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THE REQUIREMENT OF MORE THAN ONE ANTIGENIC
DETERMINANT FOR IMMUNOGENICITY*

BY K. RAJEWSKY,† M.D., V. SCHIRRMACHER,§ S. NASE,
AND N. K. JERNE, M.D.

(From the Institut für Genetik der Universität Köln and the Paul Ehrlich
Institut, Frankfurt am Main, Germany)

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A hapten will exert a primary immune stimulus only if coupled to a carrier. A secondary stimulus with the hapten is most effective if the hapten is coupled to the same carrier as that used for priming. If the secondary carrier differs from the primary carrier, the animal will usually respond poorly or not at all. The secondary stimulus, therefore, requires recognition of both hapten and carrier. If the stimulation of an antigen-sensitive cell by a hapten involves the recognition of only one antigenic determinant, we must assume that this determinant is composed of the hapten and some of the adjacent carrier surface. Alternatively, we must admit that a cell can be stimulated to respond to a haptenic determinant only if an unrelated carrier determinant, distant from the haptenic site, is likewise recognized. The experimental results reported in the present paper point to the latter alternative. A brief account of some of our findings has been reported (1).

Materials and Methods

Antigens.—Bovine serum albumin¹ (Behringwerke, Marburg/Lahn, Germany) and human gamma globulin (Serva, Heidelberg, Germany) were used as carrier proteins. Sulfanilic acid was coupled to the proteins by diazotization (2). Coupling of DNP was achieved by reaction of the protein carriers with 2,4-dinitrofluorobenzene (Serva) (3). The number of sulf groups bound per protein molecule was calculated from the results of elementary analysis. The number of DNP groups bound per protein molecule was determined spectrophotometrically (4). The coupling reactions were stopped by filtration of the reaction mixtures through columns of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with 0.067 M phosphate buffer at pH 7.2. The hapten-carrier complexes used were composed as follows: sulf₁₉-BSA, DNP₂₄-BSA, sulf₂₃-HGG, and DNP₁-HGG. Sulf₂₃-

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† Present address: Institut für Genetik der Universität Köln, 5 Köln-Lindenthal, W. Germany.

§ In partial fulfillment of doctoral thesis requirements, University of Cologne.

¹ Abbreviations used: BSA, bovine serum albumin; HGG, human gamma globulin; DNP, dinitrophenyl group; sulf, sulfanyl group.

HGG and DNP₁-HGG could not be distinguished from native HGG by an antiserum to HGG in double diffusion. This suggests that the determinants of native HGG were preserved in the sulf-carrier complex. Giant keyhole hemocyanin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Determination of Protein.—Protein was determined by the biuret method as described previously (5). This method is also applicable to sulf-proteins, if the proteins are not too heavily coupled and corrections are made for the absorbance at 550 $m\mu$ of the haptenic group in 0.02 M NaOH. For DNP-proteins, no correction is necessary. Heavily coupled sulf-proteins were determined by their dry weight.

Passive Hemagglutination.—Hapten-specific passive hemagglutination was carried out as described by Ingraham (6) and by Bullock and Kantor (3). The specificity of the reactions was verified. Antisera to DNP-proteins did not react with sulf-coupled sheep red cells nor vice versa. The hemagglutination reactions could be inhibited specifically by DNP-lysine (Mann Research Labs, New York) or by sulfanyl-chloroacetyl-tyrosine.² All assays were made in duplicate. The microtiter equipment used was obtained from Cooke Engineering Co., Alexandria, Va.

The other immunochemical techniques used have been described (7).

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

Immunization.—For primary immunization, 0.75 ml phosphate-buffered saline of pH 7.2 containing 1 mg of each antigen was mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The mixture was injected in equal amounts into each of the four footpads of the right hind leg of a rabbit. Whenever hapten-carrier complexes and uncoupled carrier protein were to be injected simultaneously, mixtures of each with Freund's adjuvant were prepared separately and either cautiously mixed immediately before injection, or injected separately, either into different footpads of the same hind leg, or into the footpads of different hind legs (see Results). Secondary injections were always given intravenously. For these injections, 1 mg of each antigen was dissolved in phosphate-buffered saline of pH 7.2 and injected intravenously in a total volume of 1 ml. The interval between primary and secondary injections was 4 and 6 wk in the two sets of experiments which have been brought together in Table I A. Serum assays at weekly intervals showed that the anti-hapten antibody titers resulting from the primary stimulus had reached a plateau at the time of secondary stimulus. Secondary titers reached their maximum 7 days after secondary injection. Anti-sulf antibodies were detected almost exclusively in the 7S region of sucrose gradients, when sera were analyzed at the time of secondary stimulus or at the maximum of the secondary response. Though all rabbits received DNP-carrier complexes together with sulf-carrier complexes, the present paper is concerned only with the response to the sulf hapten. The responses to the DNP hapten are not reported, because the majority of the rabbits did not produce a secondary response to DNP even when primary and secondary carriers were the same.

RESULTS

Presentation of the Data.—The experimental design is given in Table I A. Table I B contains the anti-sulfanilic acid hemagglutination titers of the rabbit sera 1 day before (T_1) and 7 days after the secondary stimulus (T_2). The graphical evaluation presented in Fig. 1 shows secondary vs. primary anti-sulfanilic acid titers of individual rabbits listed in Table I B.

² Inhibition was detectable at inhibitor concentrations of 10^{-6} M. Method of synthesizing sulfanyl-chloroacetyl-tyrosine will be described in a subsequent publication (V. Schirmacher. Doctoral thesis. University of Cologne).

The Use of Different Carriers for Primary and Secondary Stimulation.—In accord with previous work on carrier specificity, clear-cut hapten-specific secondary responses are obtained only if primary and secondary carriers are identical (groups A, B, C, and D in Table I; see also Fig. 1). If the secondary

TABLE I A
Experimental Design

Column in Table I B	Rabbit group	No. of rabbits	Primary injection	Supplementary injection	Secondary injection	Interval*
I	A	6	Sulf-HGG	None	Sulf-HGG	4
	B	5	Sulf-BSA	"	Sulf-BSA	6
	C	8	"	HGG‡	"	4
	D	5	"	"‡	"	6
II	E	13	"	None	Sulf-HGG	6
	F	7	"	"	"	4
	G	4	"	Hemocyanin‡	"	6
III	H	8	"	HGG§	"	4
	K	5	"	"§	"	6
	L	5	"	"	"	6
	M	5	"	"¶	"	6
IV	N	3	"	anti-HGG**	"	6
	P	4	"	"**	"	6
	R	3	"	"**	"	6
V	S	8	HGG	None	"	4

15 groups of rabbits received two injections of carrier-coupled sulfanilic acid, with an interval of 4–6 wk. The carrier was either BSA or HGG. Some groups received a supplementary injection as shown. Anti-sulfanilic acid hemagglutination titers measured 1 day before and 7 days after the secondary stimulus are given in Table I B.

* Interval between primary and secondary injection.

‡ Given twice, simultaneously with primary and secondary injection.

§ Given simultaneously with primary injection.

|| Given two wk after primary injection, in other foot.

¶ Given simultaneously with primary injection, but in other foot.

** Nature of antibody and injection schedule described in text.

carrier differs from the primary carrier, there is no clear increase of anti-hapten antibody after the secondary injection, i.e. the observed increase in titer is never more than two-fold (groups E, F, and G in Table I; see also Fig. 1).

Pretreatment with Secondary Carrier.—The experimental groups H, K, L, and M (Table I) again consist of rabbits which received the hapten coupled

to a different carrier for primary and secondary injection. In addition, however, these animals were pretreated with uncoupled secondary carrier. This pretreatment consisted of injection of free secondary carrier in complete

TABLE I B
*Anti-Sulfarilic Acid Hemagglutination Titers Resulting
From the Experiments Outlined in Table I A*

I. Same primary and secondary carrier			II. Primary carrier BSA; secondary carrier HGG			III. As II, but supplementary injection of free HGG			IV. As II, but supplementary injection of anti-HGG				
Rabbit group	T ₁	T ₂	Rabbit group	T ₁	T ₂	Rabbit group	T ₁	T ₂	Rabbit group	T ₁	T ₂		
A	16	128	E	20	20	H	10	128	N	5	4		
	40	320		20	16		1	16		20	20		
	40	80		1	2		16	32		64	40		
	20	80		20	20		2	40	P	32	32		
	32	80		40	80		2	10		16	8		
	32	128		40	64		2	32		20	16		
B	32	128	20	20	5	80	20	20					
	36	128	4	5	K	2	5	R	2.5	2.5			
	36	128	16	16		160	160		5	5			
	25	144	40	64		5	32		5	4			
	10	36	9	10		20	80	V: Primary stimulus free HGG. Secondary stimulus sulf-HGG	Rabbit group	T ₁	T ₂		
	C	4	20	F		2	6					L	10
4		20	8			8	18						128
2.5		64	20		32	16	144						
8	1024	40	22	16	288	S	<1	<1					
1	8	32	32	16	64		<1	<1					
16	80	20	20	2.5	16		<1	<1					
2.5	16	4	8	M	10	128	<1	<1					
D	1.6	16	G		4	9	16	160	<1	<1			
	8	18			20	32	2	20	<1	<1			
	4	64			56	64	5	40	<1	<1			
	16	64			20	16	16	40	<1	<1			
	5	80							<1	<1			
						1	1						

Titers of individual sera of the rabbits in the 15 groups described in Table I A. T₁: Serum titer 1 day before secondary stimulus. T₂: Serum titer 7 days after secondary stimulus.

Freund's adjuvant, either simultaneously or 2 wk after priming with the hapten-coupled primary carrier. If the action of the carrier in the secondary stimulus depends on a priming experience with both carrier and hapten, but not necessarily combined in one molecule or given at the same time, then these

pretreated animals might be expected to show a good secondary response. Inspection of the data in Table I and Fig. 1 shows that this is indeed the case. This result cannot be interpreted by invoking *in vivo* transfer of the haptenic group from BSA to HGG, since pretreatment with the secondary carrier in the other hind leg (group M) or 2 weeks after priming with the hapten coupled to the primary carrier (group L) was equally effective. The effect of pretreat-

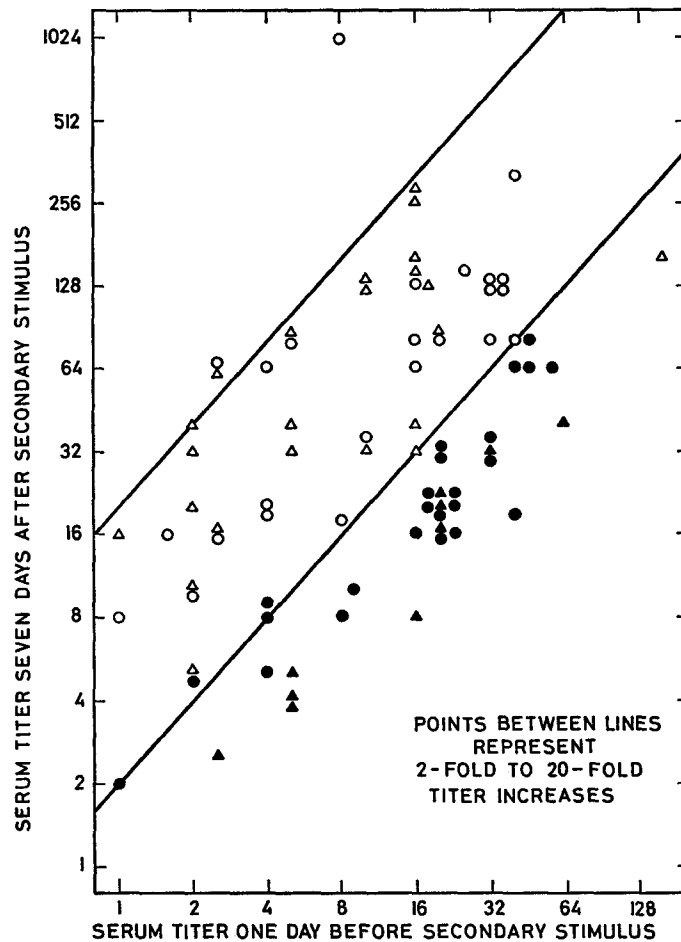


FIG. 1. Anti-sulfanilic acid hemagglutination titers from Table I B. ○, Hapten coupled to same carrier for primary and secondary stimulus (Table I B, column I). All other points: primary stimulus with hapten coupled to BSA followed by secondary stimulus with hapten coupled to HGG, as follows: △, supplementary injection of free HGG (Table I B, column III) ▲, supplementary injection of anti-HGG (Table I B, column IV) ●, no supplementary injection (Table I B, column II).

ment with free secondary carrier is seen only in animals that have also undergone a primary experience with sulf-BSA. Group S shows that the omission of this primary haptenic stimulus renders pretreatment with HGG ineffective.

The carrier dependency of the effect reported in this section is confirmed by the finding that, upon secondary injection, the secondary carrier plays its role only if chemically coupled to the hapten. Animals primed with sulf-BSA + hemocyanin and boosted with sulf-HGG + hemocyanin did not give a secondary response to the hapten (group G), though the response of the animals to hemocyanin was at least equivalent to the response to HGG as shown by precipitin tests.³

It may be noted from the data contained in Table I that anti-sulf antibody titers appear to be slightly lower in rabbits primed with sulf-BSA and HGG than in rabbits injected with the hapten-carrier conjugate alone. This reminds us of early work of Michaelis (8) and Doerr and Berger (9) who already have reported antigenic competition between serum albumin and gamma globulin.

Pretreatment with Anti-Carrier Antibody.—Rabbits primed with BSA-hapten complexes and pretreated with free HGG, will have antibodies to HGG in their serum at the time of secondary stimulation. Precipitin tests showed that the concentration of these antibodies ranged between 500 and 5000 $\mu\text{g/ml}$ serum. Are antibody molecules resulting from the pretreatment with HGG responsible for our finding that the animals respond to a secondary injection of the hapten coupled to HGG? If so, the effective antibodies could be either those that freely circulate at the time of the secondary stimulus, or they might be antibodies of a type that are adsorbed to certain cell surfaces so as to be able to act as cell-bound receptors for the secondary carrier-hapten complex. To approach these questions experimentally, the rabbits of groups N, P, and R were primed with hapten coupled to BSA, and 3–4 wk later, were given a large amount of antiserum to HGG intravenously. “Early” and “late” antiserum to HGG was used. Early antiserum was taken from rabbits 10–11 days after primary immunization with 1 mg HGG in complete Freund’s adjuvant into the footpads of a hind leg. 1 ml of the pool of early antisera precipitated 200 μg HGG. Late antiserum was obtained from the same animals, bled again 28 days after the primary injection of HGG. 1 ml of the pool of late antisera precipitated 800 μg HGG. The rabbits of group N received twice 20 ml of early antiserum on days 21 and 22 after priming; the group P rabbits were given twice 20 ml of late antiserum on days 28 and 29 after priming. The rabbits of group R were given both these pretreatments. On day 30 after priming, all animals received a secondary

³ Note added in proof. The rabbits of groups G, D, and M were injected with a sulf-hemocyanin conjugate 8 wk after completion of the experiment given in Table I. The group G animals showed a vigorous secondary response to the hapten, whereas none of the animals of groups D and M produced a booster response.

injection of hapten, coupled to HGG. It is evident from the data shown in Table I and Fig. 1 that none of the rabbits produced a detectable secondary response to the hapten in these situations.

DISCUSSION

An immunogenic molecule presents more than one antigenic determinant to the immune system. The use of an immunogenic molecule consisting of haptenic groups coupled to a protein permits us to discriminate experimentally between contributions made to the immune stimulus by haptenic determinants and by antigenic determinants that belong to the carrier surface. Free carrier molecules possess only the latter, and these, as shown by the experiments reported in this paper, are of decisive importance for the stimulus that leads to a secondary response to the haptenic determinants. Mitchison (10), in his studies of the adoptive immune response by primed cells to a secondary stimulus *in vitro*, has reached the same conclusions.

Other workers, using hapten-carrier complexes in which both hapten and carrier are of macromolecular size, have presented further evidence for cooperation between physically separate determinants in the immune stimulus. Thus, using DNP-polylysine (DNP-PLL), which is not immunogenic in certain strains of guinea pigs, Benacerraf and his co-workers (11) have shown that such nonresponder guinea pigs do respond to immunization with DNP-PLL complexed with BSA with the formation of large amounts of antibodies to the DNP determinants as well as to the albumin. These guinea pigs do not respond to DNP-PLL complexed with guinea pig serum albumin. Similar findings have resulted from studies of the immunogenicity of lactic dehydrogenase (LDH) isozymes. These are tetrameric molecules in which each of the four subunits can be either type A or type B. It was found (7, 12) that certain rabbits which produce a normal immune response to the A_4 enzyme are unable to respond to the B_4 enzyme. The type B subunits can be regarded as haptens since nonresponder rabbits do respond to the A_2B_2 hybrid enzyme with the production of similar amounts of both anti-A and anti-B antibodies. Anti-B antibodies do not react with type A subunits. The latter thus play the role of carrier in this system. Rabbits primed with a porcine A_2B_2 hybrid enzyme do not show a secondary anti-B response to A_2B_2 enzyme, consisting of porcine type B subunits hybridized with rabbit type A subunits.

Further studies of the LDH system have confirmed the finding that both hapten and carrier determinants are required for secondary stimulation. Rabbits primed with "carrier" type A subunits alone and boosted with the "carrier-hapten" hybrid produced antibodies directed to the carrier as well as to the haptenic subunits. Though approximately 20 times less anti-hapten than anti-carrier antibody appeared in the serum, both responses showed typical secondary response kinetics (12). Some workers (13-15) have reported similar

results using certain conventional hapten-carrier systems, whereas others have been unable to detect the formation of antihapten antibody in analogous experimental situations (16-18). In the experiments reported in the present paper, anti-sulf antibodies were not found in rabbits primed with HGG and boosted with sulf-HGG. We suggest that, in general, an animal pretreated with free carrier and receiving a secondary injection of this carrier complexed with a hapten will be found to produce a better antihapten response than without the pretreatment, if the experimental design is aimed at detecting this effect.

In both the LDH system and the DNP-PLL-BSA system it could be shown that the induction of tolerance to the carrier led to a reduced response to a subsequent injection of carrier-hapten complex (7, 12, 19). In this situation the reduction of the response was quantitatively similar for both antihapten and anti-carrier antibodies (7, 12). In these systems it thus appears unlikely that the role of the carrier in the haptenic stimulus is restricted to a contribution to the configuration of the complete haptenic determinant. Instead, haptenic determinants and separate antigenic determinants of the carrier appear to cooperate in some way. Mitchison, who has clearly stated this idea of cooperating antigenic determinants, came to the same conclusion in his conventional hapten-carrier system (20). He showed that, for the induction of the secondary response, the hapten could be separated from the carrier by a spacer group without abolishing the carrier effect. Schierman and McBride, in their system of chicken isoantigens, have also clearly demonstrated cooperation of antigenic determinants (21).

How can we picture the cooperation of two unrelated antigenic determinants in the stimulation of a cell? For the recognition of an antigenic determinant by the immune system, we must postulate the existence of specific receptor molecules. Some of these are free, since circulating antibody molecules obviously possess the property of antigen recognition. Others are bound to or incorporated into the surface of cells, and we should like to think that antigen-sensitive cells precommitted to the synthesis of antibody molecules of one specificity carry samples of such molecules at their surface. Experimental evidence is available (22-25) for the existence of immunoglobulin-like receptors on a majority of the lymphocytes. It is unlikely that a cell would synthesize receptor molecules of two different specificities recognizing two unrelated antigenic determinants that we have brought together in a hapten-carrier complex. If an antigenic stimulus requires the recognition of two antigenic determinants, it is therefore reasonable tentatively to assume that the two recognizing receptor molecules occur on two different cells. We may then envisage that an antigenic stimulus involves two cells each of which recognizes a different portion of the immunogenic molecule or particle. A similar two-cell pattern might have to be invoked for other immunological phenomena such as the blast-transformation of small lymphocytes, the skin reaction in delayed sensitivity, and the inhibition of

macrophage migration, for all of which the requirement of carrier specificity has been reported (26).

Assuming for a moment that the bridging of two cells by an immunogenic molecule or particle is a prerequisite for inducing antibody formation, we can ask whether this two-cell pattern is symmetrical or asymmetrical, i.e. whether each of the two cells linked by the immunogenic molecule proceeds to produce antibody. A considerable body of evidence has accumulated for the cooperation of two different cell types in the immune stimulus (27-37).⁴ Some of this evidence suggests cooperation between a macrophage and a lymphocyte, whereas later evidence suggests cooperation between two different types of lymphocytes, both originating in the bone marrow, but one type modified by having undergone several cell divisions in the thymus ("thymus-derived cells"), and the other type not thus modified ("bone marrow-derived cells"). We therefore imagine an asymmetric pattern in which the linkage of two cells by an immunogenic molecule results in the production of antibody by only one of them. Asymmetry may also pertain to the origin of the cell-bound receptor molecules. One cell may itself have produced its receptors, expressing its synthetic potentiality, whereas the other may have collected freely circulating receptor molecules at its surface. As there is some evidence that thymus-derived cells control tolerance, whereas bone marrow-derived cells become the ancestors of antibody producing cell clones, it is possible to speculate that a thymus-derived cell might transfer its synthetic potentiality perhaps by transferring an episome containing structural information (38) to any bone marrow-derived cell recipient. The receptors on the surface of the bone marrow-derived cells might then be a random collection in the sense that these receptors would not represent the inherent synthetic potentiality of the cells. Present evidence does not permit a distinction between this possibility and the more simple notion that transfer of information does not occur, because each of the two cells involved is already genetically committed to the single specificity expressed by the receptor molecules.

Our experiments have established that animals primed with a hapten-carrier complex will produce a good secondary response to the hapten, even when the hapten is coupled to a different secondary carrier, if free secondary carrier is given to the animal some weeks earlier. The most general statement we can make to interpret this finding is that the pretreatment with free secondary carrier must have resulted in the production of receptors that can recognize this carrier, and that the more abundant presence of these receptors facilitates the stimulation of antihapten antibody formation. These crucial receptors could be freely circulating or be cell-bound. The obvious candidates for freely

⁴ Mishell, R. I., R. W. Dutton, K. Hartmann, and D. J. Raidt. Cell components in the immune response. Manuscript in preparation.

circulating receptors are the antibody molecules induced by the pretreatment. Our experimental results after passive preinjection of anti-carrier antibody and similar results obtained by Mitchison (10) suggest, however, that the crucial receptors are not available in the serum. This appeared also to be true in the LDH system (12). We conclude that the effective receptors are cell-bound. It is also clear that the pretreatment with free secondary carrier can hardly have increased the number of cells committed to antihapten synthesis, nor the number of antihapten receptors. The effective cell-bound receptors for recognition of the secondary carrier could therefore be receptors produced by and situated on multiplying thymus-derived cells. The greater abundance of these cells would facilitate bridge formation by the carrier-hapten particles between such cells and the bone marrow-derived cells committed to antihapten synthesis. Alternatively, effective receptors could be produced by one type of cells and become absorbed on bone marrow-derived cells. The greater abundance of these receptors bound passively to bone marrow-derived cells would then facilitate bridge formation between such cells and the thymus derived cells that possess and can transfer the structural information for antihapten synthesis. The interpretation offered here is admittedly speculative but follows almost inescapably from the basic assumption that the crucial cells of the immune system possess a single precommitment for the synthesis of antibody molecules of one specificity. There is nothing in this picture which places carrier determinants in a different class from haptenic determinants. We would expect that the hapten-carrier molecule could bridge two cells by the attachment of a carrier determinant to one type of cell and a hapten determinant to the other, or vice versa, or by the attachment of a hapten determinant to each of the two cells, as there are about 20 hapten determinants per hapten-carrier molecule used in our studies. Admitting the latter possibility, it is puzzling why this would not permit the secondary stimulus to be carrier independent, since an abundance of hapten-recognizing cells of both types would presumably have resulted from the primary stimulus. This could either be explained by quantitative considerations, i.e. by assuming that the carrier-recognizing cells dominate in number, or we might resort to the suggestion that the determinants recognized by thymus derived cells belong to a special class, corresponding to a genetically restricted range of cell receptors. Certain determinants, such as DNP, might belong to both classes and be recognized by thymus-derived as well as bone marrow-derived cells, as suggested by the experimental results obtained by Steiner and Eisen (39) and by Paul et al. (40). This notion would permit a genetic control (11, 41, 42) of the antigenic stimulus in spite of an uncontrolled availability of potentially competent bone marrow-derived cells.

SUMMARY

Rabbits primarily stimulated with a BSA (bovine serum albumin)-sulfanilic acid complex will produce a good secondary response to the sulfanilic acid hap-

ten if the carrier used in the secondary stimulus is again BSA, and not if the secondary carrier is HGG (human gamma globulin). In the latter situation, a good secondary response is obtained, however, if the rabbits are pretreated a few weeks earlier with free HGG. We conclude that the immune stimulus involves the recognition of carrier determinants unrelated to the hapten.

As the receptors for recognition of unrelated determinants are probably situated on different cells, we suggest that the immune stimulus leading to antibody formation requires the interaction of two antigen-bridged cells.

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