RADIOIMMUNE ANALYSIS OF AN UNEXPECTED MOUSE ALLOTYPE*

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In 1974 we reported the transient appearance of an unexpected mouse allotype (1). An antigen indistinguishable from the BALB/c \( \text{IgG}_{2a} \) allotype (\( \text{Ig-la} \)) was detected in a congenic partner strain of BALB/c (C.B-17). However, C.B-17 mice were bred to exclude \( \text{Ig-la} \) and were typed homozygous for the gene that controls production of the \( \text{IgG}_{2a} \) allotype of C57BL/Ka mice (\( \text{Ig-la} \)). Because \( \text{Ig-la} \) was normally undetectable in C.B-17 mice, we referred to it as a hidden allotype (\( \text{Ig-la}' \)).

Other investigators have also noted nonallelic behavior of Ig allotypes. Lobb (2) and Rivat et al. (3) reported that human blood lymphocytes stimulated with phytohemagglutinin, or in mixed lymphocyte cultures, produce \( \text{Gm} \) allotypes not found in the serum of the donor, although it should be added that other laboratories (4, 5) have not been able to confirm this work. Pothier (6) observed that Syrian hamsters injected with human lymphoid tumors produce human allotype markers; one such recipient animal was briefly positive for \( \text{Gm} (a) \) and \( \text{Gm} (z) \), even though the injected cells came from a \( \text{Gm} (a-\) donor. For rabbits, too, there are examples in which allotypes not anticipated from genetic data have been detected. Strosberg et al. (7) first noted the appearance of three group \( a \) allotypes and three group \( b \) allotypes in a rabbit immunized against \textit{Micrococcus lysodeikticus}. Mudgett et al. (8) followed with the observation that normal rabbits of the \( a_2 \) allotype sometimes show small quantities of \( a_1 \) and \( a_3 \) in their serum (\( \approx 1\% \) of the \( a_2 \) concentration). Further, the examination of over 119 rabbits showed that very low concentrations of \( a_1, a_2, \) or \( a_3 \) allotypes could be found in rabbits of all nominal allotype combinations. Such hidden or latent allotypes appeared transiently in microgram quantities (per milliliter of serum) in \( \approx 50\% \) of the rabbits examined (8). Latent rabbit allotypes for both variable and constant regions have now been observed in several laboratories (9, 10, 11).

All of the preceding serological observations are difficult to interpret because anti-allotype sera are likely to contain subspecificities of antibody directed against different allotypic determinants. This is illustrated for rabbit allotypes by the findings of van der Loo et al. (12) and Mage et al. (13); namely, that anti-\( a_2 \) made in \( a_1a_3 \), but not in \( a_1a_1 \) or \( a_2a_2 \) rabbits, can see \( a_2 \)-like determinants in hyperimmunized \( a_1a_3 \) rabbits. This means that it is critical to distinguish whether a hidden allotype is in fact a leaky

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Abbreviations used in this paper: BSA, bovine serum albumin; CH, heavy-chain-constant; C.B-17, congenic partner strain of BALB/c mice; Ig-la, BALB/c \( \text{IgG}_{2a} \) allotype; \( \text{Ig-la}' \), a hidden allotype, indistinguishable from the BALB/c \( \text{IgG}_{2a} \) allotype; \( K' \), relative binding affinity constant of \( [\text{125I}]\text{Ig-la}' \); \( K \), relative binding affinity constant of \( [\text{125I}]\text{Ig-la} \); RH, allelic regulator; TcS, cytotoxic or suppressor T cells; VH, heavy-chain variable.
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nominal allotype derived from a repressed gene or cell, or a subspecificity (cross-
reaction) undetectable by conventional typing methods. Mindful of this problem,
Mudgett-Hunter et al. (11) have recently applied more stringent assays to measure
latent allotypes; they conclude that detection of such antigens cannot be attributed
to weak cross-reactions or to technical artifact.

Relevant to the above, we have used a radioimmune assay to analyze the Ig-1a'
found in two pooled serum samples from different Ig-la-congenic BALB/c strains, one
from C.B-17, and the other from BAB/14 mice. We report direct measurements of
competitive and noncompetitive binding of Ig-1a' to specifically purified anti-Ig-1a.
Our results indicate that Ig-1a' and Ig-1a are serologically identical.

Materials and Methods

**Mouse-Strain Allotypes.** Ig allotypes of BALB/c (Igα) and C57BL (Igβ) are noted according to
the nomenclature of Herzenberg et al. (14). Of particular interest is the IgGα subtype
of BALB/c (Ig-1a) because an antigen indistinguishable from Ig-1a (Ig-1a') was detected in
certain 7S Ig preparations of BAB/14 and C.B-17 pooled mouse sera. As allotype-congenic
partners of BALB/c mice, the latter two strains were made homozygous after 14 and 17
backcross generations for C57BL/6J genes that control production of IgG allotypes (i.e., Ig-1b,
Ig-2b, etc.)

**7S Ig Preparations.** Ig-1a containing 7S Ig of BALB/c and of C.B-17 in which Ig-1a-
producing plasmocytomas (MOPC 173, LPC-1 or UPC-10) were growing, was obtained by
precipitating serum Ig with 50% (NH4)2SO4 and passing it over DEAE-cellulose columns (2.5
× 30 cm). The columns were equilibrated with 0.02M PO4 buffer, pH 7.4. 7S Ig of C.B-17,
BAB/14, C57BL/6J (B6), and of C57BL/Ka was obtained in the same manner. BAB/14 (pool
D) and C57BL/Ka pool sera were gifts from Dr. M. Weigert of the Institute for Cancer
Research, the Fox Chase Cancer Center, Philadelphia, Pa. and Dr. M. Potter of the National
Cancer Institute, Bethesda, Md., respectively. Fc and Fab fragments of papain-digested M173
were isolated according to Fahey and Askonas (15).

**Radiolabeling of 7S Ig.** The labeling of 7S Ig (220 μg) with 131I or 125I was according to the
chloramine T method of Hunter (16). This was done in sealed V-shaped vials containing 2 mCi
of isotope (New England Nuclear, Boston, Mass.). To remove unlabeled isotope and small ra-
diodamaged protein products, each preparation was passed over a column of Sephadex G-50
(1 × 35 cm, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) topped by
a mixed bed (2 cm) of ion-exchange resin (Amberlite IR 4B, Mallinckrodt Inc., St. Louis, Mo.;
and Bio-Rad AG, 1 × 8 cm, Bio-Rad Laboratories, Richmond, Calif.). The resulting prepa-
ratations showed specific activities ranging from 8 to 12 × 10^6 cpm/μg protein.

**Radioimmune Assay**

**Construction of Ig-1a-reactive wells.** Microtiter wells (Cooke Engineering Co., Alex-
andria, Va.) were made specifically Ig-1a-reactive by a procedure described elsewhere (17).
This entailed successive additions, incubations, and washings as indicated: (a) 50 μl of 0.01%
bovine serum albumin (BSA) containing 1 μg of Ig-1a or Ig-1a Fc (from M173) for 1 h; (b) 200
μl of 0.5% BSA for 0.5 h; (c) 50 μl of a 1:200 dilution of B6 anti-Ig-1a serum (1) for 2 h; (d)
200 μl of 2.5 × 10^-3 M glutaraldehyde for 0.5 h; and (e) 200 μl of 0.5% BSA for 0.5 h. Control
wells for nonspecific binding were coated in step a with BSA only or BSA containing 2 μg of B6
7S Ig; subsequently, the wells were treated as indicated in steps b-ε.

**Noncompetitive binding of [129I]Ig-1a or [131I]Ig-1a' to Ig-1a-reactive wells.** The binding
of [129I]Ig-1a or [131I]Ig-1a' at 4°C was measured under conditions of antibody and antigen
excess. Increasing quantities of labeled antigen were added to Ig-1a-reactive wells; 16 h later,
the wells were washed, cut out, and analyzed for radioactive counts (gamma counter, IN/US
Service Corp., Fairfield, N.J.). All counts per minute values were corrected for nonspecific
binding which usually amounted to <1% of the total counts per minute added.

**Competitive binding of [125I]Ig-1a and [131I]Ig-1a' to Ig-1a-reactive wells.** Competitive
binding between a fixed quantity of [129I]Ig-1a (2.5 μg/well) and varying quantities of Ig-1a or
Ig-la’ was done at 4°C in a reaction vol of 50 μl; 16 h later, the wells were washed, cut out, and counted.

By using different labeled forms of both competitors ([125I]Ig-la and [131I]Ig-la’), direct measurements of their binding could be made. For this, increasing concentrations of each were mixed together and added in 50-μl vol to Ig-la-reactive wells.

Results

Detection of Ig-la’ in 7S Ig from Pooled Serum of C.B-17 and BAB/14 Mice. Five different preparations of 7S Ig, three from pooled sera of C.B-17 (A, B, C), and two from pooled sera of BAB/14 mice (D, E), were tested for Ig-la’. The C.B-17 pools were obtained from different groups of mice (45 or more, 3–6 mo of age) at different times in the year 1976. The two BAB/14 pools were also collected at different times: pool D came from a BAB/14 colony maintained at The Salk Institute (La Jolla, Calif.) and pool E from members of the same BAB/14 colony maintained at the Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pa. Fig. 1 shows that the A and D preparations could completely inhibit the uptake of [125I]Ig-la to Ig-la-reactive tubes. The slopes of competitive curves A and D were the same as those obtained with Ig-la myeloma protein (M173) and 7S Ig of BALB/c. However, A and D were shifted to the right of M173 by ≈3 orders of magnitude, suggesting these preparations contained only ≈0.1% of the Ig-la found in M173. Another C.B-17 preparation (B) not shown, was five times less effective as a competitor than the one

![Antigen competition in Ig-la-reactive wells between 50 μg/ml of [125I]Ig-la (from M173) and varying concentrations of unlabeled Ig-la of M173 (△); Ig-la Fab of M173 (▲); 7S Ig of BALB/c (○); 7S Ig of C.B-17, preparation A (×); 7S Ig of BAB/14, preparation D (■); 7S Ig of C57BL/ Ka (□); and 7S Ig of C57BL/6 (■). The percentage of [125I]Ig-la bound relative to control (no competitor added) is shown as a logit (ln [% control/100-% control]) vs. log of unlabeled competitor added.](image-url)
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Table I

Proportion of Ig-la' in Preparation A of Fig. 1; Estimation by Addition of Limiting Quantities of \(^{125}\text{I} \)A into Ig-la-Reactive Wells*

| Experiment | \[^{125}\text{I} \)A Nanograms added | \[^{125}\text{I} \)A Counts per minute added | \[^{125}\text{I} \)A Counts per minute bound + \[^{125}\text{I} \)A Counts per minute added |
|------------|-------------------------------------|------------------------------------------|--------------------------------------------------|
| IV         |                                    | \( \times 10^5 \)                          | \( \times 10^3 \)                                  |
|            | 40                                  | 2.32                                     | 0.35                                              |
|            | 100                                 | 5.81                                     | \( \times 10^3 \)                                 |
|            | 200                                 | 10.75                                    | 0.35                                              |
|            | 500                                 | 26.60                                    | 0.35                                              |
|            | 1,000                               | 50.33                                    | 0.26                                              |
| V          |                                    |                                          |                                                  |
|            | 27                                  | 2.21                                     | 0.11                                              |
|            | 55                                  | 4.37                                     | 0.10                                              |
|            | 109                                 | 8.63                                     | 0.12                                              |
|            | 219                                 | 16.53                                    | 0.09                                              |
|            | 438                                 | 32.91                                    | 0.11                                              |
|            | 875                                 | 65.94                                    | 0.13                                              |

* Each count per minute value is the mean of triplicate determinations. The value for counts per minute bound was corrected for nonspecific uptake.

shown (A). The remaining two preparations (C, E) were not inhibitory in this assay; neither were 7S Ig preparations of B6, C57BL/ Ka, or Fab fragments of M173. Additional controls to show that specificity of this assay system is restricted to Ig-la have been reported elsewhere (17, 18).

Noncompetitive Binding of \(^{125}\text{I} \)Ig-la' and \(^{125}\text{I} \)Ig-la to Ig-la-Reactive Wells. A direct estimate of the quantity of Ig-la' in preparation A of Fig. 1 was made as follows. Limiting dilutions of \(^{125}\text{I} \)A were added to Ig-la-reactive wells. As anti-Ig-la was in excess, the percentage of \(^{125}\text{I} \)A bound was independent of the amount added. Table I shows representative results of five such experiments. As can be seen, 0.1-0.3% of \(^{125}\text{I} \)A was bound, which is consistent with a quantitative interpretation of the results in Fig. 1. With the same kind of procedure, the percentage of Ig-la in 7S Ig of BALB/c and in preparations of M173, U10, and LPC-1 was estimated; the values were 24, 58, 64, and 91% respectively (not shown).

Competitive Binding of \(^{125}\text{I} \)Ig-la' and \(^{125}\text{I} \)Ig-la to Ig-la-Reactive Wells. To examine whether the affinity of anti-Ig-la for Ig-la' was comparable to its affinity for Ig-la, we labeled preparation A (of Fig. 1) with \(^{131}\text{I} \) ([\(^{131}\text{I} \)Ig-la']) and the Ig-la myeloma protein of U10 with \(^{125}\text{I} \) ([\(^{125}\text{I} \)Ig-la]). The simultaneous addition of both labeled antigens to Ig-la-reactive wells enabled us to measure their binding under conditions in which each would have to compete with the other. The quantity of reactive \([^{131}\text{I}]\text{Ig-la'}\) and \([^{125}\text{I}]\text{Ig-la}\) added was determined by noncompetitive assays (of the kind shown in Table I) that were carried out in parallel to the above. Experimentally determined ratios of bound \([^{125}\text{I}]\text{Ig-la'}\) to \([^{125}\text{I}]\text{Ig-la}\) were compared to those expected assuming the binding affinities of \([^{131}\text{I}]\text{Ig-la'}\) (relative binding affinity constant, K') and \([^{125}\text{I}]\text{Ig-la}\) (relative binding affinity constant, K) were equal (K' = K) or unequal (K' = K/10). The basis for these calculations is shown in the Appendix.
TABLE II

| Experiment | Nanograms [\( {^{131}I} \)Ig-la'] | Nanograms [\( {^{125}I} \)Ig-la] | Ratio of bound [\( {^{131}I} \)Ig-la']/[\( {^{125}I} \)Ig-la] |
|------------|---------------------------------|---------------------------------|---------------------------------|
|            | Added Per- | Added Per- | Observed | Expected | K' = K \( \) K' = K/10 |
|            | percentage bound | percentage bound | | | |
| I          | 0.076 100 | 0.189 100 | 0.425 | 0.402 | 0.273 |
|            | 0.193 100 | 0.479 92  | 0.459 | 0.402 | 0.267 |
|            | 0.773 88  | 1.942 77  | 0.450 | 0.402 | 0.207 |
|            | 1.925 75  | 4.968 53  | 0.346 | 0.384 | 0.101 |
| II         | 0.080 100 | 0.394 98  | 0.202 | 0.204 | 0.094 |
|            | 0.200 94  | 0.918 83  | 0.220 | 0.218 | 0.096 |
|            | 0.796 69  | 3.745 69  | 0.212 | 0.212 | 0.070 |
|            | 2.00 43  | 9.387 43  | 0.213 | 0.214 | 0.040 |

* [\( {^{131}I} \)Ig-la'] is that of preparation A of Fig. 1 and [\( {^{125}I} \)Ig-la] is that of U10 myeloma protein.

Table II summarizes the results of two competitive experiments. First to be noted is that decreasing fractions of [\( {^{131}I} \)Ig-la'] and [\( {^{125}I} \)Ig-la] were bound with increasing total antigen added, thus demonstrating both antigens had to compete for antibody. Second, the ratios of nanograms of [\( {^{131}I} \)Ig-la']/[\( {^{125}I} \)Ig-la] bound reflected the ratios of nanograms of [\( {^{125}I} \)Ig-la]/[\( {^{125}I} \)Ig-la] added as would be expected of identical antigens and as predicted from calculations assuming K' = K (see Appendix). If anything, more [\( {^{131}I} \)Ig-la'] was bound than expected; this probably reflects experimental error in our estimate of the initial concentration of Ig-la'.

Discussion

The preceding results showed that certain pools of C.B-17 (and BAB/14) serum contained detectable Ig-la'. This antigen was indistinguishable from reference Ig-la proteins and represented \( \approx \)0.1-0.3% of the 7S serum Ig fraction. Consistent with our previous observations (1), Ig-la' production appeared to be transient, as not all 7S Ig preparations of C.B-17 and BAB-14 mice had detectable Ig-la'. The following is a discussion of (a) the basis for considering Ig-la' identical to Ig-la, and (b) explanations for low, transient productions of Ig-la'.

Evidence that Ig-la' Is Equivalent to Ig-la. The first question to consider is whether detection of Ig-la' could be an experimental artifact relating to nonspecific binding or to Ig-la-like crossreacting determinants. Evidence against this idea is as follows: (a) The assay for Ig-la' was highly specific, it utilized specifically purified anti-Ig-la and homogenous Ig-la myeloma proteins; (b) Ig-la' did not act as an incomplete competitor as is typical of cross-reacting protein antigens, rather it could displace completely the binding of [\( {^{125}I} \)Ig-la] to Ig-la-reactive wells (Fig. 1); (c) The bound fraction of Ig-la' represented 0.1-0.3% of the 7S Ig added (preparation A). This compared favorably with its effectiveness as a competitor; i.e., to get comparable inhibition of the binding of [\( {^{125}I} \)Ig-la] to Ig-la-reactive wells, it was necessary to add \( \approx \)1,000-fold more 7S Ig of preparation A than Ig-la myeloma protein (Fig. 1); and (d) Direct measurements of competitive binding of [\( {^{131}I} \)Ig-la'] and [\( {^{125}I} \)Ig-la] to Ig-la-reactive wells showed these two antigens to be indistinguishable.
Possible Explanations for Low, Transient Production of Ig-la'

Deficient expression and stimulation of Ig-la' B cells. The presence of Ig-la' in Ig-1b-producing C.B-17 mice could result from leaky expression of a normally silent Ig-la' locus. Manifestation of this at the cellular level could be low and variable numbers of Ig-la'-bearing B cells.

It seems possible that heavy-chain-constant (C\text{H}) region genes for Ig-la' and Ig-1b may be expressed with different heavy-chain-variable (V\text{H}) genes. If this were not so and members of the same set of V\text{H} genes were to randomly join with either C\text{H} gene, we would expect to see early and regular production of both Ig-la' and Ig-1b. That this does not occur with Ig-la' could reflect its association with a small and distinct set of V\text{H} regions (Ig-la' locus). Serum Ig-la' would then go undetected unless the appropriate antigens were present to induce sufficient clonal proliferation of the few Ig-la'-bearing cells.

The possibility that the putative Ig-la' locus might contain additional C\text{H} genes is suggested by our preliminary radioimmune evidence for Ig-4a' in pools A and D of Fig. 1 (our unpublished data) (Ig-4a corresponds to BALB/c IgG\text{I}). This prompts us to speculate as before (18) that the mouse genome may contain several disjoint clusters of V\text{H}, C\text{H} and allelic regulator (R\text{H}) genes. The expression of a given gene cluster would then depend on which R\text{H} allele is active.

Thymus-dependent suppression of Ig-la' B cells. Not mutually exclusive of the preceding considerations, C.B-17 mice might generate thymus-dependent immunity against Ig-la'-producing B cells. Unless such B cells were to appear early and regularly in the developing C.B-17 mouse, the host might be intolerant of their presence.

Suggestive evidence for possible involvement of cytotoxic or suppressor T cells (Tcs cells) in Ig-la' regulation comes from recent experiments. Earlier, we reported (18) that normal allotype-congenic mice appear to contain T cells that can prevent adoptively transferred lymphocytes of allotype-congenic partner mice from producing their respective allotypes. The strength of such allotype barriers varies according to the allotypes in question. In the case of C.B-17 vs. BALB/c, the barrier is relatively weak. That is, after transfer of BALB/c lymphocytes to C.B-17 hosts, donor Ig-la appears and then disappears in many recipients. This does not happen when cells of the same donor are transferred to nude C.B-17 mice. In all nude recipients, donor Ig-la persists for as long as such mice have been tested (>10 wk after cell transfer) (18 and our unpublished observations). Thus, specific Tcs in normal C.B-17 mice may prevent Ig-la' B cells from producing Ig-la'. Such Tcs cells could serve as a primary or secondary regulatory system for maintaining the silence of Ig-la'. It is not clear what structures on Ig-la' B cells would be seen as foreign, although they would presumably be controlled by allotype-linked genes.

From the data presented and cited, it seems certain that hidden mouse allotypes do in fact exist, but that specific regulatory mechanisms normally prevent their production. Regulation is likely to involve both intra- and extracellular mechanisms. To understand the latter will require identification of specific recognition elements and their controlling genes in a given regulatory network of lymphocytes.

Appendix

Basis for Calculation of Table II

Noncompetitive binding of Ig-la and Ig-la' to Ig-la-reactive wells. The initial
concentration of reactive anti-Ig-1a (Abi ≈ 4.2 ng/well) was estimated from the maximum capacity of each well to bind $^{[125]}$Ig-1a in antigen excess. Similarly, initial concentrations of Ig-1a (Ag) and Ig-1a' (Ag') were estimated from the quantities of $^{[125]}$Ig-1a and $^{[125]}$Ig-1a' bound under conditions of antibody excess (Table I). Given the quantities of bound Ag (Ag·Ab) for each addition of Agi, and Abi = 4.2 ng/well, the relative affinity constant (K) of anti-Ig-1a for Ig-1a was calculated to be 7.0 × 10$^{11}$ l/m. This assumes univalent binding of Ag and Ab in Ig-1a-reactive wells.

**COMPETITIVE BINDING OF IG-1A AND IG-1A' TO IG-1A-REACTIVE WELLS.** Equilibria expressions involving only initial (Ag, Ag', Abi) and bound (Ag·Ab, Ag'·Ab) concentrations of the above terms can be written as: (1) $K = \frac{Ag·Ab}{(Abi - Ag·Ab - Ag'·Ab)} (Ag - Ag·Ab)$, and (2) $K' = \frac{Ag'·Ab}{(Abi - Ag·Ab - Ag'·Ab)} (Ag' - Ag'·Ab)$ where equilibrium concentrations are: Ab = Abi - Ag·Ab, and Ag' = Ag' - Ag'·Ab; and where $K$ is the relative affinity constant of Ab for Ag, and $K'$ is the relative affinity constant of Ab for Ag'.

Assuming $K' = K$, 7.0 × 10$^{11}$ l/m, predicted values of bound Ag and Ag' (Ag·Ab, Ag'·Ab) in expressions (1) and (2) were determined by computer given the concentrations of Agi and Agi' added to each Ag-reactive well and Abi = 4.2 ng/well. The same was done assuming $K' = K/10$.

**Summary**

We have used a radioimmune assay to confirm our earlier findings of an unexpected immunoglobulin allotype in Ig$^a$-congenic BALB/c mice. Although these mice were bred to exclude the IgG$_{2a}$ allotype of BALB/c (Ig-1a), an Ig-1a-like antigen was detected in the 7S Ig fraction of two (of five) pooled serum samples, it represented 0.1-0.3% of the total 7S protein and was indistinguishable from a reference Ig-1a.

The detection of putative Ig-1a in Ig$^b$-congenic mice is inconsistent with the notion that allotypes are products of allelic structural genes. It appears rather that expression of Ig-1a is controlled by allelic regulator genes and that its low and transient production in Ig$^b$-congenic mice results from incomplete negative regulation.

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