DETERMINATION OF ANTIBODY CLASS IN A SYSTEM OF COOPERATING ANTIGENIC DETERMINANTS*

BY VOLKER SCHIRRMACHER‡§ AND KLAUS RAJEWSKY,§ M.D.

(From the Institut für Genetik der Universität Köln, Köln, Germany)

(Received for publication 23 June 1970)

It is now well established that in the induction of the immune response, the antigenic determinants of the antigen cooperate with each other (1–5). Cooperation of antigenic determinants seems to reflect the interaction of antibody forming cell precursors and antigen specific helper cells, presumably thymus-derived lymphocytes (5–9). The roles of the two cell populations in the induction process are not yet well understood. The experiments reported in this paper show that cooperation of antigenic determinants occurs in the induction of both 19S and 7S antibodies, and suggest that the class of the emerging antibodies is determined by the antibody forming cell precursors. A brief account of some of our findings has been reported previously (10).

Materials and Methods

Antigens.—Bovine serum albumin[1] (Behringwerke, Marburg-Lahn, Germany) and human gamma globulin (Serva, Heidelberg, Germany) were used as carrier proteins. Coupling of sulfanilic acid to proteins, determinations of sulf groups per carrier molecule, and protein determinations were done as described (4). $Sulf_{19}$-BSA and $sulf_{18}$-HGG were used as hapten-carrier complexes.

Animals.—10–12-wk old randomly bred rabbits, supplied by a local breeder.

Immunization.—Details of the immunization procedure have been described (4). For primary injections, the antigens were emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Sulf-BSA was injected into the right and HGG into the left hind leg, except when otherwise indicated. Secondary injections were given intravenously. Bleedings were made 1 day before and 4 and 7 days after secondary stimulation.

Preparation of sulf-tyr.—Diazotization and coupling of diazotized sulfanilic acid to N-chloracetyl-l-tyrosine (Mann Research Labs., Inc., New York) was carried out as described for

* This work was supported by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

‡ In partial fulfillment of doctoral thesis requirement, Universität Köln.

§ Present address: Institut für Genetik der Universität Köln, 5 Köln 41. Weyertal 121, Western Germany.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; Hbc-25 (a) 10^{-7}M, hapten binding capacity at 10^{-7}M hapten (for definition see Materials and Methods); HGG, human gamma globulin; KLH, Keyhole Limpet hemocyanine; OA, Ovalbumin; sulf, sulfanyl group; sulf-tyr, mono (p-azobenzene-sulfonic acid)-chloracetyl-l-tyrosine; 1°, 2°, primary and secondary immunizations.

the arsanilic acid derivative (11). Radioactive hapten was prepared by using ^{35}S-labeled sulfanilic acid (Amersham/Searle, England). The compound had a specific activity of 1,26 Ci/mMole. It was purified by column chromatography on TEAE-cellulose (Serva, Heidelberg, Germany). Purity of the hapten was more than 90% as tested by thin layer chromatography. Details of its preparation and purification will be contained in a subsequent publication.[2]

Passive Hemagglutination.—Hapten-specific passive hemagglutination was carried out as described by Ingraham (12), one volume of diazotized sulfanilic acid (4.35 μMoles/ml) being reacted for 30 min at 4°C with two volumes of a 50% suspension of washed sheep red cells.

For HGG-specific passive hemagglutination, HGG was coupled to sheep red cells by bis-diazo-benzidine, as specified for lactic dehydrogenase in a previous publication (13). The passive hemagglutination reaction could be inhibited by free HGG at a concentration of 0.05 μg/ml. For inhibition of hemagglutination the antisera were diluted in buffer containing the inhibitor at a given concentration. All assays were made in duplicate. The microtiter equipment used was obtained from Cooke Engineering Co., Alexandria, Va.

Binding Test.—The capacity of the sera to bind ^{35}S-sulf-tyr was measured in a salt-precipitation test, based on the Farr assay (14). Antisera were initially diluted 1:10 in borate buffer, pH 8.4 (14). Further dilutions were made in borate buffer containing 10% normal rabbit serum. After incubation of the antisera with radioactive hapten for 30 min at room temperature, gamma globulin-bound radioactivity was precipitated with half-saturated ammonium sulfate. The tubes were centrifuged and the radioactivity in the supernatant was determined in a Packard scintillation counter (Packard Instrument Co., Inc., Downer's Grove, Ill.). The hapten-binding capacity of an antiserum at a certain hapten concentration was calculated from the serum dilution at which 25% of the amount of hapten in the incubation mixture was bound. Hbc-25 (a) c is defined as the amount of hapten in mmoles bound to the gamma globulin fraction of 1 ml of antiserum upon incubation at the hapten concentration c. Details of the test will be described in a subsequent publication.[2]

Separation of 19S and 7S Antibodies.—19S and 7S antibodies were separated by sucrose-gradient centrifugation as described previously (13). The gradients were divided into 24 fractions (Fig. 1). The fractions containing 19S antibodies (fractions 5–10) and 7S antibodies (fractions 12–18) respectively, were pooled, normal rabbit serum was added to a final concentration of 10% and the pools were stored at −20°C.

To determine whether antisera contained mainly antibodies of the 19S or the 7S class, a reduction assay was used. This consisted of determining the passive hemagglutination titer before and after treatment of the antisera with 0.01 M dithiothreitol (Cleland's reagent, Calbiochem, Los Angeles, Calif.) for 1 hr at 37°C. Treatment with Cleland's reagent selectively destroyed the activity of 19S antibodies as demonstrated by sucrose-gradient analysis.

Anti-sulf antibody classes were thus characterized by their sedimentation behavior and by their sensitivity to mild reduction. Since IgA antibodies sediment between 7S and 19S antibodies in density gradient ultracentrifugation (15) and are known to be partly sensitive to mild reduction (16), their presence in the 19S and 7S fractions cannot be excluded.

Activity and Specificity of 19S and 7S Anti-Sulf Antibodies in Passive Hemagglutination and the Hapten-Binding Assay.—Hapten binding could not be detected in 19S antibody fractions nor in antisera containing only 19S antibodies (see Fig. 1 and table II). It is known (17) that on a molar basis, 19S anti-hapten antibodies are 60–180 times more active in passive hemagglutination than 7S antibodies.

The hapten-binding capacity of anti-sulf antisera thus reflects their content of 7S anti-sulf antibodies. An extensive correlation analysis has shown that hapten-binding capacities and 7S hemagglutination titers of individual antisera are satisfactorily correlated.[2]

[2] Schirrmacher, V. Manuscript in preparation.

Sulf-tyr inhibited the anti-sulf passive hemagglutination reaction at low concentrations (50% inhibition at 10^{-7} M); association constants determined by the hapten-binding assay ranged from 10^{-6} to 10^{-7} moles/liter (18). In contrast, free sulfanilic acid does not effectively bind to anti-sulf antibodies and is a poor inhibitor in the passive hemagglutination assay. Sulf-tyr is therefore a suitable compound to determine anti-sulf antibodies in antisera elicited by sulf-protein conjugates, although in these conjugates the sulf group is covalently linked to tyrosyl, histidyl, and lysyl residues (19).

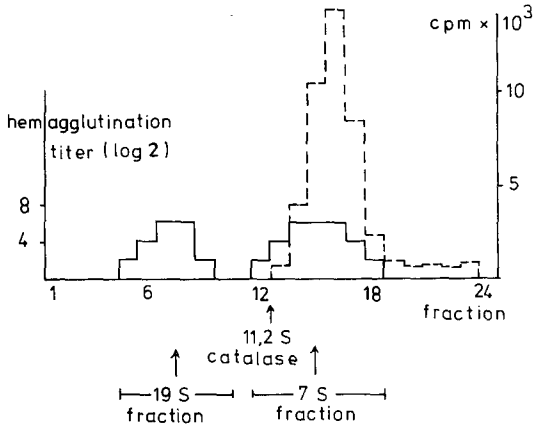

FIG. 1. Binding activity (---) and hemagglutination activity (—) of anti-sulf antibodies in sucrose gradient fractions. (*a*) A mixture of equal amounts of anti-sulf antiserum and 2×10^{-6} M ^{35}S sulf-tyr was incubated for 30 min at 20°C. 0.1 ml of the mixture was centrifuged on a sucrose gradient and the radioactivity in the fractions was measured by liquid scintillation counting. (*b*) 0.1 ml of the same serum was centrifuged on a sucrose gradient and the hemagglutination activity of the fractions was determined.

RESULTS

Experimental Design.—The experimental system used resembled the one described in a previous publication (4). Rabbits were primed with a hapten primary-carrier conjugate (sulf-BSA) and a free secondary carrier (HGG), and boosted with the hapten coupled to the secondary carrier (sulf-HGG). In some instances, the response obtained in this system was compared with the response obtained when identical hapten-carrier complexes were used for primary and secondary injection. The animals were bled before and after secondary injection and their sera were analyzed for their content of 19S and 7S anti-hapten and anti-carrier antibody. The extent of secondary stimulation at a given time after secondary injection is expressed by the ratio of antibody concentration in the serum at that time to antibody concentration at the time of secondary stimulus, the "stimulation factor". We are confident that the stimulation factor is an adequate expression for the extent of secondary stimulation, since previous data have shown (4) that in general, there is a positive

correlation between primary and secondary antibody titers, except when primary antibody titers are very high. This holds true also for the experiments described in this article (not presented explicitly).

The following questions were asked: (*a*) Can one, by increasing the dose of hapten secondary-carrier complex, substitute for priming with the secondary

TABLE I

Variation of the Dose of Sulf-HGG for Secondary Stimulation

Rabbit group	Sulf-HGG	Stimulation factor for anti-sulf*	
	μg		
A‡	1000	1,5	1,4
		1,0	
		2,0	
B	1000	7,0	7,1
		5,0	
		10,0	
C	100	6,0	4,4
		2,0	
		7,0	
D	10	3,0	5,7
		5,0	
		7,0	
		10,0	
E	1	2,5	4,0
		5,0	
		3,3	
		6,0	

1°—50 μg sulf-BSA and 10 μg HGG in adjuvant on day −28. 2°—sulf-HGG on day 0. Bleedings on day 0 and day + 7.

* Determined by passive hemagglutination assay; factors for individual animals and geometric means are given.

‡ Did not receive free HGG.

carrier? (*b*) How does variation of the doses of hapten primary-carrier conjugate and of secondary carrier affect the class distribution and the amount of secondary anti-hapten antibody? (*c*) How does variation of the time interval between secondary stimulus and priming with the hapten primary-carrier conjugate or with the secondary carrier affect the class distribution and the amount of secondary anti-hapten antibody?

Extent of the Carrier Effect.—The data presented in Table I show that, in the experimental system used, a secondary response can be elicited with very small doses of sulf-HGG, i.e., 1 μg (group E). When the dose of sulf-HGG is

increased by a factor of 10^3, carrier specificity is still fully preserved (groups A and B).

TABLE II

Variation of the Priming Dose of Sulf-BSA

Rabbit group	Sulf-BSA	Stimulation factor for anti-sulf*					
		9 S‡		7 S ‡		7 S§	
	μg						
A‖	1000	1	1,0	2	1,9	2,6	2,5
		1		1,5		2,5	
		1		2		2,7	
		1		2		2,3	
B	1000	1	1,7	6	7,5	14,7	12,7
		2		8		9,2	
		4		8		18	
		1		8		10,5	
C	100	2	2,6	6	5,1	3,9	5,1
		1,5		6		6,5	
		8		3		not done	
		2		6		5,3	
D	10	1,5¶	3,5	n**		n	
		2¶		n		n	
		3¶		n		n	
		16¶		n		n	
E	1	n**		n		not done	
		n		n			
		n		n			

1°—Sulf-BSA and 1000 μg HGG in adjuvant on day −28. 2°—1000 μg sulf-HGG on day 0. Bleedings on day 0 and +7.

* Factors for individual animals and geometric means are given.

‡ Determined by passive hemagglutination assay of sucrose gradient fractions.

§ Determined by binding assay at 10^{-8} M hapten.

‖ Did not receive free HGG.

¶ No antibody detectable before secondary injection; the value represents the reciprocal titer on day +7.

** No antibody detectable before and after secondary injection.

Determination of the Class of Secondary Anti-Hapten Antibody by the Dose of Hapten Primary-Carrier Conjugate.—The class distribution of secondary anti-sulf antibody depends on the dose of sulf-BSA used for priming. As demonstrated by the data contained in Table II, there is a shift from 19S to 7S antibody production as the dose of sulf-BSA is increased. The difference in

TABLE III

Variation of the Priming Dose of HGG

Exp. No.	Rabbit group	HGG	Stimulation factor for anti-sulf*					
			19 S‡		7 S‡		7 S§	
		μg						
I	A	1000	3‖	2,5	12	8,5	not done	
			2		6			
	B	100	1	1,9	8	8,3	7	13,3
			1,5		16		14,5	
			2		6		8,5	
			4		6		36,6	
	C	10	2	1,6	6	4,6	17,6	11,0
			1		3		11,2	
			2		3		5,7	
			1,5		8		12,6	
	D	1	1	1,3	8	4,1	11,7	4,7
			4		4		7,5	
			1		6		—	
			1		1,5		1,2	
	E	0,1	1	1,0	3	1,9	7,7	3,7
			1		2		4,1	
			1		1,5		2,8	
			1		1,5		2,0	
II	F	10	1	2,0	2	4,1	not done	
			4		16			
			1,5		6			
			4		4			
			4		4			
			1,5		8			
			1,5		6			
			1		4			

Experiment No. I: 1°—200 μg sulf-BSA and HGG in adjuvant on day −28. 2°—1000 μg sulf-HGG on day 0. Bleedings on day 0 and +7.

Experiment No. II: 1°—30 μg sulf-BSA and HGG in adjuvant on day −42. 2°—10 μg sulf-HGG on day 0. Bleedings on day 0 and +7.

* Factors for individual animals and geometric means are given.

‡ Determined by passive hemagglutination assay of sucrose gradient fractions.

§ Determined by binding assay at 10^{-8} M hapten.

‖ No antibody detectable before secondary injection; the value represents the reciprocal titer on day +7.

TABLE III *Continued*

Exp. No.	Rabbit group	HGG	Stimulation factor for anti-sulf*				
			19 S‡		7S‡		7 S§
		μg					
	G	1	1	2,2	4	3,9	not done
			3		8		
			1,5		8		
			4		4		
			1		1		
			6‖		6		
			3		8		
			2		1		
	H	0,1	1	1,2	3	3,9	not done
			n¶		n¶		
			2		2		
			1		8		
			1		2		
			1,5		16		
			1,5		6		
			1		6		
	I**	—	1	1,0	1	1,4	not done
			1		1		
			1		1		
			1		2		
			1		2		
			1		1,5		

¶ No antibody detectable before and after secondary injection.
** Did not receive free HGG.

group B between the stimulation factors determined for 7S antibodies by passive hemagglutination on the one hand, and by binding assay on the other, is presumably due to the fact that at a hapten concentration of 10^{-8} M the binding assay does not detect low affinity antibodies (see also Table III). This question will be dealt with in a subsequent publication.[2]

Variation of the priming dose of HGG does not affect the class of secondary anti-sulf antibody (Table III), except that at very low doses there is an indication of a preferential stimulation of 7S antibodies in one of the two experiments (compare groups G and H). As expected, it is the extent of secondary stimulation which depends on the dose of HGG used for priming (groups A–E).

Determination of the Class of Secondary Anti-Hapten Antibody by the Time Interval Between Priming with the Hapten Primary-Carrier Complex and Secondary Stimulus.—The class distribution of secondary anti-sulf antibody is also dependent on the time interval between the primary injection of sulf-BSA and secondary stimulus. When the secondary stimulus is given 10 days

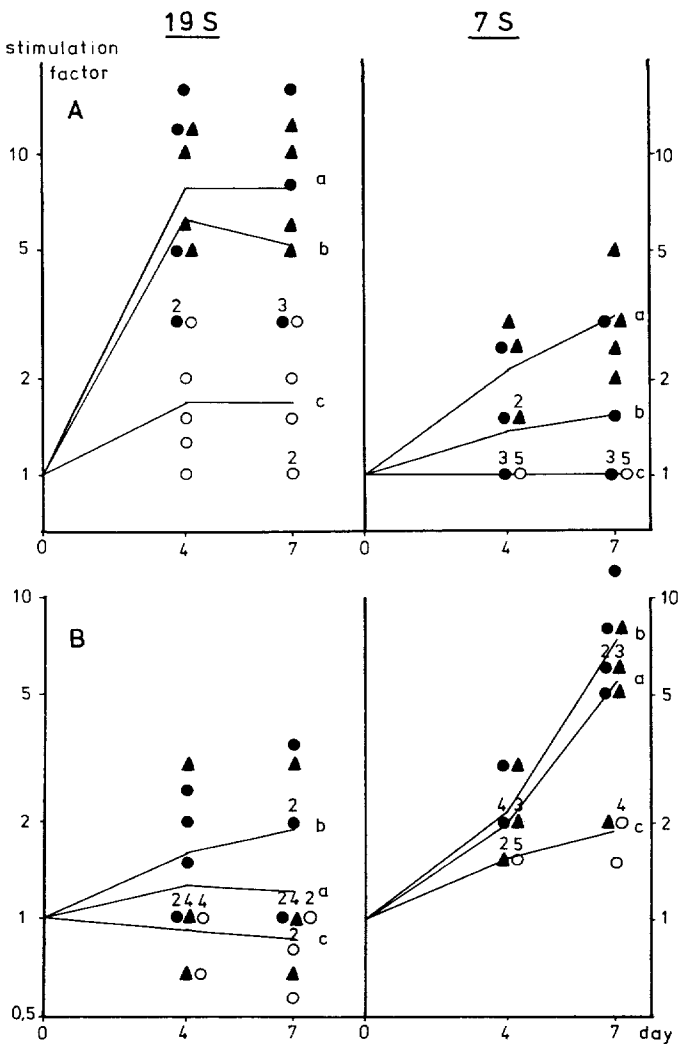

FIG. 2. Dependence of the class distribution of secondary anti-sulf antibody on the time interval between priming with sulf-BSA and secondary stimulus. (1°) 1000 μg sulf-BSA (groups *a*, *b*, and *c*) on day −10 (*A*) or −28 (*B*), and 10 μg HGG (group *b*) on day −28 in adjuvant (2°) 1000 μg sulf-BSA (group *a*) or 1000 μg sulf-HGG (groups *b* and *c*) on day 0. Bleedings on day 0, +4, and +7. 19S and 7S antibodies were separated by sucrose-gradient centrifugation and the stimulation factors were calculated from passive hemagglutination data. The diagram contains stimulation factors for individual animals. (group *a* (▲), *b* (●), or *c* (○)). Numbers of animals with identical stimulation factors are drawn above points. Geometric means of individual values are connected by straight lines. In the groups of part *A* of the experiment, no antibody was detectable before secondary stimulation. A hemagglutination titer of one was arbitrarily assigned to these sera.

after primary injection of 1 mg sulf-BSA, the secondary response consists largely of the production of 19S anti-sulf antibodies (Fig. 2 *A*, group *b*). In contrast, there is a predominant appearance of 7S anti-sulf antibodies in the secondary response, when the time interval between primary and secondary injection is 28 days (Fig. 2 *B*, group *b*). 19S and 7S secondary responses differ in their kinetics, and this has been found to be true in all experimental situations. Both responses depend on cooperation of antigenic determinants, since they do not occur in animals which have not undergone a priming experience with the secondary carrier HGG (Fig. 2, group *c*). With respect to both class and quantity of secondary anti-sulf antibody, the response obtained in the cooperating system resembles the response which is observed when sulf-BSA is used for both priming and boosting (Fig. 2, groups *a* and *b*).

In a further series of experiments, the influence of the time interval between HGG injection and secondary stimulation on the amount and class distribution of secondary anti-sulf antibodies was investigated. Table IV represents an experiment in which the animals were primed with 1 mg sulf-BSA on day −28, and with HGG either on day −28, −5, or −2. Secondary stimulation was found mainly in the 7S antibody class as tested by the passive hemagglutination reduction assay. A slight helper effect for the induction of high affinity anti-sulf antibodies is apparent in the day −5, but not in the day −2 group.[3] In a second experiment, animals were primed with 50 μg sulf-BSA on day −28 and with HGG on either day −8 or −4. The mean stimulation factors for high affinity anti-sulf antibodies 7 days after secondary injection of sulf-HGG as determined by the hapten binding assay at 10^{-8} M hapten were 13.4 in the day −8 group, 5.3 in the day −4 group, and 2.5 in the control group, which did not receive a primary injection of HGG. A third experiment showed that animals primed with 1 mg sulf-BSA on day −10 and with HGG on either day −28, −10, or −5 consistently produced almost exclusively 19S anti-sulf antibody upon secondary stimulation with sulf-HGG. The mean stimulation factors on day +4 determined by passive hemagglutination relative to control animals, which received Freund's adjuvant without HGG, were 8.7 (day −28 group), 7.8 (day −10 group), and 4.8 (day −5 group). Thus, in the experimental situations described above, variation of the time of HGG injection had no influence on the class of secondary anti-sulf antibodies. It is only the extent of secondary stimulation that is reduced when the time interval between HGG injection and secondary stimulus is short, e.g., 5 days. The rapid tempo of the development of helper activity is evident from these data. It is of interest to note that helper activity appears not to be strictly correlated with anti-carrier antibody production (compare stimulation factors for anti-sulf antibodies with anti-HGG hemagglutination titers in Table IV).

[3] The increase of relative affinities (*b*/*a*; see 25) in group *b* is similar to that in group *c* where helper activity is low.

TABLE IV

Variation of the Time of Priming with HGG

Rabbit group	HGG day	Stimulation factor for 7 S anti-sulf*				Reciprocal anti-HGG titer‡	
		a		b		Day 0	Day +4
A	—	5,2	4,5	5,9	5,9		
		4,3		4,8			
		4,1		4,9			
		5,4		8,2			
		2,2		3,2			
		7,9		11,6			
B	−28	7,5	6,6	14,7	12,5	2048	6144
		4,1		9,2		3072	8192
		6,5		14,3		2048	4096
		6,2		17,0		2048	4096
		9,1		18,0		2048	6144
		7,0		8,3		not done	
		6,6		10,0		not done	
C	−5	3,1	2,0	7,5	3,4	<1	96
		3,5		5,9		2	48
		1,7		1,9		<1	6
		1,2		3,1		<1	128
		1,9		2,2		<1	24
		1,7		2,9		<1	512
D	−2	2,0	1,9	2,9	2,4	<1	8
		2,5		2,9		<1	48
		2,0		2,7		<1	3
		1,6		2,1		<1	12
		1,5		1,9		2	16
		1,9		1,9		<1	6
E§	—	2,3	1,9	2,6	2,1	<1	6
		1,2		1,6		<1	8
		2,6		2,5		<1	12
		2,2		2,7		<1	6
		1,5		2,3		<1	6
		1,0		1,3		<1	4
		1,8		2,6		<1	4
F	—	1,6	1,5	1,6	1,8		
		2,0		2,3			
		1,6		1,9			
		1,6		1,6			
		1,3		2,2			
		1,3		2,2			
		1,2		1,7			
		1,3		1,6			
		1,4		1,4			
		1,4		1,5			

1°—1000 μg sulf-BSA in adjuvant on day −28 and 10 μg HGG in adjuvant on day −28 (group B), −5 (group C), or −2 (group D). In groups C and D, sulf-BSA and HGG were injected into the same leg. Groups A, E, and F did not receive HGG. 2°—1000 μg sulf-BSA (group A) or 1000 μg sulf-HGG (groups B–E) on day 0. Group F did not receive a secondary stimulus. Bleedings on day 0, +4, and +7.

* Determined by binding assay at hapten concentrations of 10^{-7} M(a) and 10^{-8} M(b). Stimulation factors for individual animals on day +7 and the geometric means are given.

‡ Determined by passive hemagglutination.

§ Five animals received Freund's adjuvant on day −10, two animals on dav −5

DISCUSSION

The Experimental System.—In previous papers (2, 4, 13), we have presented evidence demonstrating that the carrier effect is an expression of cooperation of distinct antigenic determinants of the antigen in the induction of the secondary response. This conclusion has also been reached by others (1, 3, 9). On the basis of the finding that cooperation could not be brought about by humoral antibody and the impressive body of evidence demonstrating cellular cooperation between thymus-derived and bone marrow-derived lymphocytes in the induction of the immune response (6–9, 20), cooperation of antigenic determinants was interpreted to reflect cellular cooperation. According to this hypothesis, the induction of antibodies requires the interaction of antigen bridged helper cells and antibody forming cell precursors. Strong evidence for the identity of helper cells and thymus-derived lymphocytes has recently been accumulated (5, 21), and we are going to interpret the data presented here on the basis of the hypothesis of cell–cell interaction taking place in the induction of the immune response. Our discussion would, however, not have to be changed essentially, should it turn out that cooperation of antigenic determinants is mediated by an unidentified class of locally secreted antibody. Antibody-mediated stimulation of the immune response has indeed been reported in a number of instances (22, 23), one of which (23) is strictly analogous to the systems of cooperating antigenic determinants discussed here. Our experimental results again argue against the idea that circulating anti-carrier antibody is responsible for cooperation of determinants in the sulf-carrier system, since definite cooperation was observed under conditions where no anti-carrier antibody was detectable in the serum at the time of secondary stimulation (Table IV and reference 18). These observations are in accord with Mitchison's finding (24) that the cooperative function of spleen cells sensitized to the carrier reaches a maximum earlier (8 days) after immunization than their capacity to produce secondary anti-carrier antibody (30 days).

The data presented in this article confirm and extend the results reported in a previous publication (4). An analysis of both 7S and 19S memory in the system is now possible and, as discussed below, provides further insight into the mechanism of cell-cell cooperation. Also, the introduction of a hapten-binding assay for anti-sulf antibodies made it possible to prove the reliability of the passive hemagglutination method and to obtain quantitative information about the amounts of 7S anti-sulf antibody present in the sera. It was found that secondary anti-sulf antisera bound up to 2×10^{-5} mmoles hapten/ml to their 7S fraction, corresponding to about 1.5 mg 7S antibody/ml (18). The inability of the 19S serum fractions to detectably bind the radioactive hapten is presumably due to the relatively low amounts of 19S antibodies produced.

Since the phenomenon of carrier specificity is the basis for the hypothesis that antigenic determinants cooperate in the induction of the immune response,

it is satisfying to see that in our system the dose of the hapten secondary-carrier complex can be varied by a factor of at least 10^3 without abolishing carrier specificity (Table I). Further experiments (25) have confirmed that carrier specificity can hardly be overcome in the sulf-carrier system. Thus, rats primed with 10 μg sulf-KLH could not be successfully boosted with up to 10 mg $sulf_{53}$-BSA, 1 mg $sulf_{204}$-HGG, or 2 mg $sulf_{36}$-OA, even when the time interval between priming and secondary injection was extended to 4–5 months. Similarly, rabbits primed with 100 μg sulf-KLH did not respond to either 1 mg $sulf_{204}$-HGG or 2 mg $sulf_{36}$-OA given 6 months after the primary injection. In all cases, vigorous secondary responses to the homologous hapten-carrier complex were observed. In other hapten-carrier systems, carrier specificity seems to be less pronounced (26–29). Particularly relevant here are Mitchison's studies of the adoptive secondary response to hapten-protein conjugates in mice, where dose-response relationships were carefully established and evidence was presented that carrier specificity could be overridden by high doses of antigen (1, 5, 26). Several mechanisms can be envisioned which would resolve these discrepancies: (*a*) certain haptenic determinants may cooperate with each other whereas others may not, depending on the spectrum of specificities present on the helper cells (4); (*b*) for some or all antigens, dose ranges may exist, where precursor cells are induced without the need of cell cooperation (this may depend on the affinity to the antigen of the receptor molecules on the precursor cells); (*c*) in the normal in vivo situation, additional mechanisms pronouncing carrier specificity may exist which do not operate in the adoptive secondary response. We will not go into these matters further at this point.

In the sulf-carrier system, as in other systems (30, 31), secondary responses occur in both the 7S and 19S antibody classes. The class distribution of secondary antibody depends on the dose of antigen used for priming and on the time interval between primary and secondary injection. Lowering the antigen dose (Table II) or shortening the time interval between priming and boosting (Fig. 2) favors the production of 19S antibodies. In the in vivo situation, the expression of 19S memory may well be inhibited by circulating 7S antibody (32, 33), and this may explain our finding that at high priming doses of sulf-BSA 19S memory was scarcely detectable (Table II).

Antibody Classes and Helper Activity.—The dependency of the class distribution of secondary anti-hapten antibody on the immunization schedule was the prerequisite for answering the two main questions which we are dealing with in this article. First, the data clearly show that hapten carrier cooperation is operating in the induction of both 19S and 7S antibodies (Fig. 2). In this respect, cooperation of determinants in the secondary response resembles thymus–marrow cooperation in the sheep red cell system (34). Second, the results presented in this paper demonstrate that the conditions of sensitizing

the animal to the hapten (not to the secondary carrier) decide whether 19S or 7S antibody is produced in the secondary response. This indicates that the class of secondary anti-hapten antibody is predetermined by hapten-primed memory cells and not by the carrier-specific helpers. In accordance with this interpretation, it can be shown (18) that in those of our experimental animals that are primed early with sulf-BSA and late with free HGG, the secondary response to sulf-HGG consists mainly of 7S anti-sulf and 19S anti-HGG antibodies and vice versa. The indication that in experimental situations where both 7S and 19S memory for the hapten is detectable in the animals there seems to be a preferential stimulation of 7S antibodies when helper activity is low (Table III) might be related to differences of affinity to the hapten of the cellular receptors on 7S and 19S memory cells. This problem can be studied further by comparing the affinity of 7S and 19S anti-sulf antibodies in the sera of our experimental animals. Apart from this slightly selective effect, the helper cells are responsible only for the extent of secondary stimulation. In this respect it is satisfying to see that in our system carrier specificity can be explained entirely by presensitization of the animal to carrier determinants (compare homologous and cooperative system in Fig. 2 and table IV).

These results together with the findings that (*a*) the allotype of the emerging antibodies is that of the hapten-primed cells and not that of the helpers (5), (*b*) the function of the carrier-specific helpers but not of the hapten-primed cell population can be suppressed with anti-θ antibody (21), and (*c*) in the sheep red cell system, the class of the emerging antibodies is determined by the bone-marrow derived and not the thymus-derived cells (34), argue strongly against any specific contribution of the helper cells to the specific product of the antibody forming cell precursors, as would be, for example, transfer of genetic material from one cell to the other. The function of the helper cells appears to be selective stimulation by antigen of memory cells which are predetermined for the specificity and the class of their antibody product. Immunological memory must thus be envisioned as being composed of two populations of antigen-specific cells.

SUMMARY

19S and 7S memory is analyzed in a system of cooperating antigenic determinants. Cooperation occurs in the induction of both 19S and 7S secondary antibodies, and for both responses carrier specificity can be entirely accounted for by presensitization of the animal to carrier determinants.

The class distribution of secondary anti-hapten antibody depends on the dose of the hapten primary-carrier conjugate used for priming, and on the time interval between priming with the hapten primary-carrier conjugate and secondary injection. The conditions of priming with the secondary carrier influence the extent of the secondary response but not the class distribution of

secondary antibody. The data confirm the cooperation hypothesis of antibody induction. Specifically, we interpret them to mean that in hapten-carrier cooperation, the hapten-specific memory cells are predetermined for the class of the emerging antibodies. Together with the hapten-specific memory cells, the carrier-specific helpers are responsible for the extent of the secondary response.

We are grateful to Mrs. N. Bikar for her able technical assistance.

BIBLIOGRAPHY

1. Mitchison, N. A. 1967. Antigen recognition responsible for the induction in vitro of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32:**431.
2. Rajewsky, K., and E. Rottländer. 1967. Tolerance specificity and the immune response to lactic dehydrogenase isoenzymes. *Cold Spring Harbor Symp. Quant. Biol.* **32:**547.
3. Mitchison, N. A. 1969. Cell populations involved in immune responses. *In* Immunological Tolerance. W. Braun and M. Landy, editors. Academic Press, Inc., New York. 149.
4. Rajewsky, K., V. Schirrmacher, S. Nase, and N. K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J. Exp. Med.* **126:**1131.
5. Mitchison, N. A., K. Rajewsky, and R. B. Taylor. 1970. Cooperation of antigenic determinants and of cells in the induction of antibodies. *In* Developmental aspects of antibody formation and structure. J. Sterzl, editor. Academic Press, Inc., New York.
6. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen reactive cells. *Transpl. Rev.* **1:**3.
7. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transpl. Rev.* **1:**43.
8. Claman, N. H., and A. E. Chaperon. 1969. Immunologic complementation between thymus and marrow cells—A model for the two cell theory of immunocompetence. *Transpl. Rev.* **1:**92.
9. Taylor, R. B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: Specific function of thymus cells. *Transpl. Rev.* **1:**114.
10. Schirrmacher, V. 1969. Die Rolle des Trägers bei der Induktion von Anti-Hapten-Antikörpern. Vortragsreferate der 1. Tagung der Gesellschaft für Immunologie. (Special issue of the European Journal of Immunology.) 7.
11. Tabachnick, M., and H. Sobotka. 1959. Azoproteins I. Spectrophotometric studies of amino acid^{azo} derivatives. *J. Biol. Chem.* **234:**1726.
12. Ingraham, J. S. 1952. Specific complement-dependent hemolysis of sheep erythrocytes by antiserum to azo hapten groups. *J. Infec. Dis.* **90:**268.
13. Rajewsky, K., E. Rottländer, G. Peltre, and B. Müller. 1967. The immune response to a hybrid protein molecule. Specificity of secondary stimulation and of tolerance induction. *J. Exp. Med.* **126:**581.
14. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I* BSA and antibody. *J. Infec. Dis.* **103:**239.

15. Onoue, K., Y. Yagi, and D. Pressman. 1966. Isolation of rabbit IgA antihapten antibody and demonstration of skin sentizing activity in homologous skin. *J. Exp. Med.* **123:**173.
16. Mäkelä, O., E. Kostiainen, T. Koponen, and E. Ruoslathi. 1967. The timing and quality of IgA, IgG and IgM responses in rabbits immunized with a hapten. *Nobel Symp.* **3:**505.
17. Onoue, K., N. Tanigaki, Y. Yagi, and D. Pressman. 1965. IgM and IgG anti-hapten-antibody: hemolytic, hemagglutination and precipitating activity. *Proc. Soc. Exp. Biol. Med.* **120:**340.
18. Schirrmacher, V. 1970. Analysis of hapten- and carrier-specific memory in a system of cooperating antigenic determinants. *In* Cell interactions in immune responses. O. Mäkelä, editor. Academic Press, Inc., New York. In press.
19. Tabachnick, M., and H. Sobotka. 1960. Azoproteins. II. A spectrophotometric study of the coupling of diazotized arsanilic acid with proteins. *J. Biol. Chem.* **235:**1051.
20. Shearer, G. M., and G. Cudkowicz. 1969. Distinct events in the immune response elicited by transferred marrow and thymus cells. I. Antigen requirements and proliferation of thymic antigen-reactive cells. *J. Exp. Med.* **130:**1243.
21. Raff, M. 1970. The role of thymus-derived lymphocytes in the secondary humoral immune response in mice. *Nature (London).* **224:**378.
22. Henry, C., and N. K. Jerne. 1968. Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J. Exp. Med.* **128:**133.
23. McBride, R. A., and L. W. Schierman. 1970. Hapten-carrier relationships of isoantigens. A model for immunological maturation based on the conversion of haptens to carriers by antibody. *J. Exp. Med.* **131:**377.
24. Mitchison, N. A. 1968. Recognition of antigen. *In* Differentiation and Immunology. K. B. Warren, editor. Vol. 7. Academic Press, Inc., New York. 29.
25. Schirrmacher, V. 1970. Untersuchungen zur Kooperationshypothese der Antikörperinduktion: Analyse der Trägerspezifität der sekundären Anti-Hapten-Antwort unter Verwendung von Sulfanilsäure gekuppelten Proteinen als Hapten-Träger Komplexen. Doctoral thesis. University of Cologne, Köln, Germany.
26. Brownstone, A., N. A. Mitchison, and R. Pitt-Rivers. 1966. Biological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitro-phenylacetic acid (NIP) and related compounds. *Immunology.* **10:**481.
27. Steiner, L. A., and H. N. Eisen. 1967. The relative affinity of antibodies synthesized in the secondary response. *J. Exp. Med.* **126:**1185.
28. Paul, W. E., G. W. Siskind, B. Benacerraf, and Z. Ovary. 1967. Secondary antibody responses in haptenic systems: cell population selection by antigen. *J. Immunol.* **99:**760.
29. Rittenberg, M. B., and D. H. Campbell. 1966. Heterologous carriers in the anamnestic antihapten response. *J. Exp. Med.* **127:**717.
30. Nossal, G. J. V., C. M. Austin, and G. L. Ada. 1965. Antigens in immunity VII. Analysis of immunological memory. *Immunology.* **9:**333.
31. Valentova, V., J. Cerny, and J. Ivanyi. 1967. Immunological memory of IgM

and IgG type antibodies. I. Requirements of antigen dose for induction and of time interval for development of memory. *Folia biol.* **13:**101.

32. Wigzell, H. 1966. The rise and fall of 19S immunological memory against sheep red cells in the mouse. *Ann. Med. Exp. Biol. Fenn.* **44:**209.
33. Kontiainen, S., and O. Mäkelä. 1968. Effect of the number of primed cells on the production of IgM and IgG antibodies in an adoptive secondary response. *Int. Arch. Allergy Infec. Dis.* **34:**417.
34. Cudkowicz, G., G. M. Shearer, and R. L. Priore. 1969. Cellular differentiation of the immune system of mice. V. Class differentiation in marrow precursors of plaque-forming cells. *J. Exp. Med.* **130:**481.