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Genetics

Human immunoglobulin allotypes: Previously unrecognized determinants and alleles defined with monoclonal antibodies

(human genetics/immunogenetics/isoallotypic markers/radioimmunoassay)

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ABSTRACT **The highly polymorphic system of serologically defined genetic markers on human IgG heavy chains (Gm allotypes) is second only to the HLA complex in terms of the large number of determinants, alleles, and haplotypes that can be used for analyses of disease associations and other genetic studies. However, present typing methods are based on the use of anti-Gm antisera that are derived mainly from fortuitously immunized human donors, often requiring processing before use, and must be used in a hemagglutination-inhibition assay that cannot be used in typing for isoallotypic determinants (currently termed "non-markers"). In studies presented here, we describe an allotyping system that utilizes monoclonal antibodies in a "sandwich" modification of the solid-phase radioimmunoassay, which is capable of reliable quantitative typing of allotypic, isoallotypic, and isotypic immunoglobulin determinants. We show that these highly reproducible, easily disseminated, and essentially inexhaustible reagents can be used for rapid, sensitive, and quantitative Gm typing. Using this system we define two previously unrecognized Gm determinants, one of which, found to date only in Caucasians, is different from all known Gm markers and thus defines previously unrecognized alleles and haplotypes. The other determinant cosegregates with the conventional G3m(b1) marker but is distinct from that marker on serological grounds. The successful preparation of mouse monoclonal antibodies that detect human Gm allotypic differences and the development of an assay system capable of typing isoallotypic as well as allotypic determinants opens the way to further dissection and application of this rich genetic system.**

Human immunoglobulin polymorphisms (Gm, Am, and Km allotypes) offer a broad range of serologically detectable markers for human genetic studies. The large number of alleles, particularly at the IgG1 and IgG3 loci, identify a series of geographically distributed immunoglobulin heavy chain (Igh) haplotypes that make the Igh region second only to HLA antigen in complexity (1, 2). Recently a number of associations of certain diseases with particular Gm markers have been reported (3–11). However, difficulties inherent in the current standard Gm typing methods limit the use of this genetic marker system. Because the typing sera are mainly derived from fortuitously immunized human donors, they are relatively scarce and sometimes impossible to replace when consumed. Furthermore, except in a few cases (12, 13), the characteristics of these reagents dictate their use in a hemagglutination-inhibition typing assay that depends on visual scoring and requires additional sources of human cells and "target" Rh antisera from a variety of donors (1).

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Most of the antisera produced by inter-species immunizations have not been useful, principally because most genetic markers detected by these antibodies cannot be scored by the standard hemagglutination-inhibition assay. These antibodies, in fact, detect "isoallotypic" determinants that are polymorphic on one of the Ig isotypes (subclasses) in human sera but are monomorphic (always present) on other isotypes. Therefore, they can only be used to identify genetic differences between individuals when the polymorphic isotype is isolated from the serum samples prior to assay. Because this prohibits the typing of large numbers of samples by current methods, these determinants are now classified as "non-markers" and named accordingly—e.g., non-G3m(g) (1). Thus, a whole series of genetic markers have been inaccessible for lack of an appropriate assay system. Past attempts to develop radioimmunoassays (RIAs) have met with only variable success, primarily due to problems in purifying and radiolabeling antibodies from conventional antisera (12, 13). The mouse monoclonal anti-Gm typing reagents and RIA typing methods described here signal an end to these difficulties. These reagents are essentially inexhaustible and can be broadly disseminated while the use of solid-phase RIA assays eliminates the need for additional cells and target antisera required in the hemagglutination-inhibition assay. Furthermore, this technology allows "in-well" isolation of individual isotypes from serum samples and subsequent scoring for allotypic (or isoallotypic) determinants on the isolated isotype. Therefore, because non-markers and markers become operationally equivalent, these reagents and methods open the way for simplification of the current Gm notation and, more significantly, for exploitation of the full genetic potential of the Gm system by allowing individuals to be typed for the hitherto inaccessible isoallotypic determinants.

The IgG heavy chain constant region structures that define the four known IgG isotypes and the multiplicity of Gm allotypes found in human sera are encoded by a series of closely linked genes in the Igh chromosome region. Alleles of a given gene code for similar but serologically distinguishable heavy chains that have the same isotypic (monomorphic) determinants and (sets of) different allotypic (polymorphic) determinants that define the individual alleles. Because crossovers between IgG loci are very rare, genetic events that create new determinants and alleles often accumulate on individual chromosomes, and recombinations that do occur tend to create new haplotypes that

Abbreviations: RISA, radioimmuno sandwich assay; RIA, radioimmunoassay; Igh, immunoglobulin heavy chain; b1, G3m(b1); b1i, G3m(b1i); b6i, G3m(b6i); P_i/NaCl, phosphate-buffered saline.

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are stably maintained. As a result, a series of distinctive Igh haplotypes have evolved (1).

In this publication, we demonstrate that mouse monoclonal antibodies against human immunoglobulins provide Gm typing reagents that can be used in radioimmuno "sandwich" assays (RISAs) to detect determinants in the human Igh chromosome region. One of these monoclonal antibodies identifies an (iso)allotypic determinant that cosegregates with the well-characterized and broadly distributed "conventional" G3m(b1) (b1) allotypic determinant. The second identifies a previously unrecognized (iso)allotypic determinant on IgG3 molecules, which is associated with b1 in most, but not all, individuals of Caucasian origin, but which rarely occurs in other human populations. The IgG3 polymorphism revealed by the latter monoclonal reagent "splits" previously known Gm alleles and haplotypes marked by the presence of b1 and provides new insights into the origins of human immunoglobulin genes.

METHODS

Solutions. The solutions were phosphate-buffered saline (P_i/NaCl) (0.15 M NaCl at pH 7.2) and RIA buffer (1% bovine serum albumin in P_i/NaCl).

Production of Monoclonal Antibodies to Human Immunoglobulins. SJL or BAB/14 (Igh-b) mice were immunized intraperitoneally with 50 μg of human myeloma IgG3 protein of known allotype on alum and 10^9 killed *Bordetella pertussis* organisms in 200 μl of sterile P_i/NaCl. To avoid generation of antibodies to idiotypic determinants, mice were given booster injections 3 days prior to fusion with a myeloma protein (10 μg) of the same allotype and isotype but of different idiotype. Spleen cells from these mice were fused with the murine myeloma cell line NS-1 (14, 15). Antibody-producing hybrids were cloned using the fluorescence-activated cell sorter with previously described methods (16). Monoclonal antibodies were purified from serum or ascites of hybridoma-injected mice by 50% ammonium sulfate precipitation and gel filtration chromatography (17). The proteins were characterized by two-dimensional gel electrophoresis (18). The production of antibody 3.18 (formerly named 18.1) has been described (19).

RIA binding assay for preliminary screening of hybridoma supernates. Human myeloma proteins (20 μg/ml in P_i/NaCl) of known isotype and allotype were adsorbed to the wells of polyvinyl chloride U-bottom microtiter plates for 1 hr at room temperature. After thorough rinsing of the plate with RIA buffer, hybridoma supernates were added and incubated for 1 hr. The plates were rinsed again, and a mixture of ^{125}I-radiolabeled monoclonal anti-mouse immunoglobulin was added to detect bound antibodies. The isotype of the monoclonal antibodies in the supernates was revealed by incubating with individual ^{125}I-labeled anti-mouse allotype antibodies, rather than with the mixture as above (20).

The RIA competition assay for analysis of monoclonal antibody reactivity pattern was performed as described (19, 20). Briefly, microtiter wells were coated with purified antibody. Graded amounts of highly purified, unlabeled myeloma proteins were then added to the well and followed immediately with a fixed amount of ^{125}I-labeled myeloma protein (sufficient to saturate the antibody binding sites coated on the well). When the test protein carries the determinant detected by the coating antibody, it competes for binding sites to prevent binding of the radiolabeled antigen.

The RISA for allotype serotyping developed for these studies provides a rapid and efficient micromethod for isolating a desired isotype from a serum sample and typing it for allotypic determinants present on that isotype. First, a monoclonal antibody that detects an isotypic determinant present on all human IgG3 molecules is adsorbed to the wells of microtiter plates at a concentration of 20 μg/ml by overnight incubation at 4°C. Serial dilutions (1:100–1:800) of serum samples in RIA buffer are then added to the coated wells and incubated for 1 hr at room temperature to allow the IgG3 to bind. Finally, the wells are washed with RIA buffer to remove the unbound serum proteins and one of the anti-allotypic monoclonal antibodies or the anti-isotypic monoclonal antibody (labeled with ^{125}I) is added to duplicate wells. The amount of bound ^{125}I present on the well after incubation and washing was assayed in a gamma counter as a measure of the amount of allotype or isotype present on the IgG3 molecules in the serum sample. The basic RIA procedures and materials used here have been described (20).

RESULTS

The RIA reactivity patterns of the monoclonal anti-Gm antibodies shown in Table 1 demonstrate that three antibodies (3.41, 3.40, and 3.18) react selectively with myeloma proteins of certain IgG3 allotypes. These antibodies also react with myeloma proteins of other IgG isotypes; however, they show isotype-specific rather than allotype-specific reactivity patterns with these latter proteins. In addition, because serum contains the invariant isotypes, these antibodies react with unfractionated serum Ig from all individuals. Thus, each of these monoclonal antibodies detects isoallotypic determinants that are polymorphic on IgG3.

The remaining two antibodies shown in Table 1 (C3-8-80 and 3.01) detect isotypic determinants—i.e., they react with all IgG3 myeloma proteins but not with myeloma proteins of other IgG isotypes. When coated onto microtiter wells, these antibodies can selectively retain IgG3 from any human serum sample applied. This rapid and reliable in-well purification method en-

Table 1. Isoallotypic reactivity of anti-G3m monoclonal antibodies

	Human myeloma proteins*										
	IgG1		IgG2		IgG3						
Monoclonal antibody	zax (5)	f (3)	n+ (2)	n− (2)	b† (5)	g (3)	IgG4 (4)	IgA1 (4)	IgA2 (4)	IgM (6)	Whole human sera‡
3.18	−	−	+	+	+	−	wk	−	−	−	+
3.41	+	+	+	+	+	−	+	−	−	−	+
3.40	+	+	+	+	+	−	+	−	−	−	NT
C3	−	−	−	−	+	+	−	−	−	−	+
3.01	−	−	−	−	+	+	−	−	−	−	+

wk, Weak reactivity; NT, not tested.
* Isotype, allotype, and number tested (in parenthesis) are shown.
† b = G3m b0,b1,b3,b4,b5.
‡ Serum samples from >35 individuals, representative of different haplotype, tested in each case.

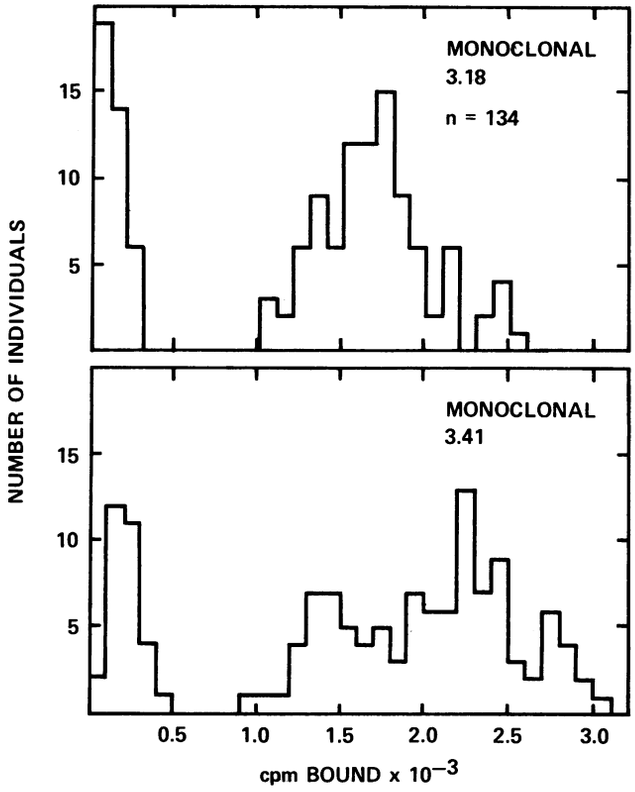

FIG. 1. Distribution of individual levels of G3m(b6i) (b6i) (*Upper*) and G3m(b1i) (b1i) (*Lower*) as measured by monoclonal antibodies 3.18 and 3.41, respectively. Samples were compared at sera dilutions that yielded 2,500-3,000 cpm bound when tested with the anti-IgG3 (isotypic) monoclonal antibody C3.

ables Gm typing with reagents such as the monoclonal antibodies discussed above that detect isoallotypic determinants on IgG3. Furthermore, by using one of the anti-isotypic monoclonal antibodies as the radiolabeled "second step," it allows accurate measurement of IgG3 serum levels. Thus, all samples can be studied at dilutions at which similar amounts of IgG3 bind to the anti-IgG3 plate-coat reagent, preventing differences in serum IgG3 levels from interfering with typing.

Data from a representative set of serum samples typed for IgG3 (iso)allotypic determinants with this RISA demonstrate the clear distinction obtained between positive and negative sera—i.e., all samples classed as positive bind substantially more radioactivity than any of the samples classed as negative (see Fig. 1). The broad range in the amount of binding of radiolabeled typing reagent by the positive samples is partly due to experimental variation, but mainly reflects the mixture of allotype homozygotes and heterozygotes in the test population as well as other possible sources of genetic variation (preliminary observations).

Typing for Currently Known IgG3 Alleles. Data from >350 individual sera typed both with the monoclonal reagents (by RISA) and conventional Gm typing antisera (by hemagglutination-inhibition) show that the expression of the IgG3 (iso)allotypic determinants detected by antibodies 3.41 and 3.40 accords uniquely and completely with that of the conventionally defined b1 specificity (see Tables 2 and 3). These monoclonal antibodies do not detect the conventional b1 epitopes, because b1 is an allotypic determinant present only on IgG3 molecules, whereas the determinant we detect is an isoallotypic determinant polymorphic on IgG3 but also present on other human IgG isotypes. Thus, the reactivity of these reagents defines a previously unrecognized determinant, provisionally named b1i. However, because b1 and b1i are expressed concordantly in all (n = 388) individuals thus far tested, the monoclonal reagents and RISA methods that detect b1i effectively substitute for the conventional antisera and hemagglutination-inhibition assays used currently to type individuals for expression of the IgG3 alleles that encode b1.

Detection of Previously Unrecognized IgG3 Alleles. The distribution of the determinant detected by the 3.18 monoclonal antibody reveals the existence of previously unrecognized alleles and haplotypes in the Gm system. As shown in Table 2, this determinant, provisionally named b6i, is found only in b1-positive individuals but is not present in all such individuals. Therefore, b6i is genetically distinguishable from b1 and must be encoded by a subset of the IgG3 alleles that code for b1.

We found allele(s) coding for b1 but not b6i (b1+ b6i−) in Oriental and African populations and, at low frequencies, in Caucasian populations. In contrast, allele(s) coding for both determinants occur mainly, or perhaps exclusively, in Caucasian populations and represent the predominant allele(s) in these populations (see Table 3). On the basis of the data we have collected thus far, individuals from Oriental and African populations seldom (if ever) produce IgG3 molecules bearing the b6i determinant.

In Caucasians, the IgG3 allele(s) that code for b1 and b6i also code for a series of other IgG3 allotypic determinants commonly referred to as the G3m b complex (b0b1b3b4b5). Like b1, all of these latter determinants can be found in b6i-negative individuals (see Table 3). Thus, b6i constitutes a unique Gm determinant whose expression on IgG3 immunoglobulins splits the currently known G3m b alleles in Caucasians into a pair(s) of alleles. Both members of each pair code for the G3m b complex but only one of each pair codes for b6i. Segregation data from family studies with >200 individuals in 25 kinships accord with this conclusion (unpublished data). Genomic DNA from the same families was studied for *Sst* I restriction enzyme site polymorphisms in regions homologous to the immunoglobulin mu-switch sequences (21). Comparison of these results with our Gm typing shows that the b1i-positive, b6i-negative haplotypes are associated only with a subset of the rarest *Sst* I haplotypes.

In our Caucasian sample there are about 69% b1i, b6i-positive, 2% b1i-positive, b6i-negative, and 29% g positive, whereas

Table 2. Typing for b1 with monoclonal antibodies

		3.40 and 3.41 phenotype*				3.18 phenotype	
Gm typing method	b1 phenotype	Positive	Negative	Gm typing method	b1 phenotype	Positive	Negative
Conventional†	+	216	0	3.41 RIA*	+	191	25
	−	0	39		−	0	39

*Measured by RISA; pooled data for 255 individuals from population and family studies; sample includes a total of 107 unrelated individuals.
†Hemagglutination-inhibition typing with conventional anti-Gm allotype sera. Nearly all typing was confirmed by a restriction-site (DNA) polymorphism analysis that correlates strongly with Gm haplotypes (20).

Table 3. Monoclonal antibody reactivity with sera from individuals expressing different combinations of IgG3 markers

Population	IgG3 phenotype												Number tested
	b0	b1	b1i*	b3	b4	b5	b6i*	g	c3	c5	s	t	
Caucasian	+	+	+	+	+	+	+	−	−		−	−	3
	+	+	+	+		+	+	−	−		−	−	6
	+	+	+	+		+	+	−					155
	+	+	+	+		+	+	+	−		−	−	4
	+	+	+	+		+	+	+					115
	+	+	+	+		+	−	+					3
	−	−	−	−	−	−	−	+			−	−	2
	−	−	−	−	−	−	−	+					31
Oriental	+	+	+	+	+	+	−	−	−		−	−	3
	+	+	+	+	+	+	−	−					3
	+	+	+	+	+	+	−	+	−		−	−	8
	+	+	+	+	+	+	−	+			−	+	1
	+	+	+	+	+	+	−	+			+	+	3
	+	+	+	−	+	+	−	+					1
	+	+	+	−	−	−	−	+					3
	+	−	−	+	−	+	−	+	−		+	+	3
	−	−	−	−	−	−	−	+			−	+	1
	−	−	−	−	−	−	−	+	−		−	−	4
	−	−	−	−	−	−	−	+					13
African	+	+	+	+			−	−	+	+	+		3
	+	+	+	+			−	−	+	+	−		8
	+	+	+	+			−	−	−	−	+		4
	+	+	+	+			−	−	−	+	−		2
	+	+	+	+			−	−	−	−	−		6
	+	+	+	−			−	−	+	+	−		2
	−	−	−	−			−	+	−	−	−		1

Blank, not tested; +, positive; and −, negative.

* Determinants detected by RISAs with monoclonal antibodies (b1i, 3.41; b6i, 3.18). Other determinants were scored by hemagglutination-inhibition.

no b6i-containing haplotypes occur in our Oriental or African samples. Thus, the b6i alleles seem to provide a sharp distinction between b1i alleles of differing ethnic origin, separating Caucasian from non-Caucasian b1i-positive individuals. Despite the small Oriental and African samples, the difference from Caucasian is highly significant.

DISCUSSION

The two Gm determinants recognized by the monoclonal antibodies described here are prototypes of the classes of determinants that future interspecific monoclonal reagents can be expected to detect—that is, some reagents will detect determinants that substitute for currently known Gm markers, whereas others will detect previously unknown determinants. For example, the b1i determinant (detected by the 3.40 and 3.41 reagents) is expressed concordantly with the classical b1 determinant. Because b1 is one of the most prevalent and well studied of the Gm determinants, this determinant provides a reference point that may facilitate transition to monoclonal-based RIA assays for Gm.

In contrast, the b6i determinant (detected by the 3.18 antibody) is distinct from any known G3m determinant and reveals a new level of complexity among Gm haplotypes. It is found only in individuals that express the G3m b complex of determinants; however, it is not found in all such individuals. As we have shown, IgG3 allele(s) that code for the G3m b complex in conjunction with b6i predominate in Caucasian populations but are absent (or are very rare) in Oriental and African populations. Thus, in addition to defining previously unrecognized IgG3 allele(s) and Gm haplotype(s), this determinant defines a previously unrecognized split among the G3m b complex-positive individuals in Caucasian populations and distinguishes the IgG3 allele(s) expressed in the majority of such individuals from the allele(s) expressed in Oriental and African populations and in a small minority of Caucasians.

Curiously, the b6i determinant is highly conserved throughout vertebrate evolution and occurs in reptiles, Aves, and mammals (19); however, its representation on immunoglobulins varies considerably. In the human, it is present as an isoallotypic determinant polymorphic on IgG3, is monomorphic on IgG2, and is absent on other isotypes. In contrast, in the mouse it is present as a simple allotypic determinant that distinguishes the Igh-4a and Igh-4b allotypes on IgG1 (22). In fact, the 3.18 antibody that detects this determinant was derived from a mouse alloimmunization (unlike the 3.40 and 3.41 monoclonal reagents) and was isolated originally because it identified the mouse allotypic difference.

We have had to break with the established World Health Organization notation for human immunoglobulins (23) in naming the isoallotypic Gm determinants identified here. The World Health Organization notation defines isoallotypic determinants as non-markers and requires placement of the prefix "non-" before the determinant name. This prefix is clearly inappropriate for determinants that, as we have shown, constitute perfectly valid genetic markers when used with an appropriate (RISA) assay. Thus, we have provisionally named the previously unrecognized determinants in accord with the World Health Organization notation for allotypic Gm markers and have added the suffix "i" to indicate that the determinant is isoallotypic—i.e., b1i and b6i.

In summary, the data we have presented define previously

unrecognized Gm determinants, alleles, and haplotypes. These will help us to understand the origins of the immunoglobulin genes in human populations. On a technical level, these findings demonstrate conclusively that the mouse is capable of producing the kinds of monoclonal reagents needed for Gm typing. Thus, they open the way for development of a bank of monoclonal anti-allotype reagents that should complement and, in some cases, replace conventional anti-Gm antisera. Further, the RISA typing assay we describe (or perhaps an equivalent enzyme-linked immunosorbent typing assay) introduces the capability for easy typing of (formerly inaccessible) isoallotypic as well as allotypic determinants from whole human sera. These easily disseminated and essentially inexhaustible monoclonal reagents provide the potential for widespread and easier investigations of human immunoglobulin and related polymorphisms.

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