SPECIFICITY OF FIXATION LOCI FOR HOMOCYTOTROPIC ANTIBODIES*

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Immediate-type hypersensitivities are known to be mediated primarily by antibodies reacting with homologous antigens. One of the biological properties of the immunoglobulins involved in immediate hypersensitivities is a capacity to sensitize skin such that subsequent exposure of the host to antigen results in immediate-type dermal reactions.

The immunoglobulins capable of passively sensitizing skin of individuals within a species (homocytotropic antibodies, 1) have been found among diverse species. Whereas one class of homocytotropic antibody has so far been described for species such as rabbit (2–5), dog (6), and monkey (7), two classes have been clearly defined for man (8–10), rat (11–13), mouse (14–19), and guinea pig (20–30).

To illustrate the two classes, among guinea pigs there exists a heat-stable IgG_1 that confers passive cutaneous anaphylaxis (PCA)[1] after a latent period of several hours (20–29). Though subdivision of this class has been suggested on the basis of different latency period requirements, i.e. 4 hr vs. 16 hr, immunoglobulins of this type share common antigenic determinants (31). The second class of antibody, IgE or reagin, is heat labile at 56°C for 1 hr and induces sensitivity persisting for many days (30, 31)

Restricted heterogeneity has not yet been described for homocytotropic antibodies arising as a consequence of immunization. Nevertheless, homogeneous populations of immunoglobulins have long been known to be synthesized in individuals with myelomas (32–35) and in those believed normal (36). Presumed to arise as a consequence of monoclonal stimulation, the antibodies or their separated chains have demonstrated restricted antigenic specificity (32–35, 37), electrophoretic mobility (38–40), allotypic and idiotypic specificity (41, 42), and isoelectric focusing mobility (41, 43). The antigens directing production of such antibodies were either well-defined haptens (39, 44) or bacterial polysaccharides (34, 38, 42, 45–48).

Dextran, one of these antigens, elicits precipitating antibodies of restricted heterogeneity in humans (34, 40, 47, 48), and, indeed, has been shown to interact with mouse myeloma macroglobulin (49). Anti-dextran antibody synthesized at 3–4 wk by guinea

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[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; PCA, passive cutaneous anaphylaxis.

pigs immunized with highly purified native dextran incorporated in complete Freund's adjuvant was found to be nonprecipitating, to be homocytotropic, and to give PCA reactions best at 18 hr (50). More recently it has been shown to be synthesized in relatively tiny amounts and to be heat stable (Budman, D., and J. R. Battisto, unpublished observations).

The novel finding reported here is that heat-stable homocytotropic antibody to dextran from individual animals sensitizes the skin of some guinea pigs but not that of others. The nonresponse was found to be due to nonfixation of the antibody in the skin. Since the Fc portion of the immunoglobulin is responsible for fixation of the molecule in the skin (51–53), these observations suggest the existence of some type of variability in the fixing sector of Fc sites. The findings also indicate that individuals within the species have different specificities for antibody binding.

Materials and Methods

Animals.—Guinea pigs of six different strains were employed as recipients of dermally deposited homocytotropic and heterocytotropic antibody in these studies. A description of animals of the Abyssinian, Pirbright, Wright XIII, and Hartley strains as well as their sources of availability has already been given (50). Guinea pigs of the Rockefeller University strain are albinos that have been pen-inbred at the Rockefeller University in New York City; those of the Weiser strain are nonalbinos maintained at the National Jewish Hospital, Denver, Colo.

Dextrans.—Three preparations of dextran were used in the work reported here. Two have already been described (50). One is whole native dextran SDD 3580-10 prepared from a sucrose-grown culture of *Leuconostoc mesenteroides* NRRL 512(F) which contains less than 0.01% nitrogen and has a molecular weight of 20–50 million. This dextran is a 1:6, 1:3 α-glucoside and was used for immunizing guinea pigs as well as for challenging guinea pigs in PCA tests.

The second dextran, a 1:6, 1:4 α-glucoside, was synthesized from dextrin by *Acetobacter capsulatum* NCTC 4943. It contains less than 0.05% nitrogenous impurity and has a molecular weight similar to that of 512(F).

The third dextran was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, Lot. No. 7600. It has a molecular weight of 5-40 million and contains nitrogenous impurities approximating 200 μg/mg. The last two mentioned dextrans were used exclusively in comparative PCA tests.

Antisera.—Dextran-specific antibodies used in these studies were derived from guinea pigs of the Abyssinian strain that had been immunized with dextran from *Leuconostoc mesenteroides* NRRL 512(F). The animals were immunized as has been described (50). Briefly, each guinea pig was injected with dextran that had been passed through a Millipore filter (pore size 0.45 μ) and emulsified in Freund's complete adjuvant. Each animal received 1 or 2 mg of dextran (in 1.5 ml saline emulsified with an equal volume of adjuvant) divided between the nuchal region (2.8 ml) and each hind footpad (0.1 ml). Routinely, the animals were skin tested with 125 or 200 μg of dextran (in 0.1 ml saline) at 20–30 days after initiation of immunization and bled 1 wk thereafter.

Rabbit serum containing antibody to pneumococcus Type 2 polysaccharide (preparation kindly supplied by Lederle Laboratories, Pearl River, N. Y.) was also used in one phase of this study. This antibody cross-reacts with dextran in precipitation reactions as well as in PCA

responses of guinea pigs. As a source of heterocytotropic antibody it resulted in PCA reactions measuring 10 × 10 mm when used in 50 ng amounts. In this quantity it was employed as a standard against which guinea pig anti-dextran sera were compared. When guinea pig sera were diluted to extinction, those dilutions provoking PCA reactions equal in size to that given by the standard were assumed to contain 50 ng of antibody.

PCA Tests.—PCA was routinely performed by injecting 0.1 ml of neat or, more often, diluted serum into the dorsal skin of young guinea pigs (250–400 g) and after an 18 hr interval, administering intravenously 2 mg of dextran in 1 ml of saline plus 1 ml of 0.5% Evans blue dye in saline. Averaged millimeters of crossed diameters and intensities of blueing were noted at 30 min.

Iodination of Anti-Dextran Homocytotropic Antibody.—With minor modifications, the method of McConahey and Dixon (54) was used to iodinate dextran-specific antibodies of two types after their adsorption to Sephadex (48). Sephadex (Grade G100, Pharmacia Fine Chemicals, Uppsala, Sweden) was washed 50 times in 0.15 M sodium chloride solution and 3 times in phosphate-buffered saline (PBS). Guinea pig sera A-5691 (type 1) and A-5708 (type 2), each containing approximately 5 μg of antibody/ml, were added in 1.5 ml amounts to separate 1 ml portions of a packed slurry of Sephadex. The mixtures were incubated at 37°C for 1 hr and at 4°C for 18 hr. This procedure was known from our prior experiments to adsorb the homocytotropic antibody. Thereafter the Sephadex was washed three times in excess PBS to rid the supernatant fluid of unadsorbed serum proteins. To achieve mild oxidation and labeling, 10 μg of chloramine-T (Eastman Organic Chemicals, Rochester, N.Y.) and 20 μCi of ^{125}I (Na^{125}I in 0.1 ml NaOH, 20 mCi/ml; carrier-free, Lot 11701, New England Nuclear Corp., Boston, Mass.) were added successively to each tube. Coupling was allowed to proceed for 0.5 hr at 4°C after which the slurry was washed three times with excess PBS. The iodinated antibody was removed from the Sephadex by adding 2 ml of 0.2 M sodium acetate buffer at pH 4.0 and stirring for 0.5 hr. The supernatant was then dialyzed against large volumes of 0.15 M NaCl over a 3 day period at 4°C.

Monitoring the dialysis fluid by scintillation counting at this time showed it contained 50 cpm/ml while the solutions within the dialysis sacs possessed 35,000 cpm/ml for serum A-5691 and 50,000 cpm/ml for serum A-5708.

Preparation of Skin for Scintillation Counting.—Guinea pigs were injected in the dorsal skin with 0.1 ml of the two preparations of ^{125}I-labeled anti-dextran antibodies (300–400 ng/ site). A fixation period of 18 hr was allowed to elapse when the animals were sacrificed by cervical dislocation. Skin directly over each injection site (2 cm^2) was excised and was subdivided into five equal pieces. The pieces were finely minced and placed into separate scintillation vials, to each of which was added 2 ml of NCS Solubilizer (Amersham-Searle Corp., Des Plaines, Ill.). Incubation at 37°C proceeded until complete dissolution of the tissue occurred (generally several days) when 8 ml of scintillation fluid (4 g of Omnifluor [New England Nuclear Corp.] per liter of toluene) was added to each vial. After an additional 24 hr the vials were read on a Beckman Model LS230 scintillation counter. The counts per minute of each of the five subdivided sections minus background counts were added to achieve the total counts per minute per injection site.

RESULTS

During the course of assaying the sera of guinea pigs immunized with highly purified dextran, guinea pigs were found that would not respond with PCA reactions to some sera known to contain heat-stable, homocytotropic, dextran-specific antibodies, while at the same time they exhibited PCA reactions to other sera containing antibodies to the same antigen. The initial observations

were made at random and subsequent reflection upon their relevance to our knowledge of immediate-type sensitivities prompted a thorough investigation of the phenomenon.

Three sera containing anti-dextran antibodies were first assayed for content of antibodies. This was accomplished by comparing their ability to elicit PCA reactions equal to that of a known quantity of rabbit antiserum to pneumococcal Type 2 polysaccharide known to cross-react with dextran. (See Materials and Methods for the manner of antibody estimation.) Equivalent quantities (100 ng) of each of the three sera were then injected into a series of guinea pigs to determine the patterns of response and nonresponse they would evoke under

TABLE I

Typical Variability of Responses to Dextran-Specific PCA Antibodies

Guinea pig		PCA diameters (mm) to anti-serum* type		
Group	Number	1	2	3
1, 2, 3	R457	19	13	25
	R458	26	15	28
1, 3	R448	15	0	26
	R446	12	0	17
2	5745	0	13	ND
3	H1	0	0	18
	H2	0	0	17

ND = not done.

* Each antiserum was first diluted to its end point to determine the quantity of antibody present and then 100 ng/site was used. Average of crossed diameters is given.

comparable conditions. After an 18 hr interval the recipient animals were intravenously injected with 2 mg of dextran and Evans blue dye to develop reactions. Some typical results given in Table I reveal that certain guinea pigs (listed as group 1, 2, 3) were capable of responding to all three sera. Some animals were found to give reactions to sera 1, and 3 but not to 2. A single animal responded to serum 2 but not to 1; and still others reacted only to serum 3 but not to 1 or 2. In this way the original random observations that certain animals supported reactions to some antibodies but not to others that had been produced to the same antigen were amply confirmed.

Of the several explanations that could account for these results, the first tested was that guinea pig recipients might be able to inactivate the homocyto-tropic immunoglobulins of certain donors. To ascertain whether this was true, 2–3 ml of blood was collected from virgin guinea pigs into sodium citrate before their use as recipients of anti-dextran antibody for the PCA test. Some animals

responded with PCA to antiserum type 1 and others did not. The whole, citrated blood of both sorts of animals was mixed in equal proportions with type 1 antiserum containing 500 ng of antibody and allowed to incubate for 20 min at 37°C before centrifugation. The clear supernatants were used for PCA tests in a new set of guinea pig recipients.

From the latter tests it was found that whole blood derived from PCA-negative donors was unable to neutralize (or remove) dextran-specific type 1 antibody (Table II). Comparable PCA reactions were achieved whether the antibody had been mixed with whole blood derived from animals that had or had not demonstrated PCA reactions to antibody type 1. From such information it was possible to eliminate the idea that PCA antibodies were being inactivated in PCA-negative animals.

TABLE II

Inability of Whole Blood from PCA (—) Guinea Pigs to Neutralize Homocytotropic Dextran-Specific Antibody

Type 1 antibody* mixed with whole blood of	PCA reactions in R 203
	(mm)
PCA(+) animal	
H 103	15
V 431	16
PCA(—) animal	
V 415	14
A 5752	20

* Final concentration of antibody injected was 100 ng/site.

An alternate hypothesis involved the possibility that antibodies possessed different specificities. The linear chains in the dextran used for immunization are 1:6 α-glucosidic groups while the branching units are 1:3 α-glucosidic bonds. The possibility existed that the combining areas of the antibodies might have specificities directed to these different areas of the dextran molecule. To test this hypothesis a dextran that lacked 1:3 α-glucosidic bonds was used for PCA tests, so that only antibody directed to the 1:6 configuration was measured.

Guinea pigs were injected with 100 ng of antibody types 1 and 2 and half were challenged with the same dextran used for immunization, i.e. that containing 1:6, 1:3 α-glucosidic bonds, while the other half received dextran composed of 1:6, 1:4 α-glucosidic bonds. Reactions of comparable size were elicited by both antigens (Table III). Thus, the antibodies responsible for divergent PCA results possess essentially a single specificity, i.e., one directed to the 1:6 α-glucosidic configuration of dextran.

A third explanation for the observed disparity in the abilities of antibodies

to evoke PCA reactions could be their differential ability to fix in skin. Fixation is known to be a property of the heavy chain portion of the molecule, the Fc fragment (51). Subtle differences in this segment could result in separate binding abilities which in turn could account for the variable PCA reactions observed.

The use of radioactively trace-labeled homocytotropic antibodies was thought best to test this supposition. Type 1 and type 2 antibodies were labeled with

TABLE III

Typical PCA Responses* Evoked by Two Dextrans with Similar Linear but Different Branching Bonds

Antibody‡		α-glucosidic bonds	
Type	Amount injected	1:6, 1:3	1:6, 1:4
	(ng)		
1	100	17	21
2	100	15	19

* Average of crossed diameters given.
‡ Synthesized to dextran with 1:6, 1:3 α-glucosidic structure.

TABLE IV

Differential Binding of ^{125}I-Labeled Homocytotropic Antibodies

cpm		Type of Antibody	
		1	2
		$(\times 10^3)$	$(\times 10^3)$
Injected dermally (0.1 ml)		3.5	5.0
Recovered* from skin of guinea pigs:			
	R 493	2.9	1.7
	H 711	1.8	1.5

* After 18 hr.

^{125}I and equal portions were injected into the skin of guinea pigs. After an 18 hr incubation, 2 cm^2 segments of dermis surrounding the injection sites were excised, dissolved in a solubilizer, and the retained counts read in a scintillation counter.

The results, shown in Table IV, indicate that preferential binding of the antibodies in some skin does indeed occur. Although an equal number of counts per minute of type 1 antibody were injected into guinea pigs R-493 and H-711, considerably more remained localized in the skin of animal R-493 than were found in the skin of animal H-711. In addition, although comparable amounts

of antibody type 2 (possessing slightly greater specific activity) were injected into the same animals, neither animal demonstrated retention of this antibody to the same extent that animal R-493 retained type 1 antibody. Thus, con-
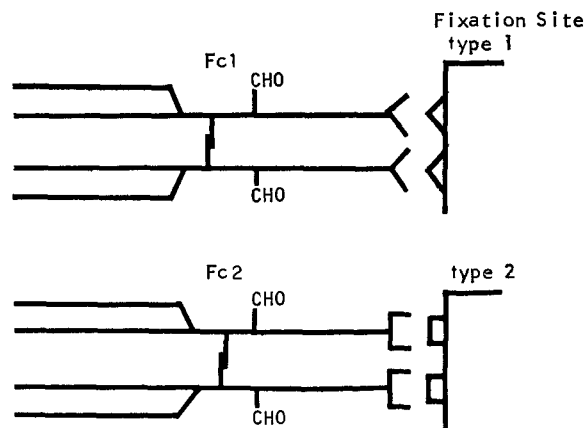

FIG. 1. Schematic representation of specificity between homocytotropic antibody Fc and fixation sites. Only for simplicity is the variability in Fc sites shown at the terminal portion of both heavy chains.

TABLE V

Distribution of Fixation Specificities for Antibody Types 1 and 2 among Guinea Pigs of Diverse Strains

Guinea pig strain	No. of animals fixing			
	Both 1 and 2	Only 1	Only 2	Neither 1 nor 2
Rockefeller University	7	7		4
Pirbright	3	3		3
Wright XIII	4	1		1
Weiser	1	1		
Abyssinian	2	1	1	2
Hartley	2			5
Totals	19	13	1	15
Per cent of total	40	27	2	31

firmation of the hypothesis that differential binding of these antibodies occurs in skin of various animals was established.

To visualize differential binding, Fig. 1 was prepared. Here are schematically shown the variability in the Fc sites of antibodies type 1 and 2 and the complementarity of loci in the skin to which the Fc fix.

To gain some idea of the distribution of antibody-fixation specificities among

guinea pigs, animals of diverse strains were tested for PCA responses to approximately equal amounts of antibodies type 1 and 2. As can be seen from the data of Table V, animals fixing both were present in every strain tested. Those with receptors only for type 1 have been found so far in all strains except Hartley. Only a single animal fixing type 2 but not 1 was detected; it was an Abyssinian guinea pig. All strains studied, except the Weiser strain, had animals incapable of fixing both types of antibodies. From the percentages given at the bottom of Table V it is apparent that about a third of all animals tested are incapable of supporting PCA reactions to the two homocytotropic, dextran-specific antibodies. Slightly more than a third of the animals were seen to respond to both antibodies and slightly less than a third were found to react to only one of the antibodies.

A search was made to determine the frequency of occurrence of the different types of dextran-specific homocytotropic antibodies. Although a large number

TABLE VI

Frequency of Occurrence and Quantity of Dextran-Specific Antibodies Characterized by Differential Binding in Skin Through Fc Sites

| | Antibodies possessing | | | | |
	Fc 1	Fc 2	Fc 3	Fc 4	Fc 5
No. of each detected	4	1	1	1	1
Greatest quantity (μg/ml)*	40	5	40	5	40

* See Materials and Methods for quantitation.

of sera were assayed (Table VI), the complexity of the assay allowed inclusion of only eight sera with certainty. Of the eight, at least half appeared similar to type 1 and the remainder were different from type 1 and from each other. An estimate of the greatest quantity of antibody detected among these sera is also given (Table VI). It is apparent that the quantities of antibody produced are consistently low; amounts greater than 40 μg/ml have not been detected.

DISCUSSION

The novel observation reported here is that individual guinea pig sera containing homocytotropic, heat-stable anti-dextran antibodies elicit PCA reactions in some individuals but not others within the homologous species. Prior experience with similar type antibodies to other more complex antigens, such as bovine gamma globulin and dinitrophenyl bovine fibrinogen, had established that they uniformly sensitize virtually all young guinea pigs for PCA. The remarkable dichotomy between these two sets of observations may be accounted for primarily by the stereochemistry of the antigens used for immunization. On the one hand, dextran represents a chemically homogeneous

substance with a maximum of two antigenic specificities, one for the linear 1:6 glucose configuration and the other for the branched 1:3 arrangement. Since 96% of the dextran molecule is composed of the former, this polysaccharide is probably recognized immunologically as essentially a single antigenic moiety. During immunization it may stimulate only a few immunocompetent cells, thereby giving rise to a restricted population of antibody molecules with a circumscribed specificity for fixing to only certain guinea pig skin. On the other hand, immunization with more complex structures such as proteins could be expected to provide multiple antigenic moieties capable of stimulating many more clones of cells to produce a heterogeneous population of antibody molecules. The latter would possess a broader range of skin-fixing specificities capable of sensitizing the skin of virtually any guinea pig.

The idea that the antibody arises from a single clone of cells stimulated essentially by a single antigenic moiety may also account for the rather small amounts of antibody that have been recoverable. Despite repeated injections of the antigen into some guinea pigs over prolonged intervals of time, amounts of antibody greater than approximately 40 μg/ml were not attained.

Should this interpretation prove to be correct, the use in immunization of synthetic molecules possessing one or two antigenic moieties might be expected to yield homogeneous immunoglobulins with skin-fixing specificities similar to those described here.

Yount et al. (47), in studying the precipitating anti-dextran antibodies elicited in humans, have commented that the striking homogeneity they have observed in these antibodies may possibly relate to the carbohydrate nature of the antigen. Curiously these antibodies were found only in the γG_2 subgroup of immunoglobulins and were virtually incapable of provoking PCA reactions in guinea pigs (55). This particular subgroup lacks the ability to act as heterocytotropic antibodies in the guinea pig primarily because the immunoglobulins do not fix in the skin of this species of animal (56).

To explain the variable sensitizability achieved with anti-dextran antibodies, several possibilities were envisioned and tested. The only one supported by the data collected thus far is that the antibodies in individual sera possess differing capacities to bind in skin. Thus, ^{125}I trace-labeled type 1 antibodies remained at 18 hr fixed in the skin of one guinea pig but not in that of another. Similarly labeled type 2 antibodies were not significantly retained in the skin of either of these animals. These results are comparable to those observed when the nonlabeled antibodies were used for PCA.

Other possibilities that were eliminated were (a) that whenever type 1 or type 2 antibodies were unable to elicit PCA in some guinea pigs they were being neutralized, and (b) that the two types of antibodies possessed different antigen-combining specificities. It was actually found that the antibodies were not neutralized by whole blood taken from PCA-negative animals and that the

antibodies used in these experiments possessed a single specificity directed to the 1:6 α-glucosidic configuration of dextran. Another possibility was (c) that the antibodies possessed different-sized antigen-combining sites. Schlossman and Kabat (57) had shown that anti-dextran–precipitating antibodies produced in humans consisted of populations of molecules heterogeneous primarily with regard to the size of the antibody-combining site. The size of the antigen-binding site of the antibody molecule was found to have an upper limit corresponding to 4, 5, or 6 glucose residues. Richter (58) has confirmed this finding in PCA-inhibition studies using antibody derived from guinea pigs immunized with *Leuconostoc mesenteroides* B512 cell debris. In the work reported here, the size of the antibody-combining sites of the PCA antibody was not studied primarily because the antibodies were always detected by dextran preparations that excluded dextran fragments with molecular weights smaller than 5 million. As the PCA differences were observed under these conditions, size of the antibody-combining site was considered an unlikely explanation.

The portion of an immunoglobulin molecule that results in its fixation in the skin is the Fc fragment (51–53, 56). Although Fc is widely regarded to be the invariant end of the heavy chain, the results reported here suggest that some subtle variability must exist to account for the different fixations observed. These small structural differences may be allotypic variations analogous to Gm allotypes on the Fc fragment of human immunoglobulins (see 59, 60). Indeed, fixation of immunoglobulins to tissue may be one of the functions served by allotypy. In the work reported here, antibodies with five different Fc sites have been distinguished, but one was encountered more frequently than the others. Thus far the exact area and composition of the Fc segment responsible for fixation of the antibody molecule in skin is unknown. However, it is known to be degradable by proteolysis and to be rendered incapable of fixation to skin after treatment of the antibody molecule with mercaptoethanol (52, 53).

The data presented here also suggest that complementariness to the variable Fc of immunoglobulins must exist at loci where antibodies become fixed. Several fixation specificities must be present in each animal. Although at least five types have been observed in guinea pigs, additional ones may exist and, indeed, the total is unknown. Whether there is heritable control over the fixation specificities and whether they are related to histocompatibility antigens are questions presently under consideration.

Differing receptor specificities for homocytotropic antibodies of the IgE class may also exist. Kisil et al. (61), studying the Prausnitz-Küstner response in volunteers observed that activity profiles obtained with the same allergic sera in different volunteers were not identical. They suggested that reagins are heterogeneous with respect to skin-fixation properties. Whether fixation specificities are present among individuals of a species for heterocytotropic

antibodies is unknown. Also unknown is whether an individual always possesses fixation specificities for the Fc of his own homocytotropic antibodies. If this were a random event, then an explanation would be at hand for the presence of sensitizing antibodies within an individual who himself lacks clinical signs of sensitivity for an homologous allergen.

SUMMARY

The heat-stable, homocytotropic antibody synthesized by guinea pigs to highly purified dextran has been studied by passive cutaneous anaphylaxis (PCA). Antibody arising in single guinea pigs was found to give PCA in some homologous recipients but not in others. Data are presented which indicate that the antibodies are bound in the skin of some animals to a greater degree than in that of others. This suggests some type of variability, perhaps allotypic, in the Fc portion of these immunoglobulins which relates to specificities of the loci where Fc becomes fixed in skin.

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