ANTIGENIC PROMOTION

Increase in Hapten-Specific Plaque-Forming Cells after Preinjection with Structurally Unrelated Macromolecules*

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The specificity of antibody and its adaptation to a determinant (1) is dependent on and reflected by the specificity of cell receptors (2–5) on bone marrow-derived cells (6). Antibody synthesis is triggered by the interaction between cell receptor and antigenic determinant. This type of interaction is not the only requirement for the induction of antibody synthesis; the involvement of other determinants (i.e. those of the carrier) is as crucial as is that of the first mentioned determinant (7–11). However, the role of these carrier determinants is not reflected by the specificity of the antibody itself. Thus the immune response appears not only to be regulated by the specificity of the determinant against which antibody is made, but also by the specificity of other determinants. In addition, the antibody response may be regulated by other processes which are unrelated to the specificity of the ultimately synthesized antibody. The latter components of regulation, though dependent on the initial interaction with a particular determinant, may have a generalized effect on responsiveness and may affect the extent of the response to many structurally unrelated antigens. Processes of this type may be implicated in such phenomena as the synthesis of nonspecific immunoglobulins after antigenic stimulation (12–15), and also in some of the phenomena which have been classified as "adjuvanticity" (16). We shall refer to processes of regulation, which may depend on an initial event of recognition but may affect the response to structurally unrelated antigens, as nonspecific regulatory processes. We shall report, here, a phenomenon which may be mediated by such a nonspecific regulatory process. This phenomenon, antigenic promotion, occurs between antigens which have no known common or cross-reacting determinants. This effect is observed in the hapten antibody response to such antigens as para-azobenzene

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arsonic acid derivatives of human albumin and of dissociated keyhole limpet hemocyanin and is detected by plaque formation with diazotized red cells as targets (17–19).

Materials and Methods

Para-Arsanilic Acid.—*p*-Arsanilic acid was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Aluminum Potassium Sulfate.—Aluminium potassium sulfate, AnalaR, was obtained from the British Drug Houses, Ltd., Poole, Dorset, England.

Agarose.—Agarose was obtained from L'Industrie Biologique Francaise S.A., Gennevilliers, France.

CMRL 1066 Medium.—CMRL 1066 medium, prepared by the method of Parker (20), was obtained from the Ontario Cancer Institute, Toronto, Canada.

Horse and Sheep Erythrocytes.—Horse erythrocytes (HRBC)[1] and sheep erythrocytes were prepared from blood obtained from Woodlyn Laboratories Ltd., Guelph, Ontario, Canada.

Human Serum Albumin.—Human serum albumin "reinst" (HA) was obtained from Behringwerke A.G., Marburg-Lahn, West Germany.

Rabbit Serum Albumin.—Rabbit serum albumin (RA) was obtained from Pentex Biochemicals, Kankakee, Ill.

Human Gamma Globulin.—Human gamma globulin (HGG) was purified by passing human immune serum globulin, Cohn fraction II, (Connaught Medical Research Laboratories, Toronto, Lot No. 36529) through a diethylaminoethyl (DEAE)-Sephadex A50 column equilibrated with 0.005 M phosphate buffer, pH 8.0. The first peak was collected and concentrated by pressure dialysis through a Diaflo membrane (UM-10) (Amicon Corporation, Lexington, Mass.). It gave a single line on immunoelectrophoresis with goat anti-human serum and goat anti-human gamma globulin.

Monomeric Human Gamma Globulin for Tolerance Induction.—HGG was centrifuged for 3 hr at 20°C and at 105,000 *g*. The top third of the supernatant was separated and, immediately thereafter, samples were injected into newborn rabbits.

Iodinated HGG.—HGG was iodinated with ^{125}I by a modification of the method of Onoue et al. (21). The concentration of HGG employed was 10 mg/1.15 ml so as to obtain a lower specific activity.

Horse Spleen Ferritin.—Horse spleen ferritin (HSF), Lot No. 900534, six times crystallized, cadmium-free, was obtained from Calbiochem, Los Angeles, Calif.

Lysozyme.—Lysozyme (Lys) of *Micrococcus lysodeikticus*, Lot No. 6904P, was obtained from Schwarz BioResearch, Orangeburg, N.Y.

Tobacco Mosaic Virus.—Tobacco mosaic virus (TMV) was kindly given by Dr. A. Kleckowski, Rothamstead Experimental Station, Harpenden, U.K.

Keyhold Limpet Hemocyanin.—Some lots of keyhole limpet hemocyanin (KLH) were the gift of Dr. W. O. Weigle; other lots were prepared by us from giant keyhole limpets obtained

[1] *Abbreviations used in this paper:* As, *p*-azobenzene arsonic acid; B cell, bone marrow-derived cell; HA, human serum albumin; HA-As, As-conjugated HA; HGG, human gamma globulin; HGG-As, As-conjugated HGG; HRBC, horse erythrocytes; HSF, horse spleen ferritin; KLH, keyhole limpet hemocyanin; KLH-As, As-conjugated KLH; Lys, lysozyme of *Micrococcus lysodeikticus*; PFC-As, plaque-forming cells detected with SRBC-As (after correction for background); RA, rabbit serum albumin; RA-As, As-conjugated RA; RBC, red blood cells; SRBC, sheep erythrocytes; SRBC-As, As-conjugated SRBC; T cell, thymus-derived cell; TMV, tobacco mosaic virus.

from Pacific Biomarine Supply Co., Venice, Calif. The crude material was purified by the method of Campbell et al. (22).

Dissociated Keyhole Limpet Hemocyanin.—Dissociated keyhole limpet hemocyanin was prepared from KLH, as described by Weigle (23).

p-Azobenzene Arsonic Acid Proteins.—*p*-Azobenzene arsonic acid (As) proteins were prepared by the method of Cinader and Dubert (24). Para-azobenzene arsonic acid human serum albumin (HA-As), *p*-azobenzene arsonic acid human gamma globulin (HGG-As), and *p*-azobenzene arsonic acid keyhole limpet hemocyanin (KLH-As) contained, respectively, 7.5, 29, and 720 atoms of arsenic per molecule of protein.

Dissociated KLH-As.—Dissociated KLH-As was prepared from associated KLH-As by the method of Weigle (23).

Alum Precipitation of Proteins and Azo Proteins.—Protein or azo protein, 10 mg/ml in 0.15 M phosphate-buffered saline (pH 7.2), was added to an equal volume of 10% w/v aluminium potassium sulfate; 0.1 N NaOH was added dropwise while the solution was stirred. Addition was completed when the pH stabilized at 7.0. The precipitates were washed twice and resuspended in 0.15 M phosphate-buffered saline.

Immunization.—New Zealand white rabbits (Rieman Fur Ranches Ltd., St. Agatha, Ontario, Canada; 2–3 kg in body weight unless otherwise specified) were used. Antigens were dissolved or suspended at a concentration such that the injected volume was always 0.5 ml. Injections were given subcutaneously into one or both hind footpads.

Induction of Immunological Tolerance to HGG.—Newborn rabbits were injected intraperitoneally with 5 mg of monomeric HGG (in 1 ml of 0.15 M NaCl) within 12 hr of birth; two similar injections were given 24 and 48 hr later.

Test for Tolerance to HGG.—On the 31st day of life, 2.5 mg of alum-precipitated HGG was injected into the right hind footpad of neonatally injected rabbits. 4 days later, ^{125}I-labeled HGG was injected intravenously (1 mg/kg body weight). Thereafter, rabbits were bled regularly; radioactivity in the serum was counted in an automatic gamma scintillation counter (Model 42245, Nuclear-Chicago Corp., Des Plaines, Ill.). Rabbits immune to HGG showed immune phase of elimination without distinguishable metabolic phase. Rabbits tolerant to HGG showed no immune phase but a prolonged metabolic phase of elimination.

Hapten-Specific Hemolytic Plaque Assay (PFC-As Assay; references 17 and 18).—Popliteal lymph nodes, draining the injection sites, were dissected out. Single cell suspensions were prepared by cutting the nodes with a pair of scissors into small pieces and squeezing the fragments through fine stainless steel gauze (100 × 100 mesh). Cells were washed once in chilled CMRL 1066 medium and were resuspended in the medium at a concentration of 3×10^7 nucleated cells/ml. Fractions of the lymph node cells were used. Each assay mixture contained 3×10^6 lymph node cells, 2×10^8 *p*-azobenzene arsonic acid-conjugated sheep erythrocytes (SRBC-As) (19) and 2.0 ml of 0.6% agarose in CMRL 1066 medium. The mixture was incubated at 37°C for 2 hr. Thereafter, guinea pig serum, as a complement source, was layered above the agarose and incubation was continued for 45 min. All assays were carried out in triplicate. Background plaques were obtained by using SRBC-As as target cells in the presence of 5.0 μmoles of *p*-arsanilic acid/incubation mixture (19). In some cases, normal sheep erythrocytes were used as target cells so as to determine the number of background plaques. There was no difference in the number of background plaques obtained by these two methods.

Enumeration of PFC-As and Nonparametric Statistical Tests.—The number of PFC-As per incubation mixture (containing 3×10^6 nucleated cells as described in the preceding section) was obtained by subtraction of the background plaques per plate from the total. The number of PFC-As per node was obtained by multiplying the number of PFC-As/3×10^6 cells with an appropriate factor based on the total number of nucleated cells in the node. When the total number of PFC per mixture did not exceed the background number by more than 2, it was

considered that there were minimally 0 PFC-As and maximally 2 PFC-As/3 $\times$ 10^6 cells. To test for the difference in response between two groups, the Wilcoxon rank sum test (25) was employed. The numbers of PFC-As per node of individual rabbits of one group were compared with that of the second group. For those rabbits whose lymph node cells gave not more than 2 plaques/3 $\times$ 10^6 cells above the background, either the minimal values or the maximal values of PFC-As per node were assigned to them for the test. It was found that a test using the maximal values always gave a lower value of P than did the corresponding test using the minimal values. The lower P values are attributable to larger cell contents of preinjected lymph nodes and hence to larger values for the plaque number of preinjected nonresponders. The results of the statistical tests, presented in the tables, are based on minimal values.

TABLE I

Hapten-Specific Plaque Response to Different p-Azobenzene Arsonic Acid Proteins

Group	Antigens injected	No. of rabbits	$\log_{10}$ (PFC-As/node)*	
			Mean	SD
a	HA-As	8	0.3	0.9
b	Dissociated KLH-As	4	0.7	1.3
c	Associated KLH-As	4	3.2	0.2
d	HGG-As (in 0.15 M NaCl)	4	1.8	2.1
e	HGG-As (alum-precipitated)	4	4.6	0.7

One of five azo proteins (0.5 mg) was injected into the right hind footpad of the rabbit. The azo proteins were HA-As (in 0.15 M NaCl), dissociated KLH-As (in 0.2 M borate buffer, pH 8.8), associated KLH-As (in 0.15 M NaCl), HGG-As (in 0.15 M NaCl), or alum-precipitated HGG-As (in 0.15 M phosphate-buffered saline, pH 7.2). 8 days later, PFC-As assays were carried out with cells of right popliteal lymph nodes of these animals.

* Comparison of individual values between groups by the Wilcoxon rank sum test:

a vs. b, $P > 0.05$; a vs. c, $P < 0.01$
a vs. d, $P > 0.05$; a vs. e, $P < 0.01$
b vs. c, $P < 0.05$; d vs. e, $P = 0.05$

To avoid presentation of the individual values, on which our statistical comparisons are based, we have summarized our data as the mean, standard deviation, and range of the $\log_{10}$ (PFC-As/node). For this purpose only, nonresponders were arbitrarily taken to have one PFC-As/node.

Passive Hemagglutination Test.—Agglutination titers with tanned red cells sensitized with HA, HGG, HGG-As, or *p*-azobenzene arsonic acid rabbit serum albumin (RA-As) were determined as described by Boyden (26) and as modified by Cinader and Pearce (27). Agglutination titers with tanned red cells sensitized with KLH or KLH-As, either in associated form or in dissociated form, were determined similarly, except that for sensitization of tanned erythrocytes one-tenth of the original concentration of protein was used.

RESULTS

Increase in the Number of Hapten-Specific Plaque-Forming Cells after Preinjection with Unrelated Macromolecules.—The magnitude of the antibody response to the hapten, *p*-azobenzene arsonic acid, depends on the identity and on

the state of aggregation of the carrier (Table I). Human albumin was the weakest of the carriers we have tested. None of eight rabbits that had received an injection with 0.5 mg of HA-As into the footpad and whose lymph nodes had been

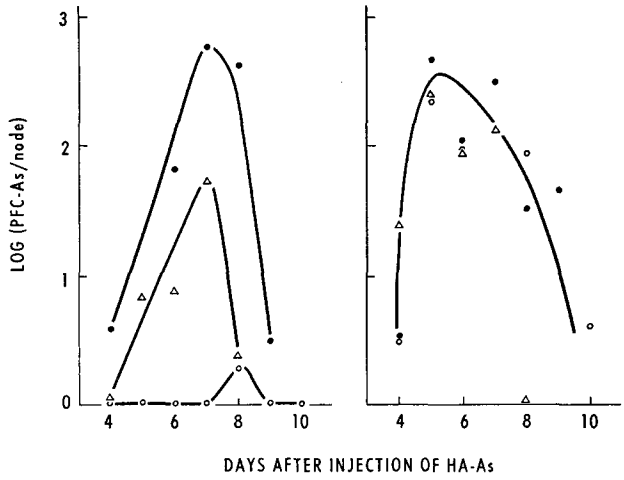

FIG. 1. The effect of preinjection with KLH on the response to HA-As–PFC-As assay. NaCl (0.15 M) or KLH (2.5 mg in 0.15 M NaCl) was injected into the right hind footpad of the rabbit. 4 days later, 0.5 mg, 1.5 mg, or 5.0 mg of HA-As (in 0.15 M NaCl) was injected into the same footpad of the rabbit. PFC-As assay with cells of the draining popliteal lymph nodes were carried out 4–10 days after the injection with HA-As. This time interval is indicated on the horizontal axis. The vertical axis represents the $\log_{10}$ of the number of PFC-As per node. Each point represents the geometric mean of the number of PFC-As per node obtained from five to nine rabbits.

Panel	1st injection	2nd injection	Symbols for geometric mean of PFC-As/node
left-hand side	NaCl; 0.15 M	HA-As; 0.5 mg	—○—
	NaCl; 0.15 M	HA-As; 1.5 mg	—△—
	NaCl; 0.15 M	HA-As; 5.0 mg	—●—
right-hand side	KLH; 2.5 mg	HA-As; 0.5 mg	—○—
	KLH; 2.5 mg	HA-As; 1.5 mg	—△—
	KLH; 2.5 mg	HA-As; 5.0 mg	—●—

assayed 5 days later had demonstrable plaque-forming cells in the draining lymph node. Even 8 days after the injection, seven out of eight rabbits did not have demonstrable plaque-forming cells in the lymph node (Fig. 1). However, more plaque-forming cells per 3×10^6 lymph node cells and per lymph node were detected if the rabbits were injected with one of several unrelated proteins 4 days before the administration of HA-As. Different proteins differed in their

capacity to increase the number of plaque-fomring cells in the draining lymph nodes of the responders (Table II, groups a–g). The increase in the hapten-specific plaque response, as a consequence of preinjection with an unrelated

TABLE II

The Effect of Preinjection with Various Antigens on Hapten-Specific Plaques to HA-As or to Dissociated KLH-As

Group	First injection	Test injection	Fraction of responders*	$\log_{10}$ (PFC-As/node)			Comparison with group	P‡
				Mean	SD	Range		
a	HSF	HA-As	8/9	2.6	1.1	0–3.5	g	<0.01
b	KLH		14/22	2.1	1.7	0–4.0		<0.01
c	Lys		4/6	2.0	1.6	0–3.8		0.05
d	HRBC		3/5	1.7	1.5	0–2.9		0.1
e	TMV		4/9	1.3	1.5	0–3.2		0.05–0.1
f	Dissociated KLH		1/8	0.4	1.1	0–3.1		>0.2
g	0.15 M NaCl		0/8	0	0	0–0		—
h	Alum-precipitated HGG	Dissociated KLH-As	18/20	2.9	1.2	0–4.7	k	<0.01
i	Alum precipitate		3/9	1.0	1.5	0–3.1		>0.2
j	HGG		2/5	0.9	1.2	0–2.3		>0.2
k	Phosphate-buffered saline		2/5	1.0	1.1	0–2.8		—

On day −4, one of the following reagents was injected into the right hind footpad of the rabbit: HSF (2.5 mg), KLH (2.5 mg), Lys (2.5 mg), TMV (2.5 mg), HRBC (6×10^9 cells), dissociated KLH (2.5 mg), 0.15 M NaCl, 0.15 M phosphate-buffered saline, pH 7.2, alum-precipitated HGG (2.5 mg), alum precipitate or HGG (2.5 mg). The first five antigens were given in 0.15 M NaCl; dissociated KLH was given in 0.2 M borate buffer, pH 8.8, and the last three reagents were given in 0.15 M phosphate-buffered saline, pH 7.2.

On day 0, HA-As (0.5 mg in 0.15 M NaCl) or dissociated KLH-As (0.5 mg in 0.2 M borate buffer, pH 8.8) was injected into the right hind footpad of each rabbit. On day 5, PFC-As assays were carried out with cells from the right popliteal lymph nodes.

* Responders are those animals whose lymph nodes give more than two plaques/3×10^6 cells above background.

‡ Individual values of each group were compared with those of group g or group k by the Wilcoxon rank sum test. Pretreatment with HSF, KLH, Lys, and alum-precipitated HGG led to PFC-As responses which were significantly higher than the response after pretreatment with salt solution.

prctein, was not only observed when HA was the carrier for the hapten but also when dissociated keyhole limpet hemocyanin was the carrier for the same hapten (Table II, groups h–k). It will be seen that the state of antigen aggregation (dissociated or associated KLH, soluble or alum-precipitated HGG) had a similar effect on carrier function (Table I) and on the promoting effect (Table II).

Increase in the Circulating Hapten Antibodies and Protein Antibodies after

Preinjection with Unrelated Macromolecules.—The effect of preinjection on the response to protein determinants of the subsequently injected antigen was investigated next in terms of circulating antibody. Though promotion of response to protein determinants was not always observed, it became apparent that promotion was in some cases not limited to the *p*-azobenzene arsonic acid response. In rabbits preinjected with KLH and challenged with the antigen HA-As, the serum hemagglutination titers against the carrier (HA) and the antigen (HA-As) were increased (Fig. 2, upper panel). In rabbits preinjected with alum-precipitated HGG and challenged with dissociated KLH-As, there was no increase in the serum hemagglutination titers against the carrier (dissociated KLH) or against the entire antigen (dissociated KLH-As). An increase was found in the

TABLE III

PFC-As Response to HA-As in Adjuvant with or without Preinjection with KLH

Group	No. of rabbits	Preinjection		Log_{10} (PFC-As/node)			
		Material	Time (days before the injection of HA-As)	Mean	SD	Range	Comparison with group I
							P
a	16	none	—	4.0	0.8	2.3–4.8	—
b	16	KLH	0.5	2.7	1.7	0–4.5	0.01–0.02
c	12	KLH	4	4.3	1.1	3.0–5.1	>0.2

HA-As (5 mg) in complete Freund's adjuvant was injected into the right hind foopad of each rabbit of group a, b, and c. 12 hr or 4 days earlier, each rabbit of group b and c had received an injection of KLH (2.5 mg in 0.15 M NaCl) into the right hind footpad. 5 days after the injection with HA-As, all rabbits were sacrificed and PFC-As assays were carried out with cells of the right popliteal lymph nodes.

* Comparison of individual values between groups by the Wilcoxon rank sum test.

titer of antibody directed against the hapten conjugated to a heterologous protein (Fig. 2, lower panel). Clearly, promotion could not only be demonstrated with plaque-forming cells from lymph nodes draining the injection site, but also with circulating antibodies.

The Effect on Promotion of the Dose of the Test Antigen and of Adjuvant.—The extent of promotion depended on the dose of HA-As which was injected. It was most marked when the dose was small (0.5 mg) and was no longer demonstrable when the dose was large (5.0 mg) (Fig. 1) or when the hapten-protein conjugate was given in complete Freund's adjuvant (Table III). In the latter instance, reduction in plaque numbers was observed if KLH was given 12 hr before the injection of HA-As (Table III).

The Effect on Promotion of the Dose of Preinjected Antigen.—Promotion depended on the dose of the promoting antigen. Promotion of response to 0.5 mg HA-As was not seen when the dose of the promoting antigen, KLH, was 0.25

mg or less, but was observed when the dose of KLH was 2.5 mg or more (Table IV, groups a–e). When 5 mg instead of 0.5 mg of HA-As was used, promotion was seen only when a large dose of KLH (25 mg) was administered (Table IV, groups f–i).

Localization of Promotion.—Promotion depended on the site of the administration of the promoting antigen. Promotion was localized to the lymph node which drained the footpad into which the promoting antigen was injected: one of several antigens was injected into the right hind footpad; after four days HA-As was injected into both the right and the left hind footpads. Hapten-

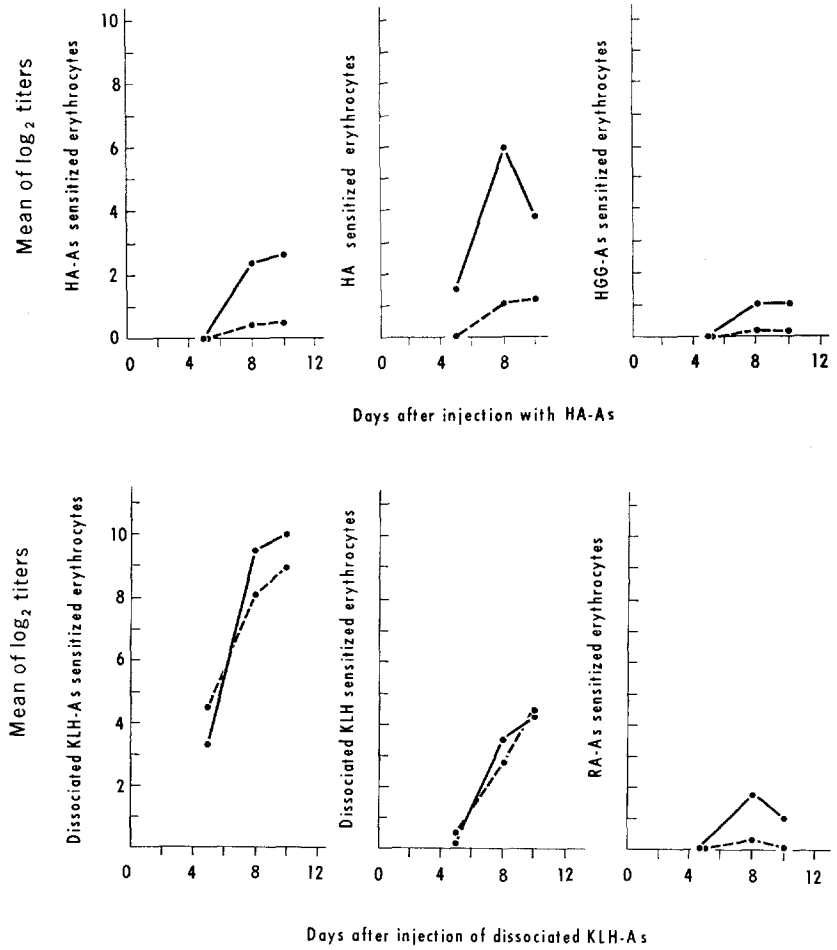

FIG. 2

specific plaques were found only in the right popliteal lymph node, but not in the left (Table V).

Inhibition rather than Promotion of Response after a Second Injection with the Promoting Antigen.—If two rather than one injection of the unrelated antigen were given before the administration of the azo protein (HA-As, 0.5 mg), promotion was not observed even though the unrelated protein and the azo protein were injected into the same footpad.

It will be seen from Table II that the plaque number was increased over that obtained by the injection of HA-As, given without pretreatment, if HSF or KLH was injected 4 days before HA-As. However, there was no promotion if HSF or KLH was given twice instead of once, whether the interval between the

FIG. 2. The effect of preinjection with structurally unrelated antigens on circulating antibody. Animals were preinjected with saline, alum precipitate, or promoting antigen 4 days before test injection with a diazotized protein. 5, 8, and 10 days after the test injection, the animals were bled.

	No. of rabbits	Preinjection	Test injection	Symbol
Upper panel	9	KLH (2.5 mg in 0.15 M NaCl)	HA-As (0.5 mg in 0.15 M NaCl)	—●—
	9	0.15 M NaCl		---●---
Lower panel	6	Alum-precipitated HGG (2.5 mg in 0.15 M phosphate-buffered saline, pH 7.2)	dissociated KLH-As (0.5 mg in 0.2 M borate buffer, pH 8.8)	—●—
	6	Alum precipitate (in 0.15 M phosphate-buffered saline, pH 7.2)		---●---

The hemagglutination titers of sera were measured with the diazotized immunizing antigens, with the carrier protein, and with a heterologous diazotized carrier as indicated on the vertical axis. A 1/10 dilution of serum was first prepared and thereafter 2-fold dilution steps were made. Titers were expressed as $\log_2$ of the reciprocal of these 2-fold dilutions. Each point represents the mean of the titers of each group on a given day. Titers from animals of paired groups were compared by a two-way analysis of variance:

	Antigens for sensitization of tanned erythrocytes	Comparison between titers of paired groups
		(*P*)
Upper panel	HA-As	0.01–0.025
	HA	<0.005
	HGG-As	0.05
Lower panel	Dissociated KLH-As	>0.20
	Dissociated KLH	>0.20
	RA-As	0.001

two injections with HSF or KLH was 7 or 60 days (Table VI, groups a–e) nor was there promotion when this interval was fixed at 60 days, and the HA-As (0.5 mg) was given 1, 4, 6, or 9 days after the second injection of KLH (2.5 mg).

The question arose whether this apparent absence of promotion was, in fact, attributable to antigenic competition which obscured the effect of promotion. We, therefore, designed experiments to test whether antigenic competition might be involved. Clearly, antigenic competition could not be demonstrated

TABLE IV

PFC-As Response to 0.5 mg or 5.0 mg of HA-As after Preinjection with Various Doses of KLH

Group	Dose of KLH	Dose of HA-As	Fraction of responding rabbits*	$\log_{10}$ (PFC-As/node)			Comparison with group	P‡
				Mean	SD	Range		
	(mg)	(mg)						
a	0	0.5	0/8	0	0	0–0		—
b	0.025		1/5	0.6	1.2	0–2.7	a	>0.1
c	0.25		2/5	1.0	1.3	0–2.6		>0.1
d	2.5		14/22	2.1	1.7	0–4.0		<0.01
e	25		6/9	2.2	1.6	0–3.7		0.02–0.05
f	0	5.0	7/7	2.8	0.6	2.4–3.9		—
g	0.25		4/4	2.6	0.3	2.3–2.8	f	>0.1
h	2.5		4/5	2.5	1.4	0–2.3		>0.1
i	25		5/5	3.9	0.2	3.6–4.1		0.01–0.02

Rabbits were divided into nine groups. On day −4, each rabbit of groups a and f received an injection of 0.15 M NaCl into the right hind footpad. Each rabbit of groups b–e and g–i received an injection with KLH (0.025 mg, 0.25 mg, 2.5 mg, or 25 mg in 0.15 M NaCl) into the right hind footpad. On day 0, all rabbits received an injection of HA-As (0.5 or 5.0 mg in 0.15 M NaCl) into the preinjected footpad. On day 5, all rabbits were sacrificed and PFC-As assays were carried out with cells of the right popliteal lymph nodes.

* Responders are those animals whose lymph nodes give more than two plaques above background/3 × 10^6 cells.

‡ Comparison of individual values between groups by the Wilcoxon rank sum test.

under the previously employed experimental conditions in which the dose of HA-As was so chosen that no plaque response occurred in the absence of promotion (Table II). Accordingly, we injected larger quantites of HA-As (5 mg) which we already knew to induce a substantial plaque response (Fig. 1). With this immunological challenge a single injection with 2.5 mg of KLH failed to affect the response. On the other hand, a preceding secondary injection with KLH inhibited the plaque response to HA-As (Table VI, groups f–h). It follows that antigenic competition might be implicated under the conditions in which we had previously failed to demonstrate promotion.

Acquired Immunological Tolerance to the Promoting Antigen.—We have, so

far, seen that promotion occurs when the promoting antigen is given for the first time, and that there was a parallel between carrier function and the promoting effect. On the basis of both these observations, it appeared as though a specific interaction between the promoting antigen and a component of the immune system might be an essential step in the initiation of the promotion which we have observed. We now tested this directly. Animals were rendered tolerant

TABLE V

Localization of Antigenic Promotion

Injection on day				PFC-As/node*	
−4		0			
Right footpad	Left footpad	Right footpad	Left footpad	Right popliteal node	Left popliteal node
HSF	0.15 M NaCl	HA-As	HA-As	⩽130	⩽120
				1000	⩽140
				1500	⩽110
H-RBC				⩽180	⩽50
				400	⩽80
				600	⩽70
TMV				⩽250	⩽110
				310	⩽20
				1200	⩽130

On day −4 one of three antigens was injected into the right hind footpad of the rabbit. The three antigens were HSF (2.5 mg in 0.15 M NaCl), TMV (2.5 mg in 0.15 M NaCl), and HRBC (6×10^9 cells in 0.15 M NaCl). On day 0, every rabbit received an injection with HA-As (0.5 mg in 0.15 M NaCl) into each of the two hind footpads. On day 5, PFC-As assays were carried out with cells from right and left popliteal lymph nodes.

* The No. of PFC-As was determined in 3×10^6 nucleated cells. It was considered to be $\leqslant 2/3 \times 10^6$ cells when the total number of plaques/3×10^6 cells did not exceed background by more than 2. The number of PFC-As/node was obtained by multiplying the number of PFC-As/3×10^6 cells with an appropriate factor based on the total number of nucleated cells in each node.

by neonatal injection with HGG (see Materials and Methods); others were left uninjected. In later life, both tolerant and normal rabbits were injected with alum-precipitated HGG, followed by dissociated KLH-As. It is apparent from Table VII that promotion occurred in animals which could respond to HGG but not in animals which were unresponsive to this antigen.

DISCUSSION

Preinjection with each of several different proteins increased the response to a hapten which was conjugated to a structurally unrelated protein. This effect was seen when the hapten-conjugated protein was given in small quantities, if

TABLE VI

Different Effects of Primary and Secondary Injections with HSF or KLH on the Subsequent PFC-As Response to HA-As

Group	Preinjection		Test injection HA-As	PFC-As	
	Material	Time of injection		per 3×10^6 cells	per node*
		(*day*)	(*mg*)		
a	HSF	0		⩽2‡	⩽110
				6	400
				12	1000
				19	1500
				21	1900
				21	3300
b	HSF	0, 60		⩽2	⩽130
				⩽2	⩽130
				⩽2	⩽170
				⩽2	⩽310
c	KLH	0		⩽2	⩽100
			0.5	⩽2	⩽150
				3	400
				3	410
				11	840
				9	940
				30	3900
				57	6700
d	KLH	0, 60		⩽2	⩽50
				⩽2	⩽140
				⩽2	⩽170
				⩽2	⩽230
e	KLH	0, 7		⩽2	⩽270
				⩽2	⩽310
				⩽2	⩽340
				⩽2	⩽400
f	0.15 M NaCl	0		3	150
				5	240
				6	280
				16	670
				20	740
				21	1100
			5.0	123	7300
g	KLH	0		⩽2	⩽110
				3	680
				12	1200
				9	1400
				10	1600
				8	1900

TABLE VI—*Continued*

Group	Preinjection		Test injection HA-As	PFC-As	
	Material	Time of injection		per 3×10^6 cells	per node*
		(*day*)	(*mg*)		
h	KLH	0, 7	5.0	$\leqslant 2$	$\leqslant 170$
				$\leqslant 2$	$\leqslant 180$
				$\leqslant 2$	$\leqslant 190$
				$\leqslant 2$	$\leqslant 200$
				$\leqslant 2$	$\leqslant 210$
				$\leqslant 2$	$\leqslant 220$
				$\leqslant 2$	$\leqslant 300$
				4	580

Rabbits were divided into eight groups. Each rabbit in four of these groups received a single injection into the right hind footpad with either HSF (2.5 mg in 0.15 M NaCl), KLH (2.5 mg in 0.15 M NaCl), or 0.15 M NaCl. Rabbits in the remaining four groups were given two injections with either HSF or KLH (2.5 mg in 0.15 M NaCl). 4 days after the primary or secondary injections, all rabbits were injected into the right hind footpad with HA-As (0.5 mg in 0.15 M NaCl for groups a–e; 5.0 mg in 0.15 M NaCl for groups f–h. 5 days (groups a–e) or 7 days (groups f–h) later, PFC-As assays were carried out with cells from the right popliteal lymph nodes.

* Comparison of individual values between groups by the Wilcoxon rank sum test:

a vs. b, $P = 0.05$; c vs. d, $P = 0.05$
c vs. e, $P = 0.05$; f vs. g, $P > 0.1$
f vs. h, $P < 0.01$;

‡ The No. of PFC-As was given as $\leqslant 2/3 \times 10^6$ cells when the total number of plaques/ 3×10^6 cells did not exceed background by more than 2.

the preinjected antigen was given for the first time, and if the animal was responsive to it.

Thus we have encountered an apparent paradox in that a nonspecific effect (i.e. promotion) appeared to depend on the high degree of specificity involved in antigenic recognition. We shall consider two alternative mechanisms which may explain this paradox: expansion of a cell population, and the release of pharmacologically active substances.

The first mechanism to be considered is the expansion of a cell population other than the cell population which is directly involved in antibody production. We shall illustrate this generality with a concrete example. The immune response to most antigens appears to involve interaction between at least two functionally different cells (bone marrow-derived cell [B cell] and thymus-derived cell [T cell]) (28, 29); it is established that the antibody is finally synthesized by the B cell (6). The T cell appears to function as helper cell, to interact with carrier determinants, and to present (directly or indirectly) the hapten to B cells (11). Interaction of antigen with both cells occurs through cell receptors which, up to now, appear to be identical with humoral antibody (compare 30–33

with 34–36). The question arises whether the range of effective cross-reactivity, as defined operationally from the study of circulating humoral antibody, is identical with the effective range of cross-reactivity of the thymus receptors (37). These cell-bound receptors might be able to bind effectively in terms of the helper functions at association constants far too low to be detected as cross-reactivity in antibody-antigen reactions. Thus the effective range of cross-re-

TABLE VII

The Effect of Tolerance on Antigenic Promotion

Group	Injections			Fraction of responding rabbits*	$\log_{10}$ (PFC-As/node)‡		
	At birth	In adult life			Mean	SD	Range
		First	Second				
a	None	Alum-precipitated HGG	Dissociated KLH-As	13/15	2.7	1.2	0–4.2
b	HGG	Alum-precipitated HGG	Dissociated KLH-As	7/13	1.6	1.5	0–3.4

Newborn rabbits were left uninjected or were given three injections with HGG intraperitoneally, each of 5 mg in 1 ml of 0.15 M NaCl. The first injection was given within 12 hr of birth and the others 24 and 48 hr later. When the rabbits were 31–50 days old, each rabbit of groups I and II received an injection with 2.5 mg of alum-precipitated HGG into the footpad. 4 days later, all rabbits were given an injection with dissociated KLH-As (0.5 mg in 0.2 M borate buffer, pH 8.8) into the preinjected footpad. PFC-As assays with cells from the draining popliteal lymph node were carried out 5 days after the last injection.

* Rabbits whose lymph node cells gave more than two plaques/3 $\times$ 10^6 cells above the background were considered as responders.

‡ Comparison of individual values between groups by the Wilcoxon rank sum test showed that group *a* was different from group *b* ($0.01 < P < 0.02$). The response of animals in group *a* (this table) is different from the response of animals which are not rendered tolerant to HGG, which are not preinjected in adult life with HGG, but which are preinjected with alum precipitate (group *i*, Table II; $P = 0.02$). The response of animals of group *b* (this table) is similar to the response of animals of group *i*, Table II ($P > 0.2$).

activity of the helper cell's receptors may be such that many unrelated proteins might cross-react at the level of the helper cell's receptors. Were this the case, division of helper cells in response to an antigen might well increase the number of helper cells (38) which would be available for the interaction between a subsequently injected hapten-conjugated protein and the B cell. Cross-reactivity of the helper function, beyond that observed with circulating antibody (i.e. beyond "classical" cross-reactivity), is the crucial feature of this working hypothesis. The detailed nature of the helper function is not important to our argument, which is as compatible with receptors on the thymus cell as it is with macrophage-attached cytophilic antibody (39, 40).

There are two observations which may, at first, appear to be inconsistent with this view: (*a*) that there is correlation between the carrier effect of a protein and its effect as a promoter, and (*b*) that proteins in different states of aggregation differ in their promotion effect.

The above inconsistencies may be more apparent than real. The first of the above two phenomena may be attributable to the number of potential determinants which can react with a helper cell; the greater their diversity, the greater the probability of interaction with helper cells, and the greater the variety of the type of cell receptor which can cross-react with another antigen. Thus, carrier effect and promotion effect would be expected to be correlated.

The different effectiveness in promotion by the same protein molecule in different states of aggregation is not attributable to the number of determinants, but may be attributable to the antigen's ability to reach the T cell receptor and to interact with it. This would affect promotion and carrier function to the same extent, if both depended on cell receptors.

Though the mediation of promotion through a cross-reaction at the level of the helper cell appears possible and even probable, a second possibility must be considered. Is it conceivable that a pharmacologically active substance was released, after specific interaction between the antigen and the receptors on the thymus-derived cells, which in some way facilitates the immune response to other antigens (41)? From the study of cell-mediated immunity, it is known that antigen-stimulated lymphocytes can release substances that have a variety of biological activities (42). Some of these activities may affect humoral as well as cellular immunity. It has been reported that specific stimulation of guinea pigs, presensitized to the purified protein derivative of tubercle bacilli (PPD), results in increased antibody response to diphtheria toxoid (43) as well as in increased delayed-type hypersensitivity to bovine gamma globulin (44).

Promotion which has been reported in this paper may be a common occurrence (45–48), even though it may appear as a rare phenomenon in terms of the burgeoning literature on antigenic competition. Indeed, it may be that antigenic promotion is the first event after exposure to two antigens and that antigenic competition is a subsequent, and sometimes, masking event. This idea is supported by Waterston's experiment (49); he observed that injection of pig erythrocytes into mice reduced the subsequent response to sheep erythrocytes. On the other hand, spleen cells from pig erythrocyte-immunized mice, when cultured in the presence of sheep erythrocytes, gave a higher response to the sheep erythrocytes than did the normal spleen cells. Cells responsible for the increase in the response to sheep erythrocytes were in the nonadherent cell fraction which contained B cells and T cells. Waterston suggested that injection of pig erythrocytes resulted in both the production of an inhibitory factor and an increase in cells involved in the response to sheep erythrocytes. The former contributed to a decrease in response while the latter contributed to an increase in response to

a subsequent injection of sheep erythrocytes. The increase in the activity of cells involved in the response to sheep erythrocytes, observed by Waterston, could be due to either of the two mechanisms discussed above: proliferation of cells due to cross-reactivity at the receptor level or proliferation of cells due to the action of a pharmacologically active substance. However, in Waterston's experiment a slight cross-reactivity between pig erythrocytes and sheep erythrocytes was demonstrated, indicating that there might be common or cross-reacting determinants, in a classical sense, between these two kinds of erythrocytes. It is, therefore, questionable whether the conclusions, based on this experiment, are applicable to other systems of antigen competition or promotion, in which classical cross-reactivity between the two antigens was not demonstrable.

So far the mechanism of antigenic competition remains unresolved. It is, therefore, futile to speculate whether or not antigenic promotion and antigenic competition have a common target.

Our failure to demonstrate promotion after two injections with a promoting antigen might be attributable to a difference between the kinetics of promotion after a single preinjection and to the kinetics after two injections with the promoting antigens. We have explored this possibility by varying the interval between the two preinjections (Table VI, groups a–e), and also by varying the interval between the second preinjection and the administration of HA-As. Promotion was not observed in any of these regimes. On the other hand, after the second preinjection, competition could be demonstrated if an immunizing dose (5 mg) of the test antigen was used (Table VI, groups f–h). We, therefore, regard it as unlikely that promotion occurs in the whole animal after a secondary response to preinjected antigen. If it does take place, it is clearly obscured by a much greater effect of simultaneously occurring antigenic competition. Indeed, increased antigenic competition has been observed upon secondary injection of a competing antigen (45, 46, 50). It is alternatively possible that a secondary injection utilizes all the helper cells so that administration of the "unrelated" antigen results in suppression (i.e. antigen competition) but not in promotion.

Injection of unrelated macromolecules, before the challenge with an antigen, increased the response to the hapten determinant of the antigen. The response to the protein determinant was increased in one case (Fig. 2, upper panel) but was not increased in another (Fig. 2, lower panel). If the lack of promotion seen in the latter case was real and was not due to an insufficiently sensitive test system, it might be considered that the response to different determinants of an antigen might be affected to different degrees by preinjection with an unrelated macromolecule. Be this as it may, promotion is clearly not limited to the chemical hapten, on which most of our work was based, since promotion affected protein determinants, at least in one experimental situation.

SUMMARY

Rabbits were immunized with *p*-azobenzene arsonic acid derivatives of human serum albumin (HA-As) or of dissociated keyhole limpet hemocyanin. The IgM response to the hapten was evaluated in terms of the number of hapten-specific plaque-forming cells in the lymph node draining the injection site. In some experiments, antibody was measured by agglutination of tanned and sensitized erythrocytes.

The hapten response of animals immunized with HA-As was increased (promoting effect) when the animals were injected with one of several structurally unrelated macromolecules: keyhole limpet hemocyanin (KLH), horse spleen ferritin (HSF), lysozyme (Lys), alum-precipitated human gamma globulin (alum-precipitated HGG). Different macromolecules differed in the magnitude of the promoting effect they induced, e.g., promotion by the associated form of KLH was greater than that by the dissociated form; alum-precipitated HGG was a better promoter than was soluble HGG. The relative magnitude of promotion by different macromolecules (associated vs. dissociated KLH, alum-precipitated vs. soluble HGG) correlated with the relative magnitude of the carrier effect, as judged by the hapten response induced by *p*-azobenzene arsonic acid conjugated to various proteins. Promotion was detected by agglutination assay of circulating antibody, by plaque assay of cells from the popliteal lymph node draining the site of preinjection, but not by plaque assay of cells from the contralateral lymph node. Promotion was dependent on the dose of the promoting macromolecule and on the dose of the hapten-protein conjugate. It was not observed in animals tolerant to the promoting macromolecule. Inhibition (i.e. antigenic competition), rather than promotion, was observed upon a secondary response to the preinjected macromolecule or when the hapten-protein conjugate was incorporated in Freund's adjuvant.

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