THE SPECIFICITY OF ALLERGIC REACTIONS

VII. Immunologic Unresponsiveness, Delayed Hypersensitivity, and Circulating Antibody to Proteins and Hapten-Protein Conjugates in Adult Guinea Pigs

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(Received for publication, January 30, 1964)

Immunologic unresponsiveness has been induced in several species of neonatal or adult laboratory animals. The maintenance of this unresponsive state depends on the continued presence of a specific antigen (1–3). This tolerant state may be prolonged or accentuated when animals are irradiated shortly before introduction of the tolerance-inducing antigen (4, 5). Also, animals "overloaded" with large doses of polysaccharide (6) or protein (5) do not respond to a later sensitization with the specific antigen. Unresponsiveness can be induced in guinea pigs by introduction of a hapten directly into the adult stomach (7, 8) or by injection of a protein into the fetus (9–11). Finally, administration of such antimetabolites as 6-mercaptopurine or cyclophosphamide in the presence of specific antigen induces unresponsiveness to that antigen (12, 13).

Since the criterion of tolerance has usually been the inability of an animal to produce circulating antibody, the specificity of tolerance often is presumed to follow the same determinants of the antigen as does the molecule of circulating antibody (2). Tolerance, however, applies to the suppression not only of circulating precipitating antibody but also of non-precipitating antibody (2), contact hypersensitivity to simple chemicals (7), and delayed hypersensitivity to proteins (11–13).

The specificity of delayed hypersensitivity has been shown to differ from that of circulating antibody (14–16). Sensitization with conjugates, such as *p*-aminobenzoic acid coupled to hen egg albumin, is followed by delayed hypersensitivity directed essentially to the protein carrier, and later by circulating antibodies to smaller groupings, as exemplified by the hapten. Since unresponsiveness applies both to delayed hypersensitivity and circulating antibody and since these two phenomena have different specificities, the question arises as to the specificity of immunologic unresponsiveness. In recent studies with contact haptens, guinea pigs made unresponsive to the contact hapten dinitro-

fluorobenzene (DFB)[1] by "gastric feeding" had this unresponsiveness modified when the animals were subsequently sensitized with the hapten conjugated to a heterologous protein, such as hen egg albumin (HEA) (3). Antibody to the DFB appeared in the "tolerant" guinea pigs, although they still did not react to contact testing with the specific hapten. These seemingly contradictory results can be explained if the specificity of unresponsiveness, like that of delayed hypersensitivity, is directed primarily toward the protein, in contrast to the reactions of circulating antibody which are oriented toward smaller antigenic configurations. Experiments were therefore initiated to examine further the specificity of unresponsiveness. The present paper offers data which indicate that unresponsiveness to protein conjugates induced in guinea pigs by simultaneous treatment with cyclophosphamide and antigen has its specificity directed toward the entire antigen molecule.

Materials and Methods

Animals.—Guinea pigs of the Hartley strain, 350 to 400 gm wt at the start of the experiments, were employed for the induction of tolerance against the γ-globulins. Adult rabbits and guinea pigs were used for hyperimmunization.

Antigens.—Bovine gamma globulin (BGG) from Armour and Company, Chicago was used primarily in the studies on tolerance, although 5 times recrystallized hen egg albumin (HEA) from K & K Laboratories Inc., Jamaica, New York, was also tried. The gamma globulins of pig, sheep, horse, and goat were prepared from fresh serum by precipitation from unbuffered 40 per cent saturated ammonium sulfate followed by purification with DEAE (cellulose *N*,*N*-diethylaminoethyl ether, Eastman Organic Chemicals, Rochester New York). A given volume of serum was diluted with one-half volume of distilled water and mixed with one volume of ammonium sulfate solution saturated at room temperature. The precipitate was dissolved in distilled water, precipitated twice more in the same way, and dialyzed against 0.01 M phosphate buffer, pH 7.0. The precipitated euglobulins were removed by centrifugation, and the supernatants were passed repeatedly through columns of DEAE until the effluents produced a single sharp band after electrophoresis on cellulose acetate strips. The same DEAE was never reused for purification of globulins from different species. The final products were the proteins which had not been retained on the columns in 0.01 M phosphate. Other fractions (referred to as "X-globulins") were eluted with NaCl up to 0.1 M and used to produce hyperimmune rabbit serum for studies on the purity of the γ-globulins. The purified fractions were lyophilized and stored at 4°C.

Conjugates of BGG, HEA, HuSA, and similar proteins were prepared by diazotization of the hapten and coupling to the protein (16). Two μmoles of diazotized hapten were used for each mg of protein. The products were precipitated by acidification with 10 per cent trichloroacetic acid (TCA) to pH 3.0 and allowed to remain in TCA solution overnight in the cold. The precipitate was redissolved by neutralization and dialyzed several days against physiologic saline.

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[1] BGG, bovine gamma globulin; BSA, bovine serum albumin; BSP, bromsulphthalein; DCB, dinitrochlorobenzene; DEAE, cellulose *N*,*N*-diethylaminoethyl ether; DFB, dinitrofluorobenzene; GGG, goat gamma globulin; HEA, hen egg albumin; HoGG, horse gamma globulin; HuSA, human serum albumin; PAAA, *p*-aminobenzene arsonic acid; PABA, *p*-aminobenzoic acid; PASA, *p*-aminobenzene sulfonic acid; PCA, passive cutaneous anaphylaxis; PGG, pig gamma globulin; SGG, sheep gamma globulin; TCA, trichloroacetic acid.

The amount of hapten in the conjugates was determined by use of C^{14}-labeled PABA for the preparation of PABA·BGG, PABA·HuSA, and PABA·HEA. After passage through sephadex G-25, followed by dialysis, the hapten content of the conjugate was determined in a liquid scintillation counter, and the protein, by the Kjeldahl method. Since all azo nitrogen may not be detected by the Kjeldahl method, its reliability for conjugates was checked by comparison with protein concentrations obtained by drying HEA and conjugates to constant weight in a vacuum oven at 100°C. 15.5 per cent was used for the nitrogen content of HEA. For PABA·HEA and PABA·BGG containing 10 per cent hapten, the nitrogen content was 14.4 ± 0.1 per cent, which would correspond to 16 per cent of the protein portion of the molecule. Therefore, the protein was calculated as 6.25 times the Kjeldahl nitrogen content of conjugates. The molecular weights of the proteins were assumed to be 45,000 for HEA, 69,000 for HuSA, and 169,000 for BGG.

Results for TCA-treated conjugates were:

	PABA per mg protein	PABA per mole protein
	μmole	*mole*
PABA·BGG	0.675	114
PABA·HuSA	0.665	46
PABA·HEA (1)	0.756	34
PABA·HEA (2)	0.815	37

DFB conjugates were prepared as previously described (19).

Induction of Tolerance.—Guinea pigs were inoculated intraperitoneally for 8 consecutive days with 10 mg of cyclophosphamide (cytoxan)[2] per day. On the 2nd day and sometimes also on the 3rd day of injection of cyclophosphamide, specific antigen in physiologic saline was administered intraperitoneally. During the 2 months following injection of the drug, 20 to 40 per cent of animals in various groups died, presumably from effects of cyclophosphamide. Hence, the number of animals described in the text as having been inoculated with cyclophosphamide refers to the number that survived and actually were used in the experiment. After the animals had been allowed to rest for 2 months, protein antigens or their conjugates were dissolved in 1 per cent normal guinea pig serum in physiologic saline, emulsified with equal volumes of Freund's adjuvant (Difco Laboratories, Inc., Detroit), and 0.5 ml of the water-in-oil emulsion was injected into the four foot-pads. The guinea pigs were immunized with 5 to 15 μg of the unconjugated protein or 15 μg of the conjugated protein.

Skin Tests.—Guinea pigs were skin tested on the flanks with 5 μg of antigen protein. The intradermal reactions were measured for areas of induration at both 4 and 18 to 24 hours.

Antibody Determinations.—Guinea pigs were bled by intracardiac puncture and the presence of antibody was determined. In passive cutaneous anaphylaxis (PCA), 0.1 ml of a test serum was injected intradermally into a normal guinea pig. About 3 hours later, the same animal was inoculated intravenously with 1 ml of 0.5 per cent Evans blue solution containing 350 μg antigen protein. The areas of blue skin were examined 30 minutes later and the results recorded. Hemagglutination titers were determined by a modification of the method of Stavitsky (17). Quantitative precipitins were done by a modification of the immunochemical procedures of Heidelberger and coworkers (18). Analysis of antigens and antibodies was also conducted by gel diffusion methods (20).

Homogeneity.—Each of the γ-globulins was assayed by agar diffusion methods against hyperimmune antisera from rabbits and guinea pigs and against sera from "control" guinea

[2] Obtained through the courtesy of Dr. D. E. Bebout, Mead Johnson Research Center, Evansville, Indiana.

pigs used in the experiments on tolerance described below. Agar plates were also prepared for tests with SGG and HoGG against rabbit antibodies to the X-globulins (anti-SXG and anti-HoXG). Single bands of precipitate developed between HoGG, PGG, and GGG and their respective homologous antisera. Two bands formed between BGG and either rabbit or guinea pig anti-BGG, and two bands were detected between SGG and guinea pig anti-SGG.

RESULTS

Unresponsiveness to Bovine Gamma Globulin (BGG).—Forty-nine guinea pigs were inoculated intraperitoneally with 10 mg cyclophosphamide (cytoxan) daily for 8 days. On the 2nd day, 20 to 40 mg BGG was also administered intraperitoneally. Forty-three control animals receiving only cyclophosphamide

TABLE I

*Response of Guinea Pigs Inoculated Intraperitoneally with 80 mg Cyclophosphamide and 20 to 50 mg BGG and 2 Months Later Administered 15 μg BGG or a Heterologous Antigen in Freund's Incomplete Adjuvant in Foot-pads**

Sensitizing antigen injected	Definite response to homologous antigen		Response to BGG	
		per cent		*per cent*
BGG	11/49‡	22	—	
PABA·BGG	27/32	84	4/16	25
PAAA·BGG	4/4		1/4	
HoGG	15/15		0/15	

* All control animals inoculated with cyclophosphamide and 2 months later with one of the specific antigens had antibodies to the injected antigen.

‡ $\frac{\text{No. of animals with circulating antibody (by PCA)}}{\text{No. of animals tested}}$

were included in the study. Two months later, all guinea pigs were inoculated in the foot-pads with 15 μg BGG in Freund's incomplete adjuvant. Groups of animals were bled at frequent intervals and the sera were assayed for anti-BGG antibody by passive cutaneous anaphylaxis.

All control animals developed detectable antibody on about the 9th day after challenge. Of the 49 guinea pigs administered antigen with cyclophosphamide, 32 were unresponsive during the test period, *i.e.* 60 days after inoculation, and 6 formed questionable amounts of antibody for a very brief period. Thus, 65 per cent of the guinea pigs remained unresponsive to the protein, BGG.

When guinea pigs treated with 20 to 50 mg BGG and 80 mg cyclophosphamide were inoculated in the foot-pads with 15 μg PABA·BGG, PAAA·BGG, or HoGG in Freund's incomplete adjuvant, antibody was readily formed to the secondary antigen, although the animals still did not respond to BGG (Table I). Thus, animals, unresponsive to a protein (BGG) after treatment

with cyclophosphamide and challenged subsequently with an altered protein (such as PABA·BGG), developed antibody to the altered protein, but tended to retain the unresponsiveness to the original protein.

Unresponsiveness to the Conjugate PABA·BGG.—Fifty-seven guinea pigs, treated as above with cyclophosphamide, were inoculated intraperitoneally on the 2nd day with 40 mg PABA·BGG. Thirty-four guinea pigs were administered cyclophosphamide without any additional antigen. About 2 months later, all animals were inoculated in the foot-pads with 15 μg PABA·BGG, and bled at frequent intervals for antibody analysis.

TABLE II

Response of Guinea Pigs Inoculated Intraperitoneally with 80 mg Cyclophosphamide and 40 mg PABA·BGG and 2 months later Inoculated in the Foot-pads with 15 μg PABA·BGG or a Variant in Freund's Incomplete Adjuvant

Sensitizing antigen injected	Response to homologous antigen	Response to PABA·BGG
PABA·BGG	0/57*	0/57
BGG	31/31	0/31
PAAA·BGG	24/27	0/27
DFB·BGG	21/21	0/21
PABA·HEA	19/19	0/19
PABA·HoGG	11/23	0/23
PABA·PGG	11/12	0/12
PABA·SGG	0/10	0/10
PASA·BGG	3/12	0/12

* No. of animals with circulating antibody (by PCA and hemagglutination) / No. of animals tested

Not one of the 57 animals, administered cyclophosphamide and PABA·BGG simultaneously and later reinoculated with PABA·BGG, developed antibody detectable by passive cutaneous anaphylaxis or hemagglutination with PABA·HEA or PABA·HuSA as antigens. Also, none showed delayed hypersensitivity on skin test with homologous conjugate. In contrast, all controls formed circulating anti-PABA·BGG antibody beginning about the 12th day after challenge.

Animals rendered unresponsive to the conjugate PABA·BGG were inoculated in the foot-pads with one of the following variants in Freund's incomplete adjuvant: BGG, PAAA·BGG, PASA·BGG, PABA·HEA, DFB·BGG, PABA·HoGG, PABA·PGG, and PABA·SGG (Table II). In all cases but two (PABA·SGG and PASA·BGG), antibody was formed to the variation of the PABA·BGG conjugate, although challenge of the unresponsive state with PABA·HoGG produced antibody in only 48 per cent of the animals. The exceptions wherein antibody could not be detected after challenge occurred in animals given PABA·SGG or PASA·BGG. SGG has extensive cross-reaction with

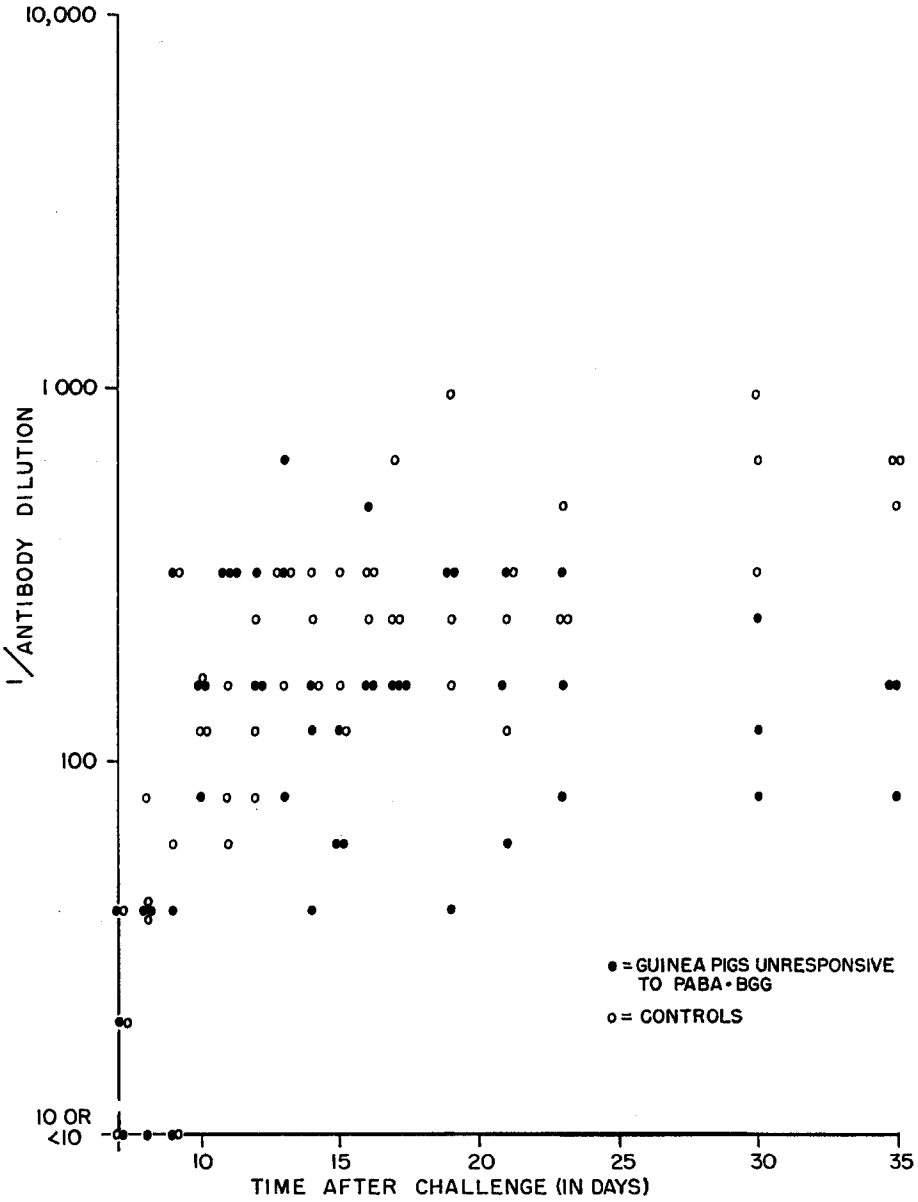

FIG. 1. Hemagglutination titers (*a*) in guinea pigs unresponsive to PABA·BGG and later challenged in foot-pads with 15 μg PAAA·BGG in Freund's incomplete adjuvant, or (*b*) in controls challenged in foot-pads with 15 μg PAAA·BGG in Freund's incomplete adjuvant.

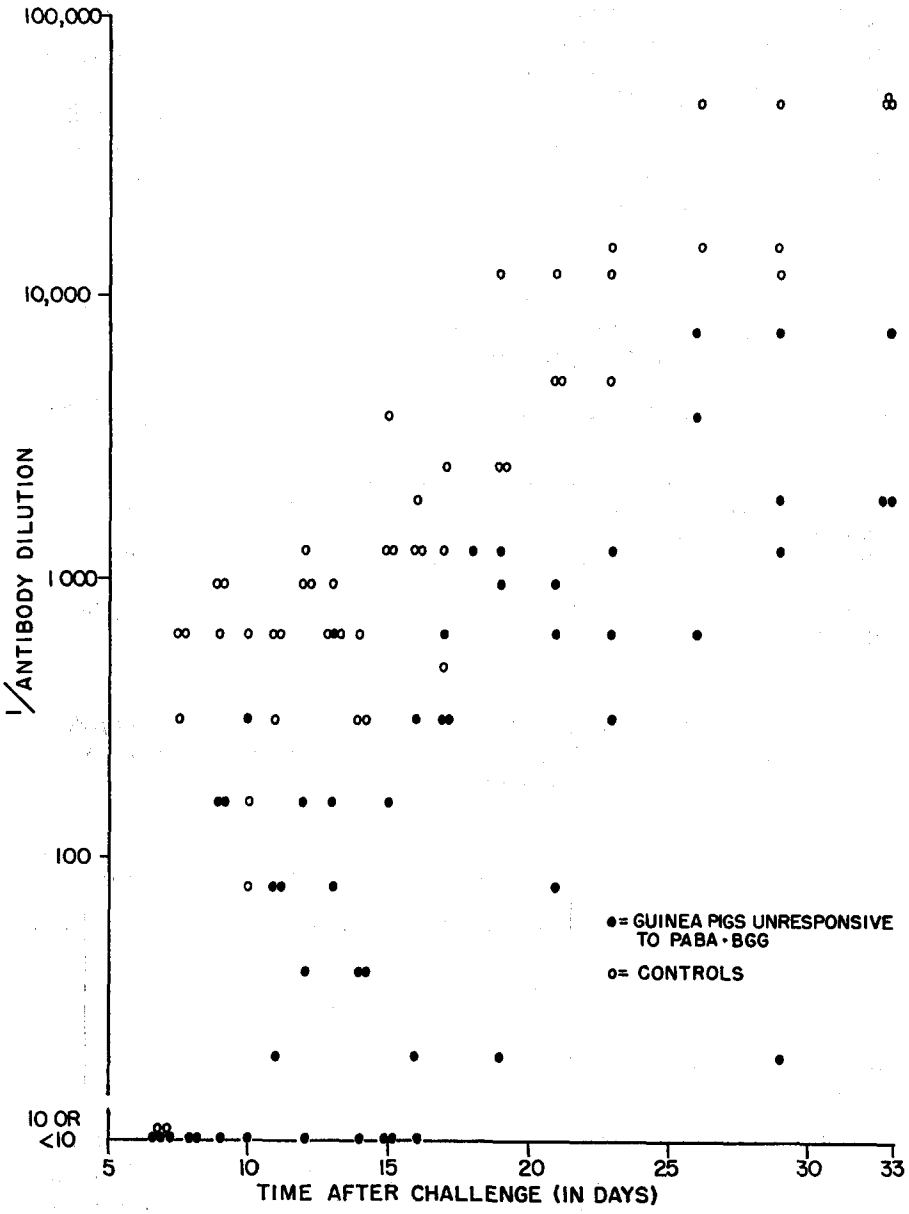

FIG. 2. Hemagglutination titers (*a*) in guinea pigs unresponsive to PABA·BGG and challenged in foot-pads with 15 μg BGG in Freund's incomplete adjuvant, or (*b*) in controls challenged in foot-pads with 15μg BGG in Freund's incomplete adjuvant.

anti-BGG, whereas HoGG and PGG do not. The tolerant animals that formed antibody to the variant of the conjugate still retained their original specific unresponsiveness. Also, guinea pigs that formed antibody to a variant of PABA·BGG but were still unresponsive to the PABA·BGG did not form antibody to the PABA·BGG when they were reinoculated in the foot-pads with that antigen in incomplete adjuvant. The controls receiving cyclophosphamide alone formed antibody to each of the different antigens injected.

TABLE III

Response of Guinea Pigs Inoculated Intraperitoneally with 80 mg Cyclophosphamide and 40 mg PABA·HoGG and Subsequently Exposed to a Heterologous Protein or Conjugate in Freund's Incomplete Adjuvant

Sensitizing antigen injected	Response to homologous antigen* (by PCA)
PABA·HoGG	0/12
HoGG	10/11
PAAA·HoGG	7/12
PABA·BGG	9/10

* None of the animals showed anti-PABA·HoGG antibody at any time during the post-challenge period of 60 days.

TABLE IV

Delayed and Arthus Hypersensitivity in 148 Guinea Pigs Sensitized in the Foot-pads with 15 μg Gamma Globulin of One Species and Skin-tested on Days 5 to 20 with Four Gamma Globulins

Sensitizing antigen	Skin-testing antigen							
	Delayed response				Arthus response			
	BGG	HoGG	PGG	SGG	BGG	HoGG	PGG	SGG
BGG	+	−	−	+	+	−	±	+
HoGG	+	+	−	+	−	+	+	−
PGG	−	−	+	−	+	+	+	+
SGG	+	−	−	+	+	−	±	+

Animals unresponsive to PABA·BGG formed antibodies to BGG, PAAA·BGG, and PABA·HoGG. During the first 14 days, the anti-PAAA titers of the sera from guinea pigs unresponsive to PABA·BGG and challenged with PAAA·BGG were approximately the same as the control animals challenged with PAAA·BGG but not made unresponsive to PABA·BGG (Fig. 1). Thereafter, the anti-PAAA titers of the control animals were higher than those of the guinea pigs unresponsive to PABA·BGG. Titers of sera from animals unresponsive to PABA·BGG and challenged with either BGG (Fig. 2) or PABA·

HoGG were lower than the titers of control animals which had not been made unresponsive to PABA·BGG. Antigen-antibody studies in agar did not show cross-reactions between rabbit or guinea pig anti-BGG and PABA·BGG or between anti-PABA·BGG and PAAA·BGG, but did show cross-reactions between anti-PABA·BGG and PABA·HoGG.

Unresponsiveness to the Conjugate PABA·HoGG.—Forty-five guinea pigs were inoculated intraperitoneally daily for 8 days with 10 mg cyclophosphamide, and on the 2nd day with 40 mg PABA·HoGG. Fifty-one control guinea pigs were inoculated daily with 10 mg cyclophosphamide. All animals

TABLE V

Response of Guinea Pigs Inoculated Intraperitoneally with 80 mg Cyclophosphamide and 20 mg BGG, and Subsequently Challenged with BGG or One of Four Heterologous Proteins

Sensitizing antigen	Response to homologous antigen	Response to BGG
BGG	2/13*	—
HoGG	15/15	0/15
PGG	13/13	13/13
GGG	14/14	12/14
SGG	15/15	11/15
Controls		
BGG	11/11	—
HoGG	11/11	1/11
PGG	10/10	9/9
GGG	10/10	10/10
SGG	10/10	10/10

* No. of guinea pigs with circulating antibody (by PCA) / No. of guinea pigs tested

were inoculated 2 months later in the foot-pads with 15 μg antigen in Freund's incomplete adjuvant (Table III).

Again, all animals were unresponsive to the original conjugate, PABA·HoGG. Any modification of this antigen for the challenge, such as HoGG, PAAA·HoGG, or PABA·BGG, resulted in the formation of antibody to the antigen used for challenge, although the original unresponsiveness to the PABA·HoGG remained. Control animals all formed antibody to the challenging antigen.

Unresponsiveness to Gamma Globulins.—The immunologic cross-reactions of gamma globulins of related mammalian species have been studied by methods involving quantitative precipitation (21). Other studies have been made on the relationship of protein antigens of different species with the aid of hypersensitivity reactions (15). Cross-reacting serum albumins have also been studied by examination of the loss of the tolerant state in rabbits injected with one of

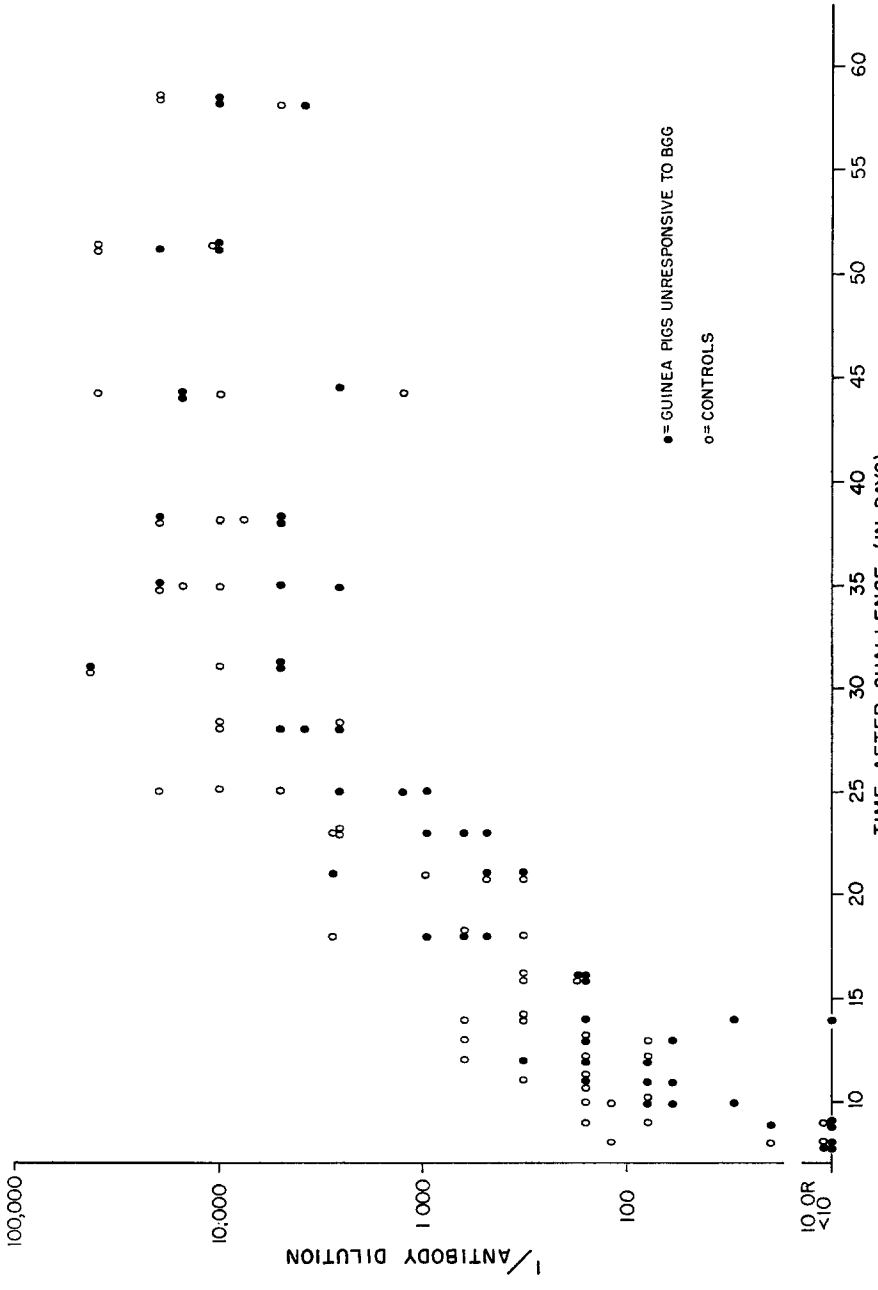

FIG. 3. Hemagglutination titers (*a*) in guinea pigs unresponsive to BGG and challenged with 15 μg PGG in Freund's incomplete adjuvant in foot-pads, or (*b*) in controls challenged with 15 μg PGG in Freund's incomplete adjuvant in foot-pads.

the proteins (22). Herein, animals made unresponsive to bovine gamma globulin were studied for their capacity to form antibodies to related heterologous globulins.

In order to determine the extent of cross-reactions in delayed and Arthus hypersensitivity between BGG, HoGG, SGG, and PGG, 148 guinea pigs were sensitized in the foot-pads with 15 μg of one antigen in Freund's incomplete adjuvant and skin tested with all four antigens at intervals from 5 to 20 days

TABLE VI

Reactions in Agar Gel between Rabbit (Ra) and Guinea Pig (GP) Antibodies and Heterologous γ-Globulins

Antibody	Antigen				
	BGG	GGG	SGG	PGG	HoGG
Ra anti-BGG	++	++	++	+	+
GP anti-BGG	++	++	++	±	−
Ra anti-GGG	+	++	++	+	+
GP anti-GGG	+	++	++	−	−
Ra anti-SGG	++	++	++	±	±
GP anti-SGG	++	++	++	±	+
Ra anti-PGG	+	+	+	++	−
GP anti-PGG	+	−	+	++	±
Ra anti-HoGG	+	+	+	+	++
GP anti-HoGG	±	−	±	+	++

++, strong; +, weak; ±, questionable; −, negative.

after inoculation. Each guinea pig was tested only once with the group of antigens. The patterns of reactions (Table IV) indicate the differences in response for delayed *vs.* Arthus types of hypersensitivity.

Seventy guinea pigs were inoculated with cyclophosphamide as described above. On the 2nd day, 20 mg BGG was injected intraperitoneally. Two months later, the animals were inoculated in the foot-pads with 15 μg of one of the following heterologous proteins in Freund's incomplete adjuvant: HoGG, GGG, PGG, and SGG (Table V). For a period of 2 months after challenge, only 2 of 13 guinea pigs administered cyclophosphamide and BGG developed anti-BGG antibodies detectable by PCA. In contrast, all 15 animals challenged with HoGG developed anti-HoGG antibodies, and none developed definite anti-BGG antibody. Of the 13 guinea pigs challenged with PGG, all formed anti-BGG and anti-PGG antibodies. Although all animals unresponsive to BGG

formed antibodies to PGG, the antibody titers to the PGG were slightly lower than those of the control animals (Fig. 3). Of the animals inoculated with GGG or SGG some remained unresponsive to BGG, although the majority did form anti-BGG antibody. The control animals receiving PGG, SGG, and GGG all formed antibodies to both the homologous antigen and BGG. Thus, it appears that those antigens closely related to BGG tend to terminate the unresponsiveness to BGG.

In studies on antigen-antibody reactions in agar gel, hyperimmune rabbit

TABLE VII

Quantitative Precipitins between Guinea Pig Antisera and γ-Globulins of Various Species

Antibody	Antigen				
	BGG	SGG	GGG	PGG	HoGG
Normal					
anti-BGG	0.96*	0.52	0.46	0.09	<0.01
anti-SGG	0.59	1.00	0.94	0.01	0.01
anti-GGG	0.25	0.30	0.36	0.02	0.01
anti-PGG	0.36	0.28	0.28	0.74	0.19
anti-HoGG	0.02	0.04	0.04	0.11	1.3
Tolerant					
anti-BGG	<0.01	0.02	0.05	0.10	<0.01
anti-SGG	0.20	0.25	0.15	0.03	0.04
anti-GGG	0.08	0.03	0.02	0.03	0.07
anti-PGG	0.11	0.04	0.07	0.59	0.06
anti-HoGG	<0.01	<0.01	0.02	<0.01	>0.25

* Mg antibody protein precipitated per ml serum.

anti-γ-globulins contained antibodies to all five of the γ-globulins in every case but one,—anti-PGG did not develop detectable precipitates to HoGG. The bands of precipitate between anti-SGG and PGG or HoGG were barely detectable. Guinea pig antisera did not have cross-reacting antibodies for a number of combinations (Table VI).

Quantitative precipitin analyses were made with sera of normal guinea pigs immunized to each one of the five antigens and with sera of guinea pigs made tolerant to BGG and then immunized with the same antigens. All the bleedings were done 5 weeks after the immunizing inoculation. Sera from single animals were used in the cases of tolerant guinea pigs sensitized to PGG and SGG and of normal guinea pigs sensitized to PGG, SGG, and GGG. The rest were pools composed of sera from 3 animals in the same experimental group. Since only 2 or 3 ml of serum of each type was available, the amount of antigen and anti-

body per tube and the number of tubes for each precipitin curve had to be reduced to a minimum. Because of these limitations, the accuracy of these results does not compare with that of the conventional precipitin method (18), but the points obtained were not scattered and fell on curves of the usual shape.

In the present case, 0.1 ml of serum and 0.1 ml of antigen were mixed in 10 x 75 mm culture tubes, precipitates were washed only twice with 0.2 ml of cold saline, and the protein determinations were obtained by a modification of the bromsulphthalein (BSP) method (23). Antigen-antibody precipitates were dissolved in 0.1 ml of M/4 acetic acid, mixed with 0.5 ml of 0.025 per cent BSP in 0.25 M citric acid, and centrifuged; 0.2 ml of the supernatant was mixed with 3 ml of M/10 NaOH and optical density was determined at 580 mμ. Amounts of protein were read from a standard curve based on BGG.

With total precipitate plotted against micrograms of antigen per milliliters of serum, the point of maximum precipitate was determined by inspection and the total antigen in the tube subtracted from the total precipitate. The error in these figures is probably about 10 per cent and antibody concentrations of 0.02 mg/ml or less are of doubtful significance. In general, the results are similar to those obtained with Arthus reactions and PCA (Table VII).

DISCUSSION

Adult guinea pigs may be made unresponsive to a variety of protein or hapten-protein antigens by simultaneous intraperitoneal administration of cyclophosphamide and a specific heterologous protein or protein conjugate. The unresponsiveness developing after treatment with cyclophosphamide has a specificity different from that characteristic of delayed hypersensitivity or circulating antibody. A change in either the protein or hapten portions of the conjugate molecule resulted in the formation of delayed hypersensitivity and circulating antibody, although the unresponsiveness to the original conjugate persisted. When a protein closely related to and cross-reacting with the original, as determined by *in vitro* precipitation (*e.g.* BGG and SGG) was substituted in the conjugate, unresponsiveness persisted to both proteins. When an animal was made unresponsive to one protein (*e.g.* BGG) and was subsequently challenged with a protein (such as SGG) which shares many, but not all, of its determinant groups, antibody developed to both antigens. When a guinea pig was made unresponsive to one protein (*e.g.* BGG) and challenged with a protein with which it has very few determinant groups in common (*e.g.* HoGG), antibody formed to the challenging protein, but only in very small amounts, if at all, to the original protein.

In previous studies of others with altered antigens in rabbits made unresponsive to proteins or protein conjugates, varied results were obtained. Thus, rabbits rendered tolerant at birth by intraperitoneal injection of bovine serum albumin (BSA) did not develop antibody to subsequent challenges of *p*-sulfanilic acid coupled to BSA (24).

The animals were challenged intravenously with conjugated BSA in saline, and antibody was determined by rate of elimination of radioactive antigen. In another set of experiments (25), rabbits, made unresponsive at birth by intraperitoneal injections of 110 to 140 mg human serum albumin (HuSA), were challenged intravenously with 2.5 mg *p*-sulfanilic acid coupled to HuSA, and antibody formation was determined by hemagglutination with tannic acid–treated red blood cells. Antibody could not be detected in any of 15 rabbits inoculated at birth with HuSA. Similarly, adult guinea pigs rendered unresponsive to dinitrochlorobenzene (DCB) by "gastric feeding" did not respond to inoculation in the foot-pads with specific contact hapten in Freund's incomplete adjuvant (3, 26). When mycobacteria were incorporated into the sensitizing emulsion, the state of unresponsiveness was less apparent. When guinea pigs gastric-fed with DCB were later inoculated in the foot-pads with an *in vitro* conjugate of the hapten combined with a heterologous protein and suspended in incomplete adjuvant, an immune response occurred both to the hapten and to the protein carrier. Again, guinea pigs, inoculated intraperitoneally with 15 mg hen egg albumin (HEA) *in utero*, failed as adults to respond to stimulation in the foot-pads with 15 μg PABA·HEA in Freund's incomplete adjuvant (11). The specificity of the unresponsiveness, therefore, seemed to be associated essentially with the protein moiety.

On the contrary, when the antigenic challenge was administered in Freund's complete adjuvant, a breakdown of tolerance was observed in many cases. When newborn rabbits were inoculated with 500 mg HuSA (27), given intraperitoneally in saline and subcutaneously "in emulsion," 2 of 6 animals developed antibodies (detected by hemagglutination) after subsequent intravenous challenge with 6 to 15 mg HuSA coupled by diazotization with *p*-sulfanilic acid (11 to 16 groups per protein molecule). In later work (28), another rabbit, after having been made tolerant to HuSA, formed antibody to PASA·HuSA. Rabbits made unresponsive to BSA by several subcutaneous injections totaling 500 mg during the first 5 days after birth lost this tolerance after challenge with 3 weekly injections each of 25 mg arsanil-sulfanil-BSA in Freund's complete adjuvant (29). Rabbits unresponsive to BSA and subsequently challenged subcutaneously with either sulfanil-BSA or arsanil-BSA in Freund's complete adjuvant formed antibody to the hapten, but not to the native protein. Apparently, when an animal unresponsive to a protein is challenged with a conjugate of the specific protein, antibody to the conjugate develops. If the challenge is made sufficiently strong by incorporation into complete adjuvant or by exposure of the animal to repeated doses, not only is antibody formed to the new conjugate but also the unresponsiveness to the original protein or conjugate may be eliminated. Further minor variations may exist depending on such factors as the species of experimental animal, the mode of induction of unresponsiveness, the route and severity of challenge, the method of conjugate synthesis, and the vehicle for antigen injection.

Unresponsiveness in rabbits following neonatal injections of BSA was terminated by a series of injections of an albumin distantly related to BSA, such as HuSA, but persisted when the animals were challenged subcutaneously with two series of injections of 110 mg each of a closely related protein, such as sheep serum albumin (22). These results are somewhat different from those described herein in guinea pigs. Unresponsiveness to BGG, induced in adults with cyclophosphamide, tended to persist after challenge in the foot-pads with 15 μg of the distantly related antigen

HoGG in Freund's incomplete adjuvant, but was terminated when the guinea pig was similarly challenged with the closely related antigen, SGG or GGG.

Recently, five types of immunological tolerance were recognized (30), although definite evidence does not exist as to whether they are or are not all part of the same basic mechanism: (*a*) that produced by exposure to antigen very early in life, (*b*) that produced by exposure of an animal to a high dose of ionizing radiation followed very shortly by exposure to antigen, (*c*) that produced by exposure of adult rabbits to high doses of soluble protein antigens, (*d*) that induced in adult guinea pigs by oral administration of certain simple chemicals or contact haptens, and (*e*) that produced by exposure of adult mice to high doses of pneumococcal polysaccharide which in lower doses would have induced immunity. In these types of unresponsiveness, the development of the capacity to react to an antigen is delayed and, in the continued presence of the antigen, is postponed indefinitely. The animal must be exposed to the antigen before the capacity to react against it becomes established. The animal is retarded in its immunologic development toward a specific antigen or antigens, although immunologic responses in general are normal. The antigen is eliminated from the blood stream in a "non-immune" pattern.

Variations in or modifications of the classical concept of unresponsiveness have been reported. Recently, for example, a partial inhibition of "delayed" hypersensitivity in mice was characterized as "immunologic unresponsiveness" (31). Mice sensitized with a single subcutaneous injection of 0.25 mg hen egg albumin in Freund's complete adjuvant were skin tested 4 days later, and an inhibition of subsequent skin tests read at 24 hours in the presence of specific anti-HEA antibody was noted, although the 3-hour readings were the same as those of the controls. Whether such specific but partial desensitization of a skin response should be included in immunologic unresponsiveness is doubtful, especially since complete and long-lasting suppression of the immune response, including delayed hypersensitivity, has been established (2, 3, 7, 11, 32, 33). Such reactions as inhibition of antibody formation to a conjugate in the presence of antibody to homologous protein was not and should not be considered as immunologic tolerance (34). Also, since immunologic unresponsiveness is specific, nonspecific suppression of the immune response should not be included.

The specificity of unresponsiveness, as demonstrated in adult guinea pigs after treatment with cyclophosphamide, is different from other phases of the immune process, such as delayed hypersensitivity and circulating antibody. Where the specificity of circulating antibody is oriented primarily toward groupings of small molecular size and where the specificity of delayed hypersensitivity is directed toward groupings of large molecular size (14–16, 35), the specificity of unresponsiveness may be oriented toward the whole antigen molecule. If unresponsiveness is considered to be associated with an early phase in the immune process and if delayed hypersensitivity is an intermediate phase, then a transition in specificity occurs in the maturation of the immunogenic cells from an orientation toward the entire antigen molecule to an orientation toward small molecular configurations. This progression in specificity may be explained in several ways. For example, as the immune process passes through

various cell types from the reticular cell to the mature plasma cell, an actual alteration or transformation in the recognition mechanism of the cell may exist, so that each cell type can identify only certain molecular types or sizes in the antigen. A second mechanism may be based on the gradual breakdown of the foreign protein antigen by cellular enzymes and on the recognition of these products as the antigen. Thus, early in the immune process, when cellular enzymes have had little opportunity to alter the original antigen, the specificity is oriented primarily toward the whole original antigen, as in immunologic unresponsiveness. As the antigen is carried from one daughter cell to another and is metabolized by the cell enzymes, the molecular size of the antigen decreases. If each cell phase recognizes and responds to the antigen fragment within, then, as the immune process progresses, the antigenic determinants become smaller and smaller. Thus, the specificity of a cell changes because of a modification in the antigen molecule within the cell and not because of a change in the recognition apparatus of a cell type.

The possibility exists that unresponsiveness may be initiated at several early phases of the immune process and that the initiation at each different stage may produce a specificity slightly different from the others. Thus, unresponsiveness induced by cyclophosphamide in adults may be associated with a slightly different stage of the immune mechanism than that produced in newborns. At any rate, the phase or phases associated with immunologic unresponsiveness does precede the phases associated with delayed hypersensitivity and antibody formation, since the latter two are both suppressed in the process.

In adult guinea pigs treated with cyclophosphamide, the more antigenic the protein or protein conjugate, the greater is the difficulty in the induction of unresponsiveness. Thus, with similar procedures, routes and doses, unresponsiveness develops more readily in animals with HoGG as antigen than with BGG as antigen, and more readily with BGG (36) than with HEA. Also, conjugates of a protein induce immunologic unresponsiveness more readily than does the protein itself, such as PABA·BGG *vs.* BGG.

SUMMARY

Adult guinea pigs were made unresponsive to a heterologous protein (*e.g.* bovine gamma globulin, or BGG) or a hapten-protein conjugate (*e.g. p*-aminobenzoic acid-bovine gamma globulin, or PABA·BGG) by intraperitoneal injection of 80 mg cyclophosphamide and the specific antigen. This immunologic unresponsiveness developed to the specific antigen administered simultaneously with the cyclophosphamide, and not to any variants. Thus, animals unresponsive to PABA·BGG remained unresponsive to the original antigen on challenge with a variant, but formed delayed hypersensitivity and circulating antibody to the variant. The specificity of immunologic unresponsiveness,

therefore, seems more closely related to the whole antigen molecule than does delayed hypersensitivity.

The authors acknowledge the technical assistance of Jane Nishio and Leroy F. Peel.

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