel (Ouchterlony technique), was employed with many samples of purified antibody using a rabbit anti-γ_M serum. Furthermore, these preparations were also precipitated by the specific antisera directed against the Fc fragments of both γ_1 and γ_2 globulins; these combined antisera precipitated 90–110% of the preparations.

TABLE I

*Percentage of L Molecules in Anti-DNP Antibodies Purified from Sera of Guinea Pigs Immunized with Different Doses of DNP_{59}-BGG and Bled at Various Times after the Immunization**

Dose of antigen	Time after immunization	Anti-DNP antibody in serum	Percentage of L molecules in purified anti-DNP antibody	K_0 ($\times 10^{-5}$)
mg	*days*	*mg/ml*		*liters.mole*
0.05	7	ND‡	17.1	
	10	0.49	5.7	
	14	2.50	1.1	12
	29	5.10	0.3	430
1.00	7	ND‡	18.4	
	10	0.56	6.9	
	14	2.00	3.3	3.2
	29	2.40	2.6	7.1

* 10 animals were injected for each dose of antigen and at several times after immunization they were individually bled and pools prepared by mixing equal volumes of each serum.

‡ Not done because amounts of antibody were too small.

RESULTS

A. *Variation of the K/L Relationship in Purified Anti-DNP Antibodies During the Course of Immunization.*—Guinea pigs, immunized with 1 mg of highly conjugated DNP-BGG in complete or incomplete Freund's adjuvant and boosted intradermally during the 2nd and 3rd wk with 0.2–0.4 mg of the antigen in saline were shown to synthesize at the end of 4 wk, anti-DNP antibodies bearing almost exclusively the K type of L chain (2). Experiments were carried out in order to verify if the immunization schedule and the dose of antigen could influence this preferential late synthesis of anti-DNP antibody molecules of the K type. Guinea pigs were immunized with different doses of DNP_{59}-BGG (0.05 mg and 1 mg) incorporated in complete Freund's adjuvant. 0.4 ml of an emulsion containing the antigen was injected into the four footpads and the animals were bled at different times after the injection.

It can be seen (Table I) that 1 month after immunization both groups of animals had synthesized anti-DNP antibodies containing a very small amount of L molecules. Early antibodies, however, contained a much higher percentage of L molecules. It is also apparent that, between the 7th and 14th day, while serum levels of anti-DNP antibodies were rising sharply, the relative concentration of L molecules diminished progressively. Another interesting observation is that the total amount of antibody synthesized until the 14th day did not differ much for the animals injected with 0.05 or 1 mg of the antigen. In contrast, at the end of 4 wk, the serum of animals which received the smaller dose of antigen not only contained a significantly higher concentration of anti-DNP antibodies but also had much higher binding affinity for the hapten.

B. *Affinity of Anti-DNP Antibodies and Concentration of L Molecules.*—As mentioned previously, the preponderance of K molecules in anti-DNP antibodies suggested that these molecules might have a greater binding affinity for the DNP hapten than L antibody molecules. An attempt was therefore made to study this problem. Unfortunately, the most direct approach, which would be the physical separation of K and L molecules from a mixed antibody population and the measurement of their binding affinities was not feasible because these two types of molecules have similar physicochemical properties. For this reason, it was decided instead to fractionate anti-DNP antibodies according to their degree of affinity for the hapten and then study the K/L ratio of the antibody in each one of these fractions. The fractionation of antibody into populations showing decreasing binding affinities for the hapten was accomplished by the method described by Eisen and Siskind (15). Antisera which had been previously studied for their content in antihapten antibody by quantitative precipitation, were incubated with a small amount of antigen, far into the antibody excess zone. After incubation at 37°C for ½ hr and 4°C overnight, the sera were centrifuged, the precipitates collected, and to the supernatants were added another small aliquot of antigen. This fractional precipitation of antibody was repeated until no more precipitation was obtained. For the last precipitations the incubation at 4°C was prolonged from 5 up to 10 days to facilitate precipitation. The precipitates obtained were washed with cold saline, and the antihapten antibodies eluted using the appropriate reagent. The purified antibodies obtained by fractional precipitation showed, as expected, a progressive decrease in binding affinity for the hapten and they were then studied for their L chain type by precipitation with specific antisera. Experiments of this type were performed on several pools of antisera as described in detail below:

1. *Fractional precipitation of anti-DNP_{44}-BSA antisera:* 18 guinea pigs (700 g) were injected with 1 mg of DNP_{44}-BSA incorporated in complete Freund's adjuvant. Six animals were each bled to death at 11 days, 14 days, and 28 days after the immunization. The anti-DNP antibody was purified from aliquots of

these individual sera; then the average binding affinities for ε-DNP-L-lysine were measured by fluorescence quenching and the percentages of L molecules determined by specific precipitation. The results are shown in Table II. It can be seen that the concentration of anti-DNP antibody doubled in the serum of these animals from the 11th to the 14th day. Furthermore, the binding affinity increased from the 14th to the 28th day. No clear correlation was noticed between the average binding affinity for the DNP-hapten and the percentage of L molecules in these individual antibodies, probably because of their relatively similar average affinities, and the considerable heterogeneity to be expected in these unfractionated preparations.

TABLE II

Antibody Binding Affinities (K_0) for ε-DNP-L-Lysine and Percentage of L Molecules in Purified Anti-DNP Antibodies Isolated from Guinea Pigs Immunized with 1 mg of DNP_{44}-BSA and Bled at Various Times after the Immunization

Days after immunization	Ident.	% L molecules in purified anti-DNP antibody	K_0 ($\times 10^{-5}$)	Mean K_0	Total anti-DNP antibody in pooled sera*
			liters/mole		*mg/ml*
11	1	9.2	3.7		
	2	9.0	5.7		
	3	14.1	2.2	3.3	0.63
	4	12.1	2.2		
	5	6.6	2.7		
14	6	5.8	3.6		
	7	5.1	3.6		
	8	ND‡	5.7	4.2	1.3
	9	4.8	4.7		
	10	7.5	3.4		
28	11	6.4	14.0		
	12	ND‡	5.9		
	13	6.6	5.0	8.0	1.2
	14	7.4	ND‡		
	15	15.6	3.3		
	16	11.8	1.2		

* Equal volumes of serum were pooled for quantitative precipitation reaction.
‡ ND, not done.

Studies were then made on fractions obtained from pooled sera from these animals. The anti-DNP antibodies in three different pools were fractionated by repeated additions of small amounts of DNP-HF, each addition amounting to about 10% of what was required for total precipitation of the antibody. The antibodies in each precipitate were isolated; their binding affinities were meas-

TABLE III

Binding Affinities for ε-DNP-L Lysine (K_0) and Percentage of L Molecules in Fractions of Anti-DNP Antibodies Isolated from Sera of Guinea Pigs Bled at Several Intervals of Time after Immunization with DNP_{44}-BSA

Ident.	Fraction No.*	Properties of anti-DNP antibody obtained			
		Mg in fraction	Percentage of L molecules	K_0 ($\times 10^{-6}$)	Heterogeneity index (a)
				liter/mole	
Pool A, 11 days after immunization	1	2.2	2.6	56.0	0.59
	2	2.3	5.1	8.7	0.52
	3	2.2	4.6	9.8	0.60
	4	2.1	6.3	6.0	0.58
	5	2.2	7.0	4.0	0.63
	6	1.4	8.2	4.5	0.62
	7	1.5	12.2	1.6	0.56
	8	1.1	11.7	2.0	0.56
Pool B, 14 days after immunization	1	1.5	0.6	23.0	0.37
	2	2.2	3.5	14.0	0.53
	3	1.9	3.9	13.0	0.46
	4	2.0	3.6	13.0	0.55
	5	2.2	3.6	8.9	0.52
	6	1.9	4.5	6.8	0.52
	7	1.8	4.2	6.0	0.53
	8	1.8	5.8	5.3	0.55
	9	1.5	6.1	4.3	0.58
	10	1.4	7.3	3.4	0.57
	11	1.6	8.1	2.2	0.64
	12	0.9	10.8	1.4	0.56
Pool C, 28 days after immunization	1	1.5	1.4	250.0	0.21
	2	1.9	2.8	40.0	0.32
	3	2.2	2.6	28.0	0.23
	4	2.0	3.4	12.5	0.39
	5	1.9	3.9	13.0	0.53
	6	1.9	4.8	8.0	0.51
	7	1.7	ND‡	ND‡	ND‡
	8	1.7	8.7	5.3	0.56
	9	2.2	10.0	4.2	0.62
	10	1.1	12.5	3.0	0.60

* Fractions obtained by successive additions of small amounts of DNP-HF. Each addition consisted of 0.025 mg of antigen per milliliter of pooled serum. The recovery of antihapten antibody in every pool was 50–70% of the amount present in serum and determined by quantitative precipitation.

‡ ND, not done.

ured; and the percentages of L molecules in the preparations were determined. The results are shown in Table III and Fig. 1. Purified anti-DNP antibodies isolated from successively formed precipitates show decreasing binding affinities for the hapten and at the same time display increasing concentrations of L

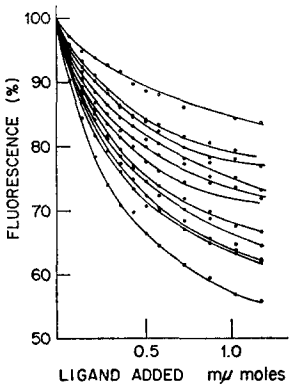

FIG. 1. Fluorescence quenching by ε-DNP-L-lysine of purified anti-DNP antibody fractions isolated from pooled sera of guinea pigs immunized with DNP_{44}-BSA, and bled 14 days after immunization. See text for procedural details.

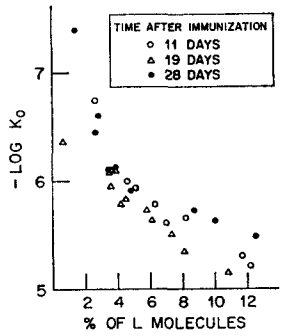

FIG. 2. Relationship between binding affinities (K_0) for ε-DNP-L-lysine and percentage of L molecules in fractions of anti-DNP antibodies obtained from sera of guinea pigs bled at several intervals of time after immunization with DNP_{44}-BSA. The data plotted correspond to those shown in Table III. For procedural details, see text.

molecules. From the curve in Fig. 2, it appears that when the binding affinities (K_0) for ε-DNP-L-lysine reach 10^7 liters/mole the percentages of L molecules in the antibody populations become minimal.

The inverse relationship between the percentage of L molecules and the binding affinity of anti-DNP antibodies for the hapten was also confirmed by studies of the L chain composition of individual anti-DNP antibody preparations selected for their widely different affinities and obtained by immunizing

guinea pigs with various doses of different DNP-protein conjugates (Table IV). Anti-DNP-BGG antibodies late in the course of immunization and anti-DNP-GPA antibodies synthesized by guinea pigs immunized with low doses of antigen (0.05 mg) show, in general, average binding affinities for ε-DNP-L-lysine 10^7 or higher and contained less than 1% of molecules bearing λ-chains. In contrast, the antibodies produced by guinea pigs immunized with 1 mg of DNP-OVA or DNP-BSA had a higher concentration of L molecules and average binding affinities for the same hapten generally between 10^5 and 10^6 liters/mole.

2. *Fractional precipitation of anti-pipsyl BGG or anti-pipsyl BSA antisera:* In contrast with guinea pig anti-DNP antibodies which have a very low percentage of L molecules, anti-pipsyl antibodies may contain a higher ratio of molecules bearing λ-chains than normal γ-globulins.

TABLE IV

*Effect of Carrier on Affinity for ε-DNP-L-Lysine and on L Chain Composition of Guinea Pig Purified Anti-DNP Antibodies Obtained 4 wk after Immunization**

Carrier protein	Immunizing dose	K_0 ($\times 10^{-6}$)	% of L molecules
	mg	*liters/mole*	
BGG	0.05	430	<1
BGG	1.00	55	<1
GPA	0.05	>100	<1
OVA	1.00	22	2–8
BSA	1.00	4.9	5–12

* These data summarize many unpublished observations, as well as the results of experiments included in previous papers (2, 16).

In the following experiments, advantage was taken of the observation that guinea pig anti-pipsyl antibodies cross-react extensively with DNP antigens[2] to investigate the relationship between the type of L chain in the anti-pipsyl antibodies and their ability to cross-react with DNP.

Anti-pipsyl antibodies can be separated into two distinct populations by specific precipitation first with the cross-reacting DNP antigen, followed by the homologous antigen. One such experiment in which the percentage of L molecules was determined in these distinct populations of antibodies from a pool of anti-pipsyl antibody is shown in Table V. An anti-pipsyl BGG serum, which contained 3.85 mg of anti-pipsyl antibody per milliliter of serum, 26% of which cross-reacted with DNP antigens, was used (Fig. 3). To a sample of this serum (pool A) an adequate amount of DNP-HF sufficient to precipitate all cross-reacting antibodies was added. The sample was then incubated at 37°C for

[2] The opposite cross-reaction, i.e., of anti-DNP antibodies with pipsyl antigens, however, is extremely poor.

TABLE V

Percentage of L Molecules in Fractions of Antihapten Antibodies Isolated from Pooled Sera from Guinea Pigs Immunized with 1 mg of Pipsyl-BGG

Sample*	Fraction	Antigen	Antibody recovered‡	
			Total	% L molecules
			mg	
A	I	DNP-HF (0.250 mg)	0.91	27
	II	Pipsyl-HF (0.250 mg)	1.33	68
	III	Pipsyl-HF (0.250 mg)	0.74	62
B	I	Pipsyl-HF (0.125 mg)	0.87	56
	II	Pipsyl-HF (0.250 mg)	1.28	57
	III	Pipsyl-HF (0.250 mg)	0.65	47

* 8 ml sample used, but data calculated in reference to 1 ml of antiserum.

‡ The total amount of anti-pipsyl antibody in this antiserum was 3.85 mg/ml. The recoveries of purified antibody were 77 and 73% in samples A and B. The amount of DNP cross-reacting antibody was 1.0 mg/ml, and the recovery 91%. The total amounts of L molecules recovered in fractions A and B were 1.61 and 1.54 mg/ml.

½ hr and 4°C overnight. The precipitate obtained was separated by centrifugation and to the supernatant two successive aliquots of pipsyl-HF were added in order to precipitate the remaining non-cross-reacting antibodies. In this way three specific precipitates were obtained. Antibodies were next purified using pipsyl-EACA as eluting hapten, and their L chain type was analyzed. The

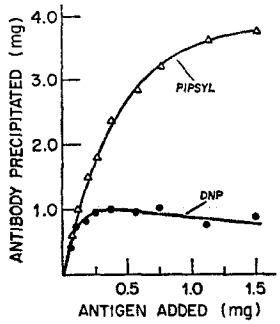

FIG. 3. Quantitative precipitation reaction of guinea pig anti-pipsyl-BGG antisera using as antigen pipsyl-HF and DNP-HF.

concentration of L molecules found in the DNP-cross-reacting antibodies (27%) was much lower than in the other two fractions of anti-pipsyl antibodies (68 and 62%), as shown in Table V.

As a control for this experiment, another aliquot of the same pool of anti-pipsyl serum (pool B) was fractionated by addition of three successive aliquots of pipsyl-HF, and the antibodies purified and analyzed in the same way. The results obtained clearly differed from the previous ones, for the successively

TABLE VI

Percentage of L Molecules in Fractions of Antihapten Antibodies Isolated from Pooled Sera from Guinea Pigs Immunized with 1 mg of Pipsyl-BSA

Sample*	Fraction	Antigen	Antibody recovered‡	
			Total	% L molecules
			mg	
A	I	DNP-HF (0.250 mg)	0.52	19
	II	Pipsyl-HF (0.250 mg)	0.40	50
	III	Pipsyl-HF (0.250 mg)	0.16	50
B	I	Pipsyl-HF (0.125 mg)	0.36	42
	II	Pipsyl-HF (0.250 mg)	0.40	40
	III	Pipsyl-HF (0.250 mg)	0.26	35

* 7.3 ml samples used but data calculated in reference to 1 ml of antiserum.

‡ The total amount of anti-pipsyl antibody in this antiserum was 1.6 mg/ml. The recoveries of purified antibody were 68 and 64% in samples A and B. The amount of DNP cross-reacting antibody was 0.57 mg/ml, and the recovery 91%. The total amounts of L molecules recovered in fractions A and B were 0.39 and 0.40 mg/ml.

obtained antibody fractions contained a similar proportion of L molecules (56%, 57%, and 47%). It is remarkable that the DNP cross-reacting antibodies contained half as many L molecules as the corresponding first fraction precipitated with the conjugate of the homologous hapten. The total yield of purified antibodies (77% and 73%) as well as the final yield of L molecules obtained in these two experiments were very similar. The preferential combination of K antibody molecules with the DNP antigen is clearly the reason for the noticeable enrichment of the non-cross-reacting fractions of pool A with L antibody molecules.

Another example of the same phenomenon is shown in Table VI. In this case

the fractionation of antibodies cross-reacting with DNP conjugates was performed with pooled anti-pipsyl BSA sera which contained 1.6 mg of anti-pipsyl antibodies, 35% of which cross-reacted with DNP. While the cross-reacting populations of antibodies contained only 19% of L molecules, the corresponding first fraction obtained with the homologous hapten contained 42% of L molecules. The same enrichment of L molecules is also noticed in the purified fractions containing only the non–cross-reacting antibody.

DISCUSSION

The data presented on the immune response of guinea pigs to hapten-protein conjugates can be interpreted as demonstrating (*a*) that the κ- and λ-types of L chains cannot form immunoglobulins with combining sites equally well adapted to certain antigenic determinants and, (*b*) that the relative adequacy of each L chain type for the formation of specific antibody combining sites in response to an antigen determines the proportion of K/L antibody molecules which are synthesized. These conclusions are based upon the following experimental evidence:

The proportion of K and L molecules in some anti-hapten antibodies is markedly different from that found in normal γ_2-globulins. Thus anti-DNP antibodies produced after 3 or 4 wk of immunization with conjugates of this hapten with BGG, BSA, ovalbumin, and guinea pig albumin contain only a small proportion (0–10%) of L molecules regardless of the mode of immunization. Furthermore, there is an inverse correlation between the amounts of L molecules in purified anti-DNP antibodies and their average binding affinity for the hapten when different DNP-protein conjugates are used for immunization. Anti-DNP antibodies produced 2 wk or more after immunization with 1 mg or 0.05 mg of DNP-BGG or of 0.05 mg of DNP-GPA have high binding affinities for ε-DNP-L-lysine and contain less than 1% of L molecules. A higher proportion of L molecules is found in anti-DNP antibodies isolated from the sera of guinea pigs immunized with 1 mg of DNP-BSA or DNP-OVA and which show a lower binding affinity for the hapten. The inverse relationship between the binding affinity of anti-DNP antibodies for ε-DNP-L-lysine and their content in L molecules was clearly demonstrated when the anti-DNP antibodies contained in pools of anti-DNP-BSA antiserum were fractionated according to their binding affinity for the hapten. L molecules constituted close to 12% of the population of anti-DNP antibody molecules with an average binding affinity for the hapten close to 10^5 liters/mole. In fractions with increased binding affinity the percentage of L molecules decreased progressively, and when a K_0 of the order of 10^7 was reached, less than 1% of molecules bearing λ-chains were detected.

In contrast with the low proportion of L molecules found in anti-DNP antibodies, anti-pipsyl antibodies isolated from guinea pigs immunized with 1

mg of pipsyl-BGG or pipsyl-BSA contain in most cases a concentration of L molecules greater than that found in normal γ_2-globulins. The relationship between the L chain type and antibody specificity is also clearly indicated by the L chain composition of the anti-pipsyl antibodies which are able to cross-react with DNP antigens. Anti-pipsyl antibodies cross-reacting with the DNP determinants show a much lower concentration of L molecules than those lacking this property. Thus, it appears that the κ-type of L chain is more satisfactory for the formation of the anti-DNP antibody combining site. If the relationship between L chain type and antibody specificity is indeed a general phenomenon, the proportion of both types of L chain synthesized in response to any antigen would be directly related to the relative adequacy of each L chain type for the formation of the corresponding sites. For example, the observation that twice as many K molecules than L molecules are found in normal guinea pig γ_2-globulins would reflect the better adequacy of K molecules for the formation of antibodies against common immunogens.

These data also lend support to the evidence (18–23, 11) that the L chains are involved together with the H chain in determining the immunological specificity of the antibody combining site and raise a problem concerning the mechanism whereby this is achieved. Two hypotheses can be proposed which are not necessarily exclusive. Both hypotheses are based on the premise that the structure of the antibody combining site is determined at least in part by the amino acid sequence of the variable segment of the L chain. (*a*) Differences in the invariant portions of the two types of L chains may restrict or modulate the tertiary or quarternary structure which the molecule may assume and thus indirectly affect the combining site. This explanation would be particularly adequate in the case that the variable portion of both L chain types is found to display precisely the same heterogeneity and assume the very same sequences. (*b*) The variable portions of the two types of L chains may be characteristically different and the two types of L chains may therefore represent two distinct and finite populations as far as the amino acid sequences in their N terminal half are concerned. This is consistent with the limited evidence available from fingerprinting studies on human Bence-Jones proteins. In this case the type of L chain would define and delimit the possible amino acid sequences that may be found in the variable portion.

The demonstration that the two L chain types can have distinct potentialities for the formation of immunoglobulins of different specificities may have important evolutionary implications considering the advantage conferred by the increased range in configurations which immunoglobulins may assume by virtue of their different L chains.

In the preceding discussion, we have considered only the structural relationship between L chain type and antibody specificity, but another important phenomenon described by Eisen and Siskind (15) has been confirmed in these

experiments, i.e., that precisely those antibody molecules which bind best the immunizing antigen increase in concentration in the course of immunization, by a process which we call "maturation." Thus anti-DNP antibodies obtained from animals immunized with DNP-BGG display increasing binding affinities for the hapten during the course of immunization, and the study of the L chain composition of these antibodies shows that simultaneously with the changes in affinities, there is a decrease in the concentration of molecules bearing the λ-type of L chain. Observations which may be of a similar nature were made recently by McGuigan et al. (24). These investigators observed that rabbit anti-DNP antibodies formed shortly after an injection of DNP-BGG differ in their amino acid composition from those synthesized months later.

These quantitative studies permit an analysis of the phenomenon of "maturation" of antibody specificity in the course of immunization at the cellular and molecular levels. This phenomenon may be explained in large part by admitting a selection of antibody-forming cells by the antigen. It is a basic underlying assumption that antibody-producing cells synthesize a single homogeneous antibody, with a single type of L chain, and this is consistent with most of the experimental data available (25–27). The observed increase in the affinities of the antibodies during the course of immunization could result from the preferential stimulation of cells synthesizing higher affinity antibody, which have a distinctive advantage in their capacity to bind antigen. This advantage would be of course particularly important in the presence of limiting amounts of antigens. The observation that sensitized cells proliferate and differentiate specifically in response to antigen, as well as the fact that continuous antibody synthesis depends in general upon the availability of antigen, favor this interpretation.

There is evidence, however, that another mechanism involving some form of immunological inhibition as postulated by Eisen (28) intervenes in determining the population of cells engaged in specific antibody synthesis. This is strongly suggested by the data on the response of guinea pigs to two different doses of DNP-BGG (Table I). The anti-DNP antibodies produced by these animals after 1 mg or 0.05 mg of this antigen differ widely in their binding affinity for the antigen. The "maturation" process appears to be distinctly slower in the animals receiving a higher dose of antigen, in terms of the concentration and binding affinities of the anti-hapten antibody found in their serum a few weeks after antigen injection. These results and similar observations by Goidl et al.[3] suggest that excess of antigen, even when administered in complete adjuvant, is still capable of selectively inhibiting cells capable of synthesizing the highest affinity antibodies.

These experiments also show that in order to interpret correctly the relation-

[3] Goidl, E. A., W. E. Paul, G. W. Siskind, B. Benacerraf. 1966. In preparation.

ship between any structural feature found in antibodies with their specificity, it is of great importance to take into account not only the changes in immunoglobulin populations which occur with time after immunization but also the effect of dose on the parameters of the antibody response.

Phenomena have been described which are responsible for the selection of L chain types in immunoglobulins based upon the specificity of the corresponding antibodies for the immunizing antigen; it is theoretically possible that similar phenomena play a role in the selection of the H chain type, and therefore of immunoglobulin classes, in the course of immunization.

SUMMARY

Two types of light polypeptide chain (κ and λ) are present in guinea pig immunoglobulins. The ratios of K/L molecules in anti-hapten antibodies differ sometimes markedly from that found in normal serum. Anti-DNP antibodies and anti-pipsyl antibodies have, respectively, a higher and a lower than normal K/L ratio. The possibility that the L chain type affects the range of configurations which the antibody-combining site may assume was investigated. Fractional precipitation of anti-DNP antibodies from serum of guinea pigs immunized with DNP_{44}-BSA was performed using limiting amounts of antigen. Antibody fractions were purified from each precipitate, their affinities for ϵ-DNP-L-lysine measured by fluorescence quenching (K_0) and the K/L ratio estimated by precipitation with specific antisera. Increasing concentrations of L molecules were found in fractions witb decreasing K_0. In other experiments, fractional precipitation and purification of antibodies which cross-react with DNP was performed in serum of animals immunized with pipsyl-BGG. The K/L ratio in antibodies isolated from these fractions was much higher than in fractions which do not cross-react with DNP. These results show that K molecules are better adapted to react with the DNP hapten than L molecules. The K/L ratio in anti-DNP antibody was shown to increase in the course of immunization, at the same time that the K_0 is increasing. This rise in K_0 is markedly delayed when larger doses of antigen are employed.

The authors would like to thank Mrs. Elinor Murphy and Mr. Robert Bloom for having written the computer program used to facilitate calculations of equilibrium dialysis, as well as to acknowledge the valuable cooperation of the staff of the Section on Communications Science of the Department of Neurology of the New York University Medical Center. We would like to thank Dr. W. E. Paul for the gift of some samples of purified anti-DNP antibody. We also acknowledge the excellent technical assistance of Miss Carolyn Fuss.

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